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

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Publication date
2013

Document Version
Final published version

Citation for published version (APA):

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Variation in sexual communication and its role in divergence of two host strains of the noctuid moth *Spodoptera frugiperda*
VARIATION IN SEXUAL COMMUNICATION
AND ITS ROLE IN DIVERGENCE OF TWO HOST
STRAINS OF THE NOCTUID MOTH

Spodoptera frugiperda

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit van Amsterdam
op gezag van de Rector Magnificus
prof. dr. D.C. van den Boom
ten overstaan van een door het college voor promoties ingestelde
commissie, in het openbaar te verdedigen in de Agnietenkapel
op vrijdag 04 oktober 2013, te 14.00 uur

door

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geboren te Rudolstadt
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Chapter 1

General Introduction
1. Sexual communication in moths

1.1. The role of sex pheromones as premating signals

Many Lepidoptera species use sex pheromones to attract a conspecific mate (Tamaki, 1985; Cardé and Minks, 1997; Cardé and Haynes, 2004). In moths, females usually attract males over long distances via a species-specific sex pheromone (Tamaki, 1985; Löfstedt and Kozlov, 1997) that is produced in a pheromone gland located terminal at the abdomen of a female (Percy-Cunningham and MacDonald, 1987). During courtship, females extrude their pheromone gland and expose the gland surface to the wind, which distributes the female pheromone in the environment (Tamaki, 1985; Percy-Cunningham and MacDonald, 1987). Males have sensitive antennae that can detect the female pheromone signal and respond to it by starting flight and following the pheromone plume (Baker et al., 1985; Mafraneto and Cardé, 1994). When the male finds the source of the pheromone signal, i.e. the female, he starts a typical calling behavior by performing wing-fluttering and releasing a male-specific pheromone through its scent brushes or hairpencils (Tamaki, 1985; Birch et al., 1990; Lassance and Löfstedt, 2009). When both mates are attracted to each other at close range, mating will occur.

Females produce a species-specific sex pheromone that is only attractive for conspecific males (Tamaki, 1985; Löfstedt and Kozlov, 1997). On the one hand, species specificity of a sex pheromone is achieved by the use of a specific combination of pheromone components. Some species, like *Cameraria ohridella*, use only one pheromone component to attract males (Svatos et al., 1999), whereas other species, like *Heliothis virescens* or *Spodoptera frugiperda*, use multiple pheromone component blends as sex pheromone (Klun et al., 1980; Tumlinson et al., 1986). Some pheromone components of a complex female blend can also repel males of closely related species to avoid hybridization (Vickers and Baker, 1997;
Groot et al., 2006; Eizaguirre et al., 2007). On the other hand, the relative amount of each pheromone component within a complex female blend contributes to the specificity of sex pheromones (Tamaki, 1985; Jurenka, 2004). The most abundant component in a female blend of one species, e.g. (Z)-9-tetradecenyl acetate in Spodoptera frugiperda (Tumlinson et al., 1986), can for example be used in lower relative concentrations by another species, like Spodoptera litura (Sun et al., 2002), to avoid cross-attraction. The specificity and diversity of sex pheromones are remarkable and to date more than 1500 different sex pheromone compounds of a large variety of species have been identified (El-Sayed, 2012).

1.2. Biosynthesis of sex pheromones in females

Moth sex pheromones can be divided in so-called Type I (75%), Type II (15%) and miscellaneous (10%) pheromones, according to their chemical structure and biosynthesis (Ando et al., 2004). While Type I pheromones consist of a long carbon chain (C$_{10}$-C$_{18}$) with an oxygen-containing functional group, i.e. an alcohol, aldehyde or acetate ester, Type II pheromones are polyunsaturated hydrocarbons and epoxides with longer carbon chains (C$_{17}$-C$_{23}$) and no terminal functional group (Millar, 2000; Jurenka, 2003; Ando et al., 2004). The majority of female moths use sex pheromone compounds belonging to Type I, and pheromone biosynthesis of these compounds is based on the products of the fatty acid synthesis, i.e. palmitic acid (C$_{16}$) and stearic acid (C$_{18}$) (Jurenka, 2003, 2004). Modification of the long chain fatty acid precursors is achieved by different enzymes, i.e. (a) desaturases, which introduce double bonds at specific positions, (b) β-oxidation enzymes, which are responsible for chain-shortening by two carbons at a time, (c) reductases, which reduce the fatty acid precursors to produce alcohols and aldehydes, (d) oxidases, which oxidize alcohols to aldehydes, and (e) acetyltransferases, which convert fatty alcohols to acetate esters (Roelofs and Bjostad, 1984; Jurenka, 2003, 2004). Due to the action of all these different
enzymes, a great variety of sex pheromone components can be produced to obtain species-specific sex pheromone blends.

Pheromone biosynthesis in moths is activated by a pheromone biosynthesis activating neuropeptide (PBAN), which is produced in the subesophageal ganglion in the brain and released through the corpora cardiaca into the hemolymph (Raina and Menn, 1987; Raina, 1993; Rafaeli, 2005). PBAN binds to specific PBAN-receptors, present in the membrane of pheromone gland cells, and leads to the activation of second messengers, i.e. calcium influx and/or cAMP production, which induce the pheromone production (Raina, 1997; Roelofs and Jurenka, 1997; Rafaeli and Jurenka, 2003). In many moths, PBAN is produced in a circadian rhythm and released at the beginning of the scotophase (Raina, 1997; Rafaeli and Jurenka, 2003), so that mates are reproducitively active at night and inactive during the day. Besides the variation in pheromone production between night and day, the female pheromone can also be influenced by many other factors.

1.3. Variation in sexual communication

Variation in the pheromone signal of a female moth is a common phenomenon and can be caused by differential environmental and endogenous factors (Raina, 1998). Many moth species for example exhibit geographic variation in the female pheromone composition (Hansson et al., 1990; McElfresh and Millar, 1999, 2001; Cardé and Haynes, 2004; Groot et al., 2009; Barrozo et al., 2010a). Furthermore, environmental factors like temperature (Delisle and Royer, 1994; Dong and Du, 2002; Raina, 2003), humidity (Royer and McNeil, 1991, 1993; Raina, 2003), photoperiod (Webster and Yin, 1997; Han et al., 1999; Gemeno and Haynes, 2001), host plant volatiles (McNeil and Delisle, 1989; Landolt and Phillips, 1997; Reddy and Guerrero, 2004), or interspecific olfactory cues (Groot et al., 2010a) can have an effect on the female pheromone. It has also been shown that endogenous
female-specific factors like age or mating status can change the sex pheromone blend of a female (Babilis and Mazomenos, 1992; Tang et al., 1992; del Mazo-Cancino et al., 2004; Lima et al., 2008).

Similar to the variation in the female pheromone signal, males exhibit variation in their response to sex pheromones, depending on endogenous, as well as environmental factors. Among other factors, the response of male moths to sex pheromones can be influenced by geographic variation (Wu et al., 1999; Groot et al., 2007; Zhu et al., 2009), humidity (Royer and McNeil, 1993), temperature (Baker and Cardé, 1979; Linn et al., 1988; Dumont and McNeil, 1992; Delisle, 1995), insecticides (Wei et al., 2004; Xu et al., 2010), predator exposure (Svensson et al., 2004; Anton et al., 2011), mating status (Gadenne et al., 2001; Barrozo et al., 2010a, b; Barrozo et al., 2011), age (Payne et al., 1970; Delisle, 1995; Altafini et al., 2010), plant volatiles (Ochieng et al., 2002; Party et al., 2009; Pregitzer et al., 2012), or pre-exposure to sex pheromone (Bartell and Lawrence, 1973; Anderson et al., 2003; Anderson et al., 2007).

Thus, although moth sex pheromones are unique and species-specific, variability in the sender of the signal, i.e. the female, and the receiver of the signal, i.e. the male, exists. Despite the fact that changes in the pheromone signal and response can reflect fitness and mating status of a partner (Johansson and Jones, 2007; Harari et al., 2011), they can also be the start of reproductive isolation between populations and contribute to the formation of new species (Phelan, 1992, 1997; Löffstedt, 1993; Cardé and Haynes, 2004; Smadja and Butlin, 2009; Wicker-Thomas, 2011). This has for example been shown in the European corn borer, *Ostrinia nubilalis*, which is described in more detail below (part 3.1.).
2. Evolution of pheromone diversity in moths

2.1. Dependence of pheromone sender and receiver

The evolution of pheromone diversity in moths is hard to understand because stabilizing selection seems to act on the sexual communication channel of a species, due to the reciprocal dependence of the sender, i.e. the female, and the receiver, i.e. the male, of a pheromone signal (Cardé and Baker, 1984; Phelan, 1992, 1997; Löfstedt, 1993; Linn and Roelofs, 1995). If only stabilizing selection would act on the sexual communication system of moths, females would emit a sex pheromone most attractive for the majority of males, and the male response would be centered according to the most common female blend within a population (Cardé and Baker, 1984; Cardé and Haynes, 2004). Such a strong reciprocal stabilizing selection would lead to the coevolution of sender and receiver and cause the reduction of variation in signal and response, which constrains directional selection and the evolution of new signal-response systems (Phelan, 1992).

When the signal of a female and the response of a male are genetically linked, changes in the signal would consequently lead to changes in the response and vice versa (Phelan, 1992). Thus, pleiotropy and/or linkage disequilibrium could contribute to the diversification of sexual communication systems. However, in the studies on moths conducted so far, genetic linkage between pheromone biosynthesis of females and pheromone perception of males was not found (Roelofs et al., 1987; Klun and Huettel, 1988; Löststedt et al., 1989; Dopman et al., 2004). Furthermore, although the species-specific signal is not variable, the female pheromone signal and the male response can vary to some extent, as mentioned above. Therefore, the question arises whether counter-active selection forces exist that may overcome stabilizing selection and contribute to the great diversity of sex
pheromones and the species richness of moths (Shields, 1989; Kristensen et al., 2007; El-Sayed, 2012).

2.2. Asymmetric tracking hypothesis

The asymmetric tracking hypotheses was proposed by Phelan (1992, 1997), who suggested that selection could act asymmetrically on sender and receiver and that evolution of pheromone diversity in moths is driven by sexual selection via differential parental investments. The main assumption of this hypothesis is the existence of sex-specific parental investment, i.e. females invest more in their offspring and value the quality, not quantity, of males, whereas males invest more in mate finding with regard to the quantity of matings (Phelan, 1992, 1997). Phelan (1992) argued that both sexes differ in their reproductive strategies to maximize their own fitness and therefore, different selection pressures act on sender and receiver. More precisely, the pheromone signal is under weak selection and influenced by “stochastic factors and by avoidance of mating mistakes”, while the male response is under strong selection and determined by the female (he “tracks” the female), as well as intraspecific competition (Phelan, 1992, 1997). The overall conclusion of Phelan’s hypothesis is that the female pheromone signal may change relatively simply due to nonadaptive forces like genetic drift, founder effects or pleiotropy, while the male will track the most common female blend due to a wide response window.

Based on the asymmetric tracking hypothesis, the following evolutionary scenario (step 1 - 4) could explain how a mutation in the female sex pheromone could lead to reproductive isolation within a population and cause the formation of new species (Phelan, 1992, 1997; Löfstedt, 1993; Roelofs et al., 2002; Roelofs and Rooney, 2003; Baker, 2008; Smadja and Butlin, 2009).
First, the female pheromone signal changes (step 1). This could be caused by adaptation to the environment via selection on new mutations or on pre-existing standing genetic variation (Barrett and Schluter, 2008). It has been shown that the female pheromone signal can change, for example, under laboratory conditions, in the noctuid moth *Trichoplusia ni* (Haynes and Hunt, 1990a, b). The mutant *T. ni* females produced a sex pheromone that was significantly different from normal females of field populations and was not attractive for normal males (Haynes and Hunt, 1990a, b).

However, some rare males exist that exhibit a broad response window and are attracted to the new pheromone signal, while they can also respond to the most common pheromone blend of normal females (step 2). Some experiments showed that moth populations can contain rare males, exhibiting a broader pheromone response spectrum than normal males (Liu and Haynes, 1994; Roelofs et al., 2002; Linn et al., 2003; Linn et al., 2007; Hemmann et al., 2008). Thus, it is possible that a mutant female finds a mating partner, i.e. a rare male, and inherits the new pheromone signal to her female offspring, while the male offspring will respond to the new signal.

As a next step, to fix the new pheromone signal in the population, hybrids between mutant females and normal males, and/or crosses between normal females and rare males, need to have a lower fitness (step 3). Due to this hybrid fitness disadvantage, assortative mating can occur between mutant females and rare males, leading to the evolution of reproductive isolation barriers between the ancestral and the derived population (step 4). Once fully established, pre- as well as post-zygotic reproductive isolation mechanisms can drive divergence and contribute to the formation of new species (Coyne and Orr, 2004).

However, it remains an open question how fixation of the new pheromone signal is achieved in the derived population and how this causes the divergence of
populations (Smadja and Butlin, 2009). Furthermore, it is unclear what selective advantage rare males have when they are broadly tuned to a female pheromone blend. If males exhibit a very wide response window, they could also be attracted to females of another species, which would be a selective disadvantage for rare males. Thus, the asymmetric tracking hypothesis describes well the evolution of pheromone divergence in Lepidoptera, although it does not explain all aspects, which leaves other selection forces to shape the mate-signaling system in moths.

2.3. Selective forces in the evolution of pheromone diversity
Other selection forces, like sexual selection (Coltman et al., 2002; Wade and Shuster, 2004; Andersson and Simmons, 2006; Irestedt et al., 2009; Sullivan-Beckers and Cocroft, 2010), host plant adaptation (Nosil, 2007; Wiklund and Friberg, 2008; Smadja and Butlin, 2009; Ohshima, 2010), or geographically varying environmental factors (Cardé and Haynes, 2004), might contribute to the diversification of moth pheromones. Furthermore, selection due to specific environmental interaction with predators and parasitoids (Stowe et al., 1987; Zuk and Kolluru, 1998; Cardé and Haynes, 2004; Anton et al., 2011; Laumann et al., 2011), or the evolution of behavioral antagonists (Vickers and Baker, 1997; Cardé and Haynes, 2004; Groot et al., 2006; Baker, 2008), could overrule stabilizing selection. To understand the evolution of sexual communication systems in moths, intra-specific studies, rather than inter-specific ones, might facilitate the identification of factors causing the divergence of pheromone signals. More precisely, the study of pheromonal strains within a species could help to understand how differentiation in sexual communication systems can arise and lead to reproductive isolation within a population.
3. Pheromone divergence within species: The first step to speciation?

3.1. The European corn borer Ostrinia nubilalis

The most prominent example of pheromone divergence within a lepidopteran species is *Ostrinia nubilalis*, which consists of two pheromonal strains, the so-called E-strain and the Z-strain (Roelofs et al., 1985; Lassance, 2010). The female sex pheromone of both strains consists of the two components (Z)-11-tetradecenyl acetate (Z11-14:OAc) and (E)-11-tetradecenyl acetate (E11-14:OAc), which are produced in a 97:3 Z/E ratio in Z-strain females and in a 1:99 Z/E ratio in E-strain females (Kochansky et al., 1975). Due to the strain-specific pheromone, both strains mate assortatively in the field and hybridization between strains is rare (Roelofs et al., 1972; Glover et al., 1987; Linn et al., 1997).

Genetic analyses revealed that different genomic regions are involved in the variation in the female pheromone and the male response (Roelofs et al., 1987; Klun and Huettel, 1988; Löfstedt et al., 1989; Dopman et al., 2004; Olsson et al., 2010). Lassance et al. (2010) showed that the fatty acyl reductase pgFAR is responsible for the opposite pheromone ratio found in E- and Z-strain females. The pgFAR gene has two strain-specific alleles that have different substrate specificities to the cis and trans isomer of the pheromone precursors (Z)- and (E)-11-tetradecenoyl and subsequently cause strain-specific pheromone blends (Lassance et al., 2010). Genes responsible for the strain-specific male response have not been identified so far. However, it was shown that the male response is determined by the resp locus, located on the sex chromosome (Roelofs et al., 1987; Dopman et al., 2004), and by loci affecting the antennal response, which are both autosomal and sex-linked (Roelofs et al., 1987; Olsson et al., 2010). Different odorant receptor genes, located on the sex chromosome, could partly cause the strain-specific male attraction (Lassance et al., 2011; Yasukochi et al., 2011).
In conclusion, strain-specific sexual communication is a strong prezygotic mating barrier between the Z- and the E-strain and leads to reproductive isolation. The pheromonal differentiation as well as genetic differences suggest that both strains are in the process of speciation and probably semispecies or sibling species (Cardé et al., 1978; Malausa et al., 2007; Lassance, 2010). As a result, O. nubilalis has become a model species to study the evolution of sexual communication (Smadja and Butlin, 2009; Lassance, 2010; Wicker-Thomas, 2011).

3.2. The fall armyworm Spodoptera frugiperda

Similar to the two strains in Ostrinia nubilalis, the fall armyworm Spodoptera frugiperda (J. E. Smith) consist of two strains that differ in their female sex pheromone composition (Groot et al., 2008; Lima and McNeil, 2009), and seem to be undergoing ecological speciation in sympatry (see Chapter 5 and 6). The two strains of S. frugiperda, the corn-strain and rice-strain, have first been described as host strains, which prefer either corn as host or smaller grasses like rice and Bermuda grass (Pashley et al., 1985; Pashley, 1986). Besides habitat isolation, both strains exhibit behavioral isolation due to strain-specific timing of mating activity in the night (Pashley et al., 1992; Schöfl et al., 2009). While the corn-strain is the “early” strain, the rice-strain is the “late” strain, calling, copulating and ovipositing approximately three hours later than the corn-strain (Schöfl et al., 2009). Behavioral isolation due to allochronic (temporal) isolation can be a strong prezygotic mating barrier (Coyne and Orr, 2004; Devries et al., 2008), because this may cause both strains to rarely encounter each other. However, the reproductively active times of both strains do overlap to some degree and allochronic isolation seems to be asymmetric and less pronounced than previously thought (Schöfl et al., 2009). To determine the role of sexual communication in the divergence of corn- and rice-strain populations, we started to investigate the strain-specific sexual communication system of both S. frugiperda strains.
3.3. Strain-specific pheromone variation in Spodoptera frugiperda

The sex pheromone of *S. frugiperda* females has first been investigated by Tumlinson et al. (1986), who showed that females emit a multi-component blend consisting of the major pheromone component (Z)-9-tetradecenyl acetate (Z9-14:OAc), the critical secondary component (Z)-7-dodecenyl acetate (Z7-12:OAc) and several minor compounds like (Z)-11-hexadecenyl acetate (Z11-16:OAc), with unknown behavioral function. Although the sex pheromone composition of *S. frugiperda* females is known since 1986, and both strains have first been described in 1985 (Pashley et al., 1985), it took more than 20 years until the existence of a strain-specific corn- and rice-strain pheromone was first identified (Groot et al., 2008; Lima and McNeil, 2009). Interestingly, both studies found different strain-specific female pheromone compositions. First, I will first describe the variation that we found (Groot et al., 2008; Marr, 2009), and then the variation that Lima and McNeil (2009) found.

We examined the pheromone composition of laboratory populations from Florida and found that corn-strain females produced significantly higher relative amounts of Z11-16:OAc than rice-strain females, while rice-strain females exhibited higher relative amounts of Z7-12:OAc and (Z)-9-dodecenyl acetate (Z9-12:OAc) than corn-strain females (Groot et al., 2008; Marr, 2009). The pheromone analysis of hybrid females revealed that the major pheromone component Z9-14:OAc, as well as Z11-16:OAc, were maternally inherited, whereas Z9-12:OAc showed a corn-strain dominant inheritance (Groot et al., 2008; Marr, 2009). Interestingly, the relative amount of the important secondary pheromone component Z7-12:OAc was significantly lower in hybrid CR (corn♀ x rice♂) and RC (rice♀ x corn♂) females than in pure strain females (Groot et al., 2008; Marr, 2009). Because Z7-12:OAc has been shown to be a critical minor component necessary for the attraction of males in the field (Tumlinson et al., 1986), the suppression of this component in
hybrid females suggests that hybrid females may not be as attractive for males as pure-strain females. Based on phenotypic correlations between all pheromone components of the female blend, we constructed a pheromone biosynthesis pathway of *S. frugiperda* and suggested candidate genes that might be responsible for the strain-specific female pheromone composition (Groot et al., 2008; Marr, 2009). Differences in the expression level or substrate specificity of a Δ11-desaturase, a Δ9-desaturase or a possible Δ7-desaturase might cause the strain-specific differences in the relative amount of Z7-12:OAc, Z9-12:OAc and Z11-16:OAc between corn- and rice-strain females (Groot et al., 2008; Marr, 2009). In conclusion, we found strain-specific pheromone differences between corn- and rice-strain females and differential inheritance of single pheromone components, which suggests the involvement of multiple genomic regions leading to differential pheromone blends (Groot et al., 2008; Marr, 2009).

Lima and McNeil (2009) examined laboratory populations that originated from Louisiana, and also found strain-specific pheromone differences between corn- and rice-strain populations. In contrast to our results (Groot et al., 2008; Marr, 2009), corn-strain females from Louisiana exhibited higher relative amounts of Z9-14:OAc than rice-strain females, whereas rice-strain females produced higher relative amounts of Z11-16:OAc than corn-strain females (Lima and McNeil, 2009). The differences between both studies suggest that geographic variation influences the strain-specific variation in the female pheromone composition of *S. frugiperda*. However, strain-specific differences in the relative amount of Z7-12:OAc were similar between females from Florida and Louisiana, namely that corn-strain females have lower amounts of Z7-12:OAc than rice-strain females (Groot et al., 2008; Lima and McNeil, 2009; Marr, 2009). Thus, although geographic variation might affect the female pheromone, similarities between both studies suggest that stabilizing selection acts on the critical sex pheromone
component Z7-12:OAc. Furthermore, Lima and McNeil (2009) showed that Z9-14:OAc was maternally inherited, which was similar to our results (Groot et al., 2008; Marr, 2009). Based on our results (Groot et al., 2008; Marr, 2009) and the research by Lima and McNeil (2009), the aim of my research was to disentangle strain-specific from geographic variation and to determine the genetic basis of the strain-specific female sex pheromone, all findings of which are described in this thesis.

4. The biology of Spodoptera frugiperda

4.1. Life cycle

The fall armyworm Spodoptera frugiperda (Lepidoptera: Noctuidae) is a noctuid moth that occurs throughout North- and South-America (Luginbill, 1928; Sparks, 1979). Depending on the climate, S. frugiperda can persist the whole year or overwinter as pupae, and one life cycle may take 4 to 13 weeks (Sparks, 1979; Andrews, 1988). During the day, adult moths usually hide within the vegetation (MU, personal observation; Fig. 1A, B). During the scotophase, receptive females sit near the top of a host plant and start calling by extruding their pheromone gland (Sparks, 1979).

One female usually attracts several males which fly towards the pheromone source and try to land near the calling female (Sparks, 1979). After landing, receptive males show a typical male calling behavior in which they extrude their hair pencils, perform wing fanning and bend their abdomen to approach a female (MU, personal observations). Females can reject unsuitable males (Sparks, 1979), which is probably caused by close range pheromonal communication between both sexes (Schöfl et al., 2011).
Figure 1. Development of *Spodoptera frugiperda* in the field. (A) Adult moths. (B) Female moth hiding in the whorl of a corn plant during the day. (C) Mating of adult moths in the night. (D) Egg clutch on a corn plant. (E) Hatching larvae. (F) Larva feeding on a corn plant. (G) Last instar larva. (H) Larva goes into pupation stage. (I) Pupae.
When a female accepts a male, both partners will mate with each other (Fig. 1C), and the duration of copulation can take several hours (Schöfl et al., 2009). During copulation, the male transfers a spermatophore, i.e. a capsule containing sperm, into the female (Lamunyon, 2000). Although *S. frugiperda* females mate only once a night (Sparks, 1979), females can mate with multiple partners in different nights and can contain more than one spermatophore (Meagher and Nagoshi, 2010).

After a successful copulation, females search for a suitable oviposition place (Fig. 1D), which includes host plants, non-host foliage as well as artificial substrates like window panes or carts (Sparks, 1979). The use of non-hosts as oviposition site for *S. frugiperda* females may facilitate neonate dispersal or avoidance of predators and parasitoids (Meagher et al., 2011). Females usually lay their eggs in clutches (Fig. 1D), which are covered with scales and contain few up to hundreds of eggs (Sparks, 1979; Meagher et al., 2011). First instars hatch from the egg clutches around two to four days after oviposition (Fig. 1E), and feed on their host plant until the sixth instar (Sparks, 1979; Fig. 1F, G).

*Spodoptera frugiperda* is known as a generalist species, which can feed on more than 80 plant species from 23 different plant families (Pashley, 1988a). Under experimental conditions, larvae feed for approximately three weeks until they reach their last instar (Fig. 1G, H), and start pupating (Groot et al., 2010b; Nagoshi, 2011). In nature, sixth instars dig themselves into the ground and pupate between one and three meters deep into the soil, where, depending on the temperate, they eclose after one to five weeks (Sparks, 1979). In some cases, larvae pupate within the stem of their host plants (Fig. 1I). After emergence from the pupal case, adults leave the soil and climb onto plants to expand their wings (Sparks, 1979). Newly emerged adults do not mate within their first night (Sparks, 1979), and are usually
reproductively active when they are two to five days old (MU, personal observation).

4.2. Identification of two host strains

In 1986, Pashley reported the presence of two strains in *S. frugiperda*, the corn- and the rice-strain, which exhibited genetic differences and a strain-specific host plant choice. Based on the first studies of both strains, Pashley (1986) suggested that both strains may be sibling species, which are reproductively isolated from each other. Since the discovery of both strains, numerous studies investigated the differences between the two morphologically identical strains, regarding genetic differentiation (Pashley, 1989; Lewter et al., 2006; Nagoshi and Meagher, 2008; Nagoshi, 2010), host plant choice (Busato et al., 2004; Nagoshi et al., 2007; Machado et al., 2008; Juárez et al., 2012), development (Pashley, 1988b; Whitford et al., 1988; Meagher et al., 2004; Groot et al., 2010b), reproduction (Pashley et al., 1992; Schöfl et al., 2009; Meagher et al., 2011; Schöfl et al., 2011), and sex pheromone differences (Groot et al., 2008; Lima and McNeil, 2009; Marr, 2009). Due to the fact that both strains differ in so many traits and occur in sympatry throughout North- and South America, *S. frugiperda* has become a model species to study the evolution of reproductive isolation between strains of the same species (Pashley, 1988a; Pashley et al., 1992; Prowell et al., 2004; Groot et al., 2008; Machado et al., 2008; Nagoshi and Meagher, 2008; Marr, 2009; Schöfl et al., 2009; Groot et al., 2010b; Schöfl et al., 2011; Juárez et al., 2012).

In addition to different possible prezygotic mating barriers, i.e. strain-specific host plant choice (Pashley, 1986; Nagoshi et al., 2006a; Nagoshi et al., 2007), timing of reproduction (Pashley et al., 1992; Schöfl et al., 2009), and sex pheromone composition (Groot et al., 2008; Lima and McNeil, 2009; Marr, 2009), both strains also exhibit postzygotic isolation (Pashley and Martin, 1987; Whitford et al., 1988;
Groot et al., 2010b). Laboratory backcross matings of *S. frugiperda* populations from Puerto Rico and Louisiana showed that hybrid RC (rice-strain♀ x corn-strain♂) females produced no fertile offspring when crossed to a pure strain corn- or rice-strain male (Pashley and Martin, 1987). Furthermore, a reduced number of fertile offspring was found when RC hybrid females were crossed to RC hybrid males, as well as in crosses between pure strain females and hybrid RC males (Pashley and Martin, 1987). Backcross experiments with laboratory populations from Louisiana (rice-strain) and Mississippi (corn-strain), conducted by Whitford et al. (1988), also showed that matings between hybrid RC (rice-strain♀ x corn-strain♂) females and males were less fertile than pure strain matings or crosses between CR (corn-strain♀ x rice-strain♂) hybrid individuals. Similarly, Groot et al. (2010b) reported a reduced fertility when laboratory RC (rice-strain♀ x corn-strain♂) hybrid females, originated from Florida, were crossed to both parental strains and hybrid males (CR and RC). On the contrary, crosses between CR (corn-strain♀ x rice-strain♂) hybrid females and pure strain (C, R) as well as CR hybrid males produced similar percentages of fertile offspring as pure strain matings (Groot et al., 2010b).

