Time-shifted reproductive behaviours among fall armyworm (Noctuidae: Spodoptera frugiperda) host strains: evidence for differing modes of inheritance

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Abstract
The noctuid moth Spodoptera frugiperda consists of two strains associated with different larval host plants (most notably corn and rice). These strains exhibit differential temporal patterns of female calling and copulation during scotophase, with the corn strain more active earlier in the night. We investigated strain-specific constraints in reproductive timing, mating interactions between the two strains, and the mode of inheritance of timing of female calling, male calling, copulation and oviposition. We observed an allochronic shift of all reproductive behaviours by approximately 3 h and a parallel shift of nonreproductive locomotor activity, suggesting involvement of the circadian clock. The corn strain was more variable in the timing of calling and copulation than the rice strain. Rice strain females were more restricted in the timing of copulation than rice strain males, while such differences between the sexes were not apparent in the corn strain. There were significant interactions between the strains affecting onset times of copulation and male calling. The four investigated reproductive traits differed in their modes of inheritance: timing of female and male calling exhibited strong maternal effects, timing of copulation was controlled by a combination of maternal effects and corn strain dominant autosomal factors, and timing of oviposition was inherited in a corn strain dominant fashion. We conclude that the allochronic separation of reproduction between fall armyworm strains is asymmetric, less pronounced than previously thought, and under complex genetic control.
polyphagous noctuid moth native to the New World, extending from Argentina to the US. Within the fall armyworm, two major genetic groups have been recognized that exhibit host-plant associated genetic differentiation at a number of mitochondrial and nuclear loci (Pashley et al., 1985; Pashley, 1986, 1989; Lu et al., 1992; Lu & Adang, 1996; McMichael & Prowell, 1999; Levy et al., 2002; Nagoshi & Meagher, 2003b; Nagoshi et al., 2006). Larvae of one group have been collected predominantly from maize, sorghum and cotton (referred to as the corn strain), whereas the other group is found mostly on rice and various pasture grasses (referred to as the rice strain) (Pashley, 1986, 1988). No diagnostic morphological features have been described to distinguish these two strains, but they differ consistently in a number of physiological, developmental and behavioural features (Pashley, 1988; Pashley et al., 1992, 1995; Groot et al., 2008). Using multilocus genotypes to examine the degree and directionality of hybridization between the fall armyworm strains in nature, Prowell et al. (2004) concluded that up to 16% of the individuals sampled were potential hybrids with many of those not being F1 in origin.

The identity and relative importance of the specific components of reproductive isolation between these two strains have not been identified unequivocally. No intrinsic hybrid inviability or sterility has yet been reported. Earlier reports of unidirectional incompatibilities in interstrain matings in the laboratory (Pashley & Martin, 1987) were not confirmed by later studies (Whitford et al., 1988; Quisenberry, 1991). The role of habitat choice and/or host fidelity as a potential isolation mechanism is contentious. The only study that specifically examined oviposition choice between the corn and the rice strain (Whitford et al., 1988) found some evidence for strain-specific preferences. Cross-attraction experiments in the field using live females as lures showed a significant strain-biased attraction, although corn strain females attracted large numbers of rice strain males as well (Pashley et al., 1992).

The mechanism that has been suggested as the most important contributor to reproductive isolation between the two strains is a strong temporal divide in mating activities throughout the night (Pashley et al., 1992). In S. frugiperda, corn strain individuals have been found to be reproductively active during the first part of the night, while rice strain individuals have been found to be active during the latter part of the night (Pashley et al., 1992). In Lepidoptera, other examples of diel differences in reproductive activity between closely related species or host races have become known in the past decades (Konno et al., 1996; Monti et al., 1997; Ueno et al., 2006). Their potential as a barrier to gene flow has only recently started to attract wider attention (Devries et al., 2008), and less is known about the genetics of temporal isolation than about any other isolating barrier (Coyne & Orr, 2004; p. 210).

To further investigate the dynamics of this isolation mechanism, we have evaluated the allochronic separation of the two host strains of S. frugiperda by assessing (i) strain-specific constraints vs. plasticity in reproductive timing, (ii) the consequences of mating interactions between the two strains on reproductive timing and (iii) the mode of inheritance of the strain-specific differences in reproductive timing, under laboratory conditions. Reproduction in moths will usually follow a behavioural sequence roughly reflected by four traits: (1) long-range mate attraction (female calling), (2) close-range courtship (male calling), (3) copulation and (4) oviposition (e.g. Alexander et al., 1997). While female calling is the emission of volatile long-range mate finding signals, males of many moth species display their abdominal hair pencils during courtship to emit odours that are important in mate acceptance and mate choice (e.g. Birch et al., 1990; Hillier & Vickers, 2004). We quantified the temporal partitioning of these four reproductive traits in within-strain matings, between-strain matings and backcross matings using hybrid individuals. We found that the two strains differed not only in the timing of the reproductive traits, but also in their temporal patterns of locomotor activity. The allochronic separation between the two strains was less pronounced than previously suggested. The corn strain was more flexible in the timing of calling and copulation than the rice strain, and significant interactions between the strains affected onset times of copulation and male calling. Surprisingly, we found evidence for differing modes of inheritance for the four investigated reproductive traits, suggesting that the timing of the reproductive behaviours is under complex genetic control.

