Of moths, mites and microbes - The role of bacteria in the life history of two arthropod herbivores
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Variability of bacterial communities in the moth *Heliothis virescens* indicates transient association with the host


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Abstract
Microbes associated with insects can confer a wide range of ecologically relevant benefits to their hosts. Since insect-associated bacteria often increase the nutritive value of their hosts’ diets, the study of bacterial communities is especially interesting in species that are important agricultural pests. We investigated the composition of bacterial communities in the noctuid moth *Heliothis virescens* and its variability in relation to developmental stage, diet and population (field and laboratory), using bacterial tag-encoded FLX pyrosequencing of 16S rRNA amplicons. In larvae, bacterial communities differed depending on the food plant on which they had been reared, although the within-group variation between biological replicates was high as well. Moreover, larvae originating from a field or laboratory population did not share any OTUs. Interestingly, *Enterococcus* sp. was found to be the dominant taxon in laboratory-reared larvae, but was completely absent from field larvae, indicating dramatic shifts in microbial community profiles upon cultivation of the moths in the laboratory. Furthermore, microbiota composition varied strongly across developmental stages in individuals of the field population, and we found no evidence for vertical transmission of bacteria from mothers to offspring. Overall, the high variability in bacterial communities suggests that the bacteria that we identified are only loosely and temporarily associated with *H. virescens*.

INTRODUCTION
Symbiotic bacteria of insects provide diverse beneficial services to their hosts, e.g., upgrading of nutrient-poor diets, digestion of refractory food sources and protection of the host against pathogens (e.g., Dillon & Dillon, 2004; Douglas, 2009; Feldhaar, 2011; Engel & Moran, 2013). Therefore, the study of bacterial communities that are associated with an organism of interest has become an integral part of the fundamental and applied research of, in particular, agricultural pests. Investigating bacterial diversity and insect-bacteria associations is greatly facilitated by high-throughput sequencing methods, especially massive parallel amplicon sequencing, that have been developed in the past decade (Margulies et al., 2005; Rothberg & Leamon, 2008; Bosch & McFall-Ngai, 2011; Loman et al., 2012).

Associations of bacterial communities with a host can be stable or dynamic. Stable associations are characterized by the temporal persistence of bacteria in the host across different life stages and across generations (Zilber-Rosenberg & Rosenberg, 2008; Rosenberg et al., 2009; Shade & Handelsman, 2012; Engel & Moran, 2013; Salem et al., 2015). The stability of the community can be indicative of the relevance of bacteria for the ecology and evolution of the host. If bacteria are transmitted vertically from one generation to the next for many generations, host and bacteria can be viewed as a unit of selection, and bacteria are likely beneficial for the host (Zilber-Rosenberg & Rosenberg, 2008; Rosenberg et al., 2009; Oliver et al., 2010).
Another criterion to assess the relevance or the functional role of bacteria for an organism is the variability of bacterial communities in the different environments in which the insect host resides (Robinson et al., 2010; Chandler et al., 2011; Colman et al., 2012; Gayatri Priya et al., 2012; Sullam et al., 2012). Variability of bacterial communities in relation to host diet is of particular interest in agricultural and forest pest insects, because the flexibility of their bacterial communities may enhance the dietary range of phytophagous insects (Dillon & Dillon, 2004; Hosokawa et al., 2007; Douglas, 2009; Tsuchida et al., 2011; Chu et al., 2013; Engel & Moran, 2013). Bacterial communities have been shown to vary depending on diet in several insect species (Broderick et al., 2004; Belda et al., 2011; Chandler et al., 2011; Gayatri Priya et al., 2012) but may also be stable across diets (Sudakaran et al., 2012). Other factors that may cause variation in bacterial communities in insects are the geographic origin of the host population or rearing history of the insect host (Xiang et al., 2006; Adams et al., 2010; Belda et al., 2011).