Interestingly, the vast majority of hybrids in the field are RC (rice-strain♀ x corn-strain♂) hybrid individuals (Nagoshi and Meagher, 2003; Prowell et al., 2004; Nagoshi et al., 2006b), which exhibit a reduced fertility when females are crossed to pure strains or other hybrids (Pashley and Martin, 1987; Whitford et al., 1988; Groot et al., 2010b). The mating incompatibilities of RC hybrid females represent a unidirectional postzygotic isolation barrier that contributes, together with the different prezygotic isolation mechanisms, to reproductive isolation between both strains (Groot et al., 2010b). Thus, *S. frugiperda* is an ideal model organism to study the evolution of pre- as well as post-zygotic mating barriers within a species.
4.3. Spodoptera frugiperda as agricultural pest species

Besides being an ideal species to study incipient speciation, *S. frugiperda* is a serious agricultural pest species that causes great damage to a large variety of plants due to its generalistic feeding behavior (Pashley, 1988a). Fall armyworm infestations of field crops in the southeastern United States can cause losses of 93 up to 297 million dollars per year which makes *S. frugiperda* one of the most damaging agricultural pests occurring in the United States (Sparks, 1986). The ability of *S. frugiperda* to overwinter in subtropical and tropical areas like Florida or the Caribbean leads to a continuous damage of larvae feeding on crop plants throughout the year (Luginbill, 1928; Sparks, 1986). Furthermore, adults can migrate large distances and annual population movements from overwintering (sub)tropical areas to temperate zones cause a spread of this pest into many regions (Luginbill, 1928; Sparks, 1979; Nagoshi and Meagher, 2008; Nagoshi et al., 2008; Nagoshi et al., 2009).

Different pest management strategies like insecticide applications (Andrews, 1988; Hruska and Gladstone, 1988; Pitre, 1988; Vergara and Pitre, 2001), trapping with sex pheromone (Mitchell et al., 1985; Andrade et al., 2000; Malo et al., 2001), biological control via predators or parasitoids (Molina-Ochoa et al., 2003; Hoballah et al., 2004; Bueno et al., 2008), resistant host plants (Sparks, 1986; Wiseman and Isenhour, 1988a, b), and the use of plant polycultures and intercrops (Andrews, 1988), have been considered to reduce the agricultural losses caused by *S. frugiperda*. Due to the fact that this pest species consists of two behaviorally and genetically different strains (Pashley, 1986), pest management treatments may have different effects on both strains. Differences between corn- and rice-strain individuals therefore also help to improve pest management strategies. The use of strain-specific pheromone lures could for example facilitate the early detection of
corn- or rice-strain adults in crops, which could then be treated with strain-specific insecticides, pathogens or parasitoids.

5. Main questions of this thesis

Similar to the case of *Ostrinia nubilalis* (Lassance, 2010), strain-specific differences in the sexual communication system of *S. frugiperda* could act as prezygotic mating barrier between both strains and cause assortative mating. The presence of strain-specific differences in the female pheromone composition of *S. frugiperda* (Groot et al., 2008; Lima and McNeil, 2009; Marr, 2009) indicates that assortative mating could occur if males of both strains are strain-specifically attracted to females of their own strain. The main aim of this thesis was to evaluate whether strain-specific sexual communication is a prezygotic mating barrier between corn- and rice-strain populations and contributes to reproductive isolation between both strains. Therefore, we investigated the differences in female pheromone composition and tested with different assays if males of both strains are distinctively attracted to strain-specific females. Furthermore, we studied the variability in the male response to pheromones in different geographic regions and examined the genetic basis of strain-specific corn- and rice-strain pheromones to understand the evolution of both *S. frugiperda* strains.

6. Thesis outline

Chapter 2 describes the pheromonal divergence between the two strains of *S. frugiperda* in Florida (Unbehend et al., 2013b). The main aim of this study was to assess whether strain-specific pheromones act as prezygotic mating barrier and cause assortative mating between both *S. frugiperda* strains in Florida. To assess strain-specific female pheromone differences, we performed pheromone gland extractions of corn- and rice-strain females of laboratory and field populations
originated from Florida. The male response of both strains towards strain-specific pheromone blends was tested in wind tunnel assays, using laboratory females, and in male trapping experiments in Florida, using self-made strain-specific synthetic pheromone lures. Dose-response experiments of different pheromone compounds were conducted in the field to evaluate the importance of single compounds for strain-specific male attraction. Furthermore, all trapping experiments were conducted in a corn-strain-specific habitat (corn field) as well as in a rice-strain-specific habitat (grass field), to determine the influence of different environments on the male response.

Similar to the results of our previous study on laboratory populations (Groot et al. 2008; Marr 2009), we found strain-specific sex pheromone differences between corn- and rice-strain females from Florida field populations in this study. The results of wind tunnel assays and field trapping experiments showed that males were not strain-specifically attracted to females of their own strain. This suggests that strain-specific female sex pheromone differences are not strong enough to cause assortative mating in Florida. Nevertheless, we found strain-specific differences in the male attraction to the critical pheromone component Z7-12:OAc, as well as differential male responses to strain-specific sex pheromone blends in different habitats. Furthermore, dose-response experiments suggested that the minor compounds Z11-16:OAc and Z9-12:OAc are not required for male attraction in Florida.

In Chapter 3, we investigated the variation in sexual attraction of S. frugiperda males in different geographic regions (Unbehend et al., 2013c), with the aim to disentangle strain-specific variation from geographic variation. Different male trapping experiments with self-made synthetic pheromone lures were carried out in Canada, North Carolina, Florida, Puerto Rico, Peru and Argentina, to evaluate variability in the response of corn- and rice-strain males. Pheromone traps were
baited with two different synthetic 4-component blends as well as with lures containing different amounts of important pheromone components like Z7-12:OAc or E7-12:OAc. The strain-identity of all trapped males was determined via molecular analysis of the cytochrome oxidase I gene, which exhibits strain-specific sequence differences.

We found that corn-strain males exhibited geographic variation in their response to our two synthetic 4-component blends and to different doses of Z7-12:OAc. In contrast, rice-strain males showed almost no geographic variation in response to different sex pheromone blends. These results suggest that corn-strain males are more restricted in their response to sex pheromone than rice-strain males. *Spodoptera frugiperda* males also showed differential responses towards the minor compound E7-12:OAc, which attracted males in North Carolina, but was unattractive for males in Peru. Furthermore, attraction of males to strain-specific pheromone blends varied between habitats within the same region, which suggests an influence of habitat-specific volatiles on the male attraction to sex pheromone blends.

In Chapter 4, we studied the genetic basis of strain-specific female pheromone differences in *S. frugiperda* (Unbehend et al., 2013a). Female-informative backcrosses were generated by hybridizing both strains and backcrossing the generated hybrid females with corn- and rice-strain males. One backcross family was used for an AFLP marker-based quantitative trait locus (QTL) analysis to determine which genomic regions are involved in the strain-specific pheromone production. We generated a genetic map for *S. frugiperda* and mapped different candidate genes that may cause differential pheromone production of both strains. To investigate the inheritance of strain-specific pheromones, pheromone gland extractions of pure strain, hybrid and backcross females were performed.
In this study, we found that multiple genomic regions are involved in the production of strain-specific female sex pheromone blends. We identified 10 QTL on 9 different chromosomes that explain the strain-specific differences in the relative amounts of Z9-14:OAc, Z11-16:OAc, Z7-12:OAc and Z9-12:OAc. A delta-11-desaturase (SfLPAQ) mapped to Sf chromosome 2, which explained a significant portion of the variance of Z9-14:OAc and Z11-16:OAc and showed the opposite-to-expected phenotypic pattern for both compounds. Furthermore, the circadian clock protein vrille (SfVRI) mapped to Sf chromosome 28, responsible for the critical secondary sex pheromone component Z7-12:OAc. Interestingly, vrille also appears to be responsible for the strain-specific differences in timing of reproduction in the scotophase (Hänniger et al., 2013). Thus, one chromosome (Sf/C28) is associated with the strain-specific variation in two prezygotic mating barriers in S. frugiperda, i.e. differential timing of reproduction and female sex pheromone composition. Analysis of the inheritance of strain-specific pheromone differences showed that Z9-14:OAc was overexpressed in CR hybrid females (C♀ x R♂) and paternally inherited in RC (R♀ x C♂) hybrid females, while Z11-16:OAc was suppressed and Z9-12:OAc overexpressed in both hybrids. The critical minor component Z7-12:OAc was rice-strain dominant inherited in CR and RC hybrid females.

Chapter 5 shows an overview of the roles and interactions of different reproductive isolation mechanisms in both S. frugiperda strains (Groot et al., 2010b). This review examines how habitat isolation and behavioral isolation through sexual communication and differential timing of reproduction affect reproductive isolation between corn- and rice-strain populations. Possible interactions between these prezygotic isolation barriers as well as their genetic bases are discussed. Furthermore, data on postzygotic incompatibilities as well as the permeability of the host strain genomes are analyzed.
The main conclusion of this review is that the separate pre- and postzygotic mating barriers seem unlikely to cause reproductive isolation between both strains, but in combination they may act additively or synergistically and thus prevent both strains from merging into one population. Furthermore, strain-specific differences in the mitochondrial mtDNA suggest that both strains separated approximately 600,000 years ago.

Chapter 6 deals with the evolution of reproductive isolation of *S. frugiperda* (Groot et al., 2013). This review comprises data on habitat isolation of both strains due to differential host plant choice, as well as behavioral isolation due to strain-specific timing of reproductive activity at night. Furthermore, the level and direction of hybridization between both strains and an evolutionary scenario on possible reproductive isolation in the two strains is discussed. In this chapter, we also summarize recent data on behavioral isolation of both strains due to variation in sexual communication. Besides inter-strain-specific pheromone differences, we found female pheromone variation within strains, which are described in relation to geographic variation in the female pheromone composition. We also present data on variation in the male response to strain-specific female pheromone blends, as well as on the male attraction to different pheromone compounds in the field.

The main conclusion of this review is that both strains are rather “timing strains” than “host strains” or “pheromonal strains”, and sterility of RC hybrid females (rice-strain♀ x corn-strain♂) is a strong postzygotic mating barrier which contributes to the divergence of the two *S. frugiperda* strains. Furthermore, we suggest a possible evolutionary scenario in which the rice-strain is the ancestral strain and the corn-strain the derived one, because the corn-strain is genetically more homogeneous than the rice-strain. This hypothesis correlates with findings that the corn-strain is more restricted in habitat occurrence and in pheromone response than rice-strain individuals.
In Chapter 7, I summarize and discuss all main findings of this thesis. First, the results are discussed with regard to the evolution of both strains and the contribution of sexual communication differences to the divergence of the corn- and the rice-strain. Secondly, the results are discussed in the light of pest management, because *S. frugiperda* is an important agricultural pest species. Based on the results of the male trapping experiments in the field, I recommended a pheromone blend that can be used to monitor both strains independent of geographic and strain-specific variation, and discussed whether it is possible to reduce *S. frugiperda* populations in the field via the use of mating disruption. A summary of the main findings of this thesis can be found on pages 166-167.

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Chapter 1


Chapter 1


Chapter 1


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Chapter 1


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Chapter 2

Pheromonal divergence between two strains of Spodoptera frugiperda

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

Key Words. Sexual communication, Male attraction, Fall armyworm, Corn- and rice-strain, Synthetic pheromone lures, Dose-response experiments, Lepidoptera, Noctuidae, Sympatric speciation.
Introduction

Many insect species produce sex pheromones that mediate sexual communication between males and females (Tamaki, 1985; Löfstedt and Kozlov, 1997). In Lepidoptera, females usually produce species-specific sex pheromones that exclusively attract conspecific males over long distances (Cardé and Baker, 1984; Tamaki, 1985; Cardé and Haynes, 2004). To find a suitable mating partner, males need to respond to the specific chemical signal that is emitted by a conspecific female (Löfstedt, 1993; Cardé and Haynes, 2004). Thus, changes in the pheromone signal of a female may result in reproductive isolation, which in turn can lead to speciation (Roelofs and Cardé, 1974; Phelan, 1992; Baker, 2002; Smadja and Butlin, 2009). A model species to study the evolution of sexual communication is the European corn borer, *Ostrinia nubilalis* (Lepidoptera: Crambidae), which consists of two strains, the Z- and the E-strain, that differ in their female-produced pheromone production and male response to pheromones (Klun and Cooperators, 1975; Smadja and Butlin, 2009; Lassance, 2010; Lassance et al., 2010; Wicker-Thomas, 2011). While Z-strain females produce 97:3 (Z)/(E)-11-tetradecenyl acetate (Z/E11-14:OAc) (Klun et al., 1973), E-strain females emit 1:99 Z/E11-14:OAc (Kochansky et al., 1975). The production of different ratios Z/E11-14:OAc is based on a strain-specific allelic variation in a fatty-acyl reductase gene (*pgFAR*), which causes different substrate specificities of the enzyme and, thus, different female pheromones (Lassance et al., 2010). Males of both strains are specifically attracted to females of their own strain, although E-strain males have a broader response to pheromones than Z-strain males (Lassance, 2010). The two *O. nubilalis* strains seem to be sibling species, which mate assortatively, and exhibit low hybridization rates in the field due to strain-specific sexual communication (Lassance, 2010).
Similar to the European corn borer, the fall armyworm, *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae), is an ideal model organism to study speciation because it also consists of two distinct strains, the corn- and the rice-strains (Pashley, 1986). Corn-strain individuals mainly occur in habitats that contain large grasses like corn and sorghum, while the rice-strain inhabits areas consist of small grasses like rice, bermuda grass, or turf grass (Pashley, 1986, 1989; Lu and Adang, 1996; Levy et al., 2002; Nagoshi et al., 2006, 2007; Machado et al., 2008). However, in most fields, both kinds of strains can be found in different proportions, and habitats containing only one strain are rare (Pashley, 1989; Meagher and Gallo-Meagher, 2003; Nagoshi et al., 2006, 2007). Although both strains are morphologically indistinguishable from each other, they exhibit several genetic differences in the mitochondrial cytochrome oxidase I (COI) and NADH dehydrogenase (ND1) genes (Pashley, 1989; Pashley and Ke, 1992; Lu and Adang, 1996; Levy et al., 2002; Meagher and Gallo-Meagher, 2003; Prowell et al., 2004; Nagoshi et al., 2006; Machado et al., 2008), esterase allozyme loci (Pashley, 1986), amplified fragment length polymorphisms (AFLP) loci (McMichael and Prowell, 1999; Busato et al., 2004; Prowell et al., 2004; Clark et al., 2007; Martinelli et al., 2007; Juárez et al., 2012), the copy number and organization of the fall armyworm rice-strain sequence (FR) (Lu et al., 1994; Nagoshi and Meagher, 2003), and in their triose phosphate isomerase (TPI) gene (Nagoshi, 2010). Furthermore, both strains differ in their timing of mating in the scotophase; corn-strain individuals call, mate, and oviposit approximately 3 hr earlier than rice-strain individuals (Pashley et al., 1992; Schöfl et al., 2009).

The pheromone composition of *S. frugiperda* females has been studied several times at different geographic regions (Tumlinson et al., 1986; Descoins et al., 1988; Batista-Pereira et al., 2006; Groot et al., 2008; Lima and McNeil, 2009). However, most studies have focused on the general composition of the female sex
Pheromonal divergence between two strains of *S. frugiperda*

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

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

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

**Methods and Materials**

*Spodoptera frugiperda Populations*

We conducted experiments with two different populations of each host-strain from Florida. The so-called laboratory populations, i.e. corn-strain (JSC3) and rice-strain (OnaR), originated from 100 to 200 larvae collected by RLM in Florida. JSC3 individuals were collected from corn plants near Homestead, Miami-Dade County in 2004, and OnaR larvae were collected from pasture grasses at the Range Cattle
Pheromonal divergence between two strains of *S. frugiperda*

Research and Education Center, Ona, Hardee County in 2003. Populations were reared on artificial pinto bean diet for 2–3 yr in a mass culture at USDA, Gainesville, Florida, after which specimens of both strains were sent to the MPICE in 2006 to establish a colony. All individuals were screened for strain-specific cytochrome oxidase subunit I (COI) markers to confirm strain-identity, and reared for another 3 yr on artificial pinto bean diet. Since these two populations have been reared up to 6 yr under laboratory conditions, they will be referred to as the laboratory populations in all experiments.

The so-called field population of both strains descended from around 300 larval specimens collected in 2010 in Florida. Corn-strain larvae (FLC) were collected from a corn field at the Everglades Research and Education Center, Belle Glade, Palm Beach County (+26° 40′ 7.20″, −80° 37′ 57.63″), and rice-strain individuals (FLR) from a grass field at the Graham Farm in Moore Haven, Glades County (+26° 53′ 3.04″, −81° 7′ 21.17″). All larvae were shipped to the MPICE, and reared until adulthood on artificial pinto bean diet. Adults were screened for strain-specific COI markers to establish strain-specific colonies. Experiments with these populations were conducted after the colony was established (2nd laboratory generation); these populations will be referred to as field populations in all experiments.

All insects were reared in climate chambers on a reversed light:dark (L:D) cycle, and a 14:10 L:D photoperiod at 26 °C and 70 % RH. Adults were fed with a 10 % honey-water solution, and random single-pair-matings were performed to avoid inbreeding and maintain both populations. Although we collected all insects from different locations in Florida, we do not assume genetic differences between populations because *S. frugiperda* is a highly migratory species (Sparks, 1979), that overwinters in Florida (Luginbill, 1928), suggesting high gene flow among
populations. Furthermore, genetic analyses of corn-strain haplotypes in different habitats indicated a genetically homogenous corn-strain population in Florida (Nagoshi and Meagher, 2008).

**Pheromone Extractions**

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

**Chemical Analysis**

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

**Preparation of Lures**

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

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

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<tr>
<th>EXPERIMENT</th>
<th>A) STRAIN-SPECIFIC BLENDS&lt;sup&gt;1&lt;/sup&gt;</th>
<th>B) Z7-12:OAc DOSE-RESPONSE&lt;sup&gt;1&lt;/sup&gt;</th>
<th>C) Z11-16:OAc DOSE-RESPONSE&lt;sup&gt;2&lt;/sup&gt;</th>
<th>D) Z9-12:OAc DOSE-RESPONSE&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHEROMONE BLENDS</td>
<td>Corn-strain blend: 2% Z7-12:OAc 13% Z11-16:OAc 1% Z9-12:OAc</td>
<td>0% Z7-12:OAc</td>
<td>0% Z11-16:OAc</td>
<td>0% Z9-12:OAc</td>
</tr>
<tr>
<td></td>
<td>Rice-strain blend: 4% Z7-12:OAc 8% Z11-16:OAc 2% Z9-12:OAc</td>
<td>2% Z7-12:OAc</td>
<td>8% Z11-16:OAc</td>
<td>1% Z9-12:OAc</td>
</tr>
<tr>
<td></td>
<td>Hexane</td>
<td>4% Z7-12:OAc</td>
<td>13% Z11-16:OAc</td>
<td>2% Z9-12:OAc</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10% Z7-12:OAc</td>
<td>18% Z11-16:OAc</td>
<td>4% Z9-12:OAc</td>
</tr>
<tr>
<td>FIELD</td>
<td>Corn field, Belle Glade, FL</td>
<td></td>
<td>Corn field, Hague, FL</td>
<td>Grass field, Moore Haven, FL</td>
</tr>
</tbody>
</table>

<sup>1</sup>All septa contained 300 µg Z9-14:OAc, which was set to 100%.

<sup>2</sup>All septa contained 300 µg (100%) Z9-14:OAc and 6 µg (2%) Z7-12:OAc

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

**Wind Tunnel Experiments**

To assess strain-specific attraction of *S. frugiperda* males in the wind tunnel, experiments were performed in November 2009 in the laboratory of Prof. Manfred Ayasse at the Institute of Experimental Ecology, University of Ulm, Germany. Strain-specific attraction of *S. frugiperda* males was tested in a wind tunnel (200×75×75 cm) at 23°C, 30 cm/s airflow, and 23% RH. To adapt males to the low humidity, we placed all males, which were located in round plastic tubes covered...
with gauze, for about 1 h in the wind tunnel before the experiments started. Attraction of males was tested with choice experiments because in nature both kinds of strains can occur within one habitat and, thus, females might be located close to each other during calling. Choice experiments were conducted with the laboratory corn-strain (JS3C, 38th generation) and rice-strain (OnaR, 49th generation). Single 2-5-d-old, virgin males and females were placed in round plastic tubes (9.5 cm, 3.5 cm diam) that were closed with gauze at both ends. One plastic tube containing a male was mounted on a stand 30 cm high, and placed downwind in the middle of the wind tunnel. After the gauze was removed, each male was able to fly upwind, and given a choice between corn-strain and rice-strain females; three females of each strain were housed separately in round plastic tubes (9.5 cm, 3.5 cm diam) on stands above each other at 30 cm, 45 cm, and 60 cm height. We used 3 females to increase the chance that at least 1 of the females would call. The stands holding females of each strain were positioned upwind 26 cm apart.

We examined the response of males on 5 consecutive nights; 18 males per night (9 corn-strain males and 9 rice-strain males) were tested repeatedly, so that every male was tested up to five times per night. After testing males one night, they were excluded from the experiment, and another subset of 18 males was tested in the following night. All males that were completely inactive for 4 min were excluded from a trial. Each active male was allowed 5 min to start upwind flight before exclusion from a trial. Active males that started upwind flight were observed until they contacted the source (i.e., one of the female tubes), and displayed courtship behavior. Various male behaviors (e.g., activity status, presence/absence zigzag flight, source contact, courtship) were recorded for 2–9 h within scotophase.
Field Experiments

To assess strain-specific attraction of *S. frugiperda* males in the field, four different male trapping experiments were performed using: (A) strain-specific blends, (B) a range of Z7-12:OAc dosages, (C) a range of Z11-16:OAc dosages, and (D) a range of Z9-12:OAc dosages (Table 1). Plastic green-yellow-white Unitraps (Pherobank, Wageningen) were baited with synthetic pheromone lures, and attached to a bamboo stick 1–2 m above the ground; traps were at least 15 m apart, as well as from field borders. All traps contained a Vaportape II insecticide strip (Hercon Environmental, Emigsville, PA, USA) to kill males after they were trapped. These males were stored at -20°C for strain-identification at a later stage (see below). All experiments were conducted using a complete randomized block design with 3 biological replicates per field. Traps were rotated and emptied every 2–3 d. Field experiments were conducted at the following sites in Florida: 1) a corn field at the Everglades Research and Education Center in Belle Glade (+26°40′7.20″, −80°37′57.63″) (experiments A and B); 2) a grass field at the Graham Farm in Moore Haven (+26°53′3.04″, −81°7′21.17″) (experiments A, B and C); 3) a corn field in Hague (+29°47′7.40″, −82°25′3.66″) (experiments C and D); and 4) in a peanut/pasture field in Williston (+29°20′ 28.72″, −82°34′18.88″) (experiment D).

DNA Extractions

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

**Strain Identification**

To determine the strain-identity of each individual, strain-specific polymorphisms at the mitochondrial cytochrome oxidase I (COI) gene, as described by Nagoshi et al. (2006), were used. PCR amplifications were conducted using 1 μl DNA, 11.92 μl dH2O, 2 μl 10x Taq buffer, 3 μl 10 mM primer mix, 2 μl 2 mM dNTPs, and 0.08 μl Taq polymerase (Metabion, Martinsried, Germany). CO1-58 (5’-GGAATTTGAGCAGGAATAG-TAGG-3’) was used as forward primer, and JM77 (5’-ATCACCTCCWCCTGCAGGATC-3’) as reverse primer (Nagoshi et al., 2006). The thermo cycler was programmed for 2 min incubation time at 94°C, followed by 35 cycles of 45 sec at 94°C, 45 sec at 56°C, 60 sec at 72°C, and a final elongation at 72°C for 10 min. The generated amplification products were further digested for 2 h at 37°C with MspI and SacI (New England Biolabs, Ipswich, MA, USA). For this digestion, 4 μl PCR product were mixed with 0.6 μl NEB buffer 4, 1 μl H2O and 0.4 μl MspI (MspI digest) as well as 0.6 μl NEB buffer 1, 0.06 μl 100x BSA, 0.94 μl H2O, and 0.4 μl SacI (SacI digest). Each digest was mixed with 3 μl loading dye, and 4.5 μl of this mix were loaded on a 1% agarose gel, and run at 110 V for 45 min. MspI digestion proved corn-strain identity, whereas SacI digestion detected rice-strain individuals.
Statistical and Graphical Analysis

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

Results

Strain-Specific Variation in the Pheromone Blend

In this study, we compared the pheromone composition of a field population with previous data from our laboratory population (Groot et al., 2008). Corn- and rice-strain females of the laboratory and field populations from Florida showed consistent strain-specific differences in their amount of Z7-12:OAc and Z9-12:OAc (Fig. 1). Rice-strain females of both the laboratory, and field populations produced significantly higher relative amounts of Z7-12:OAc and Z9-12:OAc compared to corn-strain females of both populations (Fig. 1). As for Z11-16:OAc, laboratory corn-strain females exhibited significantly higher relative amounts of Z11-16:OAc compared to laboratory rice-strain females (Fig. 1). Such a difference was not found in the field populations (Fig. 1). The relative amount of the major sex pheromone component Z9-14:OAc was not significantly different between corn- and rice-strain females in either population (P=0.918, Fig. 1).
Pheromonal divergence between two strains of *S. frugiperda*

In addition to strain-specific pheromone differences, we also found differences between laboratory and field corn-strain females, as well as between laboratory and field rice-strain females for all four pheromone components (P<0.001 for Z9-14:OAc and Z7-12:OAc, P=0.009 for Z11-16:OAc, P=0.043 for Z9-12:OAc, Fig. 1). Corn-strain females of the field population produced lower relative amounts of Z7-12:OAc than corn-strain females of the laboratory population (Fig. 1).

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

*Population-specific Variation in the Pheromone Blend*

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

Male Attraction to Females in the Wind Tunnel
Corn- as well as rice-strain males showed no strain-specific attraction in wind tunnel choice assays (Chi-square test: P=0.421, Fig. 2a). No significant strain-, choice- or strain x choice-effect was observed (Fig. 2a). Although males were flown multiple times per night, we could not detect any effect of flight experience on the male choice. Of all tested corn- and rice-strain males, 29–44% were inactive and did not respond to any of the six presented females upwind (Fig. 2a). Most of the inactive males showed no response during the whole night (i.e., in all trials of one experiment), and generally males did not become inactive when tested multiple times per experiment. All active males that reached a source did fly within the odor plume, and did not reach a female just by chance. None of the tested males showed only zigzag flight behavior without afterwards contacting a tube containing females. Active males of both strains were attracted to strain-specific females (18–22%), to females of the other strain (16–27%), as well as females of both strains (22%, Fig. 2a). We observed that most males responded to every female that was currently calling, irrespective of the female strain.
Figure 2. Attraction of *Spodoptera frugiperda* corn-strain and rice-strain males to calling females in a wind tunnel. a Data represent the sum of all males that showed source contact, and male calling within five experiments performed on five consecutive nights. n.s. not significant. b Data represent the sum of all active males that showed source contact and male calling within five experiments in the early scotophase (2–5 h), and four experiments at the end of the night (6–8 h). Different letters above the bars indicate significant differences.
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Around 80% of the active males of both strains were attracted to calling corn-strain females at the beginning of the scotophase, and to calling rice-strain females at the end of the scotophase (Fig. 2b).