Materials and methods

Experimental populations

The corn strain (C) colony used in these experiments was established from > 100 larvae collected from corn plants near Homestead in Miami-Dade Co., Florida, between October and November 2004. The rice strain (R) colony originated from > 200 larvae collected from pasture grasses from the Range Cattle Research and Education Centre, Ona, Hardee Co., Florida, between May and October 2003. Both colonies were reared in mass culture for 10 and 21 generations, respectively, on pinto bean-based artificial diet at USDA-ARS in Gainesville, Florida. Since July 2006, colonies have been maintained in our laboratory in Jena on pinto bean-based artificial diet under a single pair breeding protocol, designed to minimize inbreeding. Upon receiving the colonies from Gainesville, 48 putative corn strain and 56 putative rice strain individuals were screened for the strain-specific cytochrome oxidase I marker. All but three C individuals were confirmed to be corn strain and all R individuals were confirmed to be rice strain.
Observation of nocturnal reproductive behaviours

All nocturnal behaviours were observed in a walk-in environmental chamber at 26 °C. To facilitate observations, the environmental chamber's photoperiod was time-shifted, so that scotophase started at 12:00 h and ended at 22:00 h. Observations were conducted during the 10 h scotophase and continued for 1 h into photophase (from 12:00 to 23:00). In each experiment, one virgin female and one virgin male, each 1–4 days old, were placed together in clear 16-oz plastic cups and provided with 10% honey solution. Cups were capped with cheese cloth. Observations were performed in three different generations on (i) within-strain matings, (ii) between-strain matings and (iii) backcross matings between hybrid and pure-strain individuals. The number of mating pairs observed at each generation ranged from 320 to 363. All mating pairs observed in one generation were set up on the same day and simultaneously placed in the environmental chamber 2 h before the onset of scotophase. Observations were conducted for three consecutive nights on each set of mating pairs.

Within-strain matings

To assess strain-specific differences in the timing of reproduction, we observed 158 corn strain pairs and 162 rice strain pairs (Trial 1). We scored copulation, female calling (extrusion of pheromone glands), male calling (extrusion of male abdominal hair pencils) and oviposition. During the first 2 days, we also scored any additional actions (e.g. feeding, flight or walking) jointly as general locomotor activity. Observations were done at 30-min intervals using an electric torch fitted with red-light diodes. Each pair was observed for about 3–5 s. A single round of observation generally lasted 20 min. The order of the mating cups in the environmental chamber was randomized and strain identity was unknown to the observers.

Between-strain and within-strain matings

To assess the consequences of mating interactions between the two strains, and to determine the extent of male and female influence on the timing of reproductive behaviour, we observed 100 C-female × R-male crosses and 100 R-female × C-male crosses (Trial 2). In this trial, we also observed 80 corn strain pairs and 80 rice strain pairs, which served as a control. The between-strain matings generated the F₁ hybrids, which were used in the third trial (see below). We used the same mating procedure as described for the first trial, with the exception of monitoring general activity patterns.

Backcross matings with hybrid individuals

To assess the mode of inheritance of the strain-specific differences in the timing of reproduction and to estimate the relative maternal and paternal contributions to the timing of reproductive behaviours, we observed F₁ hybrids in all possible backcross combinations (i.e. CR<sup>♂</sup> × C<sup>♀</sup>, CR<sup>♂</sup> × R<sup>♀</sup>, RC<sup>♂</sup> × C<sup>♀</sup>, RC<sup>♂</sup> × R<sup>♀</sup>, C<sup>♂</sup> × CR<sup>♀</sup>, R<sup>♂</sup> × CR<sup>♀</sup>, C<sup>♂</sup> × RC<sup>♀</sup> and R<sup>♂</sup> × RC<sup>♀</sup>) (Trial 3). When referring to the origin of hybrids, the female parent is given first. For instance, CR refers to hybrid progeny of a cross between a corn strain female and a rice strain male.

For each cross combination, we observed 40–57 pairs, following the same observational protocol as above, with the exception of monitoring general activity patterns. A detailed breakdown of the sample sizes for all types of crosses is presented in Supporting Information Table S1.

Inference of mode of inheritance

In Lepidopterans, females are the heterogametic (ZW) sex, the Z-chromosome of the females is inherited from the father. Z-linkage can thus be demonstrated when female hybrids show trait values similar to the paternal source population in both reciprocal crosses. W-linkage, cytoplasmic, or maternal effects are demonstrated when female hybrids show trait values that are similar to the maternal source population. Reciprocal crosses that show trait values intermediate between the parental source populations indicate additive autosomal gene effects, while trait values similar to one of the parental source populations indicate nonadditive (dominance) effects.

Statistical analyses

Assumptions of normality and homogeneity of variances were not met for onset times of reproductive behaviours in the within-strain matings of Trial 1 (Shapiro-Wilk normality tests, P < 0.01 in all cases; Levene’s test for homogeneity of variances, P = 0.09 to P < 0.001). Therefore, differences between the two strains in the average onset times of reproductive behaviours were tested using nonparametric Wilcoxon signed-rank tests corrected for multiple testing (Holm, 1979). Observing the same mating pairs for three consecutive nights allowed testing for differences in onset times between the first time that a behaviour occurred and subsequent occurrences of the same behaviour. These differences were tested using nonparametric Wilcoxon signed-rank tests for each strain for each trial. To create an overall test for significance, we used Fisher’s method of combining probabilities (Sokal & Rohlf, 1995).

