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**Chasing sympatric speciation: The relative importance and genetic basis of prezygotic isolation barriers in diverging populations of *Spodoptera frugiperda***

Hänniger, S.

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# SECTION 1

## STRAIN-SPECIFIC HOST PLANT DIFFERENTIATION (OR NOT?)

*'Negative results are just what I want.  
They're just as valuable to me as positive results.  
I can never find the thing that does the job best  
until I find the ones that don't.'*

Thomas A. Edison



# 2

## **NO STRAIN-SPECIFIC DIFFERENCES IN PREFERENCE AND PERFORMANCE OF *SPODOPTERA FRUGIPERDA* ON TYPICAL HOST PLANTS**

**Sabine Hänniger<sup>1</sup>, David G. Heckel<sup>1</sup> & Astrid T. Groot<sup>1,2</sup>**

<sup>1</sup>Max Planck Institute for Chemical Ecology, Entomology, Hans-Knöll-Str. 8, 07745 Jena, Germany; <sup>2</sup>Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, Science Park 904, 1098 XH Amsterdam, the Netherlands

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**Abstract**

*Spodoptera frugiperda* is a generalist moth species occurring as two separate strains. The strains are generally referred to as host strains, as they were originally identified on different host plants, i.e. the corn strain on tall grasses and the rice-strain on small grasses. Yet field observations, oviposition studies and larval performance studies overall show inconsistent results, which induces the question of how strong the host association of the two strains is. This study investigated oviposition preference and larval preference and performance in various bioassays and aims to determine within-strain host differentiation and between-strain host differentiation in our results and previous studies. The results are very variable between different published studies and show no consistent pattern. We conclude that the currently available data suggests only a weak involvement of host-differentiation in the divergence of the two *S. frugiperda* strains.

**INTRODUCTION**

For herbivorous insects, their host plant is not only a food source, but also provides protection from predators and pathogens, as well as mating sites. Therefore, a differentiation in host plant usage between populations of one species does not only affect nutrition and traits connected to it. Habitat isolation can reduce gene flow between populations when it reduces the probability to mate between populations. With reduced gene flow, genetic differences can accumulate, which can facilitate the formation of host races and reproductive isolation, and can thus lead to the differentiation into new species (Schluter 2001; Drés and Mallet 2002; Funk et al. 2002, 2006; Coyne and Orr 2004; Rundle and Nosil 2005; Feder et al. 2012). This ecological speciation in sympatry is hypothesized as a continuous process from polymorphisms between populations to species, and the existence of host races as intermediate state supports this hypothesis (Berlocher and Feder 2002; Drés and Mallet 2002).

Specialist herbivorous insects with a narrow range of host plants, like e.g. the apple maggot fly *Rhagoletis pomonella* (Bush 1969; Feder et al. 1994), the Goldenrod ball-gall fly *Eurosta solidaginis* (Craig et al. 1993; Craig and Itami 2011) and the treehopper *Enchenopa binotata* (Wood 1980; Guttman et al. 1981) are the main focus of research addressing ecological speciation in sympatry. However, many examples also show host usage differences between populations of generalists. For example, the European corn borer *Ostrinia nubilalis* is a generalist that consists of two pheromone strains, the E-strain and the Z-strain, but in Northern France the E-strain is feeding primarily on mugwort whereas the Z-strain is feeding on maize (Thomas et al. 2003; Bethenod et al. 2005). Also the two biotypes of the larch budmoth *Zeiraphera diniana* show differences in pheromones as well as host preference, with one biotype preferring larch (*Larix* spp.) and the other preferring pine (*Pinus* spp.) (Emelianov et al. 1995, 2003, 2004). Recently, host usage differences have also been shown for two laboratory strains of the generalist tobacco

budworm *Heliothis virescens* (Blanco et al. 2008; Karpinski et al. 2014), and a QTL analysis revealed a genetic basis of the difference in larval performance on different host plants. Such differences could be the start of host specialization.

The noctuid moth *S. frugiperda* occurs as two different host strains and is potentially an ideal model organism to study the mechanisms underlying habitat isolation and ecological speciation in sympatry. The two morphologically indistinguishable strains, the so-called corn-strain and the so-called rice-strain, are found in sympatry in the Americas. The strains can be discriminated by a number of molecular markers in the nuclear and mitochondrial DNA (Pashley 1989; Lu et al. 1992, 1994; Lu and Adang 1996; McMichael and Prowell 1999; Levy et al. 2002; Nagoshi and Meagher 2003a,b; Busato et al. 2004; Nagoshi et al. 2006; Clark et al. 2007; Martinelli et al. 2007; Belay et al. 2012). Besides genetic differences, the two strains show phenotypic differentiation, and the focus of this study is the host plant associated variation between the two strains (Pashley et al. 1985; Pashley 1986). Typically, larval collections from tall grasses like corn (*Zea mays*) and sorghum (*Sorghum bicolor*) and from cotton (*Gossypium hirsutum*) consist of up to 80% corn-strain individuals and only 20% rice-strain individuals, whereas up to 95% of larvae collected from smaller grasses, like rice (*Oryza sativa*) or pasture (e.g. bermudagrass, *Cynodon dactylon*) are rice-strain larvae (Pashley 1986, 1988a, 1989; Meagher and Gallo-Meagher 2003; Prowell et al. 2004; Nagoshi et al. 2006; Machado et al. 2008; Juárez et al. 2014). However, some field collections show different patterns. For example, males caught in a cotton field in Mississippi were mostly identified as rice-strain, whereas males and larvae collected from sorghum fields in Texas and Florida revealed more rice-strain individuals than corn-strain individuals (Nagoshi et al. 2006). Also in Argentina, Paraguay, and Brazil, larvae collected from rice plants mostly consisted of corn-strain individuals, whereas larvae collected from sorghum plants consisted exclusively of rice-strain individuals (Juárez et al. 2014). Thus, host plant adaptation of the two strains may not be as strict as previously thought.