**Male Attraction to Synthetic Lures in the Field**

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

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

The male response towards different doses of Z11-16:OAc (experiment C) was equal between both strains and both corn and grass habitats. Corn-strain males were similarly attracted to binary blends (100% Z9-14:OAc and 2% Z7-12:OAc; \(n_{\text{corn field}}=153, n_{\text{grass field}}=28\)) as to three-component blends containing 8% Z11-16:OAc (\(n_{\text{corn field}}=220, n_{\text{grass field}}=51\)), 13% Z11-16:OAc (\(n_{\text{corn field}}=188, n_{\text{grass field}}=40\)), or 18% Z11-16:OAc (\(n_{\text{corn field}}=162, n_{\text{grass field}}=30\)).
Pheromonal divergence between two strains of *S. frugiperda*

**Figure 3.** Attraction of *Spodoptera frugiperda* corn-strain and rice-strain males to strain-specific synthetic pheromone lures in a corn field (a) and in a grass field (b) in Florida. Different letters above the bars indicate significant differences. n = sample size
Figure 4. Attraction of *Spodoptera frugiperda* corn-strain and rice-strain males towards different doses (0%, 2%, 4%, 10%) of Z7-12:OAc added to 100% Z9-14:OAc in a corn field (a), and in a grass field (b), in Florida. Different letters above the bars indicate significant differences. n=sample size
Although not statistically significant, corn-strain males seemed to be more attracted to the three-component blend containing 8% Z11-16:OAc in both fields. Similar to the response of corn-strain males, rice-strain males did not differentiate between binary blends ($n_{\text{corn field}}=202$, $n_{\text{grass field}}=276$) and three-component blends containing different doses of Z11-16:OAc (8%: $n_{\text{corn field}}=194$, $n_{\text{grass field}}=331$; 13%: $n_{\text{corn field}}=198$, $n_{\text{grass field}}=360$; or 18%: $n_{\text{corn field}}=161$, $n_{\text{grass field}}=252$).

In both corn and grass habitats, the addition of 1, 2, or 4% of Z9-12:OAc to the binary blend (experiment D) did not significantly increase trap catches compared to the binary blend. Corn-strain males responded similarly to binary blends ($n_{\text{corn field}}=96$, $n_{\text{grass field}}=54$) as to three-component blends containing either 1% Z9-12:OAc ($n_{\text{corn field}}=139$, $n_{\text{grass field}}=60$), 2% Z9-12:OAc ($n_{\text{corn field}}=111$, $n_{\text{grass field}}=59$) or 4% Z9-12:OAc ($n_{\text{corn field}}=111$, $n_{\text{grass field}}=70$). Like the corn-strain, rice-strain males were equally attracted to binary blends ($n_{\text{corn field}}=84$, $n_{\text{grass field}}=207$) as to three-component blends containing different doses of Z9-12:OAc (1%: $n_{\text{corn field}}=116$, $n_{\text{grass field}}=196$; 2%: $n_{\text{corn field}}=73$, $n_{\text{grass field}}=187$; or 4%: $n_{\text{corn field}}=72$, $n_{\text{grass field}}=243$). Within the corn field, males of both strains showed a slight, but not significant, increase in attraction when 1% Z9-12:OAc was added to the binary blend.

**Discussion**

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

*a) Consistent Pheromone Variation between Strains*

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

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

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

**b) Pheromone Variation within Strains**

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

c) No Differential Attraction of Males in Wind Tunnel Experiments

Wind tunnel experiments showed that males of both strains were attracted mainly to corn-strain females at the beginning of the night and to rice-strain females at the end of the night, which corresponds to the strain-specific female calling times of *S. frugiperda* (Pashley et al., 1992; Schöfl et al., 2009). Although we found no strain-specific male attraction to females of their own strain in wind tunnel choice assays, Lima and McNeil (2009) reported an experiment that showed that *S. frugiperda* males of both strains exhibited “different responses to an array of concentrations and blends in the wind tunnel” (McNeil et al. unpublished data). These data imply
that *S. frugiperda* males are able to show differential responses to pheromone blends in the wind tunnel and, thus, other factors might have influenced the male response in our experiments. It is known that lepidopteran males show differential attraction behavior in the wind tunnel depending on host plant volatiles (Landolt et al., 1994; Deng et al., 2004; Yang et al., 2004), their age (Rojas, 1999), the pheromone dosage of the source and the ambient temperature (Charlton et al., 1993), chemical noise and wind turbulences (Liu and Haynes, 1993), as well as the wind speed, flight altitude, and ground pattern (Foster and Howard, 1999). We observed that *S. frugiperda* males were highly sensitive to slight changes in the wind tunnel parameters (e.g., temperature, wind speed), and stopped their response to any stimulus when environmental conditions were inadequate. Due to the construction of the wind tunnel, it was not possible to obtain higher percentages than 23% relative humidity in the wind tunnel, which might explain why more than one third of all males were inactive and did not respond to any of the presented females. Most likely, males may have shown differential attraction in the wind tunnel if we had optimal environmental conditions and shifted the calling times of the females, so that corn- and rice-strain females would have called simultaneously.

*d) Some Differential Attraction of Males in the Field*

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

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

The fact that we found no strain-specific attraction to the four-component blends suggests either that males of both strains have a similar response range and are not differentiated in this respect, or that the blends that we tested were not strain-specific enough. Even though we and Lima and McNeil (2009) found that Z7-12:OAc is present in significantly lower amounts in corn-strain females than in rice-strain females, variation in the other two compounds Z9-12:OAc and Z11-16:OAc is not consistent between the strains and is variable within the strains. This may have confounded a possible strain-specificity of our so-called corn-strain and rice-strain pheromone blend. That such a confounding factor may have occurred
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seems to be confirmed by our dose-response experiment, varying the dose of the critical pheromone component Z7-12:OAc.

Corn-strain males were significantly more attracted when 2% of Z7-12:OAc was added to the major component compared to 4% and 10%, while rice-strain males showed a much wider response range, from 2% to 10% of Z7-12:OAc. These differences in response are in accordance with the strain-specific female pheromone production, as corn-strain females produce smaller relative amounts of Z7-12:OAc than rice-strain females. These results suggest that *S. frugiperda* males from Florida are adapted to the strain-specific Z7-12:OAc differences in the females. The fact that corn-strain males differentiated between 2% and 4% Z7-12:OAc in our dose–response experiments shows that males were able to detect minor differences of 2% between the tested synthetic lures. This in turn suggests that males similarly detected the differences between our strain-specific corn- and rice-strain blends that also differed in their amount of Z7-12:OAc by 2%. Furthermore, we found that no males of either strain were attracted when Z7-12:OAc was absent (0%), which confirmed previous findings of Tumlinson et al. (1986) that this secondary component is necessary for male attraction to the major pheromone component, Z9-14:OAc.

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

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

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

Acknowledgments

This research was funded by the Deutsche Forschungsgemeinschaft (P.S.GR 362721) and the Max Planck Gesellschaft. We thank Prof. Manfred Ayasse from the Institute of Experimental Ecology at the University of Ulm in Germany for his help with the wind tunnel experiments. Special thanks to Gregg Nuessly from the Everglades Research and Education Center in Belle Glade and to Tommy Toms, Manager of the Graham Farms in Moore Haven, which supported the male trapping experiments in Florida. We thank Antje Schmaltz and Katja Müller
(MPICE, Jena, GER) for their assistance in strain-typing trapped males, and Amy Rowley (USDA, Gainesville, FL) for assistance in the field experiments.

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Feromonal divergence between two strains of *S. frugiperda*


LANDOLT, P. J., HEATH, R. R., MILLAR, J. G., DAVIDSHERNANDEZ, K. M.,


Pheromonal divergence between two strains of *S. frugiperda*


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

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

Key Words. Sexual communication, Fall armyworm, Lepidoptera, Noctuidae, Synthetic pheromone lures, Field experiments, Dose-response experiments.
Chapter 3

Introduction

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

Geographic variation in sexual communication systems has been reported in several lepidopteran species (Tòth et al., 1992; McElfresh and Millar, 1999; Wu et al., 1999; Gemeno et al., 2000; Kawazu et al., 2005; Groot et al., 2009) and is of interest because changes in the sex pheromone signal and/or response to sex pheromones could result in reproductive isolation and subsequently may lead to speciation (Roelofs and Cardé, 1974; Phelan, 1992; Baker, 2002; Smadja and Butlin, 2009). Understanding the level and extent of intra-specific geographic variation in pheromone-mediated mating will help to identify the factors responsible for the geographic differences and provide insight in the evolution of reproductive isolation between populations.
The fall armyworm, *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae), is an excellent model system to study the evolution of reproductive isolation, as there are two genetically and behaviorally distinct strains, the corn- and rice-strain, occurring sympatrically throughout North- and South America (Pashley, 1986). Both strains appear to be undergoing ecological speciation in sympatry and reveal several possible prezygotic isolation barriers (Groot et al., 2010b). These include differential host plant choice (Pashley, 1986; Pashley, 1989; Lu and Adang, 1996; Levy et al., 2002; Nagoshi et al., 2006; Machado et al., 2008), strain-specific mating times in the scotophase (Pashley et al., 1992; Schöfl et al., 2009), as well as differences in the female sex pheromone composition (Groot et al., 2008; Lima and McNeil, 2009; Unbehend et al., 2013). The sex pheromone of *S. frugiperda* was identified by Tumlinson et al. (1986) to consists of \((Z)-9\)-tetradecenyl acetate (Z9-14:OAc) as the major sex pheromone component, and \((Z)-7\)-dodecenyl acetate (Z7-12:OAc) as critical secondary sex pheromone component. A number of other minor compounds like Z11-16:OAc and Z9-12:OAc have also been identified from the gland (Tumlinson et al., 1986; Groot et al., 2008; Lima and McNeil, 2009), but with unclear behavioral function so far (Unbehend et al., 2013). Analysis of sex pheromone gland extracts from females collected in Florida showed that corn-strain females contained significantly lower relative amounts of Z7-12:OAc and \((Z)-9\)-dodecenyl acetate (Z9-12:OAc) than rice-strain females (Groot et al., 2008; Unbehend et al., 2013). However, different male trapping experiments conducted in Louisiana and Florida showed no consistent attraction of males to females of their own strain (Pashley et al., 1992; Meagher and Nagoshi, 2013; Unbehend et al., 2013), which suggests that differences in the female pheromone are not sufficient to cause assortative mating in the field.

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

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

Male Trapping Experiments

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

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

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

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

<table>
<thead>
<tr>
<th>COUNTRY</th>
<th>LOCATION &amp; COORDINATES</th>
<th>FIELD</th>
<th>EXPERIMENT</th>
<th>EXPERIMENTER</th>
<th>DATE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canada</td>
<td>Ontario +43° 4’ 26.08&quot;, -81° 20’ 21.81&quot;</td>
<td>Corn</td>
<td>A</td>
<td>JMN</td>
<td>Sep. 2011</td>
</tr>
<tr>
<td>North Carolina</td>
<td>Plymouth +35°50’ 46.19&quot;, -76° 39’ 46.24&quot;</td>
<td>Soybean</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cotton; Grass</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Soybean; Corn</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Florida²</td>
<td>Belle Glade +26° 40’ 7.20&quot;, -80° 37’ 57.63&quot;</td>
<td>Corn A</td>
<td>A, B</td>
<td>MU, SH</td>
<td>April-May 2010</td>
</tr>
<tr>
<td>Puerto Rico</td>
<td>Santa Isabel +17° 59’ 0.93&quot;, -66° 23’ 29.88&quot;</td>
<td>Corn A</td>
<td>A</td>
<td>MU, SH, ATG,DAJ</td>
<td>April 2010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Corn B</td>
<td>A, B</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹ Experiments: A) Test of strain-specific blends, B) Z7-12:OAc dose-response, C) Z11-16:OAc dose-response, D) Importance of E7-12:OAc
² Data adapted from Unbehend et al. 2013.
To test the importance of the isomers Z7-12:OAc and E7-12:OAc in North and South America (experiment D), we added different doses of E7-12:OAc and Z7-12:OAc (0%, 1%, 2%) to 100% Z9-14:OAc (Table 2). The minimal blend (2% Z7-12:OAc + 100% Z9-14:OAc) was used as control, and as equivalent we prepared an E-blend with 2% E7-12:OAc and 100% Z9-14:OAc. To investigate a possible interaction effect of both isomers together, 1% as well as 2% of E- and Z7-12:OAc were added to 100% Z9-14:OAc. The fourth experiment (Exp. D) was carried out in North Carolina and Peru.

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

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

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

<table>
<thead>
<tr>
<th>Experiment and Lures</th>
<th>Z9-14:OAc</th>
<th>Z11-16:OAc</th>
<th>Z7-12:OAc</th>
<th>E7-12:OAc</th>
<th>Z9-12:OAc</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Blend 1</td>
<td>100%</td>
<td>13%</td>
<td>2%</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Blend 2</td>
<td>8%</td>
<td>4%</td>
<td>-</td>
<td>2%</td>
</tr>
<tr>
<td></td>
<td>Blank (Hexane)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>Z7-12:OAc</td>
<td>100%</td>
<td>-</td>
<td>2%</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C</td>
<td>Z11-16:OAc</td>
<td>100%</td>
<td>8%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13%</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18%</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D</td>
<td>Z7-12:OAc</td>
<td>100%</td>
<td>-</td>
<td>2%</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>E7-12:OAc</td>
<td>-</td>
<td>-</td>
<td>2%</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Z/E7-12:OAc</td>
<td>-</td>
<td>1%</td>
<td>1%</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Z/E7-12:OAc</td>
<td>-</td>
<td>2%</td>
<td>2%</td>
<td>-</td>
</tr>
</tbody>
</table>

1Compound concentrations were as follows: 100% = 300 µg, 18% = 54 µg, 13% = 39 µg, 10% = 30 µg, 8% = 24 µg, 4% = 12 µg, 2% = 6 µg, 1% = 3 µg.
Chemical Analysis

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

Strain Identification

The strain identity of all trapped males was determined via two strain-specific markers, i.e. MSPI- and SACI-digest of the mitochondrial COI gene, which are known to be diagnostic for strain-identification of the two fall armyworm strains in North and South-America (Meagher and Gallo-Meagher, 2003; Nagoshi et al., 2006; Nagoshi et al., 2007). DNA of all males captured was extracted as described by Unbehend et al. (2013) using CTAB (Cetyltrimethyl-ammonium bromide) and isopropanol for DNA precipitation. The extracted DNA was tested at MPICE for strain-specific polymorphisms at the mitochondrial COI gene by amplification and strain-specific digestion (Nagoshi et al., 2006; Unbehend et al., 2013). The amplified part of the COI gene was digested with MSPI as well as SACI and analyzed electrophoretically on a 1% agarose gel (Unbehend et al., 2013). MSPI digestion detected corn-strain individuals, whereas SACI digestion proved rice-strain identity (Nagoshi et al., 2006).
Chapter 3

Statistical Analysis

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

Results

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

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

The Z7-12:OAc dose-response experiments, where 0%, 2%, 4% or 10% of Z7-12:OAc was added to 100% Z9-14:OAc, showed a significant effect of geographic region and strain, as well as an interaction effect between geographic region x strain, and field crop x strain (*MANOVA*: *P* < 0.001; Fig. 2). Interestingly, corn-strain males exhibited a greater difference in their response to Z7-12:OAc than rice-strain males (Fig. 2). The highest number of corn-strain males was captured with lures containing 2% Z7-12:OAc and was significantly different from all other ratios at three field sites in Florida and Puerto Rico, while in Peru lures with 2% and 4% Z7-12:OAc were equally attractive. Furthermore, lures containing no Z7-12:OAc, considered an essential pheromone component, attracted 37 corn-strain males in Puerto Rico (Fig. 2A).
Figure 1. Attraction of *S. frugiperda* corn- and rice-strain males to two 4-component blends (Blend 1 and 2 in Table 2) in North America, the Caribbean and
Geographic variation in sexual attraction of *S. frugiperda* males

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

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

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

Testing different doses (0%, 1%, 2%) of E7-12:OAc and Z7-12:OAc, added to 100% Z9-14:OAc, showed that *S. frugiperda* males from Peru were not attracted to traps baited only with 2% E7-12:OAc added to Z9-14:OAc, but were equally attracted to all other blends tested (Fig. 3).
Figure 2. Attraction of *S. frugiperda* corn-strain (A) and rice-strain (B) males to different doses of Z7-12:OAc added to 100% Z9-14:OAc in different fields. Different letters above the bars indicate significant differences. Error bars show the variation between biological replicates (n=3). Numbers in brackets represent the total number of males caught. Data from Florida are adapted from Unbehend et al. (2013)
In North Carolina, *S. frugiperda* males were equally attracted to synthetic blends to which E7-12:OAc, Z7-12:OAc or E- and Z-7-12:OAc was added (Fig. 3). Unfortunately, we were not able to identify the strain-type of any of the trapped males because of DNA degradation of the samples.

**Discussion**

We investigated the variation in attraction of *S. frugiperda* corn- and rice-strain males in North America, the Caribbean and South America, and found a) some geographic variation in corn-strain male attraction to synthetic 4-component blends and to different doses of Z7-12:OAc, b) almost no geographic variation in rice-
strain male attraction to different synthetic blends, c) no variation in male attraction to the minor compound Z11-16:OAc, and d) some evidence of geographic variation in response to E7-12:OAc. Taken together, our results indicate that corn-strain males are more specific and restricted in their response to pheromone blends than rice-strain males and this specificity shows some geographic variation.

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

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

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

Previously, it was shown that corn-strain males varied in their attraction to sex pheromone blends in different fields with different host plants in north and south
Geographic variation in sexual attraction of *S. frugiperda* males

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

The differential attraction of corn-strain males to pheromone blends in different regions could also be explained by genetic differences between *S. frugiperda* populations from North America, the Caribbean and South America. Population genetic analyses of *S. frugiperda* samples collected throughout the Western Hemisphere reported mainly the absence of isolation by distance between populations from different regions (Clark et al., 2007; Martinelli et al., 2007; Belay et al., 2012), which indicates no geographically restricted gene flow probably due to the high migratory ability of *S. frugiperda* (Luginbill, 1928; Sparks, 1979). However, these analyses did not take into account strain-specific differences and in several cases the strain-type of captured individuals was unknown (Clark et al., 2007; Belay et al., 2012). Genetic studies on populations from Arkansas and Florida showed significant genetic variation among populations, both within and between the two strains (Lewter et al., 2006). Furthermore, corn-strain individuals exhibit different mitochondrial haplotype profiles between populations from a)
Florida, Puerto Rico, Georgia and b) Texas, Brazil, Mississippi, Alabama, Louisiana (Nagoshi et al., 2007; Nagoshi et al., 2008; Nagoshi and Meagher, 2008; Nagoshi et al., 2010). Thus, genetic differences could play a role in differential attraction of corn-strain males to synthetic blends in different regions.

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

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

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

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

In general, rice-strain males were equally attracted to Blend 1 and Blend 2 in different fields in North America, the Caribbean and South America (Fig. 1). In addition, rice-strain males from Florida and Puerto Rico were similarly attracted to 2% and 4% Z7-12:OAc and were also found in traps baited with 10% of this component (Fig. 2). Thus, rice-strain males showed a broader response spectrum to different sex pheromone blends than corn-strain males. If the pheromone response spectrum of males is determined by environmental factors like temperature and humidity, and/or by intra-specific competition, it could be possible that rice-strain males are more resistant to climate changes and/or competition, which would explain why we observed almost no geographic differences in the response of rice-strain males.
However, we did find some variation in the response of rice-strain males, and another study showed that the attraction of rice-strain males to corn- and rice-strain females varied depending on field location and season in Florida (Meagher and Nagoshi, 2013). If geographic variation in the female pheromone composition exists, the composition of our synthetic pheromone blends might not be typical for all regions we tested. Rice-strain males may have shown differential attraction in the field if we would have used region-specific female pheromone blends as baits.

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

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

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

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

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

**Conclusions**

We found some geographic variation in attraction of *S. frugiperda* corn-strain males to two synthetic 4-component blends and different doses of Z7-12:OAc in North America, the Caribbean and South America. Rice-strain males showed almost no geographic variation in their attraction to different synthetic pheromone blends. One aspect that merits further attention is the possibility that habitat-specific volatiles influence the male response to pheromone blends in different
fields. Males were equally attracted to different doses of Z11-16:OAc, but appeared to exhibit region-specific differences in their attraction to E7-12:OAc. Overall, the data show geographic variation in the response of *S. frugiperda* males to pheromone blends. If this variation coincides with geographic variation in female pheromone composition, then geographic differentiation between populations could occur.

**Acknowledgements**

This research was partly funded by the German Science Foundation (grant number GR3627/2-1), the National Science Foundation (award IOS-1052238 and NSF-DEB-1025217), and the Max Planck Gesellschaft. We thank Wilson Rivera González from Monsanto and Jose Santiago from 3rd Millenium Genetics for their help with the male trapping experiments conducted in Santa Isabel in Puerto Rico, and Sofía Torres, Carmen Urueña, and Alejandro Vera at EEAOC for their help in the trapping experiment in Tucumán, Argentina. We thank Raul Blas, Jorge Tobaru, German Arellano, Antonio Oblitas, Daniela Gálvez, and Jose Meléndez from Universidad Nacional Agraria La Molina (UNALM), and José Galarza from Servicio Nacional de Sanidad Agraria (SENASA), for their assistance with male trapping experiments and DNA extractions conducted on UNALM campus in Peru. Special thanks to Antje Schmaltz, Susanne Neumann, Katja Müller, Sarah Behr, Simone Sölltl, Maximilian Hemmerling, and Susanne Donnerhacke for their assistance in strain-typing trapped males at the MPICE.

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Chapter 3


Chapter 4

Genetic basis of strain-specific female sex pheromone differences in *Spodoptera frugiperda* (Lepidoptera: Noctuidae)

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Abstract. The fall armyworm, *Spodoptera frugiperda*, consists of two distinct strains, the corn- and the rice-strain, which exhibit different prezygotic isolation mechanisms and seem to be in the process of ecological speciation in sympatry. Recent studies found that females of both strains showed significant differences in their sex pheromone composition and strain-specific sexual communication is considered as possible prezygotic isolation barrier between both strains. In this study, we investigated the inheritance of strain-specific sex pheromone blends by comparing the sex pheromone of pure strain, hybrid and backcross females. Furthermore, we conducted a QTL analysis and mapped different candidate genes to QTLs involved in the strain-specific production of (Z)-9-tetradecenyl acetate (Z9-14:OAc), (Z)-11-hexadecenyl acetate (Z11-16:OAc), (Z)-7-dodecenyl acetate (Z7-12:OAc), and (Z)-9-dodecenyl acetate (Z9-12:OAc). We found that all four pheromone compounds were differentially inherited and multiple factors influenced their inheritance. The QTL analysis showed that multiple genomic regions on 9 different chromosomes determined the strain-specific female sex pheromone. For three pheromone compounds, i.e. Z9-14:OAc, Z7-12:OAc and Z9-12:OAc, we found the involvement of one minor QTL each, whereas a total of seven different QTLs were significantly correlated with differential relative amounts of Z11-16:OAc. A delta-11-desaturase (*Sf*LPAQ) mapped to one chromosome that explained a significant proportion of the variance in Z9-14:OAc and Z11-16:OAc, and showed the opposite-to-expected phenotypic pattern for both components. Interestingly, the circadian clock gene *vrille* (*Sf*VRI), which appears to be responsible for strain-specific differences in the onset time of mating in the night, mapped to another chromosome that was involved in the production of the critical secondary sex pheromone component Z7-12:OAc. Our results suggest that two different prezygotic mating barriers in *S. frugiperda*, i.e. sexual communication and allochronic separation, may be genetically linked. If there is
genetic coupling of differential reproductive traits, evolution of prezygotic isolation in *S. frugiperda* could be facilitated.

**Key Words.** Fall armyworm, Corn- and rice-strain, QTL analysis, Pheromone extractions, Delta-11-desaturase, Delta-9-desaturase, *Vrille*, Prezygotic isolation, Timing of reproduction.

**Introduction**

Sex pheromones are commonly used amongst animals as premating signal to attract conspecific individuals for mating (Cardé and Minks, 1997; Rasmussen et al., 1997; Achiraman and Archunan, 2002, 2005; Rajanarayanan and Archunan, 2011; Buda et al., 2012). Within the order Lepidoptera, female moths usually produce species-specific sex pheromones in a pheromone gland to attract males over long distances (Cardé and Baker, 1984; Tamaki, 1985; Cardé and Minks, 1997; Cardé and Haynes, 2004). Because most adult moths are short-lived, a reliable sexual communication system between females and males is essential for the mating success and fitness of a species (Löfstedt, 1993; Cardé and Haynes, 2004). When the female pheromone signal changes due to mutations, the response of males needs to adapt to those changes or the mating partners will not find each other and reproduce. Due to this dependence of sender (female) and receiver (male), pheromone premating signals are expected to be under stabilizing selection (Löfstedt, 1993; Linn and Roelofs, 1995; Phelan, 1997). However, evolution solely under stabilizing selection processes is not able to explain the great diversification of moth species (Mitchell et al., 2000; Kristensen et al., 2007) and sex pheromones (El-Sayed, 2012). Other evolutionary forces like sexual selection (Coltman et al., 2002; Wade and Shuster, 2004; Andersson and Simmons, 2006; Irestedt et al., 2009; Sullivan-Beckers and Cocroft, 2010), genetic drift (Masel, 2011; Mendez et
Genetic basis of strain-specific female sex pheromone differences

al., 2011; Keller et al., 2012; Velo-Anton et al., 2012), or selection due to specific environmental factors like predators (Stowe et al., 1987; Haynes et al., 2002; Anton et al., 2011) or host adaptation (Nosil, 2007; Smadja and Butlin, 2009; Felix et al., 2011) may be able to act against stabilizing selection and generate diversification of moth pheromones. To understand the variability and the evolution of sexual communication systems in moths, intra- and inter-specific behavioral and genetic studies of sexual communication systems (premating signals and responses) are required.

An ideal model organism to study the evolution of sexual communication is the fall armyworm *Spodoptera frugiperda* (Lepidoptera: Noctuidae), which consists of a so-called corn-strain and rice-strain (Pashley, 1986). Both strains occur in sympatry in North and South America and hybridization rate between strains in the field of up to 16% (Prowell et al., 2004). Corn- and rice-strain populations seem to be in the process of sympatric speciation and exhibit several genetic and behavioral differences (Groot et al., 2010). Besides differential host plant preferences (Pashley, 1986; Meagher and Gallo-Meagher, 2003; Nagoshi et al., 2006; Nagoshi et al., 2007; Machado et al., 2008), both strains differ in their timing of mating in the night (Pashley et al., 1992; Schöfl et al., 2009), as well as in their female sex pheromone composition (Groot et al., 2008; Lima and McNeil, 2009; Unbehend et al., 2013).

The multi-component sex pheromone blend of *S. frugiperda* was first described by Tumlinson et al. (1986), consisting of the major pheromone component (Z)-9-tetradecenyl acetate (Z9-14:OAc), the critical secondary pheromone component (Z)-7-dodecenyl acetate (Z7-12:OAc), as well as other minor compounds like (Z)-11-hexadecenyl acetate (Z11-16:OAc). More than two decades later, Groot et al. (2008) reported the presence of strain-specific pheromone differences between
corn- and rice-strain females of laboratory populations. Corn-strain females had significantly higher relative amounts of Z11-16:OAc than rice-strain females, while rice-strain females exhibited higher relative amounts of Z7-12:OAc and (Z)-9-dodecenyl acetate (Z9-12:OAc) than corn-strain females (Groot et al., 2008). Similarly, pheromone extractions of *S. frugiperda* field populations confirmed the presence of corn- and rice-strain specific female pheromone blends (Unbehend et al., 2013). Based on a proposed pheromone biosynthesis pathway of *S. frugiperda* (Groot et al., 2008), different candidate genes like delta-9- or delta-11-desaturases could explain the pheromone differences between corn- and rice-strain females. Determining the genetic basis of strain-specific pheromone differences may help to understand how variability in a premating signal can arise.