For the within- and between-strain matings of Trial 2, onset times of female calling, copulation and oviposition departed from normal distributions. Box-Cox transformations (package MASS 7.2–42) were used to improve the fit to normality. We analysed the transformed onset times of female calling, copulation and oviposition...
(λ = 1.3, 1.5 and 0.5 respectively), and untransformed onset times of male calling by fitting linear mixed-effects models (package \texttt{nlme} 3.1–89) (Pinheiro \& Bates, 2000). To determine the effects of different types of crosses on the timing of reproduction, type of cross and the order of occurrence of a behaviour (i.e. whether a behaviour was observed for the first time in a pair vs. any subsequent time) were modelled as fixed effects. To determine the effects of the female and the male mating partners on timing of reproduction, strain identity (C or R) of the female and the male, their interaction, and the order of occurrence of a behaviour were modelled as fixed effects. Repeated observations within pairs were modelled as a random effect. Because we found significant heteroscedasticity between types of crosses in the timing of female calling and copulation (Levene’s test for homogeneity of variances: female calling \( P < 0.001 \), copulation \( P << 0.001 \)), which could not be resolved by transformations, we fitted heteroscedastic models when dealing with these two traits. These models equalize residual variances across groups by weighing them using the VariCov function implemented in the \texttt{nlme} package (Pinheiro \& Bates, 2000). The significances of specific differences between cross types were tested post hoc by Tukey’s honest significant difference (HSD) tests using the \texttt{multcomp} package (1.0–2).

For matings involving hybrids (Trial 3), Box-Cox transformations removed departures from normality, except for onset time of oviposition. Therefore, we analysed untransformed onset times of oviposition by nonparametric Kruskal–Wallis \texttt{anovas} and performed post hoc tests for specific differences between types of crosses via nonparametric multiple comparison based on relative contrast effects (package \texttt{npaRCOMP} 1.0–0). We analysed Box-Cox transformed onset times of female calling and copulation (\( \lambda = 1.3 \) and 1.5, respectively), and untransformed onset times of male calling by fitting (heteroscedastic) linear mixed-effects models as described above. Specific differences between types of crosses were tested by Tukey’s HSD tests. Models fitting the strain identity (C, R, CR or RC) of the female and the male as a fixed effect could not be estimated directly because of linear dependencies in the model matrix. Therefore, we used likelihood ratio tests (LRT) to determine the respective effects of the mating partners on timing of female calling, male calling and copulation as follows. We compared full models fitting types of crosses (i.e. all eight female \( \times \) male combinations) as a fixed effect, to reduced models fitting only the strain identity of the male or of the female mating partner as a fixed effect. For onset time of oviposition, the effects of the mating partners were tested using the Scheirer-Ray-Hare extension of the Kruskal–Wallis test as a nonparametric alternative (Sokal \& Rohlf, 1995: pp. 445–447). All analyses were performed using the R 2.8.0 statistical software (R Development Core Team, 2008; \url{http://www.r-project.org/}).

**Results**

**Within-strain matings (Trial 1)**

We observed significant differences between the corn and rice strains in the onset times of all nocturnal reproductive activities studied (i.e. female and male calling, copulation and oviposition), even though onset times of female calling and male calling were highly variable in the corn strain (Fig. 1a,b and Table 1). On average, rice strain females and males started to call 3.0 and 2.6 h later than corn strain females and males, respectively. In both strains, onset time of female calling was significantly correlated with onset time of male calling (in rice strain, only after the removal of two outliers; Fig. 2).

In the corn strain, onset of copulations peaked between 3 and 6 h into scotophase. In the rice strain, copulations were largely restricted to the last 4 h of the scotophase (Fig. 1c). Only 5% of all rice strain pairs started copulating earlier. On average, rice strain pairs started to copulate 3.2 h after corn strain pairs. Despite the significantly earlier average onset time of copulation in corn strain (Table 1), there was a considerable overlap between the two strains: 37% of all corn strain copulations were started during the last 4 h of scotophase.

Considering only pairs that copulated at least twice in different nights, the average within-individual range in onset times of copulation was markedly higher in corn strain (corn strain: 3.4 ± 2.3 h, \( N = 103 \); rice strain: 1.6 ± 1.4 h, \( N = 102 \)). 53% of the corn strain pairs initiated copulation at least once during the first 6 h and at least once during the last 4 h of the night; 35% started copulating exclusively during the first 6 h of the night; 12% started copulating exclusively during the last 4 h of the night. In contrast, in the rice strain 90% of the multiply copulating pairs initiated copulation exclusively during the last 4 h of the night, while none started copulations exclusively during the first 6 h of the night.

Ovipositions were only observed on the second or third night after at least one copulation had taken place. Rice strain females started ovipositing throughout the night, while a majority of corn strain females initiated egg-laying during the first few hours of the scotophase (Fig. 1d). On average, rice strain females initiated oviposition 2.4 h later than corn strain females.