Different mechanisms could underlie the distributional differences of the two strains observed in the field. Generally, host use biases may be due to different time points in insect/plant-interaction, i.e. by a) preference for oviposition sites in adult females, b) larvae that accept or do not accept the host they emerge on, c) differences in larval development and viability on different hosts, d) larval preference for different hosts, or through a combination of these preferences and performances. For the two *S. frugiperda* strains, behavioral differences in host use remains unclear, even though many assays have been conducted to investigate oviposition preferences of *S. frugiperda*, as well as the influence of host plants on development and viability of larvae (see Table 1). For example, Whitford et al. (1988) found that the corn-strain preferred to oviposit on corn and sorghum compared to bermudagrass, whereas the rice-strain preferred bermudagrass over

corn or sorghum in one of two conducted assays. Similarly, Meagher et al. (2011) found a preference of the rice-strain for pasture grass (*Cynodon nlemfuensis*) over corn in two oviposition assay, but the corn-strain did not show a preference for either plant. Many more studies have addressed larval performance differences on different host plants, but have found inconsistent results (e.g. Pashley 1988b; Meagher et al. 2004; Groot et al. 2010). Together, the inconsistent findings in field observations, oviposition studies and larval performance studies induce the question of how strong the host association of the two strains is.

In this study, we aimed to assess oviposition preference and larval preference and performance of the two strains of *S. frugiperda*. We conducted a number of bioassays, following the sequence of insect/plant-interaction events in nature: after females choose a plant as oviposition site (i. oviposition preference), larvae hatch and accept or do not accept the host plant (ii. larval host acceptance). When accepting and feeding on the host, larval development and viability may be influenced by the host plant (iii. larval performance). At different life stages, the larva can choose to move to a different plant (iv. larval preference). The ultimate aim of these assays was to verify or falsify the following two hypotheses: I. There is host-plant differentiation *within* the two strains, and II. There is a difference in performance and/or preference *between* the two strains. If both hypotheses are correct, host plant differentiation may underlie the strain differentiation in *S. frugiperda*.

## MATERIALS AND METHODS

### *Insects and rearing*

The bioassays in this study were conducted using three laboratory corn- and rice-strain populations. The oldest population originated from larval collections in Florida in 2003 and 2004. For the corn-strain, >100 corn-strain larvae were collected from sweet corn fields in Miami-Dade County (25°38'42", 80°27'18") in 2004. This population is referred to as JS3C. More than 200 rice-strain larvae were collected from different *Cynodon* pasture grasses at the Range Cattle REC near Ona (27°23'50", 81°56'40") in 2003 to establish the rice-strain population, referred to as OnaR. These populations were reared for 10 (corn-strain) and 21 (rice-strain) generations in mass culture at the USDA-ARS in Gainesville before shipment to the Max Planck Institute for Chemical Ecology in Jena, Germany (MPICE) in 2007. Larvae of these populations were used after 37 and 48 generations at MPICE, respectively.

The second population originated from larval collections of >120 individuals by Carlos A. Blanco in Mississippi in August 2008. Corn-strain larvae were collected from a corn field in Stoneville (+33°15'8.59", -90°31'59.765") and will be referred to as MSC. Rice-strain larvae were collected from a grass field in Raymond (+32°9'51.883", -90°13'29.406") and are referred to as MSR. After

collection, larvae were shipped to MPICE and reared on artificial diet. Larvae of these populations were used after 8 and 7 generations at MPICE, respectively.

The youngest population originated from field collections of 300 specimens in April 2010. Corn-strain larvae were collected in a corn field in the Everglades Research and Education Centre in Belle Glade, Florida (+26°40'7.20", -80°37'57.63") and are referred to as FLC. Rice-strain larvae were collected in a pasture field at the Graham Dairy Farm in Moore Haven, Florida (+26°53'3.04", -81°7'21.17") and are referred to as FLR. All larvae were shipped to MPICE and reared on artificial diet in the laboratory since then. Larvae of these populations were used after two generations at MPICE. Upon arrival at the MPICE, all individuals were screened for strain-specific COI markers (Nagoshi et al. 2006), and separated accordingly into strain-specific colonies. All populations were reared in incubators 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 set up to maintain the populations and minimize inbreeding. Larvae were fed on artificial diet based on pinto beans (PBD).

### **Plants**

Seeds of sweetcorn hybrid SWEET G 90 (*Zea mays*) were obtained from Syngenta Seeds, Inc. (Boise, Idaho) and seeds of bermudagrass (*Cynodon dactylon*) were obtained from B&T World Seeds (Paguignan, France). Both plant species were cultivated in the greenhouse under L:D 14:10, 19 °C (night) – 24 °C (day) and 50-60% RH in 1-l pots. One corn plant was planted per pot, whereas for the bermudagrass enough grass plants were planted to gain a dense coverage of grass foliage in the pot (~50 plants per pot).

### **Bioassays**

#### *i) Oviposition preference*

Female preference for oviposition sites was investigated in three different bioassays, presenting 1) plant leaf parts, 2) whole plants and 3) whole plants with and without gauze to mated females as potential oviposition sites.

Experiment 1: To determine oviposition preference in many females simultaneously, we first used leaf parts of corn and grass plants. 40 Single pair matings per strain were set up in plastic boxes (28 × 20 cm, Savelock). For the corn-strain matings, individuals from the JS3C population were used, whereas for the rice-strain matings the OnaR population was used. All matings were set up simultaneously in a walk-in climate chamber (L:D 14:10, 26 °C, 65% RH). Insects were provided with a 10% honey solution on a cotton ball. Boxes were covered with gauze and leaf parts of freshly cut corn and grass were placed in randomly chosen opposite corners on the gauze and covered with a moist paper towel. Leaf parts were renewed daily. Males and females were kept in the boxes and egg masses were counted daily for 4 days.



Experiment 2: To test oviposition preference using whole plants, 8 single pair matings per strain (FLC and FLR) were set up in mesh wire cages (60 × 60 × 60 cm) and provided with 10% honey solution. Additionally, one corn plant in a one liter pot and a one liter pot with grass plants were placed randomly in opposite corners of the cage. The set-ups were placed in a walk-in climate chamber (L:D 14:10, 24 °C, 55% RH). Egg masses were counted every day for 4 days in total.

Experiments 3: To investigate whether volatile cues of the plant are more important than tactile cues for stimulating oviposition, we repeated the above experiment, but covered the plants with gauze in 6 cages per strain and left the plants uncovered in additional 6 cages per strain. The gauze was sturdy, so it did not bend the plants, and the holes in the mesh were 1 mm<sup>2</sup> to allow insects to perceive plant volatiles. The experiment was performed in the same way as above, however this time with individuals from the FLC and FLR populations in their 4<sup>th</sup> laboratory generation.