A quantitative trait locus (QTL) analysis is a frequently used tool to determine the genetic basis of specific phenotypic traits in plants and animals (Gleason et al., 2005; Sheck et al., 2006; Groot et al., 2009; Manceau et al., 2011; Beecher et al., 2012; Merah et al., 2012). This genetic method has a wide area of applications ranging from the determination of genes responsible for favorable agricultural traits (Santos et al., 2012; Tsukazaki et al., 2012; Gao and Lin, 2013), up to the investigation of genes related to the evolution of species (Gleason et al., 2009; Groot et al., 2009; Gould et al., 2010; Lassance et al., 2010; Limousin et al., 2012). The advantage of working with a Lepidopteran model organism like *S. frugiperda* is the absence of crossing over in females (Heckel, 1993), which facilitates the genetic analysis of premating signals like sex pheromones because backcross females can be generated in which all markers on one chromosome co-segregate as one unit.

A recent QTL analysis of one prezygotic isolation barrier between both *S. frugiperda* strains, i.e. differential timing of reproduction in the night, showed that
the circadian clock protein *vrille* appears to be involved in the shifted onset time of mating between both strains in both sexes (Hänniger et al., 2013). Similar to the study conducted by Hänniger et al. (2013), the aim of this study was to investigate the genetic basis another potential prezygotic mating barrier, i.e. the strain-specific differences in pheromone composition of *S. frugiperda* corn- and rice-strain females. Therefore, we a) examined the inheritance of pheromone blends by comparing the pheromone phenotype of pure strain females (C, R), hybrid females (CR, RC) and backcross females (CR-R, CR-C, RC-R, RC-C); b) conducted a QTL analysis; and c) mapped different candidate genes to the QTLs involved in the differential production of four pheromone components (Z9-14:OAc, Z11-16:OAc, Z7-12:OAc, Z9-12:OAc).

**Methods and Materials**

*Spodoptera frugiperda populations*

To determine the genetic basis of strain-specific pheromone differences, we conducted experiments with a laboratory corn-strain and rice-strain population. Our laboratory corn-strain population originated from over 300 *S. frugiperda* larvae, which were collected in April 2010 from two corn fields in Santa Isabel in Puerto Rico (corn field 1: +17° 59' 0.93", -66° 23' 29.88"; corn field 2: +17°57' 30.65", -66° 23' 32.43"). Our laboratory rice-strain population descended from around 300 larval specimens collected in May 2010 from a grass field at the Graham Farm in Moore Haven, Glades County in Florida (+26° 53' 3.04", -81° 7' 21.17"). All larvae were shipped to the MPICE and reared until adulthood on artificial pinto bean diet. To establish strain-specific colonies, the adults were screened for strain-specific COI markers (Nagoshi et al., 2006). Both populations were reared in climate chambers with reversed light:dark (L:D) cycle and 14:10 L:D photoperiod at 26°C.
and 70% RH. Adults were fed with a 10% honey-water solution and random single-pair-matings were performed to maintain both populations.

**Generation of backcrosses**

We generated female-informative backcrosses to determine a) the pheromone composition of pure strain, hybrid and virgin females, as well as b) the genetic basis of strain-specific pheromone differences. Single pair matings between pure strain individuals were performed to generate F\textsubscript{1} hybrid females, which were then backcrossed to pure corn- and rice-strain males to produce different backcross families (Table 1). In the case of F\textsubscript{1} hybrid females, we crossed laboratory corn-strain females (6\textsuperscript{th} generation) with rice-strain males (5\textsuperscript{th} generation) to obtain CR hybrid females (first letter thus always referring to the female, second letter to the male), and rice-strain females (5\textsuperscript{th} generation) were mated with corn-strain males (6\textsuperscript{th} generation) to produce RC hybrid females (Table 1). One generation later, adult CR hybrid females were mated with either corn-strain males or rice-strain males to produce CR-C and CR-R backcross females (the first two letters of the backcross females refer to the mother, the last letter to the father of the female). Furthermore, RC hybrid females were backcrossed to corn-strain males and rice-strain males to generate RC-C and RC-R backcross females (Table 1). In total, we obtained five fertile CR-C backcross families, five CR-R families, one RC-C family and one RC-R family (Table 1). All individuals of two CR-C families (CR-C 2, CR-C 3), two CR-R families (CR-R 5, CR-R 19), the one RC-C family (RC-C 34) and the one RC-R family (RC-R 26) were reared until adulthood and used for pheromone extractions (see below). One CR-R backcross family (CR-R 19) was used for further genetic analysis.
Table 1 Generation of female informative *Spodoptera frugiperda* backcrosses

<table>
<thead>
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<th>Female strain</th>
<th>Male strain</th>
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</tr>
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<td>RC</td>
<td>16</td>
<td>5</td>
<td>31</td>
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<tr>
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<tr>
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<tr>
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<td>Rice</td>
<td>RC-R</td>
<td>34</td>
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</table>

Pheromone extractions

To determine the pheromone phenotype of intrastrain- and interstrain-specific crosses, pheromone extractions of virgin pure strain females (C and R), hybrid females (CR and RC) and backcross females (CR-R, CR-C, RC-C, RC-R) were performed. Pheromone glands of 3 days old corn-strain (6th generation) and rice-strain (5th generation) females were extracted during the scotophase according to strain-specific female calling times (corn-strain: 4 h, rice-strain: 6-7 h). CR hybrid females were extracted 3.5-5.5 h into scotophase at the age of 3 days and pheromone glands of 3 days old RC hybrid females were extracted 5.5 h into scotophase. Pheromone extractions of 2-3 days old backcross females were performed 4-7 hours into scotophase. Pheromone glands were excised from the female abdomen and singly placed into a glass vial containing 50 μl hexane and 125 ng pentadecane as internal standard. After an extraction time of 30 min, the gland was removed from the vial and the extract was stored at -20°C until gas chromatography analysis (see below).
Chapter 4

Chemical analysis
Gas chromatography (GC) analysis was performed according to methods used by Unbehend et al. (2013), using a HP7890 gas chromatograph with a polar capillary column (DB-WAXetr; 30 m × 0.25 mm × 0.5 μm) and a flame-ionization detector (FID). Female pheromone extracts were reduced from 50 µl to 2 µl under a gentle stream of nitrogen. Together with 2 µl octane, the reduced pheromone extracts were singly transferred into a glass vial and injected into the gas chromatograph. Female pheromone compounds were identified by comparing retention times with synthetic standards of Z9-14:OAc, Z11-16:OAc, Z7-12:OAc and Z9-12:OAc, which were bought from Pherobank (Wageningen, the Netherlands).

DNA extraction and AFLP marker analysis
Genetic analysis was performed with one backcross family (CR-R 19), which was most fertile amongst all backcross matings and produced 159 offspring females. Out of all CR-R 19 backcross females, we selected 88 females for further AFLP analysis based on the amount of Z7-12:OAc found in the female glands. We chose 36 females which exhibited low amounts of Z7-12:OAc (1-2%), 16 females with medium amounts of Z7-12:OAc (~2.5%), and 36 females with high amounts of Z7-12:OAc (>3.5%). We extracted DNA of the 88 selected CR-R females, their parents (CR hybrid female and rice-strain male) and their maternal grandparents (corn-strain female and rice-strain male). DNA extractions were performed as described by Unbehend et al. (2013), and 200 ng DNA of each sample was digested with EcoRI and MseI (New England Biolabs, Ipswich, MA, USA) at 37°C for 2 h, according to Wilding et al. (2001). After the restriction digest, EcoRI- and MseI-adapters were ligated to the EcoRI and MseI restriction sites and all DNA fragments that contained an adapter were preamplified (Wilding et al., 2001).
Table 2 Number of informative AFLP-makers scored per primer combination

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<th>MseI primer</th>
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<th>No. markers</th>
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<tr>
<td></td>
<td>ACG</td>
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</tr>
<tr>
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</tr>
<tr>
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<tr>
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<tr>
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<tr>
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<tr>
<td>CTG</td>
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</tr>
<tr>
<td><strong>Total markers</strong></td>
<td></td>
<td><strong>303</strong></td>
</tr>
</tbody>
</table>

The preamplified DNA was diluted with ddH$_2$O (1:50) and selectively amplified with 26 specific EcoRI- and MseI-primer combinations, consisting of a core sequence (EcoRI-primer: 5’-GACTGCGTACCAATTC; MseI- primer:5’-GATGAGTCCT-GAGTAA) plus three selective bases at the end of each primer (Table 2). The generated AFLP fragments were analyzed on a 6.5% polyacrylamide gel using a LI-COR 4300 DNA analyzer (LI-COR Biosciences,
Lincoln, NE, USA) according to methods by Groot et al. (2009). AFLP gels were scored with AFLP-Quantar Pro 1.0 (KeyGene, Wageningen, the Netherlands). To identify corn-strain specific markers, we searched for AFLP markers that were present in the grandmother (C female), the mother (CR hybrid female) and half of the offspring females (heterozygote CR-R females), but absent in the grandfather (R male), the father (R male) and the homozygote CR-R females. For identification of rice-strain specific markers, we scored markers present in the grandfather (R male), the mother (CR female) and half of the offspring females (homozygote CR-R females), but absent in the grandmother (C female) the father (R male) and the heterozygote CR-R females.

**Genetic map construction and QTL analysis**

We scored in total 303 AFLP-markers (Table 2), and constructed a linkage map with MapMaker 3.0 (http://www.broadinstitute.org/ftp/distribution/software/mapmaker3/). Using a LOD score of 10, 30 linkage groups were identified which refer to the 30 autosomes in the CR-R19 backcross family, because there is no crossing over in Lepidoptera females (Heckel, 1993). The chromosome names (chromosome 1 to 30) were chosen arbitrarily. To identify candidate QTL, we tested for each chromosome whether the homozygote CR-R backcross females had significantly different relative amounts of Z9-14:OAc, Z11-16:OAc, Z7-12:OAc and Z9-12:OAc than the heterozygote CR-R backcross females (see statistical analysis).

**Mapping of candidate genes**

To determine whether candidate genes that are likely involved in the pheromone biosynthetic pathway may be responsible for strain-specific pheromone differences, we mapped a delta-11-desaturase and a delta-9-desaturase to our
generated QTL map. Furthermore, we mapped the circadian clock protein \textit{vrille} to our map, which appears to be involved in the strain-specific differences of timing of reproduction in the night (Hänniger et al., 2013). Based on \textit{Spodoptera} sequences (ESTs) published at NCBI (http://www.ncbi.nlm.nih.gov/), we designed primers for a delta-11-desaturase (\textit{SfLPAQ}) and a delta-9-desaturase (\textit{SfKPSE}). Based on \textit{S. frugiperda} \textit{vrille} sequences obtained from SH (Hänniger et al., 2013), we designed primers for \textit{vrille} (\textit{SfVRI}) (Table 3).

To identify SNPs in the candidate genes that could be used to map them onto our genetic map, PCR amplification of the different genes were conducted with the grandparents (C female, R male), the parents (CR hybrid female, R male) and 12 to 24 backcross females of the backcross family CR-R 19. PCR amplifications were performed using 1 µl DNA, 11.92 µl dH₂O, 2 µl 10x Taq buffer, 2 µl 2 mM dNTPs, 3 µl 10 mM primer mix (Table 3) and 0.08 µl Taq polymerase (Metabion, Martinsried, Germany). The thermo cycler program started with 2 min incubation time at 94°C, followed by 35 cycles of 45 s at 94°C, 45 s at the primer-specific annealing temperature \(T_a\) (Table 3), 60 s at 72°C and a final elongation at 72°C for 10 min. The generated amplification products were mixed with 3 µl loading dye and ran on a 1.5% agarose gel at 120 V for 2 h.

The obtained products were cut out of the gel and extracted with a QIAGEN gel extraction kit (QIAGEN, Hilden, Germany). After gel extraction, all products were sequenced using Sanger-sequencing, according to methods described by Vogel et al. (2011), and analyzed with Sequencher 4.10.1 (Gene Codes Corporation, Ann Arbor, MI, USA).
Table 3 Primer combinations and annealing temperatures (Tₘ) of candidate genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Tₘ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delta-11-desaturase SfPAQ</td>
<td>Forward: 5’AACATTGGGGGAAAGGTTTCC Reverse: 5’CAAATGCAACATTATAAAACTTCA</td>
<td>53°C</td>
</tr>
<tr>
<td>Delta-9-desaturase SfKPSE</td>
<td>Forward: 5’TCATTATGCCCCAAGGTGATT Reverse: 5’ATGACAGTGAAAGGAAGACAT</td>
<td>53°C</td>
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<tr>
<td>Vrille SfVRI</td>
<td>Forward: 5’GAGGCCTGCTTTATGCTGAA Reverse: 5’GGCTCCTGCTTTATGCTGAA</td>
<td>60°C</td>
</tr>
</tbody>
</table>

Statistical analysis

Statistical analysis was performed with R 2.5.0 (R-Development-Core-Team, 2007). Data of the female pheromone extractions were log transformed to stabilize the variance and analyzed using a generalized linear model (GLM). To identify candidate QTL, we conducted a 2-sided t-test, and we used a GLM to assess how much of the variance can be explained by the different QTL (R² value).

Results

Strain-specific pheromone differences

Corn- and rice-strain females exhibited significant differences in the relative amount of Z9-14:OAc, Z11-16:OAc, Z7-12:OAc and Z9-12:OAc (Fig. 1). While corn-strain females produced significantly higher relative amounts of Z11-16:OAc than rice-strain females, rice-strain females had larger relative amounts of Z9-14:OAc, Z7-12:OAc and Z9-12:OAc than corn-strain females (Fig. 1).

Inheritance of pheromone compounds

We found differential modes of inheritance for all four pheromone compounds of *S. frugiperda*, i.e. Z9-14:OAc, Z11-16:OAc, Z7-12:OAc and Z9-12:OAc (Fig. 1).
Figure 1. Relative percentage of A) Z9-14:OAc, B) Z11-16:OAc, C) Z7-12:OAc, and D) Z9-12:OAc in *Spodoptera frugiperda* pure strain (C/R), hybrid (CR/RC)
and backcross (CR-C/CR-R/RC-C/RC-R) females. Each box plot shows the median (black line), the smallest/largest observations (dashed lines) as well as outliers (circles). Different letters above the box plots indicate significant differences. N= sample size. Information about the crossing schemes used to generate the different females can be found in Table 1.

The major sex pheromone component Z9-14:OAc was present in similar relative amounts in rice-strain and hybrid CR (C♀ x R♂) females, suggesting a paternal inheritance (Fig. 1A). Hybrid RC (R♀ x C♂) females and hybrids that were backcrossed to corn-strain males, i.e. CR-C and RC-C backcross females, produced significantly higher relative amounts of Z9-14:OAc than all other female crosses, while corn-strain females produced overall the lowest amount of Z9-14:OAc (Fig. 1A). In contrast, CR-R and RC-R females, i.e. backcrosses of hybrid females with rice-strain males, produced lower relative amounts of Z9-14:OAc than rice-strain or CR (C♀ x R♂) hybrid females (Fig. 1A).

The relative amount of Z11-16:OAc was similar in rice-strain females, hybrid CR (C♀ x R♂) and CR-R/RC-R backcross females, suggesting rice-strain specific paternal inheritance (Fig. 1B). In contrast, hybrid RC (R♀ x C♂) and CR-C/RC-C backcross females, i.e. crosses to corn-strain males, resulted in the overall lowest amount of Z11-16:OAc in all crosses (Fig. 1B).

The critical minor component Z7-12:OAc was inherited as a dominant trait from the rice-strain in hybrid CR and RC females (Fig. 1C). Backcross CR-R and RC-R females, i.e. crosses between hybrids and rice-strain males, exhibited significantly higher relative amounts of Z7-12:OAc than all other crosses (Fig. 1C). On the contrary, crosses between hybrids and corn-strain males, i.e. CR-C and RC-C backcross females, produced relative amounts of Z7-12:OAc which were intermediate between those of corn- and rice-strain females (Fig. 1C).
The relative amount of the minor compound Z9-12:OAc was significantly higher in CR/RC hybrids and backcrosses to rice-strain males (CR-R, RC-R) than in pure strain females of both strains (Fig. 1D). Whenever a hybrid female was backcrossed to a corn-strain male, backcross CR-C/RC-C females had similar relative amounts of Z9-12:OAc than rice-strain females (Fig. 1D).

**Genetic mapping of strain-specific pheromone differences**

A total of 303 informative markers were used to identify 30 linkage groups (chromosomes) of *S. frugiperda*. Each linkage group consisted of at least two markers (from different primer combinations) up to a maximum of 22 markers. On average, each linkage group contained around 10 different markers. A total of 7 markers did not map to any linkage group. QTL analysis of strain-specific pheromone differences showed that multiple genomic regions were involved in the production of strain-specific pheromone blends (Fig. 2).

In total, one minor QTL was found which explained the strain-specific differences in the relative amount of the major sex pheromone component Z9-14:OAc (Fig. 2A), a different minor QTL was found for the critical sex pheromone component Z7-12:OAc (Fig. 2B), yet another minor QTL was found for Z9-12:OAc (Fig. 2C), and a total of seven minor QTL were found for the variance in Z11-16:OAc, i.e. C01, C02, C03, C17, C22, C25, C30, (Fig. 2D). Chromosome 2 (C02) explained 4% and 7% of the variance of Z9-14:OAc and Z11-16:OAc, respectively (Fig. 2A, D). C28 was associated with higher percentages of Z7-12:OAc in homozygous (RR) females than in heterozygous (CR) females (Fig. 2B), and C11 affected the production of Z9-12:OAc, which was found in higher proportions in homozygous (RR) females than in heterozygous (CR) females (Fig. 2C).
Seven out of the ten above-described QTL affected the pheromone production in the expected direction, i.e. heterozygous (CR) females produced smaller amounts of Z7-12:OAc and Z9-12:OAc as well as higher amounts of Z11-16:OAc than homozygous (RR) females (Fig. 2). Contrary to expectation, C02 and C30 showed
the opposite-to-expected pattern for Z9-14:OAc (C02) and Z11-16:OAc (C02, C30), i.e. heterozygous (CR) females had higher proportions of Z9-14:OAc and lower amounts of Z11-16:OAc than homozygous (RR) females (Fig. 3A, D), while these compounds are present in lower and higher amounts in C females than in R females, respectively (Fig.1).

**Candidate genes**

A delta-11-desaturase (SfLPAQ) mapped to the QTL chromosome 2, which explained a significant portion of the variance of Z9-14:OAc and Z11-16:OAc, and showed an opposite-to-expected phenotypic pattern for both components (Fig. 2). A similar delta-11-desaturase can be found on chromosome 23 in *Bombyx mori* (ID: BMgn011563 on KAIKObase, http://sgp.dna.affrc.go.jp/KAIKObase/), which suggests that our Sf C02 could be homologous to *Bm* C23. A delta-9-desaturase (SfKPSE) mapped to chromosome 5, which was not associated with strain-specific differences in any of the four pheromone components (Fig. 2).

Interestingly, the circadian clock protein *vrille* (SfVRI) mapped to chromosome 28, which affected the production of the critical secondary sex pheromone component Z7-12:OAc (Fig. 2). *Vrille* is located on chromosome 27 in *B. mori* and our Sf C28 is homologous to *Bm* C27 (Hänniger et al., 2013).

**Discussion**

In this study, we investigated the inheritance and genetic basis of strain-specific pheromone differences between both *S. frugiperda* strains and found a) differential inheritance of all pheromone compounds, b) the involvement of multiple genomic regions determining the female pheromone composition c) the potential contribution of a delta-11-desaturase (SfLPAQ) in strain-specific pheromone
production of Z9-14:OAc and Z11-16:OAc, and d) a possible genetic linkage between two prezygotic mating barriers of \textit{S. frugiperda}, i.e. strain-specific production of the critical secondary sex pheromone component Z7-12:OAc and the onset time of mating in the night, through the circadian clock gene \textit{vrille}.

\textit{a) Differential inheritance of pheromone compounds}

To assess the inheritance of each pheromone compound of \textit{S. frugiperda}, we performed pheromone extractions of pure strain, hybrid and backcross females and found that all four pheromone compounds, i.e. Z9-14:OAc, Z11-16:OAc, Z7-12:OAc and Z9-12:OAc, were differentially inherited. The inheritance of the major pheromone component Z9-14:OAc seemed to be influenced by strain-specific paternal genes, which caused either overproduction of Z9-14:OAc (in RC, CR-C and RC-C females), or reduced amounts of Z9-14:OAc (in CR-R and RC-R females). Similarly, the amount of Z11-16:OAc in hybrid and backcross females was influenced by corn- and rice-strain male-specific factors. The critical secondary sex pheromone component Z7-12:OAc was rice-strain dominant in hybrid females, and paternal factors seemed to influence the amount of this component in backcross females. In contrast, the inheritance of Z9-12:OAc appeared to be influenced by different factors and a higher production in heterozygotes was found in hybrid and most backcross females.

The results of this study are significantly different compared to results of our previous study on the inheritance of strain-specific sex pheromone differences (Groot et al., 2008). In our previous study, where we examined the pheromone composition of pure strain and hybrid females, we found that Z9-14:OAc and Z11-16:OAc were both maternally inherited, while Z7-12:OAc was suppressed in hybrid females and Z9-12:OAc showed a corn-strain dominant inheritance (Groot et al., 2008). In contrast, this study showed that Z9-14:OAc was overexpressed in
CR hybrid females (C♀ x R♂) and paternally inherited in RC (R♀ x C♂) hybrid females, while Z11-16:OAc was suppressed in both hybrids, Z7-12:OAc was rice-strain dominant in CR and RC females, and Z9-12:OAc was overexpressed in both hybrids. These differential results could be explained by differing experimental procedures and/or by different S. frugiperda populations used in both studies. In our previous study, pheromone gland extractions were performed using females that were previously injected with pheromone biosynthesis activating neuropeptide (PBAN), to induce pheromone production and perform pheromone extractions in the photophase (Groot et al., 2008). In this study, we extracted only untreated females within the scotophase, i.e. their natural time of pheromone production, because PBAN was shown to influence the pheromone composition of S. frugiperda females (Groot et al., 2008). Concerning the populations used, the first study used laboratory corn- and rice-strain populations from Florida (Groot et al., 2008), while experiments in this study were conducted with a corn-strain population from Puerto Rico and a different rice-strain population from Florida. Although both studies showed different results, they also confirmed that multiple genomic regions and differential modes of inheritance are involved in the heredity of strain-specific sex pheromone differences in corn- and rice-strain females.

b) Multiple genomic regions influence the female pheromone composition

We investigated the genetic basis of strain-specific pheromone differences in both S. frugiperda strains and found that multiple genomic regions on 9 different chromosomes were involved in the production of corn- and rice-strain specific female pheromone blends. A delta-9-desaturase (SfKPSE), a delta-11-desaturase (SfLPAQ) and the circadian clock gene vrille were mapped to a generated S. frugiperda map, whereupon the last two genes were associated with strain-specific pheromone differences. Interestingly, for three pheromone compounds, i.e. Z9-14:OAc, Z7-12:OAc and Z9-12:OAc, we found the involvement of one QTL each,
whereas a total of seven different QTLs were significantly correlated with the amount of Z11-16:OAc. This result suggest that Z11-16:OAc might not be under strong stabilizing selection, compared to Z9-14:OAc, Z7-12:OAc and Z9-12:OAc.

c) Involvement of desaturases in strain-specific pheromone production

To identify candidate genes which may be responsible for the strain-specific pheromone differences in *S. frugiperda*, we mapped two desaturases to our generated *S. frugiperda* map and found that the delta-11-desaturase *SfLPAQ* mapped to chromosome 2, which was involved in the production of Z9-14:OAc and Z11-16:OAc. Thus, strain-specific differences in this desaturase (*SfLPAQ*) could, at least partly, explain why corn-strain females produce higher relative amounts of Z11-16:OAc and lower percentages of Z9-14:OAc than rice-strain females.

Based on a proposed pheromone biosynthesis pathway of *S. frugiperda*, pheromone production starts with one product of the fatty acid synthesis, i.e. 16-carbon acyl-CoA (16:CoA), which can be modified by a delta-11-desaturase to produce Z11-16:CoA, that can further be reduced and acetylated to produce Z11-16:OAc (Groot et al., 2008). However, the precursor Z11-16:CoA may also be modified by chain-shortening enzymes to produce Z9-14:CoA, the precursor of Z9-14:OAc (Groot et al., 2008). Because Z11-16:OAc and Z9-14:OAc are linked via their biosynthetic pathway, overproduction of one component leads to the reduction of the other component (Groot et al., 2008). If corn-strain, but not rice-strain females, would posses a higher activity of a delta-11-desaturase that could convert 16:CoA to Z11-16:CoA, e.g. *SfLPAQ* on *SfC02*, it could explain why corn-strain females exhibited higher amounts of Z11-16:OAc and lower percentages of Z9-14:OAc than rice-strain females.
However, chromosome \textit{Sf}C02 showed the opposite-to-expected pattern for both components, i.e. heterozygous (CR) females had higher proportions of Z9-14:OAc and lower amounts of Z11-16:OAc than homozygous (RR) females. This suggest that \textit{Sf}LPAQ, if it exhibits strain-specific sequence or expression differences, may act together with another enzyme to produce strain-specific differences in the relative amount of Z9-14:OAc and Z11-16:OAc. We found that seven different chromosomes were significantly correlated with the relative amount of Z11-16:OAc and \textit{Sf}C02 accounted for only 7\% of the variance, while all seven chromosomes explained together 48\% of the variance of Z11-16:OAc. The involvement of multiple genomic regions in the strain-specific production of Z11-16:OAc suggests that, either different enzymes, and/or different activating or inhibiting regulatory elements act together to produce strain-specific Z11-16:OAc and Z9-14:OAc differences.

In addition to the delta-11-desaturase \textit{Sf}LPAQ, we mapped another desaturase, i.e. a delta-9-desaturase (\textit{Sf}KPSE) to \textit{Sf}C05, which was which was not associated with strain-specific pheromone differences. Thus, the delta-9-desaturase \textit{Sf}KPSE is probably not responsible for strain-specific differences in any of the four pheromone components. To verify whether the delta-11-desaturase \textit{Sf}LPAQ is responsible for strain-specific differences in the amount of Z9-14:OAc and Z11-16:OAc, further experiments will be necessary. Structure elucidation will help to determine strain-specific sequence differences and heterologous expression studies can proof whether \textit{Sf}LPAQ is able to strain-specifically convert 16:CoA to Z11-16:CoA.
d) Genetic linkage between pheromone production and timing of reproduction

To examine whether strain-specific differences in two prezygotic mating barriers of *S. frugiperda*, i.e. sex pheromone composition and differential timing of reproduction, may be determined by the same genomic regions, we mapped the circadian clock protein *vrille* to our generated *Sf* map, which was recently found to be responsible for strain-specific differences in timing of reproduction (Hänniger et al., 2013). Interestingly, we found that *vrille* (*SfVRI*) mapped to chromosome 28 (*Sf C28*, homologous to *Bm C27*), which affected the production of the critical minor component Z7-12:OAc, that is known to be essential for male attraction to the major sex pheromone component Z9-14:OAc (Tumlinson et al., 1986; Unbehend et al., 2013). Thus, genes involved in strain-specific Z7-12:OAc production and timing of mating in the night are located on the same chromosome. This suggests that these two prezygotic mating barriers might be genetically linked and influenced by the same set of genes and/or regulatory elements.

Genetic analysis of strain-specific differences in timing of reproduction showed that one *S. frugiperda* chromosome, which is homologous to *Bm C27*, explained around 30% of the variance in the differential onset time of mating between both strains (Hänniger et al., 2013). Hänniger et al. (2013) found that the circadian clock gene *vrille* mapped to this chromosome and although *vrille* showed no strain-specific sequence differences, its relative expression levels cycled in a clock-dependent manner and *vrille* expression peaks were shifted between both strains in both sexes. Hänniger et al. (2013) suggested that differential *vrille* expression levels might be caused by strain-specific differences in a cis-regulatory enhancer element, which still needs to be investigated.
If strain-specific differences in a cis-regulatory element do exist and influence 
vrielle expression, it could be possible that the same regulatory element also 
influences another gene, responsible for differential production of Z7-12:OAc in 
females. Due to the fact that Z7-12:OAc consists only of 12 carbons and 
pheromone production starts with 16:CoA, many different enzymes could be 
responsible for the production of Z7-12:OAc, i.e. desaturases, chain-shortening 
enzymes, reductases and acetyl transferases (Groot et al., 2008). If a cis-regulatory 
enhancer element strain-specifically affects the expression and/or substrate 
specificity of one of these genes, it could explain why corn-strain females produced 
lower relative amounts of Z7-12:OAc than rice-strain females. Further genetic 
analysis will be necessary to evaluate which genes are responsible for the strain-
specific production of Z7-12:OAc.