The onset time of female calling was earlier on subsequent nights (‘non-first callings’) than on the night of first occurrence (‘first callings’) (Table 1). Comparable trends were found in Trials 2 (within- and between-strain crosses) and 3 (backcrosses) (data not shown). Combining data from all three trials showed that this difference was significant only for the rice strain (Fisher’s combined probability for corn strain, \( \chi^2 = 7.4 \), d.f. = 6, \( P = 0.28 \); for rice strain \( \chi^2 = 18.5 \), d.f. = 6, \( P = 0.005 \)). The opposite pattern was observed for timing of copulation: both corn and rice strain pairs copulated later the
second and third time than the first time they copulated (Table 1). Combining data from all three trials showed that this difference was significant in the corn strain (Fisher’s combined probability, $\chi^2 = 20.9$, d.f. = 6, $P = 0.002$) and marginally significant in the rice strain (Fisher’s combined probability, $\chi^2 = 12.2$, d.f. = 6, $P = 0.06$). Therefore, order effects were included as fixed effects in subsequent linear mixed-effects models analyses for female calling and copulation. Too few observations of multiple occurrences of male calling and oviposition precluded meaningful comparisons for these behaviours (data not shown).

The temporal profile of general activity patterns (feeding and locomotion) for the two strains paralleled the

### Table 1 Timing of nocturnal behaviours of Spodoptera frugiperda host strains, averaged over 3 consecutive nights of observation.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Strain</th>
<th>N</th>
<th>Mean ± SD (h)</th>
<th>$\Delta t$ (h)*</th>
<th>$P$-value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Onset time of female calling (first callings)</td>
<td>C</td>
<td>76</td>
<td>4.8 ± 3.0</td>
<td>2.5</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>88</td>
<td>8.0 ± 1.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Onset time of female calling (nonfirst callings)</td>
<td>C</td>
<td>44</td>
<td>4.5 ± 2.4</td>
<td>2.6</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>25</td>
<td>7.1 ± 2.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Onset time of male calling</td>
<td>C</td>
<td>72</td>
<td>4.5 ± 2.7</td>
<td>2.6</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>45</td>
<td>7.1 ± 2.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Onset time of copulation (first copulations)</td>
<td>C</td>
<td>123</td>
<td>4.9 ± 2.6</td>
<td>3.2</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>119</td>
<td>8.1 ± 1.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Onset time of copulation (nonfirst copulations)</td>
<td>C</td>
<td>87</td>
<td>5.4 ± 2.1</td>
<td>3.2</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>81</td>
<td>8.6 ± 1.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration of copulation (first copulations)</td>
<td>C</td>
<td>117</td>
<td>2.7 ± 1.9</td>
<td>1.1</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>113</td>
<td>1.5 ± 1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration of copulation (nonfirst copulations)</td>
<td>C</td>
<td>85</td>
<td>2.4 ± 1.6</td>
<td>1.1</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>77</td>
<td>1.3 ± 0.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Onset time of oviposition</td>
<td>C</td>
<td>115</td>
<td>2.9 ± 2.5</td>
<td>2.4</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>97</td>
<td>5.2 ± 2.7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Average difference in onset times between the two host strains in hours.
†Wilcoxon rank sum test with continuity correction; $P$-values adjusted for multiple testing (Holm, 1979).
temporal patterns of reproductive behaviours (Fig. 3). After an initial peak in activity after lights-off, corn strain individuals remained active at relatively high levels 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 lights-on. The difference in activity between the two strains was highly significant for both females (Kolmogorov–Smirnov two-sample test, $D_{2063,1823} = 0.222, P < 0.001$) and males ($D_{2311,2100} = 0.217, P < 0.001$). We detected no significant differences between the sexes within strains (corn strain: $D_{2063,2311} = 0.038, P = 0.08$; rice strain: $D_{1823,2100} = 0.034, P = 0.22$).

**Between-strain matings (Trial 2)**

Comparison of within-strain matings and between-strain matings allowed estimation of the influence of each strain on the timing of reproductive behaviours of the other. For onset time of female calling, the linear mixed-effects model revealed a significant main effect of female strain identity ($t_{106} = 4.98, P < 0.001$), but not of male strain identity ($t_{106} = -0.17, P = 0.86$). There was no significant interaction effect between male and female strain identity ($t_{106} = 0.24, P = 0.81$). Thus, males did not influence the onset time of female calling (Fig. 4a).

With onset times of male calling, the linear mixed-effects model revealed significant main effects of female strain identity ($t_{68} = 2.18, P = 0.03$) and male strain identity ($t_{68} = 2.86, P = 0.006$), but no significant interaction effect between male and female strain identity ($t_{68} = -0.57, P = 0.57$). This shows that both males and females influenced onset time of male calling (Fig. 4b).