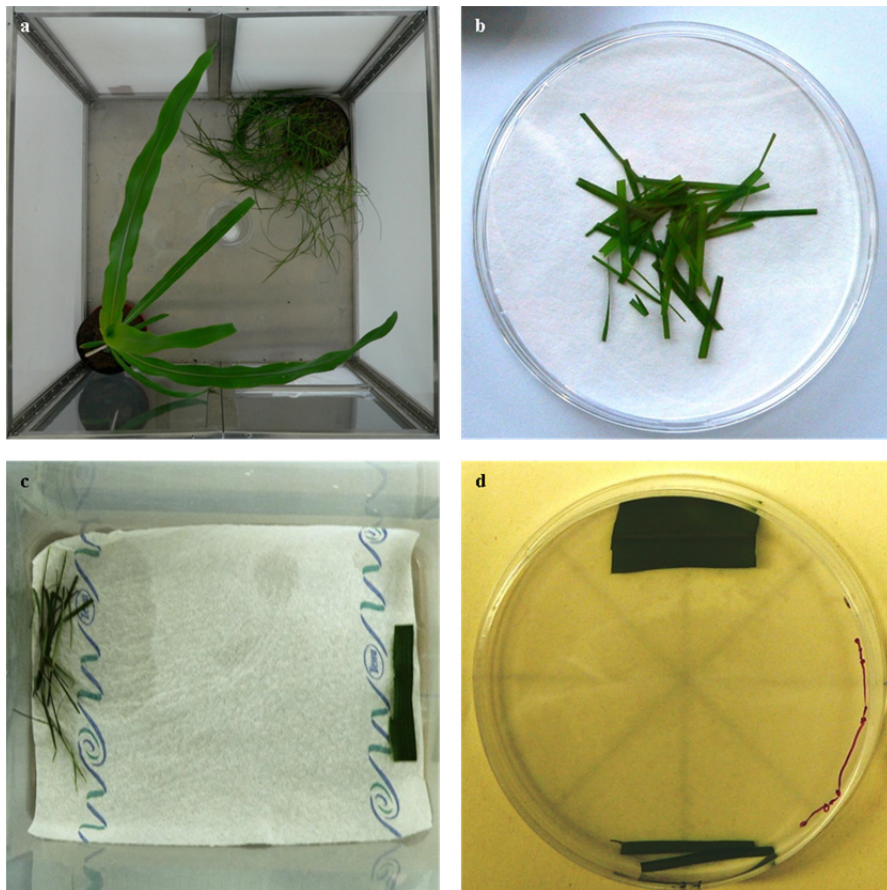
All statistical analyses were conducted using R Studio (RStudio 2012). The oviposition assays were analyzed individually and as a combination of experiment 1-3, using GLM with quasibinomial error structure.

#### *ii) Larval host acceptance*

As in nature, larvae are rarely in choice situations between plants, but can choose to start or not start feeding on the plant that they emerged on, we conducted two types of host acceptance assays: 4) larvae could move to and feed on leaf parts in a no-choice situation and 5) larvae were placed directly on the leaf part and feeding commencement was observed (Figure 1b.).

Experiment 4, where larvae needed to move to a leaf part, was conducted in small arenas (Petri dishes (Ø 9 cm, GBO, Frickenhausen, Germany)) or large arenas (plastic boxes (28 × 20 cm, Savelock)). Moist filter paper or paper towel were placed in the arenas and renewed prior to every assay for every new larva. Plants were always cut fresh for every assay and every new larva, and immediately placed in the arena. The tested corn-strain larvae originated from the MSC population and the rice-strain larvae from the MSR population and were of comparable size, 2<sup>nd</sup> to early 3<sup>rd</sup> instar. Larvae that were about to molt were excluded from the experiment. Such larvae can be recognized by typically having a darker skin and showing less or no movement until molting. A corn leaf part or some grass leaf parts, representing the approximate biomass of the corn leaf, on one side of each arena. The larva was placed in the center of the arena, i.e. 3.5 cm away from the plant in small arenas or 13 cm from the plant in large arenas. In the larger arenas, a directed movement towards the plant was more easily observable. Larvae were observed continuously for 30 minutes. In small arenas, four larvae were observed simultaneously by one observer. In the large arenas, two observers continuously observed 10 larvae

simultaneously. Since early instar larvae are rather small and several insects were observed at a time, a green gut content that was visible in the translucent larvae was also used as an indication for larval feeding on a plant in the larger arenas.



**FIGURE 1.** Setup of different bioassays. **a.** Oviposition preference with whole potted plants without gauze. **b.** Larval host acceptance. **c.** Larval host preference in large arena. **d.** Larval host preference in small arena.

Experiment 5, where larvae were placed directly on the plant, was carried out in small arenas. Fresh cut leaf material of corn or grass plants was placed in the middle of the arena on moist filter paper, 2<sup>nd</sup> to early 3<sup>rd</sup> instar were placed on the leaf parts and the time taken until feeding commencement was noted. Four larvae in individual arenas were observed simultaneously.

The variation within the strains was analyzed with Chi-squared tests. The between-strain variation was analyzed using a binominal comparison of proportions. The variation of the time taken to start feeding was analyzed using ANOVA on log-transformed data.

### *iii) Larval performance*

Experiment 6: To determine whether different host plants have a different effect on the viability and the development of *S. frugiperda* larvae, we investigated larval performance of both strains on different plant-based diets. These diets were used, because plant parts dry out very quickly and need to be renewed every day, which causes a high larval death rate, and whole plants need a large amount of space, especially when testing >100 larvae per strain on each plant species.