Acknowledgements
This research was funded by the Deutsche Forschungsgemeinschaft (P.S.GR 
362721), the National Science Foundation (award IOS-1052238), and the Max 
Planck Gesellschaft. We thank Susanne Donnerhacke for her help with the AFLP 
analysis and Anne Karpinski for helping with the AFLP marker scoring. Thanks to 
Gerhard Schöfl for his support with the statistical analysis. We thank Domenica 
Schnabelrauch for sequencing of the candidate genes.

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Chapter 5

The roles and interactions of reproductive isolation mechanisms in fall armyworm (Lepidoptera: Noctuidae) host strains

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Abstract. 1. The moth *Spodoptera frugiperda* presents an interesting opportunity to study the evolution of reproductive isolation, because it consists of two host races that may be in the process of speciation. 2. The two races exhibit habitat isolation through host-plant preference, and two types of behavioural isolation, i.e. differences in sex pheromone composition and timing of mating activity at night. 3. In this paper, we review the selection pressures acting upon these three barriers as well as their genetic bases, to address the question of how divergence of the two strains may have evolved. 4. We also address possible interactions between the three barriers, whether and how they may have evolved in concert, and we view the evolution of these three prezygotic isolation barriers in the light of postzygotic isolation.

Key words. Behavioural isolation, Fall armyworm, Habitat isolation, Postzygotic isolation, Reproductive isolation, *Spodoptera frugiperda*.

Introduction

The generation of biological diversity by speciation is one of the central themes of evolutionary biology (Dobzhansky, 1937; Mayr, 1963; Howard & Berlocher, 1998; Coyne & Orr, 2004). To learn about the generalities of this process we will need to identify the factors causing divergence between natural populations in many case studies. Generally, the main factors likely to be important in the evolution of reproductive isolation between populations include a gradual accumulation of genetic incompatibilities by drift, adaptive divergence in response to environmental variation, and rapid genetic changes associated with founder events (Tregenza, 2002). Barriers to reproduction can be divided into prezygotic versus postzygotic isolation (e.g. Coyne & Orr, 2004). Comparisons between closely related Drosophilid species suggested that their relative importance may depend on
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whether populations diverge in geographic isolation or in sympatry (Coyne & Orr, 1989, 1997). However, interspecific comparisons have the disadvantage that we cannot distinguish between divergence contributing to speciation and divergence occurring afterwards. Important insights in speciation are likely provided by examining contemporary patterns of reproductive isolation among partially isolated populations (e.g. Berlocher & Feder, 2002; Drés & Mallet, 2002; Via, 2002; Via & West, 2008). Understanding the process of speciation is important for explaining the diversity of life in general and, from a more applied perspective, crucial for conservation biology as well as pest management.

In this review we discuss the contributions of ecology, behavioural mechanisms, and genetic incompatibilities to reproductive isolation between fall armyworm host strains. We aim to assess whether and how these isolating barriers may act in concert to minimize gene flow between the two strains in sympatry and review the current state of knowledge on the underlying genetics of these traits.

**Fall armyworm host strains**

In the noctuid moth *Spodoptera frugiperda* J. E. Smith (fall armyworm; Lepidoptera: Noctuidae) two sympatrically occurring, major genetic groups have been recognized that show host-plant associated genetic variation (Pashley et al., 1985; Pashley, 1986). Larvae collected from maize, sorghum, and cotton typically represent the corn-strain, whereas larvae collected from rice and various pasture grasses represent the rice-strain (Pashley, 1986, 1989). Originally, a diagnostic esterase allozyme marker and three other significantly strain-biased protein variants were discovered by an electrophoretic survey of allozyme variation in samples from corn fields and rice paddies (Pashley et al., 1985; Pashley, 1986). Subsequently, several additional strain-biased or strain-diagnostic molecular markers have been identified: the two strains differ in several DNA sequence
variants in the mitochondrial cytochrome oxidase I and ND1 genes (Pashley, 1989; Pashley & Ke, 1992; Lu & Adang, 1996; Meagher & Gallo-Meagher, 2003; Nagoshi et al., 2006a), strain-biased and strain-specific amplified fragment length polymorphism (AFLP) markers (McMichael & Prowell, 1999; Busato et al., 2004; Prowell et al., 2004), and the FR repetitive nuclear DNA sequence extensively present in the rice strain and mostly absent from the corn strain (Lu et al., 1994; Nagoshi & Meagher, 2003b; Nagoshi et al., 2008). To date, no diagnostic morphological features have been described that distinguish these two strains, but they differ in a number of physiological, developmental, and behavioural features (Pashley, 1988b; Pashley et al., 1992, 1995; Veenstra et al., 1995; Groot et al., 2008; Lima & McNeil, 2009; G. Schöfl, A. Dill, D. G. Heckel, A. T. Groot, unpubl. res.). It has been suggested that these two genetically differentiated groups may actually be reproductively isolated cryptic sibling species (Pashley, 1986; Drés & Mallet, 2002). More recently, however, the detection of individuals non-concordant for two or more strain-specific markers (e.g. the esterase genotype of one strain and the mtDNA of the other) suggests hybridization rates in the field of up to 16% (Prowell et al., 2004) or even more (Nagoshi & Meagher, 2003a; Nagoshi et al., 2006b; Machado et al., 2008). Such high hybridization rates argue for incipient, rather than completed speciation.

Despite these relatively high hybridization rates in at least some of the areas of occurrence, the two strains have been found throughout South and North America (Meagher & Gallo-Meagher, 2003; Busato et al., 2004; Prowell et al., 2004; Nagoshi et al., 2007; Machado et al., 2008; Vélez-Arango et al., 2008) and can be identified using the same strain-specific mitochondrial markers (Nagoshi et al., 2007). Apparently, there is an array of reproductive isolating barriers active throughout the Western Hemisphere that prevents these strains from merging into one panmictic population. Apart from habitat isolation, two behavioural isolation
mechanisms may contribute to reproductive isolation in *S. frugiperda*: differences in the female pheromone composition (Groot *et al.*, 2008; Lima & McNeil, 2009) and differential timing of reproductive activity at night (Pashley *et al.*, 1992; Schöfl *et al.*, 2009b G. Schöfl, A. Dill, D. G. Heckel, A. T. Groot, unpubl. res.). No intrinsic hybrid inviability has been found for the two host strains (G. Schöfl, unpubl. res.), but extrinsic ecological or behavioural sterility may affect overall reproductive isolation.

**Habitat isolation**

In phytophagous insects, habitat isolation may arise if genetically-based biases in habitat or host use decrease the probability of heterospecific encounters (Funk *et al.*, 2002; Coyne & Orr, 2004; Rundle & Nosil, 2005). Biases in host use may be caused either by differential performance on their respective host plants, by specific ovipositional preferences in adult females, or by a combination of both. There is also evidence in some species for ‘natal habitat preference induction’, in which early experience in a particular habitat increases the probability that habitat is chosen after adult dispersal (Davis & Stamps, 2004). Once a bias in host use is established, it may contribute to reproductive isolation, either directly through spatial or temporal segregation of the preferred host plants or pleiotropically by, for example, host-plant-mediated mate attraction or post-mating isolation if hybrid offspring show decreased viability because of a reduced capacity to use either parental host (Coyne & Orr, 2004; Rundle & Nosil, 2005). (Potential pleiotropic effects are discussed below.)

Analysing populations from Louisiana, Florida, Puerto Rico, Guadeloupe, and French Guiana, Prowell *et al.* (2004) found relatively high frequencies of rice-strain individuals (19%) on corn plants, and lower frequencies of corn-strain individuals (5%) on pasture grasses or rice plants. However, host use varied from
region to region as well as from year to year (Prowell et al., 2004). In Brazil (Rio Grande do Sul), Machado et al. (2008) found that all larvae collected from rice carried the rice-strain haplotype, while 83% of the larvae collected from corn carried the corn-strain haplotype. In Colombia, similar results were obtained; the corn strain was found exclusively on corn or cotton, while the rice-strain was found mainly on rice and in low proportions on corn and cotton (Vélez-Arango et al., 2008). Finally, similar patterns of asymmetric host use of the two strains were reported for a number of populations from Florida, Texas, and Brazil (Meagher & Nagoshi, 2004; Nagoshi et al., 2006b; Nagoshi et al., 2007). Thus, these studies indicate a similar differentiation in host use of the two strains throughout their range. The corn-strain is largely restricted to corn, cotton, or sorghum. The rice-strain predominates on pasture grasses and rice but may also utilize typical corn-strain habitats to variable degrees. However, the proximate causes for the differential host use between the host-associated strains in fall armyworm remain elusive.

**Proximate causes of differential host use**

Even if larvae of the two strains are able to utilize the same host plants, differences in their competitive abilities may lead to selection against ‘immigrants’ and thus promote host differentiation (Nosil et al., 2005). *Spodoptera frugiperda* is polyphagous and can feed on many different plant species (Pashley, 1988a). For the fall armyworm host strains, a number of reports have described differential effects of plant hosts on viability and development of the two strains, but often their results are not consistent (Pashley, 1988b; Whitford et al., 1988; Pashley et al., 1995; Meagher et al., 2004; Stuhl et al., 2008). For example, concerning the rice strain, Pashley (1988b) and Pashley et al. (1992) found a negative effect on larval weight and larval developmental time when larvae were reared on corn plants as compared to rice plants, while pupal weight was significantly higher on
corn, and larval survivorship was similar on both host plants. Whitford (1988) also reported a negative effect on larval weight and larval developmental time, but on pupal weight as well, when rice-strain larvae were reared on corn or sorghum, and found no effect of host plant on larval survival either. Concerning the corn-strain, developmental differences with respect to host plants were less pronounced and more variable across these studies. Overall, both strains performed equally well on rice, but corn-strain tended to outperform rice-strain on corn plants (Pashley, 1988b; Whitford et al., 1988; Pashley et al., 1995). In contrast, Meagher et al. (2004) found that rice-strain larvae were significantly larger and developed faster on corn and sorghum–sudangrass hybrids (both corn-strain hosts) than corn-strain larvae, although typical rice-strain host plants were not tested.

We observed a similar pattern as Meagher et al. (2004) in a feeding assay that assessed larval weight gain and developmental time of both strains on corn and rice plants, compared to a control using artificial diet. We reared both strains from neonates in individual cups on leaves of either plant, which were refreshed every 2–3 days. We did not find any difference in larval weight of either strain on the three diets until day 16–18 (Fig. 1). Rice-strain individuals were heavier when reared on corn leaves (although not significantly, likely due to sample size) than on rice leaves or artificial diet, while corn-strain larvae were heaviest when reared on artificial diet. Rice-strain individuals developed significantly faster when reared on corn plants compared to the other food sources as well as compared to the corn-strain (Fig. 1).

The variability across studies in the physiological responses of larvae of the two strains to different host plants may reflect differences in the geographic origin of the samples used, or they may reflect genetic and phenotypic variation in wild populations (Stuhl et al., 2008).
Figure 1. Mean weight gain (± SEM) of *Spodoptera frugiperda* corn- and rice-strain larvae on different diets (corn leaves, rice leaves, artificial pinto bean diet). The eggs of nine fertile JS3C and 16 fertile OnaR families were transferred onto Petri dishes containing artificial pinto bean diet. The hatched larvae lived on diet for 4–6 days, after which they were transferred to plastic cups comprising fresh plant material or diet. Corn (*Zea mays*) and rice (*Oryza sativa*) plants were reared in the greenhouse and their leaves were cut into pieces and fed to the larvae. We started with 90 rice-strain and 60 corn-strain individuals per treatment (diet, corn leaves, rice leaves), so that a total of 270 OnaR rice-strain and 180 JS3C corn-strain individuals were used for the experiment. At the beginning of the experiment the plant material was changed every 3 days. After 10 days of the experiment the plant material was changed every day. The artificial pinto bean diet was cut into cubes [2 cm (h) × 2 cm (l) × 2 cm (d)] and exchanged every 9–12 days. Each larva was weighed every third day of the experiment until it pupated. The date of
pupation, the date of eclosion, the sex of the eclosed individual, and the mortality rate up to pupation and eclosion was also examined.

No consistent effects of host plant on survival have been found, and larval performance does not translate into differential fecundity (Pashley et al., 1995). It thus seems unlikely that the consistent host differentiation found in field samples from a wide range of populations can be explained by differential host-plant adaptations of the larvae.

Adult oviposition preference can also contribute to host-plant specificity. The only study to date that examined oviposition preference of corn- and rice-strain females (Whitford et al., 1988) showed a preference of corn-strain females to oviposit on corn and sorghum in two replicate trials, while rice-strain females preferred to oviposit on bermudagrass in only one of the two trials. While this finding is in line with the overall distribution of the strains in nature, more oviposition preference studies are needed, preferably with populations of several geographic origins. The possibility that post-natal experience could influence adult habitat choice should also be investigated, as evidence for this phenomenon exists in Spodoptera littoralis (Anderson et al., 1995).

Host differentiation and reproductive isolation

How much the bias in host use directly contributes to reproductive isolation between fall armyworm host strains is unknown. The fall armyworm is known to migrate long distances annually (Luginbill, 1928; Nagoshi & Meagher, 2008), which makes it unlikely that micro- or even macro-spatial segregation of suitable habitats alone can restrict encounters between the two strains. Many herbivore species mate on or near their host plants and oviposition sites, a tendency that may restrict encounters between heterospecifics (Funk et al., 2002; Rundle & Nosil,
2005). We know of no reports that indicate whether fall armyworm females preferentially call or mate in proximity to their host plants or whether they seek out oviposition sites after having mated.

Two studies suggest a strong seasonal component to host use. In samples from Southern and Central Florida from corn (Nagoshi & Meagher, 2004) and from the Mato Grosso (Brazil) from sorghum (Nagoshi et al., 2007), the corn-strain dominated in spring collections while the rice-strain dominated in autumn collections. This is likely due to seasonal differences in the availability of host plants, which may affect the abundance of the two strains and thus cause some seasonal temporal isolation (Pashley et al., 1992).

**Behavioural isolation**

*Behavioural isolation through sexual communication*

The most common type of behavioural reproductive isolation in moths is through sexual communication. Females attract males from a distance by emitting a species-specific sex pheromone, usually consisting of two or more volatile compounds, that is released from the sex pheromone gland in the scotophase (e.g. Cardé & Haynes, 2004). So far the sex pheromones of about 1600 moth species have been identified (El-Sayed, 2008). The sex pheromone of *S. frugiperda* was identified in 1986 (Tumlinson et al., 1986) and field experiments have been conducted in several regions (Mitchell et al., 1985; Tumlinson et al., 1986; Andrade et al., 2000; Malo et al., 2001; Batista-Pereira et al., 2006). However, none of these studies mentioned whether corn- or rice-strain females were analysed or whether corn- or rice-strain males were attracted to the different blends, even though both strains have been recognized in North America (Pashley et al., 1985) as well as in Brazil (Busato et al., 2004). One field experiment assessed strain-
specific attraction: Pashley et al. (1992) used 1–2 day old live virgin females of each strain as bait in pheromone traps, one female per trap, in fields that contained host plants of both strains in Louisiana in two consecutive years. In this study, a slight but significant strain-specific attraction was found, although corn-strain females also attracted a large number of rice-males. This may not be so surprising, because a total of only 77 corn-strain males were caught against a total of 727 rice-strain males, indicating a much larger abundance of a rice-strain population at the time of the experiment.

To assess whether corn and rice-strain females differ in their sex pheromone composition, we analyzed the sex pheromone from both strains (Groot et al., 2008). The corn strain (JS3C) was obtained from corn plants near Homestead, Florida, in 2004, while the rice-strain (OnaR) colony originated from pasture grasses in Ona, Florida, in 2003. Glands were extracted from 2–3-day-old virgin corn- and rice-strain females, as well as from hybrid female offspring (see Groot et al., 2008 for a detailed description). Our analysis included the four compounds that have been found to be attractive for S. frugiperda males: the major component Z9-14:OAc; the crucial secondary pheromone component Z7-12:OAc without which S. frugiperda males are not attracted (Mitchell et al., 1985; Tumlinson et al., 1986; Descoins et al., 1988; Andrade et al., 2000; Malo et al., 2001; Fleischer et al., 2005; Batista-Pereira et al., 2006); Z11-16:OAc, the addition of which attracted significantly more males in Pennsylvania (Fleischer et al., 2005), Mexico and Costa Rica (Andrade et al., 2000; Malo et al., 2001), but not in Florida (Tumlinson et al., 1986); and Z9-12:OAc, which also increased attraction of S. frugiperda males, at least in Pennsylvania (Fleischer et al., 2005).
Considering these four components, we consistently found that Z11-16:OAc was significantly higher, while Z7-12:OAc and Z9-12:OAc were significantly lower in corn-strain than in rice-strain female glands (Fig. 2). The major component Z9-14:OAc was present in similar amounts in both females; however, significantly lower amounts were found in corn-strain females when they were injected with pheromone biosynthesis activating neuropeptide (PBAN). Hybrid females contained similar amounts of Z9-14:OAc and Z11-16:OAc as found in their mothers, indicating a maternal inheritance or maternal effect, while the other two components showed a corn-dominant inheritance (Groot et al., 2008).

Lima and McNeil (2009) assessed strain-specific sex pheromone differences in three of the above-mentioned components (Z9-14:OAc, Z11-16:OAc, and Z7-12:OAc) in strains originating from Louisiana. They found that corn-strain females

**Figure 2.** Sex pheromone composition of the corn- and rice-strain when considering the four components that have been shown to be important in the attraction of males. The major pheromone component Z9-14:OAc is present in similar amounts in both strains, however corn-strain females contain significantly more Z11-16:OAc and significantly less Z9-12:OAc and Z7-12:OAc than rice-strain females. See Groot et al. (2008) for further details.
contained a higher amounts of the major component Z9-14:OAc and lower amounts of Z11-16:OAc and Z7-12:OAc than rice-strain females. Thus, strains from Louisiana gave contrasting results to strains from Florida. Perhaps strain-specific differences in pheromone composition vary across geographic regions, as has been found in many other moth species (Klun, 1975; Cardé et al., 1977; Guerin et al., 1984; McElfresh & Millar, 1999, 2001; Gemeno et al., 2000; Gries et al., 2001; El-Sayed et al., 2003; Groot et al., 2009b). Interestingly, the major component Z9-14:OAc showed maternal inheritance in Louisiana hybrids as well as in Florida hybrids. To determine the biological significance of these sex pheromone differences, it will be important to evaluate whether different pheromone blends are differentially attractive to corn- and rice-strain males in the field across the geographic regions.

In addition to the long-range sex pheromones of females, male moths may also emit short-range pheromones by extruding abdominal hairpencil scales (Birch et al., 1990; Cardé & Haynes, 2004). Although these hairpencils are usually displayed extensively during courtship, their effects can range from no detectable influence on female behaviour (Gothilf & Shorey, 1976) to being a key stimulus in determining mate acceptance and mate choice (Conner et al., 1981; Löfstedt et al., 1989; Hillier & Vickers, 2004). In a number of noctuid species, benzaldehyde has been identified as a component of the volatile male hairpencil secretions (Birch, 1974; Weatherston & Percy, 1977; Fitzpatrick et al., 1985). Other chemicals commonly found in plants (e.g. linalool, cresol) have been identified in noctuid male hairpencils as well (Birch & Hefetz, 1987). Birch and Hefetz (1987) suggest that females may already have receptors for plant-derived compounds since they show behavioural responses to them (Birch & Hefetz, 1987). However, it is not known whether these compounds emitted from the male hairpencils are sequestered from plant hosts (as in e.g. Arctiids; Eisner & Meinwald, 1995) or synthesised de
novo. In *S. frugiperda*, male pheromones have not yet been identified. Even so, males do possess abdominal hair pencils and display them overtly before mating (G. Schöfl, A. Dill, D. G. Heckel, A. T. Groot, unpubl. res.). Mate choice experiments in the laboratory indicate that non-random mating between the two host strains depends not only on the allochronic separation of reproduction during the night (see next section), but also involves a time-independent component of female choice, possibly influenced by male pheromones emitted during courtship (Schöfl et al., 2009a).

**Behavioural isolation through differential timing of reproduction**

*Spodoptera frugiperda* offers a rare example of host-strain isolation by differential timing of reproductive activity at night. The two strains are allochronically separated in their timing of female calling (extrusion of the pheromone gland) and mating at night: the corn-strain is active early at night, while the rice-strain is active late at night (Pashley et al., 1992; G. Schöfl, A. Dill, D. G. Heckel, A. T. Groot, unpubl. res.). The only other example in lepidopterans where differences in the timing of reproductive activity at night have been described for two host strains is the rice stem borer *Chilo suppressalis* (Konno et al., 1996; Samudra et al., 2002). Differences in the timing of female calling have been found between closely related species, such as *Spodoptera latifascia* and *S. descoinsi* (Monti et al., 1997), four sympatric limacodid moth species (Sasaerila et al., 2000), two species of plume moths (Haynes & Birch, 1986), and the two closely related species *Heliothis virescens* and *H. subflexa* (Heath et al., 1991). Recently, a study on a large number of co-occurring species of Neotropical skippers (Hesperiidae), demonstrated significant temporal partitioning of diurnal flight activity among subfamilies, genera, and species (Devries et al., 2008). This suggests that temporal displacement of activity may be an important but hitherto largely neglected factor in reproductive isolation.
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When the temporal displacement of reproductive activity between the two strains of *S. frugiperda* was first described (Pashley *et al.*, 1992), hardly any overlap in the timing of copulations at night was observed. Under similar conditions, but using populations of different geographic origin and a much larger sample size, we found that both strains were significantly differentiated for female calling and copulation times as well, but also showed a considerable overlap in the onset times of mating activity (G. Schöfl, A. Dill, D. G. Heckel, A. T. Groot, unpubl. res.). Specifically, onset of female calling and copulation in the rice-strain was restricted to the last third of scotophase, while the corn-strain was more flexible and would start calling and mating throughout the night. Interactions between the sexes in heterogamic pairings implied that rice-strain females are in general more restricted in the timing of copulation than rice-strain males (G. Schöfl, A. Dill, D. G. Heckel, A. T. Groot, unpubl. res.). This suggests that the allochronic reproductive isolation between the strains may be asymmetric between strains and sexes, and less pronounced than previously thought.

**Possible interactions between the prezygotic isolation barriers**

Viewed in isolation, each of the above-mentioned prezygotic isolation barriers seems unlikely to be sufficiently strong to prevent the two strains of *S. frugiperda* from merging into one panmictic population. However, the total reproductive isolation achieved in nature may potentially be much higher than the individual evaluations of habitat isolation, differential attraction to sex pheromones, and allochronic separation of reproductive activity suggest. Barriers may act simultaneously and could therefore contribute multiplicatively rather than cumulatively to reproductive isolation, or barriers may enhance each other through pleiotropic interactions (Fig. 3).
Figure 3. Possible interactions between the different reproductive isolation barriers. Double arrows, potential reciprocal interaction; single arrows, one-way effect; dashed lines, potential extrinsic postzygotic effects, i.e. hybrids may be less effective in their sexual communication, not able to find their host plants, or timing reproduction differently than the parental strains.

Habitat isolation and differential mate attraction via sex pheromones may interact pleiotropically if females preferentially call on or near their oviposition sites and if males tend to alight on their larval host plants. This effect may be further enhanced by a spatially clumped distribution of the hosts. For instance, in two host races of the larch budmoth, assortative mate attraction is strongly affected by the tree species used as a calling substrate by the females and by the host neighborhood structure (Emelianov et al., 2001). This has been shown to be due to a genetically-based preference of adults of both sexes to alight on their on native host trees (Emelianov et al., 2003). Adult attraction to native host plants has also been demonstrated in tephritid flies (Feder et al., 1994) and aphids (Via, 1999). An association of females with host plants can be caused if, for example, female
pheromone production is dependent on females perceiving the odour of a host plant (McNeil & Delisle, 1989; Raina et al., 1992, 1997) or if pheromone release is affected by plant odours (Hendrikse & Vos Bunnemeyer, 1987; McNeil & Delisle, 1989; Landolt & Phillips, 1997). In females, the coordination of pheromone production or release with the availability of a suitable host for oviposition is considered adaptive (Raina et al., 1992).

Male attraction to host plant for the fall armyworm is more difficult to explain. In the cases of apple maggot flies and aphids, males are thought to rely on host cues to find mates (Feder et al., 1994; Via, 1999). In the larch budmoth, mate attraction is through host-independent sex pheromones. Nevertheless, males show heritable alighting preferences for their respective host plants independent of potential mating partners (Emelianov et al., 2003), which may also be the case in the fall armyworm. For this species, we do not know (yet) whether mating occurs mostly on or near host plants, and/or whether males are attracted solely to the female sex pheromone or to a combination of host-plant volatiles and strain-specific sex pheromone.

Host-plant chemistry might also directly affect pheromone production and/or the male response to female pheromones (reviewed by McNeil & Delisle, 1989; Landolt & Phillips, 1997). In Noctuidae, female pheromone is produced de novo every night and the components do not have host-plant precursors (Bjostad et al., 1987; Tillman et al., 1999; Jurenka, 2004). However, male courtship pheromones that are active at close range have been found to be affected by the host plant on which the males were raised (reviewed by Birch et al., 1990). At the receiver side, males may be more attracted to a combination of pheromone and plant volatiles than to pheromone alone (Dickens et al., 1990, 1993; Landolt et al., 1992; Hardie et al., 1994). One study has been conducted to assess a possible synergistic effect
between the commercial Trécé sex pheromone lure of *S. frugiperda* and phenylacetaldehyde (Meagher & Mitchell, 2001). This compound has been isolated from many flowering plants, including corn (Cantelo & Jacobson, 1979). However, when comparing traps baited with pheromone alone to traps baited with pheromone plus this flower compound in a corn field in Florida, *S. frugiperda*, males were more attracted to traps with pheromone alone than to traps where phenylacetaldehyde was added (Meagher, 2001).

Under field conditions, differential mate attraction via sex pheromones and temporal displacement of reproduction are also expected to reinforce each other as a barrier to reproduction. Corn-strain females will tend to attract males early in the night while rice-strain females attract males late at night. The only study so far to test for cross attraction between the two strains in the field, using live females as a lure (Pashley *et al*., 1992), did not record the time of night at which potential mating partners homed in on the calling females.

**Genetic basis of the prezygotic isolation barriers**

*Habitat isolation*

The study of the genetic basis of host-plant preference is still in its infancy (Berlocher & Feder, 2002; Feder *et al*., 2005) and nothing is known about the genetic architecture of either larval performance differences or oviposition preferences in *S. frugiperda*. One quantitative trait locus (QTL) analysis has been described for *Drosophila sechellia* where two QTL were identified to be involved in oviposition site preference (Jones, 1998). In F1 hybrids, intermediate as well as dominant preferences for one of the parental plants have been found (e.g. Sheck & Gould, 1995; Sezer & Butlin, 1998; Via *et al*., 2000; Emelianov *et al*., 2003). Some studies found sex linkage of oviposition preference (Thompson, 1988; Scriber *et
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al., 1991), while others did not (Sheck & Gould, 1995). All studies so far indicate that host-plant utilization is a trait governed by multiple, mostly autosomal loci (Hagen, 1990; Thompson et al., 1990; Sheck & Gould, 1995). Recently, two genes were identified that affect the oviposition site preference in *Drosophila sechellia* (Matsuo et al., 2007): odorant binding protein Obp57d and Obp57e. This finding makes the class of odorant binding proteins possible candidate genes that may be involved in oviposition preference in other species as well.