With onset time of copulation, the linear mixed-effects model revealed significant main effects of female strain identity ($t_{304} = 12.7, P < 0.001$) and male strain identity ($t_{304} = 3.86, P < 0.001$). In this case, there was also a significant interaction effect between male and female strain identity ($t_{304} = -3.57, P < 0.001$). Rice strain females paired with different male partners did not differ in their onset time of copulation, while corn strain females copulated significantly later when paired with rice strain males than when paired with corn strain males (Tukey’s HSD, $z = 3.86, P < 0.001$; Fig. 4c). The magnitude of the effect predicted from the linear mixed-effects model was considerably larger for female strain identity ($\hat{\beta}_{\text{female}} = 8.53; 95\% \text{ CI} = 7.21–9.85$), than for male strain identity ($\hat{\beta}_{\text{male}} = 3.22; 95\% \text{ CI} = 1.58–4.86$) or the interaction between female and male strain identity ($\hat{\beta}_{\text{female} \times \text{male}} = -3.51; 95\% \text{ CI} = -5.44$ to $-1.57$).

For onset time of oviposition, we found a significant main effect of female strain identity ($t_{184} = 4.88, P < 0.001$), but not of male strain identity ($t_{184} = 1.79, P = 0.08$). There was no significant interaction effect between male and female strain identity ($t_{184} = -1.31, P = 0.19$). Thus, males did not influence onset time of oviposition (Fig. 4d).

**Backcross matings with hybrid individuals (Trial 3)**

In hybrid × parental strain pairings (CR♀ × C♂, CR♀ × R♂, RC♀ × C♂, RC♀ × R♂, C♂ × CR♀, R♀ × CR♂, C♂ × RC♀ and R♂ × RC♂), the strain identity of the matting partners affected reproductive timing in a similar way as found in crosses between parental strains (previous paragraph). Only females significantly influenced timing of female calling (LRT for the female effect, $LR = 53.5, P < 0.001$; for the male effect, $LR = 8.5, P = 0.08$). Both males and females significantly influenced timing of male calling (LRT for the female effect, $LR = 15.1, P = 0.005$; for the male effect, $LR = 14.6,$
Inheritance of onset time of female calling

Onset times of female calling were not statistically different between pure corn strain females and CR-hybrid females, or between pure rice strain females and RC-hybrid females (Fig. 5a). CR- and RC-hybrid females differed significantly from each other (Tukey’s HSD, $z = 2.97, P = 0.02$). This suggests that the strain-specific differences in onset time of female calling are controlled by a maternal pattern of inheritance. The analysis of patterns of inheritance can be taken to a deeper level by directly modelling the effects of the parental strain identity of each female on onset time of female calling (cf. Nygren et al., 2006). Taking into account all possible hybrid × parental strain pairings of Trial 3 as well as the within-strain pairings of Trial 2 simultaneously, this approach allows estimating the relative maternal and paternal contributions to the timing of a female calling. Employing a linear mixed-effects model, the effect of the female’s maternal strain identity was highly significant ($P < 0.001$) and showed the largest predicted effect size, while the effect of the female’s paternal strain identity was marginally significant ($P = 0.06$) and the predicted effect size considerably smaller (Table 2). There was no significant interaction between the female’s maternal and the female’s paternal strain identity. This corroborates that the strain-specific differences in onset time of female calling are controlled by a predominantly maternal pattern of inheritance, which may be modulated by some additive effects.

Inheritance of onset time of male calling

The analysis of onset time of male calling was complicated by the fact that both sexes significantly influenced male calling times. Disregarding the biasing effect of female mating partners of different strain identity, Fig. 5b suggests an overall pattern of maternal inheritance similar to what we observed for female calling. Statistical support for maternal inheritance provides the observation that CR-hybrid males called earlier than RC-hybrid
males in the presence of rice strain females (Tukey’s HSD, \( z = -4.88, P < 0.001 \)). This notion was further supported when testing for the effect of the parental strain identity of each female and of each male using all possible backcross combinations of Trial 3 and the within-strain pairings of Trial 2. We found significant effects for the male’s maternal strain identity but not for the male’s paternal strain identity (Table 2). Onset time of male calling was also significantly affected by the female’s paternal strain identity (Table 2).

### Inheritance of onset time of copulation

Analyses of between-strain matings suggested that females exerted more control over onset times of copulation than males (see above). Therefore, we considered this trait from the females’ perspective (Fig. 5c). With onset time of copulation, the two reciprocal hybrid categories did not differ significantly from each other. However, while RC-hybrid females were significantly different from both parental strains, CR-hybrid females (when paired with C-males) did not differ significantly from pure corn strain females (Fig. 5c), suggesting some degree of corn strain dominance. Estimating the maternal and paternal contributions in both sexes to the timing of a copulation revealed significant effects of both the female’s and the male’s maternal strain identity, as well as the female’s and the male’s paternal strain identity (Table 2). The predicted effect size of the female’s

![Table 2 Effects of parental strain identity of females (and males) on the onset times of reproductive behaviours.](image-url)