The plant-based diets were based on lyophilized plant material. For these diets, corn plants and bermudagrass plants were grown in the greenhouse (L:D 14:10, 19 °C (night) – 24 °C (day), 50-60% RH), freshly harvested without roots and directly cut into pieces of ~10 cm length, immediately frozen at -80 °C in a chest freezer and lyophilized (Gefriertrocknungsanlage ALPHA 2-4 LD, CHRIST®, Germany). The lyophilized and dry plant material was powdered, after which 168 g plant powder, 2,200 ml water, 35 g agar, vitamins, tetracycline, sorbic acid and methyl paraben were mixed to produce the plant-tissue based diets (Blanco et al. 2008). The Pinto bean diet (PBD) that we generally use for our rearing was used as the control. All diets were irradiated for 1 h with UV light to kill microorganisms. Cubes of each diet, measuring ~2 cm<sup>3</sup>, were placed in small (5 oz.) plastic cups. Eggs from both the FLC and FLR populations were collected and larvae were reared on Pinto bean diet until 2<sup>nd</sup> instar. 315 larvae of each strain were weighed and evenly distributed among the different diets, one larva per cup. Larvae were weighed every 3<sup>rd</sup> day until pupation, and pupae were weighed within a day after pupation. The date of eclosion was recorded, and adults were weighed within a day after eclosion. Also, larval and pupal death was documented. The growth rate between day 1 and day 4 of the experiment, i.e. after the larvae were placed on the plant-based diet, was calculated:

$$GR = (\log_{10}(\text{weight day4}) - \log_{10}(\text{weight day1})) / (3 \text{ (days)}) .$$

This period was chosen, as some individuals had already pupated when the weight was measured at day 7. The differences between growth rates were analyzed using ANOVA. The survival rate was analyzed using Cox's proportional hazard and a parametric model.

### *iv) Larval host preference*

Experiment 7: To investigate whether larvae prefer the host plant that they perform on best, we conducted choice assays in small or large arenas, described above for

the acceptance assays with movement (experiment 4). To examine a host preference, the larvae were presented with a choice between a corn leaf part and grass leaf parts, resembling the biomass of the corn leaf part. The different leaf parts were placed on opposite sides of each arena, i.e. ~7 cm apart, 3.5 cm away from the larva in small arenas or ~26 cm apart, 13 cm from the larva in large arenas. The tested larvae originated from the MSC and MSR population and were of comparable size, 2<sup>nd</sup> to early 3<sup>rd</sup> instar. As in the acceptance assays, larvae that were about to molt were excluded. Larvae were observed continuously for 30 minutes, as described in the acceptance assays above.

## RESULTS

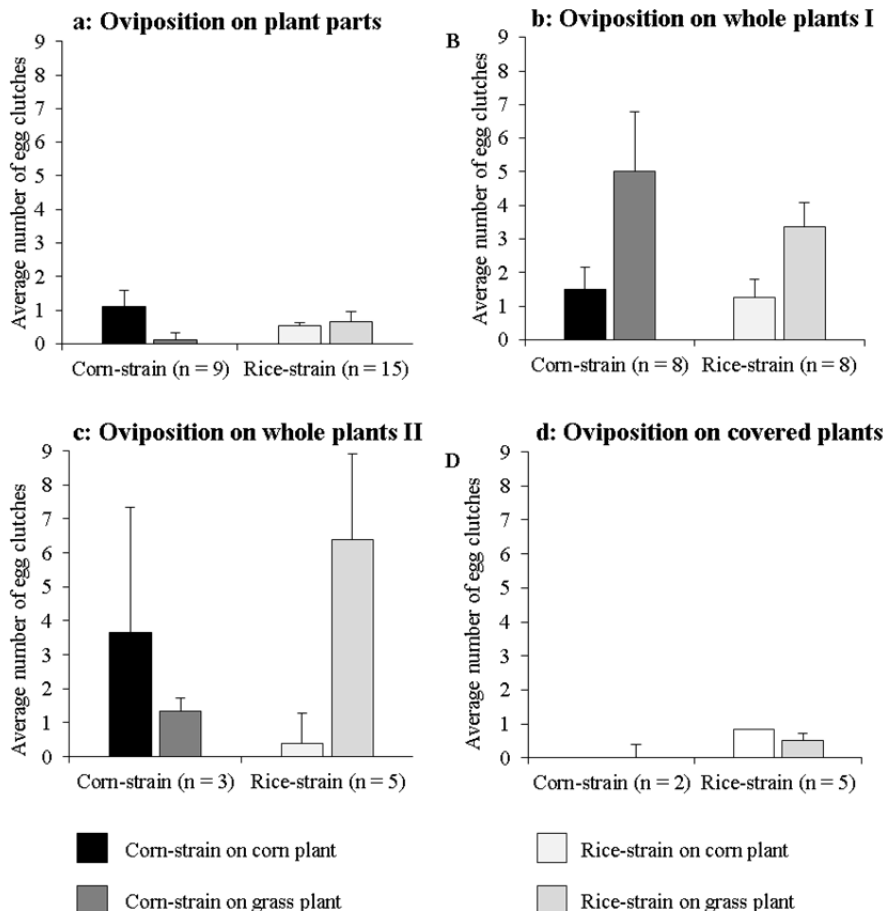
### *i) Oviposition preference*

When females were offered leaf parts on gauze for oviposition (experiment 1, Figure 2a), only 9 of 40 (22.5%) of the tested corn-strain females laid eggs, and only 15 of 40 (37.5%) of the rice-strain females laid eggs. The corn-strain females oviposited on average  $1.11 (\pm 0.48 \text{ SEM})$  egg clutches per female under the gauze under the corn leaf parts, compared to only  $0.11 (\pm 0.11 \text{ SEM})$  egg clutches under the gauze under the grass leaf parts, whereas  $2.56 (\pm 0.77 \text{ SEM})$  eggs were laid on the cage surfaces. Comparably, the rice-strain females only laid  $0.53 (\pm 0.21 \text{ SEM})$  egg clutches under corn leaf parts,  $0.67 (\pm 0.29 \text{ SEM})$  under grass leaf parts and  $5.8 (\pm 1.04 \text{ SEM})$  on other surfaces of the cage. Thus, the majority of females of both strains did not lay eggs on gauze under the leaves. There was no significant difference between the corn- and rice-strain females in their oviposition behaviour in this first experiment.