Sexual communication

Most studies on moth pheromone genetics have concentrated on the genetic architecture of differences in sexual communication that can be explained by one gene (e.g. Jurenka et al., 1994; Roelofs & Rooney, 2003; Dopman et al., 2004). Analyses of the genetic basis of sex pheromone differences between two closely related noctuid moths, *Heliothis virescens* and *H. subflexa*, shows variation that cannot be explained by a single genetic change (Groot et al., 2004, 2009a; Sheck et al., 2006). These analyses revealed that five chromosomes and thus at least five autosomal loci are involved in the different pheromone blends between the two species. Since the sex pheromone of *S. frugiperda* is also a multi-component blend and differences between the two strains are complex (Groot et al., 2008), these differences are likely caused by multiple genes as well. In addition to the multiple genes that probably affect the pheromone composition of multiple component blends, other studies have found that male response genes are located in different genomic regions than female pheromone genes, at least in Lepidoptera (Butlin & Ritchie, 1989; Butlin & Trickett, 1997; Smadja & Butlin, 2009). Genetic correlations that have been found in Lepidoptera between long distance mate-signalling traits in one sex and response to those signals in the opposite sex appeared to be due to linkage disequilibrium and not pleiotropy (e.g. Linn et al., 1997; Zhu et al., 1997; Gray & Cade, 1999). Rare genotypes with a novel signal or
response phenotype have been shown to be less attractive (e.g. Linn et al., 1997; Zhu et al., 1997). Thus, at least two independent mutations, one in sexual signal and one in response, are needed for a possible fitness advantage to arise, so that sexual communication signals can be considered complex traits.

Genes that are likely involved in the pheromone differences between moth races and species can be deduced from the biosynthetic pathways of sex pheromone production (Gould et al., 2009; Groot et al., 2008, 2009a). These biosynthetic pathways have been elucidated in a number of moth species (Tillman et al., 1999; Jurenka, 2004) and typically share common characteristics: most moth sex pheromones consist of multi-component blends, synthesised \textit{de novo} through the fatty acid biosynthetic pathways, followed by a variety of modifications within the pheromone gland. However, so far genes encoding the enzymes involved in the biosynthetic pathway have not been identified, except for a family of desaturases in several moths (Knipple et al., 2002; Roelofs & Rooney, 2003; Serra et al., 2007) and a reductase in \textit{Bombyx mori} (Moto et al., 2003). Linking the pheromone differences that we found between the two strains of \textit{S. frugiperda} to the hypothetical biosynthetic pathway of these compounds, we suggest that a $\Delta_{11}$-desaturase and $\Delta_{9}$-desaturases, and possibly a $\Delta_{7}$-desaturase (not yet identified in insects) are candidate genes that may be differentially active between the two strains (Groot et al., 2008). In the genus \textit{Spodoptera}, $\Delta9$- and $\Delta11$-desaturases have been characterised in \textit{S. exigua}, \textit{S. littura} (Knipple et al., 2002), and \textit{S. littoralis} (Rodriguez et al., 2004). These identifications will facilitate the assessment of whether and which of these genes vary between the two strains.

Differential timing of reproduction

A genetic analysis of temporal separation of sexual activity between the two closely related \textit{Spodoptera} species, \textit{S. descoinsi} and \textit{S. latifascia}, suggested a
polygenic control with mainly additive autosomal gene effects for onset time of female calling (Monti et al., 1997). For the *S. frugiperda* host strains, different components of time-shifted reproductive behavior (female calling, male courtship, copulation, and oviposition) have been found to show differing modes of inheritance: the timing of female and male calling was controlled mainly by maternal effects, the timing of copulation was controlled by a combination of maternal effects and corn-strain dominant autosomal factors, and the timing of oviposition was inherited in a purely corn strain dominant fashion (G. Schöfl, A. Dill, D. G. Heckel, A. T. Groot, unpubl. res.). This suggests that the differential timing of reproductive behaviours between the two host strains is under complex genetic control.

In *S. frugiperda*, a temporal profile of general activity patterns (feeding and locomotion) parallels the temporal profile of reproductive behaviours between the two strains. Corn-strain individuals were active at relatively high levels from the beginning of scotophase until shortly before the end of scotophase. Rice-strain individuals were less active than corn-strain individuals during the first half of the night, but increased activity during the latter part of the night until shortly before photophase (G. Schöfl, A. Dill, D. G. Heckel, A. T. Groot, unpubl. res.). Such a coordinated time shift between reproductive traits and locomotor activity may suggest an involvement of genes associated with the central circadian system (Miyatake et al., 2002; Tauber et al., 2003). For example, it has been demonstrated that mating behaviour is gated by a circadian clock in *Drosophila* (Sakai & Ishida, 2001) and a cockroach (Rymer et al., 2007). Differences in mating time between populations of melon flies and two sibling species of tephritid fruit fly have been correlated to differences in circadian fluctuations of two clock genes (*period* and *cryptochrome1*) (Miyatake et al., 2002; An et al., 2004). In *S. frugiperda*, three genes of the core circadian clock (*period, timeless*, and *cryptochrome 2*) have been
found to exhibit significant strain-specific differences in their daily transcription profiles (G. Schöfl, unpublished). Since central circadian clocks are driven by negative transcriptional feedback loops, the actual molecular difference(s) between the two strains may be located in any of the genes involved in the circadian clockwork.

Postzygotic incompatibilities

Postzygotic barriers to gene flow come into play only when prezygotic barriers are incomplete or absent. Prezygotic barriers are often thought to evolve rapidly in response to divergent selection in different habitats (Schluter, 1998), while intrinsic hybrid inviability and/or hybrid sterility commonly arise from sets of loci that interact epistatically and potentially accumulate much slower (Coyne & Orr, 1989, 1997; Orr, 1995). If one takes an ecological view on speciation and focuses on the order in which barriers to gene flow tend to evolve, intrinsic postzygotic effects may be of relatively little relevance to the early stages of the speciation process (Mallet et al., 1998; Via, 1999; Funk et al., 2002). On the other hand, for a large fraction ($\approx 2/3$) of sympatrically occurring Lepidopteran species that still hybridise in nature, intrinsic hybrid incompatibilities have been reported (Presgraves, 2002). This suggests that intrinsic postzygotic isolation may contribute to the maintenance of genetic integrity in many partially reproductively isolated species.

Although less extensively studied, extrinsic postzygotic isolation (i.e. reduced hybrid fitness due to ecological disadvantages of hybrid phenotypes in the parental environments) can be a direct result of adaptive evolution in different habitats and therefore more commonly observed during early stages of speciation (Coyne & Orr, 1998, 2004; Schluter, 1998). Another form of postzygotic barrier arises when hybrids are unable to secure mates (Coyne & Orr, 2004). This form of post-mating isolation may be intrinsic if, for example, behavioural dysfunctions render hybrids
incapable of courtship, or extrinsic if, for example, intermediate courtship phenotypes render hybrids unattractive to members of the parental populations (Servedio & Noor, 2003; Coyne & Orr, 2004).

In the fall armyworm, Pashley and Martin (1987) found that in no-choice matings between corn females and rice males (C × R) no spermatophores were transferred, while the reciprocal cross (R × C) produced viable offspring. However, a second study could not replicate these results (Whitford et al., 1988), since the strains crossed successfully in both directions at similar rates. Pashley (1988a) suggested that the discrepancy between the two studies was due to the age of the laboratory colonies used. Pashley and Martin (1987) had used colonies that had been reared for one to five generations in the laboratory, while Whitford et al.’s (1988) colonies were at least 3 years (~30 generations) old. This objection was tested using colonies of different age ranging from two generations to 25 years in the laboratory (Quisenberry, 1991). Again, no reproductive incompatibilities between the strains were found. Independent of colony age, both cross directions proved fertile to a similar extent (Quisenberry, 1991). Nevertheless, it is unlikely that these differences in results are due to chance. Possibly there is geographic variation in the success rate between the two reciprocal cross directions; Pashley and Martin (1987) used individuals collected either in Puerto Rico or Louisiana, while Whitford et al. (1988) used fall armyworm collected in Louisiana and Mississippi. When considering backcrosses, results between different studies are more consistent. Pashley and Martin (1987) found that RC-hybrid females mated moderately successfully only with their hybrid brothers, but not with males of either parental strain (RC-hybrid males mated successfully with females of either parental strain). Whitford et al. (1988) found that interhybrid crosses between either CR- or RC-hybrids did produce fertile clutches, although RC × RC pairs
were significantly less successful than CR × CR pairs (Fisher’s exact test, \( P<0.001 \), reanalysis of data from Whitford et al., 1988).

Similarly, when we performed between-strain crosses between rice-strain and corn-strain individuals originating from Florida, we found no reproductive isolation in either cross direction (Table 1), but we did consistently find that backcrossing RC-hybrid females to males of either parental strain mostly failed, while all other backcross combinations, including RC-hybrid males, yielded fertile clutches (Table 1). RC-hybrid females did mate successfully with their RC-hybrid brothers, although at significantly reduced rates (Table 1), similar to what was reported previously (Pashley & Martin, 1987; Whitford et al., 1988).

### Table 1

<table>
<thead>
<tr>
<th>Female strain</th>
<th>Male strain</th>
<th>No. paired</th>
<th>No. fertile</th>
<th>% fertile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn</td>
<td>Corn</td>
<td>181</td>
<td>109</td>
<td>0.60&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Corn</td>
<td>Rice</td>
<td>70</td>
<td>38</td>
<td>0.54&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Rice</td>
<td>Corn</td>
<td>60</td>
<td>28</td>
<td>0.47&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Rice</td>
<td>Rice</td>
<td>124</td>
<td>75</td>
<td>0.60&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>(C&lt;sup&gt;♀&lt;/sup&gt; × R&lt;sup&gt;♂&lt;/sup&gt;) hybrid</td>
<td>Corn</td>
<td>35</td>
<td>28</td>
<td>0.80&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>(C&lt;sup&gt;♀&lt;/sup&gt; × R&lt;sup&gt;♂&lt;/sup&gt;) hybrid</td>
<td>Rice</td>
<td>27</td>
<td>18</td>
<td>0.67&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>(R&lt;sup&gt;♀&lt;/sup&gt; × C&lt;sup&gt;♂&lt;/sup&gt;) hybrid</td>
<td>Corn</td>
<td>31</td>
<td>5</td>
<td>0.16&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>(R&lt;sup&gt;♀&lt;/sup&gt; × C&lt;sup&gt;♂&lt;/sup&gt;) hybrid</td>
<td>Rice</td>
<td>31</td>
<td>1</td>
<td>0.03&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>30</td>
<td>28</td>
<td>0.93&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Rice</td>
<td>(C&lt;sup&gt;♀&lt;/sup&gt; × R&lt;sup&gt;♂&lt;/sup&gt;) hybrid</td>
<td>23</td>
<td>15</td>
<td>0.65&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Corn</td>
<td>(R&lt;sup&gt;♀&lt;/sup&gt; × C&lt;sup&gt;♂&lt;/sup&gt;) hybrid</td>
<td>29</td>
<td>23</td>
<td>0.79&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Rice</td>
<td>(R&lt;sup&gt;♀&lt;/sup&gt; × C&lt;sup&gt;♂&lt;/sup&gt;) hybrid</td>
<td>22</td>
<td>14</td>
<td>0.64&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>(C&lt;sup&gt;♀&lt;/sup&gt; × R&lt;sup&gt;♂&lt;/sup&gt;) hybrid</td>
<td>(C&lt;sup&gt;♀&lt;/sup&gt; × R&lt;sup&gt;♂&lt;/sup&gt;) hybrid</td>
<td>73</td>
<td>66</td>
<td>0.90&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>(C&lt;sup&gt;♀&lt;/sup&gt; × R&lt;sup&gt;♂&lt;/sup&gt;) hybrid</td>
<td>(R&lt;sup&gt;♀&lt;/sup&gt; × C&lt;sup&gt;♂&lt;/sup&gt;) hybrid</td>
<td>22</td>
<td>12</td>
<td>0.55&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>(R&lt;sup&gt;♀&lt;/sup&gt; × C&lt;sup&gt;♂&lt;/sup&gt;) hybrid</td>
<td>(C&lt;sup&gt;♀&lt;/sup&gt; × R&lt;sup&gt;♂&lt;/sup&gt;) hybrid</td>
<td>22</td>
<td>5</td>
<td>0.23&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>(R&lt;sup&gt;♀&lt;/sup&gt; × C&lt;sup&gt;♂&lt;/sup&gt;) hybrid</td>
<td>(R&lt;sup&gt;♀&lt;/sup&gt; × C&lt;sup&gt;♂&lt;/sup&gt;) hybrid</td>
<td>37</td>
<td>13</td>
<td>0.35&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Different letters indicate significant differences (unplanned \( G \)-tests of independence, \( \alpha = 0.05 \)).
Since RC-hybrid females hardly ever mate successfully with males of either parental strain but may mate successfully with RC-hybrid males, this behavioural sterility could be extrinsic in nature. On the other hand, the significantly lower rate of RC interhybrid matings as compared to CR interhybrid matings suggests some intrinsic component to behavioural sterility. In either case, the behavioural sterility of RC-hybrid females in the laboratory predicts that gene flow across strains boundaries will be sex-biased with reduced female-mediated gene flow relative to male-mediated gene flow.

For the fall armyworm, assessments of rates and directionality of hybridisation in the field are based on discordancies between strain-specific mitochondrial haplotypes and nuclear markers (esterase genotypes, AFLPs or FR-repeat). Prowell et al. (2004) estimated that 16% of 162 field-collected individuals were potential hybrids with the majority being second or later generation. The observed discordancies between mitotype on the one hand and esterase or AFLPs on the other hand suggest that roughly equal proportions of hybrids derive from C × R and R × C matings. However, in a series of recent publications Nagoshi and co-workers (Nagoshi & Meagher, 2003a; Nagoshi et al., 2006b; Machado et al., 2008) used discordancies between mitotypes and the sex-linked tandem repeat sequence (FR-repeat) to infer directional interstrain mating biases. The FR-repeat is abundantly present in rice-strain genomes (FR⁺) but absent or present only in low copy numbers in the corn strain (FR⁰) (Lu et al., 1994; Nagoshi & Meagher, 2003b) and can be diagnosed by PCR amplification patterns. Surprisingly, in samples from Florida, Texas, and Brazil, substantial proportions of mt⁰FR⁰ genotypes were discovered, which can only be accounted for by RC-hybrid females backcrossing to corn males. In Florida and Texas, FR⁰ was found in the majority of all individuals that carried a rice-strain mitochondrial haplotype, while the reciprocal
configuration, $m^C{FR}^+$, was rarer (Nagoshi & Meagher, 2003a; Nagoshi et al., 2006b; Machado et al., 2008).

Since an $m^R{FR}^0$ hybrid combination can only arise as a result of RC-hybrid female × C-male backcrosses or RC-hybrid female × RC-hybrid male matings, these results disagree with the findings that under laboratory conditions this type of backcross happens only at a very low frequency, if at all (Pashley & Martin, 1987; Whitford et al., 1988, Table 1). Nagoshi and Meagher (2008) suggest that this high frequency genotype class might represent an additional hybrid subpopulation, besides the two recognized host strains, which could show different patterns of host use, behaviours, or susceptibilities to pesticides. While this is an intriguing possibility, an alternative explanation could be that the $m^R{FR}^0$ samples are not of hybrid origin after all. Z chromosomes of the rice strain harbour an about 100-fold lower copy number of the FR-repeat than the W chromosome (Lu et al., 1994; Nagoshi & Meagher, 2003a) and may additionally be naturally polymorphic with respect to copy number. Thus, rice-strain variants of the Z chromosome could exist that give rise to an amplification pattern similar to what is usually classified as the corn-strain variant. To clarify whether the observed $m^R{FR}^0$ genotypes indeed represent hybrids between the rice- and the corn-strain, it needs to be tested whether these individuals also exhibit discordant patterns with respect to the other known diagnostic markers, specifically the Z-linked Est3 allozyme and the strain-specific AFLP patterns.

**Permeability of the host strain genomes**

Overall, surprisingly few fixed nuclear genetic differences have been found between the two strains. Sequencing the nuclear ITS1 gene (Prowell, 1998) and an intron at the para sodium channel (Adamczyk et al., 1996) revealed no strain-specific or strain-biased variation. Studies using nuclear AFLP markers to identify
strain-specific markers found that only a small fraction of the AFLP loci exhibited diagnostic allelic differences (McMichael & Prowell, 1999; Busato et al., 2004; Prowell et al., 2004). For example, McMichael and Prowell (1999) investigated more than 1000 loci to find 10 strain-specific AFLP loci; Busato et al. (2004) examined more than 200 loci and detected two strain-specific loci. This suggests that less then 1% of the nuclear genome may be fixed for different alleles between the two strains. Loci that show such an excessive level of genetic differentiation between populations may identify parts of the genome that resist the homogenizing effects of between-strain gene flow, perhaps indicating regions that are under divergent selection between the two strains (Beaumont, 2005). Such a pattern is generally expected under divergence-with-gene-flow models of speciation (e.g. Emelianov et al., 2004; Via & West, 2008), and emphasizes that probably just a few key traits act to reduce inter-strain mating and reproduction between fall armyworm host races.

By contrast, large and consistent differences exist in the mitochondrial genome which forms two distinct strain-specific clusters of haplotypes (Lewter et al., 2006). When comparing two corn-strain populations from Florida (Homestead, Miami-Dade Co.; Hague, Alachua Co.) and two rice-strain populations from Florida (Ona, Hardee Co.), and Mississippi (Washington Co.), we found 11 fixed differences between rice- and corn-mitotypes in the 16S-ND1 mitochondrial region, (Tables 2 and 3), which corresponds to a mean pairwise sequence divergence (uncorrected) of 2.1% between the two strains. Combining this estimate with a previous estimate of observed divergence between strains for the mitochondrial cytochrome oxidase I and II genes (0.66%, Lewter et al., 2006), the average estimate of sequence divergence for a 1.1 kb portion of the mitochondrial genome is 1.3%, whereas within-strain mitochondrial haplotypes differed almost exclusively due to variation at a fast-evolving microsatellite locus.
**Table 2.** Table of polymorphic sites among fall armyworm haplotypes at a 516 bp fragment of the 16S-ND1 mitochondrial region*

<table>
<thead>
<tr>
<th>Position</th>
<th>0000111111</th>
<th>1111111111</th>
<th>1112222244</th>
<th>5</th>
</tr>
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<tbody>
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<td>Position</td>
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<td>0000111112</td>
<td>7883578977</td>
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</tr>
<tr>
<td>Position</td>
<td>3838012345</td>
<td>6789012346</td>
<td>6589898114</td>
<td>3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Sequence</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>A<em>T</em>T***** ****T TTTTAAACGA T</td>
<td></td>
</tr>
<tr>
<td>C2</td>
<td>..........T ATATATATAC AGAA.......</td>
<td></td>
</tr>
<tr>
<td>C3</td>
<td>.......... TATATA ..........</td>
<td></td>
</tr>
<tr>
<td>C4</td>
<td>.......... TATA ..........</td>
<td></td>
</tr>
<tr>
<td>R1</td>
<td>GTA*ATATAT ATATATATAC ....GGTGTAG A</td>
<td></td>
</tr>
<tr>
<td>R2</td>
<td>GTA*....... ....TA ......GGTGTAG A</td>
<td></td>
</tr>
<tr>
<td>R3</td>
<td>GTA*........ ....TA ......GGTGTAG A</td>
<td></td>
</tr>
</tbody>
</table>

*Sequences were obtained from 83 individuals from two corn-strain and two rice-strain populations (see text). Triangles identify fixed differences between corn-strain and rice-strain populations. Differences between mitochondrial haplotypes within strains are almost exclusively due to variation at a microsatellite locus.

**Table 3.** Frequency of fall armyworm corn- and rice-strain haplotypes at a 516 bp fragment of the 16S-ND1 mitochondrial region in two corn-strain and two rice-strain populations

<table>
<thead>
<tr>
<th>Location</th>
<th>Host</th>
<th>C1</th>
<th>C2</th>
<th>C3</th>
<th>C4</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homestead, Miami-Dade Co., FL</td>
<td>Corn</td>
<td>–</td>
<td>–</td>
<td>13</td>
<td>2</td>
<td>–</td>
<td>3</td>
<td>–</td>
</tr>
<tr>
<td>Hague, Alachua Co., FL</td>
<td>Corn</td>
<td>8</td>
<td>12</td>
<td>2</td>
<td>–</td>
<td>–</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td>Ona, Hardee Co., FL</td>
<td>Pasture</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>14</td>
<td>5</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Washington Co., MS</td>
<td>Pasture</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>23</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>
A preliminary estimate of divergence time between the corn- and the rice-strain can be based on a molecular clock rate estimate of 2.3% pairwise mtDNA sequence divergence per million years for various arthropod taxa (Brower, 1994). By assuming tentatively that the *S. frugiperda* mtDNA is evolving neutrally, the maximum uncorrected pairwise interstrain sequence divergence corresponds to an age of separation of the two strains of approximately 600,000 years. The difference in permeability between the nuclear genome and the mitochondrial genome supports the notion that female-mediated gene flow between the two strains may be more reduced than male-mediated gene flow.

**Conclusions**

Even though *S. frugiperda* has been the subject of numerous studies since the discovery of the two host strains in the 1980s, there are still many unresolved issues in how the two strains maintain their identity. Now that different reproductive isolation barriers have been recognized, it is important to assess their relative contribution to the reproductive isolation between the strains. The main questions that need to be resolved are the following. Is the occurrence on different host plants due to differential oviposition preference? What is the relative importance of the female sex pheromone differences versus the differential timing of reproduction in the assortative attraction of males in the field? Is there geographic variation in host preference and/or sexual communication and/or differential timing of reproductive activity and/or postzygotic incompatibility? What is the genetic basis of the reproductive isolation barriers? And finally, how do the different reproductive isolation barriers interact? Elucidating these questions will give insight into the speciation process that seems to be ongoing in this intriguing species.
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The roles and interactions of reproductive isolation mechanisms


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The roles and interactions of reproductive isolation mechanisms


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The roles and interactions of reproductive isolation mechanisms


Chapter 5


The roles and interactions of reproductive isolation mechanisms


Thompson, J.N. (1988) Evolutionary genetics of oviposition preference in swallowtail


Chapter 6

Evolution of reproductive isolation of *Spodoptera frugiperda*

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Book chapter in preparation

*In: Pheromone Communication in Moths: Evolution, Behavior and Application*, Jeremy Allison & Ring Cardé (eds.)


Introduction

*Spodoptera frugiperda*, the fall armyworm, is a noctuid moth occurring in North and South America with two host strains (a corn- and a rice-strain) identified in the 1980s (Pashley et al. 1985; Pashley 1986). These two strains were originally characterized by a polymorphism in an esterase allozyme marker and three other strain-biased protein variants in larvae collected from corn fields and rice paddies in Puerto Rico (Pashley et al. 1985; Pashley 1986). Since then, several additional strain-biased or strain-diagnostic molecular markers have been identified: the two strains differ in mitochondrial DNA sequences in the cytochrome oxidase I (COI) and NADH dehydrogenase I (ND1) genes (Pashley 1989; Pashley and Ke 1992; Lu and Adang 1996; Levy et al. 2002; Meagher and Gallo-Meagher 2003; Prowell et al. 2004; Nagoshi et al. 2006a; Machado et al. 2008). There are also strain-biased and strain-specific amplified fragment length polymorphisms (AFLP) (McMichael and Prowell 1999; Busato et al. 2004; Prowell et al. 2004; Clark et al. 2007; Martinelli et al. 2007; Juárez et al. 2012), restriction length fragment polymorphisms (RFLP) (Lu et al. 1992), a so-called Frugiperda Rice (FR) repetitive nuclear DNA sequence, present in high copy number in the rice-strain and mostly lower copy number in the corn-strain (Lu et al. 1994; Nagoshi and Meagher 2003b; Nagoshi et al. 2008) and nucleotide polymorphisms within the triose phosphate isomerase gene (Tpi, Nagoshi 2010).

Recently, sex pheromone differences have been found among populations of the two strains (Groot et al. 2008; Lima and McNeil 2009; Unbehend et al. 2012). However, these differences were not consistent among studies, suggesting that geographic variation may be confounded with strain-specific variation, or that pheromones may vary within strains as well. The relative importance of the pheromone differences between the two strains still needs to be established, i.e. are all pheromone compounds in the pheromone glands behaviorally important and/or
are males of the two strains differentially attracted to the different pheromone blends? Since other physiological, developmental, and behavioral differences have been found among the strains (Pashley and Martin 1987; Pashley 1988b; Pashley et al. 1992; Pashley et al. 1995; Veenstra et al. 1995; Meagher et al. 2004; Schöfl et al. 2009; Groot et al. 2010; Meagher et al. 2011; Schöfl et al. 2011; Meagher and Nagoshi 2012), this overview integrates strain-specific variation in sexual communication (variation in the pheromone gland composition as well as variation in male response) with other possible pre- and postmating barriers that likely contribute to isolation of the two strains. First, we will show that the naming of the two strains is somewhat misleading, as the host specificity of the two strains is not as clear-cut as the names suggest. Then we will focus on the two types of prezygotic isolating mechanisms that have been demonstrated to differ between the two strains: a) the diel pattern of reproductive activity, and b) variation in sexual communication (both signal and response). In addition to the premating barriers, we also consider postmating barriers that may isolate the two strains. Finally, based on recent findings, we discuss a possible evolutionary scenario for the evolution of the two strains of *S. frugipera*da.

**I. Are the two strains really host strains?**

Allozyme differences at five loci, including one apparently strain-specific esterase allele, provided the first evidence of partial genetic differentiation of populations collected from adjacent corn and rice fields in Puerto Rico and Louisiana (Pashley et al. 1985; Pashley 1986). Differences in mitochondrial DNA RFLP patterns were also found among these populations (Pashley 1988a). Subsequently, the same genetic differences were found in populations collected from other host plants and localities, and used to assign them to either the corn- or rice-strain. The so-called corn-strain was found to infest mainly corn (i.e. maize, *Zea mays*), sorghum,
(Sorghum bicolor subsp. bicolor), and cotton (Gossypium hirsutum), whereas the so-called rice-strain was found mostly in rice (Oryza sativa), sugar cane (Saccharum officinarum), and grasses such as Johnson grass (Sorghum halepense), and Bermuda grass, (Cynodon dactylon). Genetic differentiation between these two strains has been confirmed in several regions in North and South America, using different molecular markers. The host associations of the two strains are summarized below.

1a. Host associations based on mitochondrial COI polymorphism

Among all molecular markers available to distinguish the two strains, the most widely used target mitochondrial DNA. For example, the two strains show differences in their cytochrome oxidase I (COI) gene and can be identified by a polymorphism in the restriction sites for SacI and AcI (both present in rice-strain and absent in the corn-strain), and for Hinfl, BsmI and MspI (all present in corn-strain and absent in the rice-strain). The polymorphisms in SacI and MspI are used in most studies (Lu and Adang 1996; Levy et al. 2002; Meagher and Gallo-Meagher 2003; Nagoshi et al. 2006a). Based on the restriction site polymorphisms mentioned above, especially in the double digestion with SacI and MspI, the identity of the strains has been evaluated for different habitats and has been shown that the association is not always absolute.

Approximately 80% of individuals collected from corn habitats were identified as corn-strain and the remaining 20% as rice-strain (Pashley 1989; Lu and Adang 1996; Levy et al. 2002; Nagoshi et al. 2006a; Nagoshi et al. 2007b). However, exceptions from this percentage of distribution have been found as well: Prowell et al. (2004) identified samples collected from corn predominantly (i.e. 50% or more of the individuals) as rice-strain in French Guiana as well as in Louisiana. Nagoshi et al. (2006a) also found mostly rice-strain individuals in a sorghum field in Texas,
which is considered a corn-strain habitat. In the case of larvae collected from rice fields, up to 95% of individuals have been identified as rice-strain (Nagoshi and Meagher 2003a, 2004; Machado et al. 2008; Velez-Arango et al. 2008). Recently, Juárez et al. (2012) did not find a consistent pattern between the two strains and their respective host plants either (especially, in rice habitats), when using COI markers in South American populations.