<table>
<thead>
<tr>
<th>Model terms</th>
<th>Effect</th>
<th>SE</th>
<th>d.f.</th>
<th>t</th>
<th>P-value</th>
</tr>
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<tr>
<td>Onset time of female calling</td>
<td>Female’s mother</td>
<td>3.49</td>
<td>0.85</td>
<td>167</td>
<td>4.08</td>
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<tr>
<td></td>
<td>Female’s father</td>
<td>1.09</td>
<td>0.59</td>
<td>167</td>
<td>1.86</td>
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<td></td>
<td>Female’s mother × female’s father</td>
<td>0.60</td>
<td>1.07</td>
<td>167</td>
<td>0.56</td>
</tr>
<tr>
<td>Onset time of male calling</td>
<td>Female’s mother</td>
<td>0.63</td>
<td>0.41</td>
<td>158</td>
<td>1.56</td>
</tr>
<tr>
<td></td>
<td>Female’s father</td>
<td>1.08</td>
<td>0.36</td>
<td>158</td>
<td>2.97</td>
</tr>
<tr>
<td></td>
<td>Male’s mother</td>
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<td>0.35</td>
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<td>Male’s father</td>
<td>-0.01</td>
<td>0.33</td>
<td>158</td>
<td>-0.4</td>
</tr>
<tr>
<td>Onset time of copulation</td>
<td>Female’s mother</td>
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<td>0.39</td>
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<td>7.68</td>
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<tr>
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<td>0.43</td>
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<td>2.92</td>
</tr>
<tr>
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<td>0.34</td>
<td>398</td>
<td>4.72</td>
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<td>0.31</td>
<td>398</td>
<td>2.96</td>
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<tr>
<td>Onset time of oviposition</td>
<td>Female’s mother</td>
<td>-14.7</td>
<td>39.6</td>
<td>262</td>
<td>-0.37</td>
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<tr>
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<td>-4.09</td>
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<td>262</td>
<td>-0.24</td>
</tr>
<tr>
<td></td>
<td>Female’s mother × female’s father</td>
<td>104.9</td>
<td>43.3</td>
<td>262</td>
<td>2.42</td>
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</table>

Effect and SE indicate the predicted effect sizes of each term on (transformed/ranked) onset times of reproductive behaviours and their standard errors obtained from linear mixed-effects models fit by `REML`. For onset time of male calling and copulation interaction terms could not be estimated jointly because of linear dependencies in the model matrix.
maternal strain identity ($\hat{\beta} = 2.96; 95\% \text{ CI} = 2.20–3.71$) was significantly greater than the predicted effect sizes of the female’s paternal strain identity ($\hat{\beta} = 1.25; 95\% \text{ CI} = 0.41–2.10$) and both the male’s maternal ($\hat{\beta} = 1.61; 95\% \text{ CI} = 1.05–2.17$) and paternal strain identity ($\hat{\beta} = 0.92; 95\% \text{ CI} = 0.40–1.43$). This supports the notion that onset time of copulation is controlled to a larger degree by females than by males, and that onset time of mating is influenced by a combination of maternal and corn strain dominant effects.

**Inheritance of onset time of oviposition**

Neither CR-hybrid-females nor RC-hybrid females differed significantly from pure corn strain females in onset time of oviposition, whereas both F$_1$ hybrid classes differed significantly from pure rice strain females (Fig. 4d). In a model testing for the effect of the parental strain identity of each female, only the interaction between the females’ maternal and paternal strain identity was significant (Table 2). This suggests an autosomal corn strain dominant mode of inheritance for onset time of oviposition.

**Discussion**

Our observations of a total of 480 pure strain pairs, 200 between-strain pairs and 320 pairs involving hybrids for three consecutive nights have given us a detailed picture of timing differences of the four reproductive traits (female calling, male calling, copulation and oviposition) between the two strains in *S. frugiperda*. As we observed pairs in all possible cross combinations, we could determine the effects of the mating partner, as well as of their parents, on these traits. We first summarize each of the traits separately, then we discuss the interaction effects between the mating partners and the mode of inheritance of the traits.

**Individual traits**

The average initiation time of female calling differed significantly between the two strains, even though corn strain females showed a very broad distribution of onset time of calling throughout the night (Fig. 1a). When first-time calling was compared with subsequent nights, rice strain females started calling earlier the second or third time, which further reduced the average gap between the two strains. We found no indication that the timing of female calling was affected by the presence of the male mating partner. The onset time of female calling was predominantly maternally inherited: C$_C^C \times R_2^C$-hybrids behaved similar to the pure corn strain females, while R$_C^C \times C_2^C$-hybrids behaved similar to pure rice strain females (Fig. 5a).

The average differences in timing of male calling between the two host strains, as well as their distributions, matched those of female calling (Fig. 1b), and the initiation times of male and female calling were closely correlated within strains (Fig. 2). Onset time of male calling was controlled not only by the males but also by the females (further discussed below). Surprisingly, the onset time of male calling was also maternally inherited (Fig. 5b and Table 2).

Copulations in the corn strain peaked between 3 and 6 h into scotophase (Fig. 1c), although roughly 37% of all corn strain copulations coincided with the later rice strain copulations. The rice strain copulated mostly in the last part of the night, only 5% of all rice strain matings occurred earlier than during the last 4 h of scotophase. Considering only multiple copulations of the same pairs at different nights, the corn strain showed large within-individual variability, i.e. there were no distinct groups of ‘late copulating pairs’ and ‘early copulating pairs’. Onset time of copulation seems to be controlled by a combination of corn-strain dominant and maternal effects: CR-hybrid females and RC-hybrid females copulated significantly earlier than pure rice strain females, but CR-hybrid females (mated to C males) were not significantly different from pure corn strain females (Fig. 5c), indicating some degree of corn strain dominance. The RC-hybrids were significantly different from both parental strains in their timing of copulations (Fig. 5c), suggesting a maternal contribution to onset times of copulation. Both the female’s and male’s maternally inherited genotype exhibited a greater influence over onset time of copulation than the two sexes’ paternally inherited genotypes, confirming the suggestion of a maternal contribution to onset time of copulation (Table 2).