When females were offered whole plants in planting pots for oviposition (experiment 2, Figure 2b), all females of both strains laid eggs, and all but one corn-strain female laid at least part of her egg masses on plants. On average, corn-strain females laid  $1.5 (\pm 0.65 \text{ SEM})$  egg masses on corn plants, compared to  $5 (\pm 1.78 \text{ SEM})$  egg masses on grass, and  $1.75 (\pm 0.49 \text{ SEM})$  egg clutches on the cage surfaces. Similarly, rice-strain females laid an average of  $1.25 (\pm 0.56 \text{ SEM})$  egg masses on corn plants,  $3.38 (\pm 0.71 \text{ SEM})$  egg clutches on grass plants, and  $0.5 (\pm 0.38 \text{ SEM})$  egg clutches on the cage surfaces (Figure 2b). Thus, both strains laid most eggs on the grass plants. However, there were no significant differences between the strains or between plants.

When the same populations were tested one generation later in the same setup (experiment 3, Figure 2c), only 50% of the corn-strain females laid eggs, whereas 83% of the rice-strain females laid eggs. The corn-strain females laid on average  $3.67 (\pm 3.67 \text{ SEM})$  egg clutches on corn plants,  $1.33 (\pm 0.88 \text{ SEM})$  egg clutches on grass plants and none on the cage. In contrast, the rice-strain oviposited only  $0.40 (\pm 0.40 \text{ SEM})$  egg clutches on corn, but  $6.40 (\pm 2.50 \text{ SEM})$  egg clutches on grass and  $1.6 (\pm 1.17 \text{ SEM})$  egg clutches on the cage surfaces. Thus, the corn-strain laid most

of the eggs on corn, whereas the rice-strain laid most eggs on grass. However, none of the differences were significant, probably due to low sample size.



**FIGURE 2.** Oviposition choice (mean + SEM) of corn-strain and rice-strain female adults on **a** corn and grass plant parts, **b+c** whole corn plants and grass plants and **d** corn and grass plants covered in gauze. Figures show average number of egg masses laid per female on different plants per strain. n = number of females laying eggs. GLM with binomial error structure.

When plants were covered with gauze to determine the importance of tactile cues (experiment 3, Figure 2d), only 30% percent of the corn-strain females oviposited, whereas 100% of the rice-strain females laid eggs. The corn-strain females that did oviposit, laid an average of 1.50 ( $\pm$  0.5 SEM) egg clutches on the cage walls and no eggs on either plant. In contrast, the rice-strain females laid on

average  $0.83 (\pm 0.40 \text{ SEM})$  egg clutches on the corn plant,  $0.5 (\pm 0.22 \text{ SEM})$  egg clutches on the grass plant and  $1.17 (\pm 0.60 \text{ SEM})$  on the cage surfaces. Thus, oviposition was generally lower in cages with covered plants compared to the cages with uncovered plants (Figure 2d). Also, whereas both strains laid more eggs on their typical host plant in cages without gauze, both strains laid few egg masses, preferably on the cage, when the plants were covered with gauze.

A combined GLM analysis of oviposition experiments 1-3 (with leaf parts and with whole plants without gauze) shows that the strains chose different oviposition sites in each experiment and that there was no clear strain-specific choice for either plant.

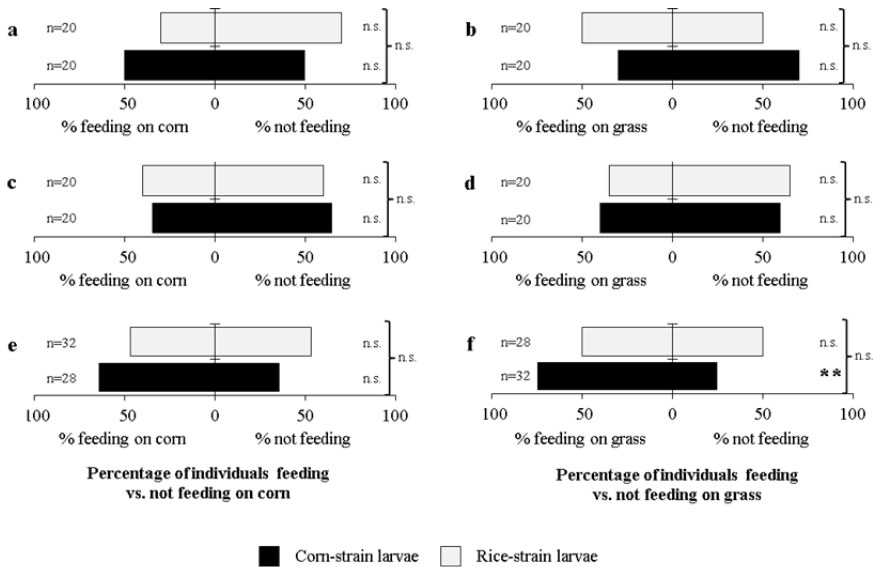
### *ii) Larval host acceptance*

In the acceptance assays where larvae needed to move towards a leaf part in small arenas, no significant differences were observed between the two strains and between the plant parts tested (Figure 3a, b): 50% of the corn-strain larvae fed on corn whereas 30% of the rice-strain larvae fed on corn, within 30 min. When offered grass leaf parts, 30% of the tested corn-strain larvae started feeding, compared to 50% of the rice-strain larvae. In the large arenas, we found no difference of host plant acceptance between the two strains either (Figure 3c, d): 35% of the corn-strain larvae and 40% of the rice-strain larvae fed on corn leaf parts within 30 minutes. On the grass plant parts, 40% of the corn-strain larvae and 35% of the rice-strain larvae started feeding within 30 min (Figure 3b). When larvae were directly placed on the leaf parts, more corn-strain than rice-strain larvae started feeding on either plant leaf parts (Figure 3e, f): On the corn plant, 64% of the corn-strain larvae started feeding compared to 47% of the rice-strain larvae. On the grass plant, 75% of the corn-strain larvae and 50% of the rice-strain larvae started to feed. Thus, the two strains did not differ significantly from each other in this host acceptance assay.

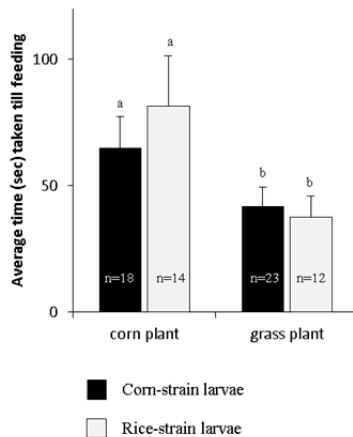
Larvae that did start feeding within 5 minutes, did so significantly later on the corn-plant than on the rice plant in both strains (Figure 4) ( $P < 0.001$ ), with no difference between the two strains.