Some of these shifts in strain distributions may be due to seasonal and temporal variation in the distributions of the two strains and in the distribution of available plant hosts or by different migration patterns of the two strains (Nagoshi et al. 2007a). For example, Nagoshi et al. (2007c) showed that the corn-strain predominated in collection from sorghum in the fall (March-June) in Brazil and reduced in frequency in spring (September-November), while in Florida rice-strain larvae predominated in collections made in the fall (September-November), and corn-strain larvae were mostly present in the spring season (February-April). In Louisiana, Pashley et al. (1992) found that corn strain populations were detected in the corn fields in the spring, while rice strain populations remained at low density on various grasses until late summer when they increased in number. Together, these findings suggest that the migration pattern of the two strains may not be the same (Nagoshi and Meagher 2004).

In Figure 1 we provide an overview of collections of the fall armyworm over a period of 27 years (from 1983 until 2010) from a number of different habitats. Most individuals from 17 out of 20 populations, sampled from predominantly rice habitats (rice and pasture/Bermuda grass), were identified as rice-strain, whereas most individuals from 29 out of 44 populations from predominantly corn habitats (corn, cotton and sorghum) were identified as corn-strain.
**Figure 1.** Distribution of *Spodoptera frugiperda* host strains in different habitats and geographic regions. Each bar shows the percentage of the identified strains per collection, based on mitochondrial markers. Habitats are indicated on the right. Numbers in [ ] indicate total n of collection. MS-Mississippi, BR-Brazil, AR-Argentina, LA-Louisiana, FL-Florida, PY-Paraguay, PR-Puerto Rico, NC-North.
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Although mitochondrial markers generally show a strong correlation between strain type and host plant, in many of collections this association is lacking, especially in predominantly corn habitats (see Figure 1).

\textit{1b. Host associations based on genome-wide AFLP markers}

Although some studies have found a close association between the two strains and their host plants using AFLP markers (e.g., McMichael and Prowell 1999; Busato et al. 2004 in the USA and Brazil, respectively), others have not (e.g., Martinelli et al. (2007) in Mexico, Brazil, Argentina and the USA). Recently, we found that individuals from populations collected from corn plants tended to cluster together and showed a high degree of homogeneity (Juárez et al. 2013). This finding thus contrasts the trend found in the COI marker, where 15 out of 44 populations collected from corn (see Figure 1) showed a significant portion of rice-strain individuals. Individuals from the populations collected from rice formed three distinct groups and showed a much higher level of heterogeneity in their AFLP markers (Juárez et al. 2013). Overall, individuals collected from corn-strain habitats were clustered separately from individuals collected from rice-strain habitats, although there were some marked exceptions (Juárez et al. 2013).
1c. Host association based on mitochondrial and nuclear markers

Combining mitochondrial and nuclear markers with their different modes of inheritance, the rate and directionality of hybridization between the strains in the field can be identified. Prowell et al. (2004) analyzed populations from Louisiana, Florida, Puerto Rico, Guadeloupe, and French Guiana with different molecular markers (mitochondrial haplotype, esterase genotypes, AFLPs) and reported that 16% of the samples were potential hybrids due to discordance for at least one marker. The authors found evidence of crosses between the strains in both directions: when using mtDNA and esterase markers, 66% of the hybrids were inferred to be derived from rice-strain females mated with corn-strain males, i.e. RC hybrids, while in multilocus comparison using the three markers, 54% of the hybrids were RC hybrids and 46% were from the reciprocal cross, i.e. CR hybrids. In addition, Prowell et al. (2004) found that these hybrids occurred mostly in the corn habitats. Similar results were found by Saldamando and Vélez-Arango (2010) with Colombian populations. In contrast, Nagoshi and Meagher (2003b) and Nagoshi et al. (2006b), using mitochondrial haplotypes and the nuclear FR tandem-repeat sequence, found mainly RC-hybrids (56%), while CR-hybrids were rare or absent, and hybrids occurred in both corn and rice habitats.

Recently, Nagoshi (2010) identified 10 polymorphic diagnostic sites in the Z-linked (sex-linked) triose phosphate isomerase (Tpi) gene that can be associated with the corn- or rice-strain of the fall armyworm (as in Lepidoptera females are the heterogametic sex, ZW). With this marker, Nagoshi (2012) analyzed 12 populations (9 collected from corn and 3 from rice) with the COI marker, and then reanalyzed the same samples with the Tpi marker, and found that 60% and 7% of the COI-R typed individuals were Tpi-C in the corn and rice habitats, respectively (i.e. RC hybrids). The reverse constellation, COI-C and Tpi-R (i.e. CR hybrids), occurred in 8% and 22% of the COI-C typed individuals from corn and rice
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habitats, respectively. Like Nagoshi (2012), when we combined the COI marker with the Z-linked Tpi marker, we also found a high percentage of hybrids (43%) (Juárez et al. 2013). These hybrids consisted of four different combinations: RC (33% of all hybrids), CR (5% of all hybrids), CI (25% of all hybrids), and RI (37% of all hybrids). The I stands for a Tpi-intermediate haplotype, i.e. individuals in which corn and rice SNPs were present in similar proportions or heterozygous individuals in which SNPs showed the two alternative nucleotides. The latter individuals must be hybrid males, as in Lepidoptera the females carry only one copy of the Z-linked Tpi gene. Nagoshi (2010) and Nagoshi et al. (2012) also found this intermediate configuration in a very low frequency and proposed that they may represent hybrid individuals as well.

In summary, both types of hybrids seem to occur in nature, although recent studies suggest that the RC-hybrids are more common. These hybrids are mostly found in corn habitats, while other hybrids (CR, CI, RI) are mostly found in rice habitats. Overall, the two strains seem to be predominantly found in the habitats from where they were originally described, but significant exceptions have been found with all markers used. Therefore, our preliminary conclusion is that divergence between the strains is not likely due to host plant specialization, or at least not alone. We hypothesize that an interaction between ecological and behavioral mechanisms has contributed to reproductive isolation between the two strains (Groot et al. 2010).

II. Behavioral isolation mechanism 1: Timing of reproductive activity at night

Differences in the diel pattern of mating activity between strains would create a powerful barrier to hybridization. Strain-specific differences in the timing of reproductive activity of the two strains have been consistently found, independent
of the geographic origin of the strains (Pashley et al. 1992; Schöfl et al. 2009; Schöfl et al. 2011): the corn-strain is active early in the scotophase, while the rice-strain is active late in the scotophase. Schöfl et al. (2009) showed that different reproductive behaviors (calling, copulation and oviposition) are differentially inherited and thus under complex genetic control, and suggested the involvement of the circadian clock.

When testing whether allochronic separation causes assortative mating in the laboratory, Schöfl et al. (2011) found an interaction between strain-specific timing of mating and time-independent intrinsic preferences that influenced the mating choice of both strains. Furthermore, mate choice changed over time in consecutive nights and was influenced by the timing of introduction of the mating partners, i.e. at the onset of the scotophase or 6 hours after the onset of scotophase (Schöfl et al. 2011). In general, females were more restricted in their mate preference than males and approximately 30% of the isolation between both strains was generated by female mate preference, suggesting the involvement of a male-specific sex pheromone that mediate close-range courtship behavior (Schöfl et al. 2011). Also, this mate-choice experiment indicates that the level of assortative mating caused by allochronic separation alone is not strong enough to cause reproductive isolation between strains. Although the importance of differential timing of reproduction is probably not as strong as suggested by Pashley et al. (1992), the consistent timing differences between the strains, independent of the geographic origin, suggests that this behavioral difference could have a stronger influence as prezygotic isolation barrier than host plant choice. Therefore, we are tempted to argue that both strains are “timing strains” rather than “host strains.”
III. Behavioral isolation mechanism 2: Variation in sexual communication

In the early 1990s, Pashley et al. (1992) found that males of both strains showed a slight preference for females of the same strain, 60-65% of corn- and rice-strain males being attracted to corn- and rice-strain females, respectively. These findings indicate that pheromone differences might be important for mate choice and cause assortative mating in the two strains, although Pashley et al. (1992) suggested that “pheromone chemistry may play a small role (if any) in strain separation”. The sex pheromone composition of *S. frugiperda* females has been studied in different geographic regions (Mitchell et al. 1985; Tumlinson et al. 1986; Descoins et al. 1988; Batista-Pereira et al. 2006; Groot et al. 2008; Lima and McNeil 2009; Unbehend et al. 2013). While earlier studies mainly focused on the general composition of the female sex pheromone without distinguishing the two strains, later studies investigated strain-specific differences in the female pheromone composition (Groot et al. 2008; Lima and McNeil 2009; Unbehend et al. 2013).

In general, the fall armyworm sex pheromone consists of the primary sex pheromone component Z9-14Ac and the critical secondary sex pheromone Z7-12Ac (Tumlinson et al. 1986; Batista-Pereira et al. 2006; Groot et al. 2008; Lima and McNeil 2009). The behavioral effect of other secondary compounds in the female gland, is not clear so far (Tumlinson et al. 1986; Andrade et al. 2000; Fleischer et al. 2005; Groot et al. 2008; Unbehend et al. 2013). However, twice as many males were caught when Z11-16Ac or Z9-12Ac were added to the binary blend (Fleischer et al. 2005), suggesting at least a synergistic effect of these compounds. It has been shown that corn- and rice-strain females exhibit strain-specific differences in their relative amount of Z7-12Ac (relative to the amounts of other gland compounds), as well as in the relative amount of Z9-14Ac, Z11-16Ac
and Z9-12Ac, although the type of variation found seems to vary in different geographic regions (Groot et al. 2008; Lima and McNeil 2009; Unbehend et al. 2013).

**Disentangling geographic from strain-specific variation**

Extractions of the pheromone glands of females from a colony, that was initiated with larvae collected in Florida, revealed that rice-strain females produce significantly higher relative amounts of Z7-12Ac and Z9-12Ac, and lower relative amounts of Z11-16Ac, than corn-strain females (Groot et al. 2008). However, laboratory rice-strain females originating from Louisiana contained lower relative amounts of the major component Z9-14Ac, as well as larger relative amounts of Z7-12Ac and Z11-16Ac, compared to laboratory corn-strain females from Louisiana (Lima and McNeil 2009). Taken together, only Z7-12Ac showed consistent strain-specific variation in females from Florida and Louisiana (Groot et al. 2008; Lima and McNeil 2009; Unbehend et al. 2013). This is not too surprising, as Z7-12Ac is a critical component for male attraction and thus likely to be under stabilizing selection. The importance of Z11-16Ac and Z9-12Ac in the attraction of fall armyworm males is not completely understood yet, but their variation suggests that these components are not under strong stabilizing selection.

Geographic variation in the strain-specific pheromone composition of females from Florida and Louisiana may be related to different haplotype profiles in Floridian and Louisianan corn-strain populations. There seem to be two main migration routes of the fall armyworm, based on haplotype patterns in the corn-strain (Nagoshi et al. 2008; Nagoshi et al. 2010). These patterns suggest an eastern migration route, i.e. populations originating from Puerto Rico and Florida move northwards to Georgia, and a western migration route, i.e. populations from Texas move northeastwards to Louisiana, Mississippi, Alabama and Pennsylvania.
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(Nagoshi et al. 2008; Nagoshi et al. 2009). If no other geographic effects influence the female pheromone, then pheromone profiles of females from Texas, Louisiana, Mississippi, Alabama and Pennsylvania may be more similar to each other than to pheromone profiles of females from Florida, Puerto Rico and Georgia.

In fall armyworm females from Brazil, another minor sex pheromone component, E7-12Ac, was identified to be attractive to Brazilian males in the field (Batista-Pereira et al. 2006). Addition of E7-12Ac to binary blends, containing Z9-14Ac and Z7-12Ac, significantly increased the number of males captured in Brazil, i.e. from an average of 70 males per trap to an average of 100 males per trap (Batista-Pereira et al. 2006). The fact that E7-12Ac has not been found in females from Florida, Louisiana or French Guyana (Descoins et al. 1988; Groot et al. 2008; Lima and McNeil 2009) suggests the existence of geographic variation in female pheromone production. In conclusion, the two *S. frugiperda* strains do differ in their female sex pheromone composition (Groot et al. 2008; Lima and McNeil 2009; Unbehend et al. 2013), but geographic variation seems to influence the strain-specific pheromone production. To disentangle geographic from strain-specific variation, additional strain-specific pheromone extractions of different populations from North and South America will be necessary.

**Variation in pheromone composition within the strains**

In addition to strain-specific and geographic variation in the pheromone composition, pheromone differences within females of both strains have been observed between artificially-reared and field-collected females (Unbehend et al. 2013). Females of both laboratory strains produced significantly lower relative amounts of the major pheromone component Z9-14Ac and usually higher relative amounts of Z7-12Ac, Z11-16Ac and Z9-12Ac, compared to the field-collected females, although strain-specific pheromone variation was maintained (Unbehend
To estimate how much within-strain variation occurs in nature, we analyzed the pheromone composition of females from seven different corn-strain families, originating from single pair matings of individuals that were collected one generation earlier from a corn field in Florida (Marr 2009). The females of these families exhibited significant differences in their pheromone composition compared to our laboratory populations (Marr 2009). The variation of Z9-14Ac, Z7-12Ac, Z11-16Ac and Z9-12Ac was strongly heritable and a broad-sense heritability analysis showed that the variation in gland compounds within the different families is determined mainly by genetic rather than environmental effects (Marr 2009). However, the within-strain variation found in laboratory- and field-females, in addition to the geographic variation, indicates that laboratory rearing and environmental factors influence the pheromone composition of females. The challenge is to determine which factors may cause variation in the pheromone composition and why. Understanding the cause of variation in the pheromone composition and its genetic control will be important to understand how variation in sexual communication influences reproductive isolation and how sexual communication systems may evolve (Baker and Cardé 1979; Löfstedt 1993; Butlin and Trickett 1997; Ritchie 2007; Bergen et al. 2012).

**Male response to strain-specific pheromone**

The existence of strain-specific sex pheromone blends can only contribute to differentiation between the strains if this leads to differential attraction of fall armyworm males in the field. Although several trapping experiments of *S. frugiperda* males have been conducted in the field (Mitchell et al. 1985; Tumlinson et al. 1986; Meagher and Mitchell 1998; Andrade et al. 2000; Batista-Pereira et al. 2006), only one investigated strain-specific differences in the male attraction toward different pheromones (Pashley et al. 1992). In Louisiana fields containing both host plants, 60% of all rice-strain males trapped in pheromone traps were
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attracted to a virgin rice-strain female, while 65% of all trapped corn-strain males were caught in traps baited with virgin corn-strain females (Pashley et al. 1992). Thus, males of both strains exhibited only a slight bias toward females of their own strain in mixed habitats, suggesting that strain-specific sexual communication is a weak prezygotic isolation barrier (Pashley et al. 1992). Similarly, Lima and McNeil (2009) argued that is quite unlikely that strain-specific sex pheromone differences alone “would be sufficient to ensure reproductive isolation of the two strains.”

To evaluate whether fall armyworm males exhibit strain-specific attraction towards females of their own strain, we conducted wind tunnel choice assays and male trapping experiments in Florida (Unbehend et al. 2013). Wind tunnel experiments without plant volatiles revealed that *S. frugiperda* males from laboratory populations show no strain-specific attraction to virgin females of their own strain. Interestingly, males of both strains were mainly influenced by the timing of female calling, and did not discriminate among calling females (Unbehend et al. 2013). However, when testing pheromone lures mimicking the four-component pheromone blend of Floridian corn-strain females (i.e. 100% Z9-14Ac, 13% Z11-16:Ac, 2% Z7-12Ac, 1% Z9-12Ac), 74% of all trapped corn-strain males in a corn field were attracted to this corn-strain lure, and only 26% to the rice-strain lure, i.e. 100% Z9-14:Ac, 8% Z11-16:Ac, 4% Z7-12:Ac, 2% Z9-12:Ac (Figure 2). In rice fields, such a strong strain-specific attraction was not found, and only 59% of all trapped corn-strain males were attracted to the synthetic corn-strain lure, while 41% were attracted to the rice-strain lure (Figure 2). This result suggests that strain-specific attraction to different lures depends on the respective (volatile) environment, and hints to a synergistic effect of sex pheromones and host plant volatiles. However, similar to corn-strain males, rice-strain males were also mostly attracted to the synthetic corn-strain lure in the corn field and 76% of all trapped rice-strain males were caught in traps baited with the corn-strain lure (Figure 2).
The pheromone traps that were baited with the so-called rice-strain lure (100% Z9-14:Ac, 8% Z11-16:Ac, 4% Z7-12:Ac, 2% Z9-12:Ac) did not specifically attract rice-strain males in a grass field and only 49% of all trapped rice-strain males were attracted to the rice-strain lure (Unbehend et al. 2013). Together, these results

Figure 2. Mean percent of corn-strain and rice-strain males caught in sex pheromone traps baited with synthetic pheromone lures in a corn field and a grass field in Florida. The corn-strain blend consisted of 300 µg Z9-14:OAc, which was considered 100%, 6 µg (2%) Z7-12:OAc, 39 µg (13%) Z11-16:OAc and 3 µg (1%) Z9-12:OAc. The rice-strain blend was constructed in a similar way, only with 12 µg (4%) Z7-12:OAc, 24 µg (8%) Z11-16:OAc and 6 µg (2%) Z9-12:OAc. Numbers in the bars indicate total number of males caught. See Unbehend et al. (2013) for more details. ** P < 0.01; * P < 0.05; NS: not significant.
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indicate that corn-strain lures are most attractive for both strains in a corn habitat, while there is no preference for a corn- or rice-strain lure in a rice habitat.

**Importance of different pheromone components for male attraction in the field**

To assess strain-specific male response towards the different pheromone components, we also evaluated the importance of single pheromone components in the attraction of corn- and rice-strain males in a corn and a grass field in Florida (Unbehend et al. 2013). As mentioned earlier, fall armyworm males can vary in their attraction towards E7-12Ac, but show stable geographic-independent attraction towards binary blends containing Z9-14Ac and Z7-12Ac (Tumlinson et al. 1986; Andrade et al. 2000; Fleischer et al. 2005; Batista-Pereira et al. 2006; Unbehend et al. 2013). We tested different doses of the critical secondary component Z7-12Ac and found that corn-strain males were most responsive to lures containing 2% Z7-12Ac, whereas rice-strain males showed a wider response range, as they were attracted to lures containing 2%, 4% or 10% Z7-12Ac (Unbehend et al. 2013; Figure 3). This strain-specific male response is consistent with the strain-specific female pheromone production in Florida, at least in the corn-strain, because corn- and rice-strain females produce around 2% and 4% Z7-12Ac, respectively (Groot et al. 2008; Unbehend et al. 2013). These results suggest that fall armyworm corn-strain males in Florida are adapted to the strain-specific female pheromone differences in the amount of Z7-12Ac, i.e. 2% versus 4%. To our knowledge, this is the first time that such a small change in a pheromone component has such a pronounced effect on male attraction.
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The relative importance of Z11-16Ac is still unclear. In Costa Rica, the ternary blend of Z11-16Ac, Z9-14Ac and Z7-12Ac captured marginally more males than the binary blend of Z9-14Ac and Z7-12Ac in one test and marginally fewer in another, although neither effect was statistically significant (Andrade et al. 2000). Similarly, addition of Z11-16Ac to binary blends did not significantly increase trap catches in Brazil (Batista-Pereira et al. 2006) or Florida (Tumlinson et al. 1986; Unbehend et al. 2012). However, trapping experiments in Pennsylvania indicate that the addition of Z11-16Ac, together with Z9-12Ac, enhances male attraction to Z9-14Ac and Z7-12Ac (Fleischer et al. 2005), and field experiments in Florida showed that addition of Z11-16Ac, together with Z9-12Ac, to Z9-14Ac and Z7-12Ac changed the response of corn- and rice-strain males in different fields (Unbehend et al. 2013).

Figure 3. Strain-specific response of Spodoptera frugiperda males towards different doses of Z7-12:OAc added to 300 µg Z9-14:OAc in a corn and grass field in Florida. Different letters next to the bars indicate significant differences. Numbers in the bars indicate total number of males caught. See Unbehend et al. (2013) for more details.
The compound Z9-12:Ac has been reported to occur in glands of females from North and South America (Descoins et al. 1988; Batista-Pereira et al. 2006; Groot et al. 2008). In Costa Rica and Florida, fall armyworm males were attracted to traps containing only Z9-12Ac (Jones and Sparks 1979; Andrade et al. 2000). When conducting experiments where we added different relative amounts of Z9-12Ac to the binary blend of Z9-14:Ac and Z7-12:Ac, we found that all tertiary blends containing Z9-12Ac were similarly attractive as the binary blends without Z9-12Ac, both in corn-and rice-strain habitats in Florida (Unbehend et al. 2013). However, since the addition of Z9-12Ac and Z11-16Ac to binary blends also changed the attraction of corn- and rice-strain males in Florida (Unbehend et al. 2013), a synergistic effect of Z9-12Ac in combination with other components cannot be excluded.

In summary, corn- and rice-strain males in Florida were mostly attracted to a corn-strain pheromone blend, at least in corn fields. Thus, there may be synergistic effects of host plant volatiles and sex pheromone components in corn fields. In grass fields, we did not find a preference for a corn- or a rice-strain pheromone blend in either strain. Strain-specific responses were found towards different doses of Z7-12Ac added to the major pheromone component Z9-14Ac, where corn-strain males were mostly attracted to 2% Z7-12Ac and rice-strain males were attracted to a wider range (2-10%). Together, these data suggest that strain-specific differences in the sexual communication of both strains do not cause assortative mating in Florida and thus are a weak prezygotic isolation barrier between the corn- and the rice-strain.

**IV. Level and direction of hybridization between the two strains**

The fact that hybridization between the two strains can be observed in the field raises the question: are these strains in the process of divergence or convergence?
RC-hybrid females have been found to be less likely to mate with any kind of male (C, R, RC or CR) and to produce a lower number of egg masses when they do mate (Pashley and Martin 1987; Whitford et al. 1988; Groot et al. 2010). Interestingly, RC-hybrid males did not show this deficiency and mated readily with all types of females (C, R and CR) (Groot et al. 2010). The fact that RC hybrid females are found to be mostly sterile in laboratory experiments seems to conflict with the field observation where mainly RC hybrids are found (see Section I). However, this contradiction makes the “reproductive problem” of RC hybrid females a perfect postzygotic isolation barrier: if the most abundant individuals are at the same time the least fertile ones, gene flow is maximally prevented at this stage. This thus indicates that these strains are in the process of divergence rather than convergence. Since reproductive isolation barriers are believed to act sequentially in the life cycle of hybridizing taxa (Schemske 2000; Ramsey et al. 2003), postzygotic phenomena like the evolution of RC hybrid female sterility do not appear to be the first step in speciation. However, given the existence of RC hybrid females in the field, while in the laboratory these hybrid females are hardly able to reproduce, this hybrid incompatibility represents an essential contribution to the process of speciation between the two strains.

V. Possible evolutionary scenarios on reproductive isolation in the two strains

Since the host association of the two strains does not seem to be as strict as early studies indicated, ecological specialization does not seem the most likely cause of differentiation between the two strains in S. frugiperda. Other factors may have influenced a host association between the strains. One of these factors may be the presence of competitors or natural enemies on the ancestral host as has been suggested for other phytophagous insects (Berlocher and Feder 2002). Pashley et
al. (1995) reported that over a two year period, fall armyworm larval mortality caused by parasites, predators and pathogens was higher in pastures than in corn fields. For this reason, the corn habitat may constitute a more protected environment than the rice habitats.

On the basis of the distribution of the two strains, particularly the distribution of the respective hybrids, and the behavioral differences between the two strains, we hypothesize that the rice strain is the ancestral strain and corn the derived strain (Juárez et al. 2013). Higher levels of genetic and behavioral homogeneity observed in the corn- than the rice-strain suggests that the corn-strain went through a bottleneck, i.e. that the corn-strain arose from a few individuals. Additionally, in corn fields a significant portion of rice-strain individuals as well as hybrids are found, specifically RC hybrids, while in rice fields the percent of corn-strain individuals or hybrids is generally much lower (Prowell et al. 2004; Saldamando B and Vélez-Arango 2010). The observation that males of both strains are mostly attracted to a corn-strain sex pheromone blend in corn fields, while this preference is not found in rice fields, is consistent with these results. Hybrid incompatibility is between R mothers and C fathers and not vice versa, i.e. RC hybrids are incompatible with any kind of male, whereas CR hybrids produce fertile and viable offspring. Together, these findings suggest that the rice-strain is the ancestral strain and the corn-strain is the derived strain.

**Conclusion**

In reviewing many studies on the host plant association of the two strains, host associations do not seem to be consistent when the mitochondrial COI marker is considered. In corn fields, more rice strain individuals seem to be found than vice versa, and RC hybrids are also mostly found in corn habitats. Thus, habitat isolation alone does not seem to be strong prezygotic isolation barrier between the
corn- and the rice-strain. Similarly, strain-specific differences in the sexual communication system of both strains alone do not appear strong enough to cause assortative mating between strains. However, differences in diel patterns of reproductive behaviors seem to be much more consistent than host-plant associations or differential sexual communication between the strains. Since a shift in timing can immediately inhibit gene flow, the strains may be “timing strains” rather than “host strains” or “pheromone strains”. Furthermore, the postmating barrier of RC hybrid female sterility seems to be most likely a key element in the divergence of these two strains.

Acknowledgements
This research was funded by the Deutsche Forschungsgemeinschaft (P.S.GR 362721), the National Science Foundation (award IOS-1052238), and the Max Planck Gesellschaft.

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Chapter 6


Evolution of reproductive isolation of *S. frugiperda*


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Evolution of reproductive isolation of *S. frugiperda*


Chapter 7

Discussion
1. Importance of sexual communication differences in strain divergence

The main aim of my thesis was to investigate sexual communication differences between corn- and rice-strain individuals in order to assess whether strain-specific pheromonal communication acts as prezygotic mating barrier between the two S. frugiperda strains. Another aim was to distinguish strain-specific variation from geographic variation and to determine the genetic basis of female pheromone differences. Based on the strain-specific variation found in signal and response, the importance of sexual communication as a prezygotic isolation barrier will be discussed and I will propose how sexual communication differences may have evolved.

1.1 Variation in the female sex pheromone signal

1.1.a Strain-specific female pheromone composition

Pheromone extractions showed that corn-strain females from Florida produced consistently lower relative amounts of the important secondary sex pheromone component Z7-12:OAc (and of Z9-12:OAc) than rice-strain females, while we found no strain-specific differences in the amounts of Z9-14:OAc and Z11-16:OAc (Chapter 2). A QTL analysis of the female sex pheromone showed that the strain-specific pheromone composition is under complex genetic control and multiple genomic regions are involved in the production of Z7-12:OAc, Z9-12:OAc, Z9-14:OAc and Z11-16:OAc (Chapter 4). Interestingly, the circadian clock protein vrille, which appears to be responsible for the strain-specific differences in the onset time of mating in the scotophase (Hänniger et al., 2013), mapped to one QTL for Z7-12:OAc (Chapter 4). This result suggests that two different prezygotic mating mechanisms, i.e. sex pheromone production of Z7-12:OAc (Groot et al., 2008; Chapter 2), as well as strain-specific timing of reproduction in the night
(Schöfl et al., 2009; Hänniger et al., 2013), might be genetically coupled, which could facilitate the divergence of both strains.

Although we showed that sex pheromone differences can, at least partly, be explained by genetic differences between strains (Chapter 4), the fact that we only found minor QTL ($R^2 \approx 4\% - 10\%$) suggests that other factors, like environmental variation, also influence the strain-specific sex pheromone composition. So far, it is unclear whether environmental factors like temperature, humidity, photoperiod, host plant volatiles, and/or interspecific olfactory cues can influence the sex pheromone composition of *S. frugiperda*, as it has been shown for several other moth species (McNeil and Delisle, 1989; Royer and McNeil, 1991, 1993; Delisle and Royer, 1994; Landolt and Phillips, 1997; Gemeno and Haynes, 2001; Groot et al., 2010a). Most likely, all these factors could have an effect on *S. frugiperda* females of both strains, because the pheromone composition of corn- and rice-strain females appears to be quite variable depending on different factors, as discussed below.