Onset times of oviposition were biased towards the first 4 h into scotophase in the corn strain, while they were roughly uniformly distributed throughout the scotophase in the rice strain (Fig. 1d). Onset time of oviposition showed no indication of maternal effects. Instead, this trait was inherited in a purely corn strain dominant fashion: CR-hybrid females and RC-hybrid females oviposited significantly earlier than pure rice strain females, but were not different from pure corn strain females (Fig. 5d). When testing for the effects of the parental strain identity of the females on the onset times of oviposition, only the interaction between the female’s maternally and paternally derived genotypes was significant. This indicates nonadditive effects.

**Overlap in reproductive timing between the strains**

The behaviour of the corn strain in our study differed from the behaviour of the corn strain in the first report on differential timing of nocturnal activities in *S. frugiperda* (Pashley et al., 1992). In the previous study, corn strain copulations were clearly restricted to the first 6 h of a 10-h scotophase and did not overlap with rice strain copulations (see Fig. 1 in Pashley et al., 1992).
Three explanations are possible for this discrepancy. First, the strains used here have been reared in the laboratory for much longer (15 and 26 generations for the corn and the rice strain, respectively) than the strains used in Pashley et al.’s (1992) study (field collection, 3 and 10 generations for two corn strain samples and a rice strain sample, respectively). However, this does not explain why constraints should relax because of prolonged rearing in the laboratory in the corn strain alone, and not in the even older rice strain colony. Second, the sample sizes employed in the previous study may not have been large enough to observe the full range of variation in these timing traits in the corn strain of the fall armyworm. Pashley et al.’s (1992) data for the corn strain were based on observations on 16 mating pairs, while our data is based on observations on 123 corn strain pairs. Finally, there may be geographic variation in these behavioural traits. In our study, the corn strain derived from South Florida while in the previous study eight corn strain pairs derived from Louisiana and eight pairs came from Florida (the precise place of origin within Florida was not given).

Effects of an interaction between the sexes on the timing of reproductive behaviours

In the behavioural sequence of reproduction, onset times of female calling and oviposition are expected to be determined by the female alone, because under field conditions neither behaviour requires (or will usually be performed in) the presence of a male. As males of many moth species have been found to extrude their hair pencils in the close proximity of the female (Birch et al., 1990), the timing of male calling is likely to be the result of an interaction between the two sexes. The onset time of copulation clearly depends on an interaction between the female and the male. While it is difficult to directly determine the extent of male and female influence over a behaviour such as onset time of copulation, cross-strain mating experiments can be used to address this issue, because the average activity peaks of the two mating partners are different (see, e.g. Holwell, 2008).

As predicted, the timing of male calling was not only dependent on the male but also strongly affected by the strain identity of the female mating partner. In interstrain crosses, C-males paired with R-females, and R-males paired with C-females, called on average at a time intermediate between pure corn strain and rice strain pairs (Fig. 4b). The correlation between the onset times of female and male calling (Fig. 2) suggests that the interaction with the female may be elicited by the female sex pheromone. Even though this pheromone may not be confined to the mating cups but could also emanate into the climate chamber, this ‘chemical background noise’ did not affect the timing of male calling in pure-strain crosses: R-males paired with C-females called earlier than R-males paired with R-females. Hence, the concentration of the background pheromone is most likely too low in comparison to that inside the mating cups to elicit a response from males. In addition, other time-shifted cues of female receptiveness may affect the temporal control of male calling. This may explain why males sometimes started calling before females (cf. Fig 2).

While both sexes exerted a similar influence on the timing of male calling, the pattern was more complicated for the timing of copulation. C-females paired with R-males copulated on average 1.5 h later than when paired with C-males, whereas copulation times of R-females were not affected as much by the strain of the males they were paired with (see Fig. 4c). Interestingly, model predictions of effect sizes of female and male strain identity on onset times of copulation implied that females exert a greater influence over the onset of copulation than males. The asymmetric interaction between the strains suggests that (i) the corn strain is more flexible in the timing of copulation relative to the rice strain and (ii) the mating phase is more restricted for R-females than for R-males. Therefore, rice strain females rather than rice strain males seem to cause the restriction of rice strain matings to the last four hours of the scotophase.

Differing modes of inheritance and the genetic basis of differential timing of reproduction

All four reproductive behaviours measured here appear to be shifted co-ordinately by approximately 3 h between the two strains (Fig. 1). Moreover, their time shift is paralleled by a temporal shift in general locomotor activity (Fig. 3). This suggests that a single common genetic factor controls the timing of these activities, mostly likely a gene involved in the circadian system (e.g. Sakai & Ishida, 2001; Miyatake et al., 2002; Tauber et al., 2003; An et al., 2004; Rymer et al., 2007).