### *iii) Larval performance*

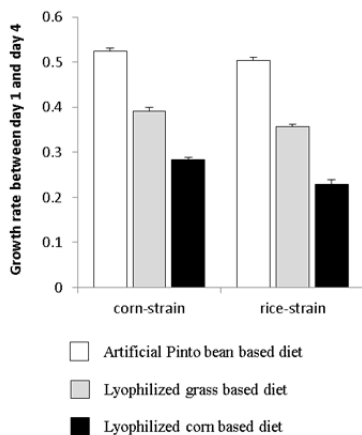
Larvae of both strains showed significantly different growth rates when placed on the three different diets (Figure 5): on artificial pinto bean diet both larval strains grew fastest and on the lyophilized corn diet both larval strains grew slowest (Figure 5,  $P < 0.001$ ). The corn-strain grew faster than the rice-strain on each diet, including the control diet ( $P < 0.001$ ). There was no significant interaction effect between strain and diet, thus the differences between the strains on the different diets were due to the corn-strain generally performing better in this experiment.



**FIGURE 3.** Larval host acceptance of corn-strain and rice-strain larvae. a-d No-choice assays with plant parts in small arenas (a+b; a corn plants, b grass plants) and big arenas (c+d; c corn plants, d grass plants). Figures show percentage of individuals moving to and feeding on the plant within 30 min vs. percentage of non-feeders. Within-strain variance: Chi-squared test. n.s. >0.05. Between-strain-variance: binominal comparison of proportions. n=individuals tested. e+f Food acceptance assays (e corn-plant, f grass plant). Figures show percentage of individuals starting to feed within 5 min vs. percentage of non-feeders. Within-strain variance: Chi-squared test, \* P<0.05. Between-strain-variance: binominal comparison of proportions. n=number of individuals tested.

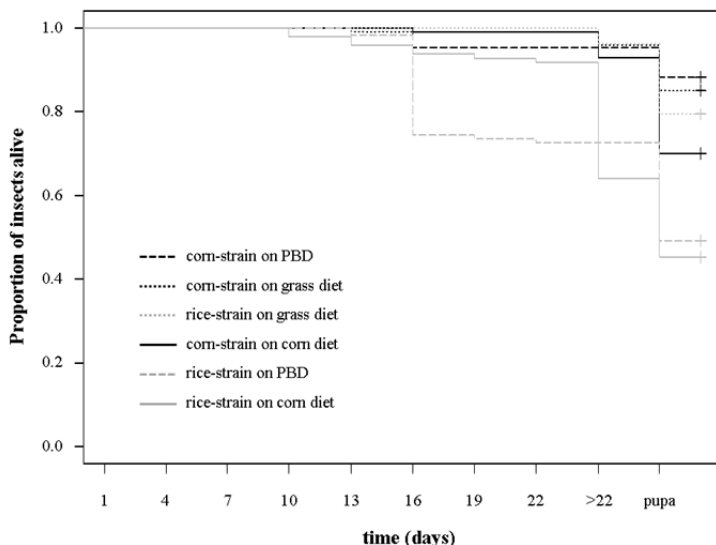


**FIGURE 4.** Food acceptance (mean + SEM) of corn-strain and rice-strain larvae on corn plant parts and grass plant parts. Figures show seconds taken till feeding starts. n= individuals feeding. Different letters indicate above the bars indicate significant differences (P<0.01).



**FIGURE 5.** Larval growth rate of both strains on artificial pinto bean based diet (white bars), grass based diet (grey bars) and corn based diet (black bars). Error bars represent SEM.

Both strains showed the highest survival to adulthood on the diet based on lyophilized grass compared to lyophilized corn (Figure 6). Both strain and diet had a significant influence on the survival rate (both  $P < 0.001$ ). i.e. the corn-strain had a significantly better survival rate than the rice strain and overall survival was best on grass based diet and poorest on corn-based diet.

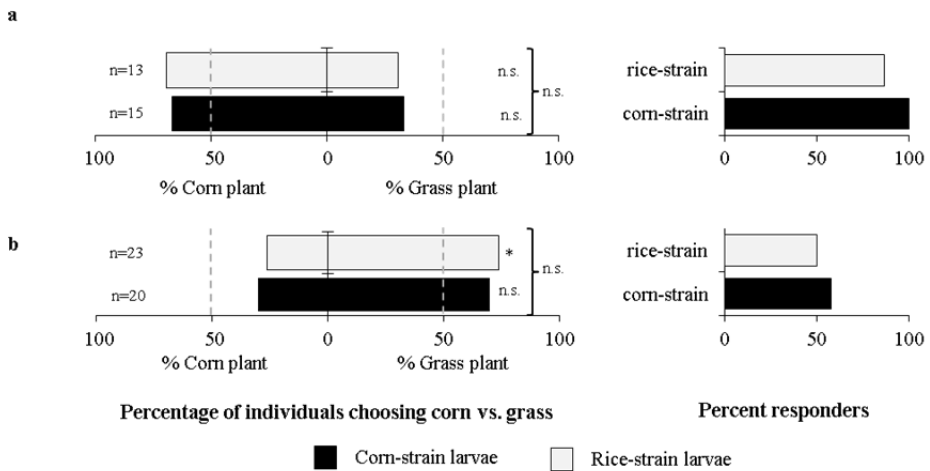


**Figure 6.** Kaplan-Meier survival plot of corn-strain (black line) and rice-strain (grey lines) larvae on three different diets based on lyophilized corn leaves (solid lines), lyophilized grass leaves (dotted lines) and on pinto beans (dashed lines).



## iv) Larval preference

When larvae were given a choice between corn and grass leaf parts in small arenas, more individuals of both strains chose the corn leaf part (Figure 7a). All corn-strain larvae responded and 67% chose corn leaf parts, whereas 33% chose grass leaf parts. Of the rice-strain larvae, 87% responded, of which 69% chose corn leaf parts and 41% chose grass leaf parts. As these differences were not significant, the strains did thus not differ in their preference. In large arenas, 58% of the corn-strain larvae responded, of which 30% chose corn and 70% chose grass, whereas 50% of the rice-strain larvae responded, of which 25% chose corn and 74% chose grass (Figure 7b). Thus, the strains did not differ significantly in their preference in this assay either.