### 1.1.b Geographic variation in the female pheromone signal

In addition to strain-specific variation, geographic variation seems to be an important factor that can influence the sex pheromone of corn- and rice-strain females (Tumlinson et al., 1986; Batista-Pereira et al., 2006; Groot et al., 2008; Lima and McNeil, 2009; Chapter 2). Strain-specific differences between corn- and rice-strain females from Florida (Groot et al., 2008; Chapter 2) and Louisiana (Lima and McNeil, 2009) indicate geographic variation in Z9-14:OAc and Z11-16:OAc, whereas the critical secondary component Z7-12:OAc did not vary between these regions. In addition, geographic variation has been found in the relative amount of E7-12:OAc (Tumlinson et al., 1986; Descoins et al., 1988; Batista-Pereira et al., 2006; Groot et al., 2008). So far, it is unclear whether females
of both strains differ in their level/degree of geographic variation and thus additional research is required to disentangle variation caused by strain-specific differences from variation based on geographic differences.

1.1.c Effect of PBAN on the female pheromone composition

Analysis of pheromone extracts of laboratory corn- and rice-strain females from Florida showed that the pheromone biosynthesis activating neuropeptide (PBAN) can influence the female sex pheromone composition (Groot et al., 2008; Marr, 2009). More precisely, PBAN injection into the abdomen of corn- and rice-strain females, 1-2 hours before the pheromone gland extraction, significantly increased the relative amount of Z9-14:OAc and Z11-16:OAc in rice-strain females, and decreased the relative amount of Z7-12:OAc and Z9-12:OAc in corn- and rice-strain glands, compared to extractions in the scotophase without PBAN (Groot et al., 2008). In general, it appears that PBAN injections have greater and more consistent effects on the relative amount of minor abundant pheromone compounds (Z7-12:OAc, Z9-12:OAc) than on highly abundant pheromone compounds (Z9-14:OAc, Z11-16:OAc) (MU, unpublished data). Thus, although the use of PBAN has been described as functional to determine the native pheromone phenotype in moths like *Heliothis virescens* or *H. subflexa* (Groot et al., 2005), pheromone extractions of *S. frugiperda* should be conducted only under natural conditions, i.e. within the scotophase at strain-specific female calling times (Pashley et al., 1992; Schöfl et al., 2009).

The fact that PBAN injections can change the relative amount of different pheromone compounds in corn- and rice-strain females suggests that strain-specific differences in the female pheromone composition, e.g. in the relative amount of Z7-12:OAc (Groot et al., 2008; Lima and McNeil, 2009), could be caused by strain-specific PBAN differences, if PBAN would strain-specifically interact with
pheromone biosynthesis enzymes in corn- and rice-strain females. Groot et al. (2008) suggested that PBAN could act on the reduction step of fatty acids, i.e. on the fatty acyl reductase (FAR), or could alternatively activate strain-specific Δ11-desaturases, which still needs to be investigated.

1.1.d Intra-strain specific variation and effect of laboratory rearing

Pheromone gland extractions of corn-strain females from field Florida populations showed that females of different families exhibited significant differences in their relative amount of Z9-14:OAc, Z11-16:OAc, Z7-12:OAc and Z9-12:OAc (Marr, 2009). Thus, corn-strain females exhibit intra-strain between-family variation in their pheromone composition. An intra-strain pheromone study on rice-strain field females has not been conducted so far and therefore, we cannot state that this kind of variation also occurs in rice-strain females, although this will most likely be the case. Genetic analyses showed that the intra-strain specific variation in corn-strain females appears to be mainly determined by genetic effects, although environmental variation also seems to influence the female pheromone composition (Marr, 2009).

When we compared the pheromone composition of corn- and rice-strain females from laboratory and field population from Florida, we found significant differences within both strains between both populations, i.e. a significant increase in the amount of Z7-12:OAc, Z11-16:OAc and Z9-12:OAc, in at least one of both laboratory strains compared to field females (Chapter 2). The fact that laboratory rearing can change the pheromone composition of females has been shown for some moth species (Miller and Roelofs, 1980; Löfstedt et al., 1985; Haynes and Hunt, 1990), and is probably caused by changes in selection pressures, e.g. in the laboratory, females and males will get mated without the need to attract or be attracted. We used single pair matings to maintain our laboratory populations, but
thus forced a female to mate with one random male, which was the only mating partner available. Mating experiments with laboratory corn- and rice-strain populations showed that females are more restricted in their mate choice than males, which suggests the possible involvement of close-range male sex pheromones (Schöfl et al., 2011). Thus, the use of single pair matings might reduce possible selection pressures linked to mate choice in *S. frugiperda*, which could in turn cause a change in the female pheromone signal.

1.2 Variation in the male response to sex pheromone signals

1.2.a Habitat and geographic variation in the male response

Wind tunnel assays and field experiments in Florida showed that corn- and rice-strain males exhibit no consistent attraction to strain-specific female pheromone blends, but habitat-specific differences seem to influence the response of males to pheromone blends (Chapter 2 and 3). Most likely, host plant volatiles mediate the sexual attraction of corn- and rice-strain males, due to the fact that both strains exhibit some host-plant preferences (Pashley, 1986, 1989; Nagoshi et al., 2007; Machado et al., 2008; Groot et al., 2010b), and show differential electro-antennographic (EAG) responses to different plant volatiles (Malo et al., 2004b).

When we tested the male response towards different pheromone lures in different regions, we found that mainly corn-strain males, not rice-strain males, exhibited geographic variation in their attraction (Chapter 3). This suggests that rice-strain males exhibit a broader response spectrum towards sex pheromone blends than corn-strain males. However, due to a limited number of experiments conducted so far (Chapter 2 and 3), we cannot exclude that some environmental factors also affect the response of rice-strain males. Observations during wind tunnel experiments showed that this might be the case, because we observed that males of
both strains were highly influenced by specific experimental conditions, i.e. temperature, humidity and wind speed (Chapter 2). In addition, geographic variation in the male response to E7-12:OAc has been reported (Batista-Pereira et al., 2006; Chapter 3), although it is unclear whether males of both strains differ in their response to E7-12:OAc, and if geographic variation influences a possible strain-specific attraction to E7-12:OAc.

1.2.2 Strain-specific male response to the critical secondary component Z7-12:OAc

Trapping experiments in Florida showed that males of both strains differ significantly in their response to different amounts of Z7-12:OAc, i.e. corn-strain males are mainly attracted to 2% Z7-12:OAc, while rice-strain males mostly respond to 2-4%, but also to 10% Z7-12:OAc (Chapter 2). This strain-specific response to Z7-12:OAc is probably the most important factor that differentiates corn-and rice-strain males, and corn-strain males seem to be more restricted and specific in their response to Z7-12:OAc than rice-strain males, independent of the geographic region (Chapter 2 and 3). Differences in the olfactory pheromone receptors (ORs) on the male antennae of both strains could explain the strain-specific differences in the response to Z7-12:OAc. If corn-strain males exhibit more specifically tuned ORs for Z7-12:OAc than rice-strain males, it could explain why corn-strain males mainly respond to 2% Z7-12:OAc and rice-strain males to 2% up to 10% Z7-12:OAc. Genetic analyses of *S. frugiperda* field populations showed that corn-strain populations are genetically much more homogenous than rice-strain populations (Juárez et al. 2013), which could be correlated to possible strain-specific differences in ORs responding to Z7-12:OAc.
1.3 Strain-specific sexual communication as weak prezygotic isolation barrier

Although we found consistent strain-specific differences in the female pheromone composition of both strains, corn- and rice-strain males were not exclusively attracted to females of their own strain in Florida (Meagher and Nagoshi, 2013; Chapter 2). Similarly, Pashley et al. (1992) showed that corn- and rice-strain males from Louisiana were not strain-specifically attracted to traps baited with virgin corn- and rice-strain females. These data indicate that strain-specific sexual communication is a weak prezygotic isolation barrier and is unlikely to be a major force in the divergence of both S. frugiperda strains, at least not in North America (Florida and Louisiana).

On the other hand, geographic variation influences the male response (Chapter 3), which could mean that strain-specific sexual communication might drive divergence of both strains in other regions than North America, i.e. in South America. Two different field studies suggest that the role of E7-12:OAc is especially important in regions in South America, i.e. in Brazil (Batista-Pereira et al., 2006), and Peru (Chapter 3). If rice-strain males in South America would be attracted to E- and Z-7-12:OAc, while corn-strain only respond to Z7-12:OAc, assortative mating could occur. However, so far we can only speculate that sexual communication might contribute to the divergence of the two strains in South America.

1.4 Evolution of strain-specific sexual communication differences

Genetic analyses of S. frugiperda field populations showed that corn-strain populations are genetically much more homogenous than rice-strain populations, suggesting that the corn-strain went through a bottleneck (Juárez et al. 2013). In
Chapter 7

Chapter 6, we proposed an evolutionary scenario with the rice-strain as ancestral strain and the corn-strain as derived strain that arose from a few individuals. A corn-strain specific genetic homogeneity could explain why corn-strain males are more restricted in their response to a female sex pheromone than rice-strain males (Chapter 2 and 3). Interestingly, we found a congruence between the production of and the response to Z7-12:OAc in corn-strain populations from Florida (Chapter 2). These data suggest that a change in the pheromone production of the ancient rice-strain population might have been fixed due to bottleneck effects, which in turn has led to a small change in the sexual communication system of the derived corn-strain population. QTL analysis of the female sex pheromone of S. frugiperda showed that multiple genomic regions are involved in the production of strain-specific pheromone blends (Chapter 4), which indicates that changes in the pheromone composition might have occurred in multiple minor steps. This would explain the fact that a total of 10 minor QTLs explained the strain-specific pheromone differences (Chapter 4).

With all our experiments, we focused on 5 different compounds that were found in the pheromone glands of corn- and rice-strain females, i.e. Z9-14:OAc, Z7-12:OAc, E7-12:OAc, Z11-16:OAc and Z9-12:OAc (Chapter 2-4). Based on all results, I will now speculate how strong or weak selection pressures might act on these 5 different compounds.

Z9-14:OAc. The major pheromone component Z9-14:OAc seems to be under strong selection, because it has been found as main abundant component in glands of both strains in different regions (Tumlinson et al., 1986; Batista-Pereira et al., 2006; Groot et al., 2008; Lima and McNeil, 2009; Chapter 2), and it is necessary for male attraction in the field (Tumlinson et al., 1986; Andrade et al., 2000; Batista-Pereira et al., 2006; Chapter 2 and 3). Due to the fact that corn- and rice-strain females from Florida show no strain-specific differences in their relative
amount of Z9-14:OAc (Chapter 2), we did not conduct a Z9-14:OAc dose-response experiment. However, Lima and McNeil (2009) reported that corn-strain females from Louisiana produce higher amounts of Z9-14:OAc than rice-strain females, and thus male response to Z9-14:OAc might differ between strains from this region.

**Z7-12:OAc.** Corn- and rice-strain females exhibit significant differences in their relative amount of Z7-12:OAc (Groot et al., 2008; Lima and McNeil, 2009), and males show strain-specific responses to different doses of Z7-12:OAc (Chapter 2 and 3). Thus, it seems that the critical secondary sex pheromone component Z7-12:OAc is under relatively strong strain-specific selection. Interestingly, our QTL analysis suggests that the sex pheromone differences in Z7-12:OAc are genetically linked to strain-specific timing of mating via *vrille* (Chapter 4). Such a genetic linkage could be achieved via a cis-regulatory element that influences both the expression of *vrille* and pheromone biosynthesis enzymes like desaturases or PBAN.

**E7-12:OAc.** Female production of and male response to E7-12:OAc varies significantly between regions (Tumlinson et al., 1986; Batista-Pereira et al., 2006; Groot et al., 2008; Lima and McNeil, 2009; Chapter 3), and E7-12:OAc appears to be especially important for the attraction of males in western South America. Thus, selection pressures acting on E7-12:OAc production and response could be strong in some regions, while in other regions this component is not produced by females and therefore unnecessary for male attraction.

**Z11-16:OAc and Z9-12:OAc.** Our trapping experiments showed that Z11-16:OAc has only a small, if any, effect on the response of corn- and rice-strain males (Chapter 2 and 3). This suggests that Z11-16:OAc is under weak selection, if at all, which is supported by the fact that seven genomic regions are involved in the production of Z11-16:OAc (Chapter 4). Our experiments also evidence that Z9-12:OAc is not required for attraction of corn- and rice-strain males (Chapter 2),
which suggests minor or no selection on the relative amount of Z9-12:OAc in the pheromone blend.

1.5 Importance of different reproductive mating barriers

Our and many other studies have shown that both *S. frugiperda* strains exhibit genetic and behavioral differences, and so far three possible prezygotic mating barriers, as well as one postzygotic isolation mechanism, have been described (Pashley et al., 1992; Schöfl et al., 2009; Groot et al., 2010b; Juárez et al., 2012). Although both strains were first considered to exhibit strong host-associations, more recent studies show that host associations are not as clear-cut as previously thought, as both strains can often be found together in many habitats (Pashley, 1989; Meagher and Gallo-Meagher, 2003; Nagoshi et al., 2007; Juárez et al., 2012; Chapter 6). Thus, reproductive isolation solely due to host plant differences seems to be a weak prezygotic isolation mechanism between the two strains. Similarly, strain-specific sexual communication does not appear to be a strong prezygotic mating barrier between the strains (Pashley et al., 1992; Meagher and Nagoshi, 2013; Chapter 2). Instead, consistent strain-specific, geographically independent, differences in the timing of reproductive behaviors (female/male calling, copulation, oviposition) seem to be the strongest prezygotic mating barrier that drives divergence between strains (Pashley et al., 1992; Schöfl et al., 2009; Schöfl et al., 2011; Hänniger et al., 2013). Even though strain-specific timing of mating is probably the strongest of all prezygotic barriers (Figure 1), strain-specific mating times do overlap to some degree (Schöfl et al., 2009), and this barrier alone is most likely also not sufficient to completely prevent hybridization (Schöfl et al., 2009, 2011).
Besides prezygotic isolation, postzygotic isolation seems to be important in the divergence of both strains, i.e. the partial sterility of RC (rice-strain♀ x corn-strain♂) hybrid females (Groot et al., 2010b; Kost et al. unpublished; Figure 1). This one-sided hybrid mating incompatibility appears to be a strong postzygotic
barrier, which probably acts together with the prezygotic isolation barriers to reduce gene flow between strains (Groot et al., 2010b; Figure 1). Together, all data suggest that both *S. frugiperda* strains exhibit several incomplete reproductive isolation barriers that prevent both strains from merging into one mixed population (Groot et al., 2010b). Therefore, both *S. frugiperda* strains seem to have acquired some elements of species rank, but exhibit incomplete reproductive isolating mechanisms and are still able to hybridize. Alternatively, it could be possible that geographic isolation caused divergence and partial reproductive isolation mechanisms developed, while now ongoing hybridization is merging both strains.

2. Sexual communication and pest management of *Spodoptera frugiperda*

The strain-specific differences that we found in the sexual communication system of *S. frugiperda* corn- and rice-strain individuals may be useful to improve different pest management strategies and/or to optimize the control of this agricultural pest species. Based on the results of Chapter 2 and 3, I recommend a general pheromone blend to monitor both strains independent of geographic and strain-specific variation, and try to assess whether it is possible to reduce *S. frugiperda* populations in the field via the use of mating disruption.

2.1 Monitoring

Although we found strain-specific differences in the attraction of males to different amounts of Z7-12:OAc, responses of both strains do overlap to some degree. Also, corn- and rice-strain males from different regions show no consistent strain-specific attraction to female pheromone blends in the field (Chapter 2 and 3). Thus, the use of strain-specific pheromone lures to trap only *S. frugiperda* corn- or rice-
strain males is probably not doable, meaning that the same pheromone lures can be (more or less efficiently) used for both strains.

The results of our male-trapping experiments showed that two pheromone components, i.e. Z9-14:OAc and Z7-12:OAc, are required for the attraction of corn- and rice-strain males in different regions in North and South America (Chapter 2 and 3). Thus, binary blends containing Z9-14:OAc and Z7-12:OAc are probably the best choice to monitor *S. frugiperda*. More precisely, blends containing 100% Z9-14:OAc (300µg) and 2% Z7-12:OAc (6µg) could be most effective for monitoring of both strains within different habitats, because males of both strains show an equally high response to such blends (Chapter 2 and 3). Higher amounts of Z7-12:OAc, i.e. 5% (Andrade et al., 2000) or 10% (Chapter 2 and 3), can reduce the response of *S. frugiperda* and should therefore be avoided when monitoring this species.

An universal trap design that can be used for our monitoring blend (100% Z9-14:OAc + 2% Z7-12:OAc) cannot be concluded from our experiments, because we used the same green-yellow-white bucket traps in all experiments (Chapter 2 and 3). Trapping experiments of *S. frugiperda* showed that male attraction can vary depending on different factors like color and shape of the pheromone traps, and also geographic variation seems to be involved in this variation (Mitchell et al., 1989; Meagher and Mitchell, 2001; Malo et al., 2004a). In Florida, green-yellow-white bucket traps seem to be most efficient to catch *S. frugiperda* (Meagher and Mitchell, 2001), while in Mexico cone traps are more attractive than bucket traps (Malo et al., 2004a). To what extent *S. frugiperda* males can be repelled from a pheromone trap due to visual cues is so far unknown and still needs to be investigated.
2.2 Control via mating disruption

The saturation of the environment with synthetic sex pheromone, i.e. mating disruption, is used to confuse males with the aim to reduce/delay matings with females, because the location of conspecific females is masked by the smell of synthetic pheromones (Taschenberg and Roelofs, 1978; Witzgall et al., 2008; Cocco et al., 2013; Trematerra and Spina, 2013). The use of mating disruption has been shown to be effective in decreasing pest populations of moth species like *Cydia pomonella*, *Grapholita lobarzewskii* or *Lobesia botrana* (Witzgall et al., 2008; Gambon et al., 2009; Ioriatti et al., 2011). In *S. frugiperda*, synthetic binary pheromone blends containing 100% Z9-14:OAc and 2% Z7-12:OAc could be used as blends in mating disruption, due to the consistently high and geographically independent attraction of corn- and rice-strain males to this blend (Chapter 2 and 3). Interestingly, high amounts (10%) of Z7-12:OAc can reduce the attraction of corn- and rice-strain males (Chapter 2 and 3), which suggests that saturation of a field with high dosages of solely Z7-12:OAc might confuse males of both strains enough to lead to a reduction of matings. If this would be case, the costs of synthetic pheromone dispensers could be drastically reduced when only Z7-12:OAc is used, instead of Z9-14:OAc and Z7-12:OAc.

One great advantage of mating disruption, in contrast to trap-and-kill methods, is that it does not require any pheromone traps or killing agents because males are solely repelled from a field. Besides the fact that this makes the mating disruption cheaper, one does not need to worry about possible geographic variation in the trap color and design, which was described above. However, a major drawback of this method is that it works most efficiently when population densities are low and field sites isolated (Witzgall et al., 2008). The fact that *S. frugiperda* is a generalist species (Pashley, 1988), that can infest large areas of agricultural land (Luginbill, 1928; Sparks, 1979, 1986), suggests that this might lead to problems in the
efficiency of this method. Nevertheless, I think it will be worthwhile to develop the use of mating disruption to reduce the density of and damage caused by *S. frugiperda* corn- and rice-strain populations in agricultural crops.

References


Chapter 7


Summary

The main aim of my research was to determine the level and importance of strain-specific sexual communication as prezygotic isolation barrier in *Spodoptera frugiperda*. Specifically, the first aim was to evaluate whether strain-specific sexual communication acts as prezygotic mating barrier between corn- and rice-strain populations and contributes to reproductive isolation between both strains. The results of our studies showed that differences in the sexual communication system of both strains are probably not strong enough to cause assortative mating in the field (Chapter 2), which suggests that sexual communication is a weak prezygotic mating barrier between both strains, at least in Florida. However, our data also indicate that the sexual communication system of *S. frugiperda* varies between North- and South America (Chapter 3), and thus we cannot exclude that sexual communication might play an important role in strain divergence in some regions. Therefore, more research, especially in regions in the Caribbean and South America, will be needed to rule out that strain-specific sexual communication alone is not capable of driving divergence between the two *S. frugiperda* strains.

The second objective of this thesis was to differentiate strain-specific variability in the male response from geographic variation that might influence both strains. We studied the response of corn- and rice-strain males in different geographic regions and found that geographic variation interferes with strain-specific variation, at least in the corn-strain (Chapter 3). In contrast, we found almost no geographic variation in the response of rice-strain males (Chapter 3). However, in most regions, we tested only a limited set of different synthetic pheromone blends, and E7-12:OAc, which seems to be important for the attraction of *S. frugiperda* males in South America, was not part of our strain-specific female pheromone blends (Chapter 3). More studies on the response of males towards different doses of E7-12:OAc in different regions are required because it is still unclear whether males of both strain
Summary
differ in their response to E7-12:OAc. Furthermore, additional pheromone analyses of females from different regions should be performed, with the aim to prepare synthetic pheromone lures that mimic a specific regional strain-specific female pheromone blend. Testing such regional synthetic pheromone lures to attract males in the field could help to further disentangle geographic from strain-specific variation in the corn- and the rice-strain. Besides additional information to understand the evolution of both strains, further research might also help to improve pest management of *S. frugiperda* in different regions.

The third aim of this thesis was to examine the genetic basis and inheritance of strain-specific corn- and rice-strain female pheromone blends. We conducted a QTL analysis and found that multiple genomic regions are involved in the production of strain-specific pheromone blends (Chapter 4). A delta-11-desaturase (SfLPAQ) could be involved in the strain-specific production of the major pheromone component Z9-14:OAc and the minor compound Z11-16:OAc (Chapter 4). Interestingly, we found a possible genetic link between pheromone production of Z7-12:OAc and strain-specific timing of reproduction in the night, via the circadian clock protein *vrille* (Chapter 4). Additional genetic studies could help to understand whether a possible genetic coupling of different reproductive isolation mechanism exists and facilitates the divergence of corn- and rice-strain populations in different regions. Genetic studies on the response of corn- and rice-strain males will be useful to determine if a possible linkage between different prezygotic mating mechanisms, i.e. the pheromone response and the strain-specific reproductive activity of males, also exist in males.

The results of this thesis suggest that strain-specific sexual communication alone is not sufficient to prevent hybridization between both strains in the field, although it seems that sexual communication might act together with other prezygotic
reproductive isolation barriers like strain-specific host choice and differential timing of mating in the night (Chapter 5 and 6). Interactions between these different prezygotic mating barriers, together with one postzygotic isolation mechanism, i.e. the reduced mating success of RC hybrid (rice-strain♀ x corn-strain♂) females, seems to prevent both strains from merging into one mixed populations and thus preserves strain-identity of both S. frugiperda strains (Chapter 5 and 6). However, hybridization between both strains exists and more research will be required to estimate how differentiated and separated both strains are in different regions. Area-wide studies on all different pre- and post-zygotic isolation mechanisms found so far will help to understand the strength and interactions of different isolations barriers in S. frugiperda in North and South America. So far, it seems that both strains are mainly separated by the strain-specific timing of reproduction and the partial sterility of RC hybrid (rice-strain♀ x corn-strain♂) females, and less so by strain-specific sexual communication via sex pheromones (Chapter 5 and 6). However, none of these isolation barriers alone are likely to explain the divergence between the two strains of S. frugiperda. Therefore, these two sympatrically occurring strains are a good model system to study how the evolution of ecological divergence interacts with the evolution of sexual communication.
Acknowledgements

First of all, I want to thank my supervisor Astrid T. Groot for all her help and support during my diploma and PhD thesis. She was the reason I got interested in moths and she showed me how beautiful the world of sexual communication in insects can be. Besides fruitful and vivid discussions, she helped me to become a scientist, to find collaborators, and to deal with all kinds of trouble one experiences when working with insects and scientists. Thank you very much for all the effort you put into this project! I learned a lot from you as a great scientist and person and I’m looking forward to fruitful collaborations with you in the future. I’m also really grateful that I was allowed to work in the entomology department under the supervision of David G. Heckel who fully supported my ideas and my project, gave me the opportunity to present my work at international conferences, and shared a lot of his amazing knowledge with me. Thanks to my supervisor Steph B. J. Menken from the University of Amsterdam for all his helpful comments on my thesis and for supporting my defense in all possible ways.

Within our little Spodoptera group in Jena I want to especially thank my colleague and best friend Sabine Hänniger who was more than once the reason I came to work with a smile. Thank you for all the nice field trips we did together, for listening to and helping me with all of my scientific and private issues, and for calming me down whenever things went wrong. I’m really glad that I have you as a friend! Thanks to Gerhard Schöfl for providing me with insects when I started this project, for all productive discussions and for his remarkable statistical knowledge he shared with me. I want to thank Maria Laura Juárez who worked in our Spodoptera group for a short while and was so kind to conduct field work for me in Argentina. Thanks to Silvia Kost for all kinds of discussions, especially the ones about postzygotic isolation, for the good company during our retreat and for the fun with Edda.
This work would not have been possible without the help of different collaborators that either conducted or supported the field experiments in North and South America. Thanks to Robert L. Meagher and his technician Amy Rowley who helped me to find infested field sites in Florida and also conducted experiments for me in the sunshine state. Furthermore, I want to thank Gregg Nuessly and Tommy Toms for letting me perform trapping experiments at their experimental and agricultural field sites in South Florida. I want to thank David A. Jenkins for the nice collaboration in Puerto Rico, for all his support during the field work and for providing a lovely place to stay at his father’s house. I also thank Wilson Rivera González from Monsanto and Jose Santiago from 3rd Millennium Genetics for giving me access to their experimental field sites in Santa Isabel in Puerto Rico. Special thanks to Gissella M. Vásquez who performed field work, genetic analysis and pheromone gland extractions in Peru. I want to thank Jeremy N. McNeil for performing field work for me in Canada and for being a member of my thesis committee, and thanks to Dominic Reisig for conducting male trapping experiments in North Carolina. I also want to thank all of my collaborators for their helpful comments on our joined manuscript about geographic variation in *Spodoptera* males.

I want to thank Carlos Blanco for providing our lab with *Spodoptera* populations from Mississippi and for sharing his home with me when I visited Washington. Thanks to Manfred Ayasse and his group at the University of Ulm for letting me use their wind tunnel setup and for the warm welcome during our collaboration. Special thanks to Christer Löfstedt for his comments on my first manuscript and for being part of my thesis committee. I also want to thank Peter H. van Tienderen, Peter Roessingh and Joris M. Koene who are so kind to be members of my thesis committee.
Acknowledgements

I thank all members of the entomology department in Jena for the great working atmosphere and all helpful discussions during my time as a PhD student. Many thanks to my colleagues and friends Anne Karpinski, Andrea Barthel, David Neunemann and André Busch for the great company during the last years, and special thanks to Anne for her help with the AFLP analysis. I also want to thank our technicians Antje Schmaltz and Susanne Donnerhacke for all their support in the lab and for helping me with DNA extractions, strain identifications, and the AFLP analysis. Thanks to Regina Seibt for her support with the insect rearing and thanks to Domenica Schnabelrauch for providing a great sequencing service.

I want to thank all student helpers that took care of the *Spodoptera* rearing the last years and helped me in the molecular lab with DNA extractions and strain identification of thousands of moths. Special thanks to Kerstin Pohl, Doreen Jahn, Stephanie Ley, Anne Karpinski, David Neunemann, Camilla Kressner, Harald Zimmer, Rebecca Herold, Florian Bredy, Christine Pöllath, Raik Minkenberg, Jörgen Unbehend, Simone Söltl, Sarah Behr, Susanne Neumann, Max Hemmerling and Katja Müller.

Besides scientific advice and help, this thesis was only possible because my friends supported me all the time and took care of me after working hours. Therefore, I want to thank my best “non-biologist” friend Franziska for the great company since I was six years old, the great conversations about everything, and for being my maid of honor. No matter what happens, I know we will be best friends forever. I want to thank my former flat mates Torsten and Björn for the awesome time we spend together during studies and for being good friends since then. Thanks to their families Corinna, Levi and Martha, as well as Franziska and Enna for all the great days we spent together and for their deep friendship.
Curriculum vitae

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