Surprisingly, the results of our crosses indicate differing modes of inheritance for the differences in timing of the four investigated reproductive behaviours. Two traits (timing of copulation and oviposition) showed evidence for autosomal corn strain dominant control. Three traits (timing of female calling, male calling and copulation) showed varying degrees of maternal inheritance. As female lepidopterans are the heterogametic sex (ZW) and males are the homogametic sex (ZZ), a maternal mode of inheritance can be caused either by the (maternally transmitted) W-chromosome, by maternal effects (cytoplasmatic factors) or both. In traits limited to the female sex (onset time of female calling), these factors are confounded. In a trait expressed in both sexes (onset time of copulation) or limited to the male sex (onset time of male calling), these factors can be separated. For male calling and copulation, our results imply that cytoplasmatic effects rather than W-linked factors cause the maternal inheritance.

The idea of a single genetic factor controlling the timing of reproduction and other activities in the S. frugiperda host
Temporal isolation of *S. frugiperda* host strains

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Differential timing of reproduction as a prezygotic barrier to gene flow

As isolating barriers act sequentially in the life cycle of hybridizing taxa, early-acting barriers may contribute more to total isolation than later-acting barriers, even if they are of equal strength (Schemske, 2000; Ramsey *et al.*, 2003). The temporal partitioning of reproduction in *S. frugiperda* has been suggested to be the major prezygotic isolation barrier between the two strains (Pashley *et al.*, 1992). However, our study shows that, although being significantly differentiated, there is a large overlap in the timing of both female calling and copulation between the two strains. Our data indicate that rice strain females are more restricted in the timing of copulation than rice strain males. Therefore, if the differences in onset time of copulation were a major contributor to prezygotic isolation, this should translate into *R* × *C* reciprocals being rarer than the reciprocals. Molecular evidence for directness of hybridization in the field does not support this notion, but suggests a roughly equal representation of CR- and RC-hybrids (Prowell *et al.*, 2004) or even an under-representation of CR-hybrids (Nagoshi & Meagher, 2003a).

Our results do not invalidate the idea that temporal partitioning of reproduction is an important prezygotic isolation barrier, but they do call for a re-evaluation of other probable prezygotic isolating barriers in the fall armyworm, and their potential interactions. Studies testing for habitat isolation of the *S. frugiperda* host strains, either through differences in oviposition preferences or differential larval survival on different host plants, have been inconclusive to date (Pashley, 1988; Whitford *et al.*, 1988; Pashley *et al.*, 1995; Veenstra *et al.*, 1995; Prowell *et al.*, 2004) and need to be further investigated. The other most obvious behavioural prezygotic barrier is differential mating preferences because of sex pheromone differences (Pashley *et al.*, 1992; Groot *et al.*, 2008). Recently, we (Groot *et al.*, 2008) found significant differences in the female sex pheromone composition between the two host strains. Mate attraction tests in the field using live females of both strains as a lure showed a significant strain-biased preference, although corn strain females still attracted large numbers of rice strain males (Pashley *et al.*, 1992). These data considered only total trap catches throughout the night and not the potential interaction of pheromone chemistry with differences in the timing of female calling. The total reproductive isolation achieved in nature is potentially much higher than the individual evaluations of allochronic separation and differential attraction to sex pheromones suggests. Both may act simultaneously and could therefore contribute multiplicatively rather than cumulatively to reproductive isolation. To test this, the next step is to assess to what extent these different pheromone blends are differentially attractive to corn and rice strain males, and to take into account the timing of mate attraction during the night.

In summary, the two strains of the fall armyworm differ in the nocturnal timing of four reproductive traits and also in their locomotor activity. The allochronic separation of reproduction between the two strains is less pronounced than previously thought. The corn strain is more flexible in the timing of calling and copulation relative to the rice strain. Interactions between the sexes suggested that rice strain females were more restricted to the timing of copulation than rice strain males. In the corn strain, differences between the sexes were not apparent. The co-ordinated time shift between reproductive traits and locomotor activity suggests an involvement of the circadian clock. Surprisingly, however, the four investigated reproductive traits showed evidence for 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. This indicates that the different reproductive behaviours are under complex genetic control. The effectiveness of allochronic isolation needs to be tested in interaction with other isolation mechanisms in the laboratory and the field.

Acknowledgments

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References


**Supporting information**

Additional supporting information may be found in the online version of this article:

**Table S1** Sample sizes for all three trials.

**Table S2** Tukey’s honest significant difference (HSD) post hoc tests between crosses in Trial 2 (within- and between-strain matings).

**Table S3** Tukey’s HSD post hoc tests (nonparametric comparison of relative contrast effects in the case of oviposition) between crosses in Trial 3 (backcrosses).

**Table S4** Tukey’s HSD post hoc tests (nonparametric comparison of relative contrast effects in the case of oviposition) between hybrid classes of females (males, in the case of male calling) in Trial 3 (backcrosses).

**Figure S1** Average onset times of (a) female calling, (b) male calling, (c) copulation and (d) oviposition in crosses of females of either strain (males in the case of male calling) with pure strain and hybrid males (females in the case of male calling).

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