**FIGURE 7.** Larval preference (left) and response rate (right) of corn-strain and rice-strain larvae. Choice assays with plant parts in **A** small and **B** large arenas. Within-strain variation: Chi-squared test, significance levels: \* $P < 0.05$ . Between-strain variance: binominal comparison of proportions. n= individuals responding.

**DISCUSSION**

The main objective of this study was to identify the level and extent of host plant differentiation in the two strains of *S. frugiperda* to test the following two hypotheses: I. There is host-plant differentiation within the two strains, and II. There is host-plant differentiation between the two strains.

As we neither found an oviposition preference, nor a difference in larval performance or preference and results of other studies are not consistent either (see Table 1), it seems that host plant differentiation is not strongly developed in these two strains.

***i) Oviposition preference***

If *S. frugiperda* females of the two strains would choose different oviposition sites, this could cause a differential distribution of the two strains in the field, even if larvae would not perform differently on the different hosts. However, we found no significant differences in oviposition preference within or between the two strains. Even though our sample sizes were small, the same populations in the same setup showed variable results between experiments, so that a larger sample size would probably not have yielded different results. Whitford et al. (1988) also found different results for different experiments in the same study. In one experiment, corn-strain females preferred corn and sorghum as oviposition sites, whereas rice-strain females preferred bermudagrass, and the strains differed significantly from each other. However, in the second experiment, the two strains did not exhibit a difference (Whitford et al. 1988). Meagher et al. (2011) did consistently find that the rice-strain chose pasture grass over corn plants, whereas the corn-strain did not show a preference. However, if only rice-strain females show an oviposition preference, whereas corn-strain females do not, oviposition preference seems to play only a minor role, if any, in the host association of the two strains of *S. frugiperda*. In conclusion, only one of two experiments in the study of Whitford et al. (1988) verifies both hypothesis I (within-strain differentiation) and hypothesis II (between-strain differentiation), thus host plant differentiation between the two strains due to oviposition preference cannot be concluded from the available data.

Tactile cues do seem to play an important role as oviposition cues in *S. frugiperda*, because we found a generally much lower oviposition when we had covered the plants with gauze, to eliminate tactile cues, compared to when plants were uncovered. Similarly, Rojas et al. (2003) found that *S. frugiperda* females showed a strong preference for grooved or pitted surfaces over smooth surfaces, although the females did not prefer surfaces with host plant leaf extracts over control surfaces and were even repelled by high doses of extract. Thus, tactile cues rather than volatile cues seem to be involved in oviposition choice, at least at short distances.

***ii) Larval host acceptance***

If oviposition does not play a role in determining the distribution of the two strains in the field, the host acceptance of the larvae may contribute to host differentiation. In our experiments 4 and 5, corn-strain larvae showed a higher acceptance of the grass plant, i.e. the untypical host, and also accepted the grass plant faster than the corn plant, but only when directly placed on the leaf parts. When the larvae had to move to leaf parts in no-choice situations, the corn-strain did not show a differential acceptance of either plant. The rice-strain larvae accepted the grass plant significantly faster than the corn plant when directly placed on the leaf parts, but did not show a higher acceptance of either host in the other assays. Thus, one of three

assays addressing larval acceptance showed a difference within both strains (but the corn-strain favoured the ‘wrong’ host), verifying hypothesis I (within-strain differentiation). However, none of the larval host acceptance assays revealed a significant difference *between* the strains, so hypothesis II cannot be verified. Thus, we cannot conclude that larval host acceptance underlies the differential distribution of the two strains in the field. We are not aware of other studies testing *S. frugiperda* host acceptance.

### ***iii) Larval performance***

Since eggs of both strains are laid on the same plants and larvae do not differ in their host acceptance, the differential distribution of the two strains may be caused by a difference of larval development or viability on the different hosts. Both strains were heavier when reared on the grass plant diet and there was no difference between the strains beyond an overall higher weight of the corn-strain on all different diets. Both strains also performed better on a typical rice-strain host plant, and there was no difference between the strains. Thus, it cannot be concluded from our results that a differential larval performance on different plants underlies the observed host association in the field. Other studies have found contradictory results (see Table 1): some did find a difference between the strains (Pashley 1988b; Whitford et al. 1988; Pashley et al. 1995; Meagher et al. 2004), whereas others did not (Groot et al. 2010; Meagher and Nagoshi 2012). Larval developmental time, pupal weight and survival rates also differ widely between studies. Some studies show within-strain variation favouring the ‘typical’ host of the strains, i.e. corn or sorghum plants for the corn-strain and rice or different pasture grasses for the rice-strain (Pashley 1988b; Whitford et al. 1988; Pashley et al. 1995; Meagher and Nagoshi 2012), whereas other studies show between-strain variation in these developmental traits (Pashley 1988b; Whitford et al. 1988; Pashley et al. 1995; Veenstra et al. 1995; Meagher et al. 2004; Groot et al. 2010; Meagher and Nagoshi 2012). In summary, none of the studies, including our own, verifies both hypothesis I and II for larval performance, so that larval performance differences between the two strains of *S. frugiperda* strains cannot be concluded.

### ***iv) Larval host preference***

If neither oviposition preference, nor larval host acceptance or larval performance are likely to underlie a host association of *S. frugiperda*, the differential distribution of the strains in the field could still be caused by strain-specific larval choice for a particular host. In this study, larvae were tested in choice situations between leaf parts of corn plants and bermudagrass in two different arenas. In the larger arena, rice-strain larvae showed a preference for bermudagrass, their typical host, whereas the corn-strain did not prefer either plant. Though not significant, also more corn-strain larvae chose to feed on the bermudagrass. Thus, there was no difference

between the strains. In the smaller arenas, more larvae of both strains chose the corn leaf part, but the difference was not significant. There was also no difference between the strains. Our experiments cannot verify Hypothesis I (within-strain variation) or Hypothesis II (between-strain variation). Thus, a larval preference is also unlikely to be involved in the host association of *S. frugiperda*. We are not aware of other studies testing larval preference between typical corn-strain host plants and typical rice-strain host plants.

The existence of two distinct *S. frugiperda* strains is unquestioned, as proven by many studies identifying two distinct strains based on molecular markers (e.g. Nagoshi et al. 2006, 2008, 2012a,b; Nagoshi 2010; Kergoat et al. 2012; Juárez et al. 2014). The question thus remains what separates the two strains, if not their host association, and prevents them from forming one panmictic population. There are two additional potential isolation barriers: differences in the composition of the female sex pheromone (Groot et al. 2008; Unbehend et al. 2013a,b; Hänniger et al. 2015) and allochronic separation of mating activity at night (Pashley et al. 1992; Schöfl et al. 2009, 2011; Hänniger et al. 2015). Host plants could act together with pheromonal divergence and/or allochronic separation to form isolation mechanisms that are not addressed in our bioassays or in the referenced studies. For example, it is possible that different host plant volatiles enhance the attraction of males to the pheromone composition of one or both strains, as described for example for *Grapholita molesta* (Varela et al. 2011). If corn plant volatiles would enhance the attraction of males to corn-strain females, and females would prefer corn plants as oviposition sites, this would facilitate a bias of mated corn-strain females in corn fields and could possibly lead to oviposition bias without an oviposition site preference. Another possibility of how host plants could interact with an isolation barrier is the synchrony of rhythms between different plants and the different strains. The volatile emission of flowers as well as leaves shows circadian rhythms in plants (e.g. Loughrin et al. 1991, 1994; Staudt et al. 1997). A possible scenario would be that corn plants emit certain attractive volatiles earlier in the night than pasture grasses and are thus attracting the early active corn-strain, whereas the grasses attract the later active rice-strain. This may not be the case when the plants are in close vicinity to each other in bioassays, as their volatiles may be mixed too much. Also, tactile cues may be more important at close range than volatile cues, as our experiment (with gauze covered plants) and Rojas et al. (2003) suggest.

### ***Summary of studies on host plant preference and performance of the two strains of *S. frugiperda****

Table 1 summarizes all published studies that address host plant use of the two *S. frugiperda* strains to verify or falsify our two main hypotheses: I. There is host-plant differentiation *within* the two strains, and II. There is a host-plant differentia-

**TABLE 1:** Verification or falsification of the two main hypotheses by the different experiments of this and other studies.

Experiment	H1: Differentiation within strains		H2: Differentiat ion between strains	H1+H2
	Corn- strain	Rice- strain	Corn-strain vs. rice- strain	Can underlie host differentiation?
Oviposition preference on plant parts	-	-	-	no
Oviposition preference on whole plants I	-	-	-	no
Oviposition preference on whole plants II	-	-	-	no
Oviposition preference on whole plants with gauze	-	-	-	no
Oviposition preference I (Whitford et al. 1988)	C	R	+	yes
Oviposition preference II (Whitford et al. 1988)	C	C	-	no
Oviposition preference cage (Meagher et al. 2011)	-	R	+	no
Oviposition preference pool (Meagher et al. 2011)	-	R	+	no
Larval host acceptance small arena	-	-	-	no
Larval host acceptance large arena	-	-	-	no
Larval host acceptance placed on plant	R	-	-	no
Larval host acceptance timing	R	R	-	no
Larval developmental time (Groot et al. 2010)	-	C	+	no
Larval developmental time (Meagher et al. 2004)	n.a.	n.a.	+	n.a.
Larval developmental time (Meagher and Nagoshi 2012)	C	C	+	no
Larval developmental time (Pashley 1988)	-	R	-	no
Larval developmental time (Pashley et al. 1995)	R	R	+	no
Larval developmental time (Veenstra et al. 1995)	n.a.	n.a.	-	n.a.
Larval developmental time (Whitford et al. 1988)	-	C	+	no
Larval survival	R	R	+	no
Larval survival (Meagher et al. 2004)	n.a.	n.a.	+	n.a.
Larval survival (Pashley 1988)	-	R	+	no
Larval survival (Pashley et al. 1995)	-	-	-	no
Larval survival (Veenstra et al. 1995)	n.a.	n.a.	-	n.a.
Larval survival (Whitford et al. 1988)	-	-	+	no
Larval weight	R	R	-	no
Larval weight (Groot et al. 2010)	-	-	-	no
Larval weight (Meagher et al. 2004)	n.a.	n.a.	+	n.a.
Larval weight (Meagher and Nagoshi 2012)	C	C	-	no
Larval weight (Pashley 1988)	R	R	+	no
Larval weight (Pashley et al. 1995)	R	R	+	no
Larval weight (Whitford et al. 1988)	-	-	+	no
Larval weight day 8 (Veenstra et al. 1995)	n.a.	n.a.	+	n.a.
Larval weight II(Veenstra et al. 1995)	-	-	-	no
Larval weight last molt (Veenstra et al. 1995)	n.a.	n.a.	-	n.a.
Pupal weight (Meagher et al. 2004)	n.a.	n.a.	+	n.a.
Pupal weight (Meagher and Nagoshi 2012)	C	-	-	no
Pupal weight (Pashley 1988)	C	C	-	no
Pupal weight (Pashley et al. 1995)	C	C	+	no
Pupal weight (Veenstra et al. 1995)	n.a.	n.a.	+	n.a.
Pupal weight (Whitford et al. 1988)	-	R	-	no
Larval choice small arena	-	-	-	no
Larval choice large arena	-	R	-	no
Total fecundity (Pashley et al. 1995)	-	-	-	no

tion *between* the two strains. Grey rows summarize experiments of this study. ‘C’ indicates preference for or better performance on host plants typical for the corn-strain (e.g. corn, sorghum), ‘R’ indicates preference for or better performance for host plants typical for rice-strain (e.g. rice, pasture grasses), ‘+’ indicates verification, ‘-’ indicates falsification, n.a. = not available due to experimental design or missing statistical analysis. Only the oviposition preference study by Whitford et al. (1988) shows both levels of host plant differentiation.

In conclusion, the inconclusive results of this and other studies suggest that host plants only have a minor influence, probably in interaction with other isolation barriers, on the divergence of the two *S. frugiperda* strains. Possibly, as already indicated by Juárez et al. (2014), the strains should be called host forms instead of host strains.

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