Studies of bacterial communities in Lepidoptera have accumulated in the past decade, mostly because larvae of many Lepidoptera are major agricultural or forest pests (Broderick et al., 2004; Xiang et al., 2006; Belda et al., 2011; Gayatri Priya et al., 2012; Xia et al., 2013). Bacteria from the genus Enterococcus have been repeatedly found in Lepidoptera (Jarosz, 1975; Broderick et al., 2004; Xiang et al., 2006; Brinkmann et al., 2008; Tang et al., 2012; Hammer et al., 2014) and have been shown to be metabolically active in Manduca sexta (Brinkmann et al., 2008). Other bacterial candidates that could serve an ecological role in Lepidoptera were isolated from saturniid butterflies and the silkmoth Bombyx mori (Pinto-Tomas et al., 2007; Prem Anand et al., 2010). These bacteria exhibited, among others, cellulyolytic, pectinolytic and xylanolytic activities that might help caterpillars to digest plant material (Pinto-Tomas et al., 2007; Prem Anand et al., 2010). Also, a recent metagenomic study of the bacterial midgut community in the pyralid moth Ostrinia nubilalis revealed the presence of bacterial cellulase, amylase, β-galactosidase and β-glucosidase encoding genes, indicating the potential of the gut bacterial community to support digestion in its lepidopteran host (Belda et al., 2011). However, when the midgut is damaged by insecticidal toxins in spores of Bacillus thuringiensis, formerly benign, resident bacteria may breach the midgut barrier, enter the hemocoel and participate in the destruction of the host (Broderick et al., 2006, 2009).

The noctuid moth Heliothis virescens is a major agricultural pest in North and South America (Fitt, 1989). Its larvae are polyphagous and feed on over 37 plant species from 14 families (Barber, 1937; Stadelbacher, 1981; Fitt, 1989; Waldvogel & Gould, 1990; Sheck & Gould, 1993; Blanco et al., 2007). Among these plants are important economic crops such as cotton (Gossypium hirsutum), tobacco (Nicottiana tabacum) and chickpea (Cicer arietinum) (Barber, 1937; Neunzig, 1969; Fitt, 1989; Blanco et al., 2007). Since bacteria can facilitate host-plant use by herbivorous insects, investigating the bacterial community of H. virescens and its variation in
relation to diet could lead to the identification of bacterial candidates that enhance or
inhibit its development and thereby its impact as an agricultural pest.

In this study, we identified and compared bacterial communities in different
developmental stages (eggs, larvae and female adults) of *H. virescens* from the field
and laboratory, and reared on different plant species (cotton, chickpea and tobacco).
Additionally, we investigated the transmission of the bacterial community from one
generation to the next.

**MATERIAL AND METHODS**

**Ethics statement**

*Heliothis virescens* was collected in North Carolina, USA, where this species is a
pest and not protected by law. Eggs of *H. virescens* were collected on private
property, for which no permits were required.

**Effect of host plant species on bacterial community composition of laboratory-
reared larvae**

To investigate whether the bacterial community composition in *H. virescens* larvae
changes depending on the food plant species, we used the long-term laboratory-
reared ARS strain. This strain was started in 1971 in Washington County, MS, and
since then reared in the laboratory at USDA-ARS in Stoneville, MS, and transferred
to the Max Planck Institute for Chemical Ecology (MPI-CE), Jena, Germany in
2010, where the larvae were reared on pinto bean diet containing the antibiotic
tetracycline hydrochloride (Sigma-Aldrich, The Netherlands) (Burton, 1970). Adults
were provided with a 10% (vol/vol) honey water solution. All life stages were kept
in climate chambers at a temperature of 25 °C, 60% relative humidity and a
light/dark cycle of 16h:8h.

We placed 30 first instars of the ARS strain on whole cotton (*Gossypium
hirsutum*), chickpea (*Cicer arietinum*) and tobacco plants (*Nicotiana attenuata*),
which were grown in the greenhouse of MPI-CE, Jena, Germany. After nine days,
when the larvae were in the second, third or fourth instar, they were taken off the
plants and starved for 8 h to empty their gut content and to reduce the amount of
chloroplasts, as chloroplast DNA may be amplified in the procedure of 16S rRNA
amplicon sequencing. To remove bacteria that resided on the outer cuticle,
experimental larvae were washed by submerging them for 10 s in 1% (wt/vol)
sodium dodecyl sulfate (SDS) solution and for 10 s in sterile water. Larvae were
then put in 1.5-ml Eppendorf tubes, immediately placed on ice and then stored at
-80 °C.

**Effect of host plant species on bacterial community composition of field larvae**

*Heliothis virescens* eggs were collected in July 2011 in North Carolina, USA, on
commercially grown tobacco, *N. tabacum*. Experiments were conducted with larvae,
referred to as field larvae, as well as females that developed from these larvae, and
eggs that were laid by these females. The field-collected eggs were divided into three
groups in plastic cups (100 ml) which were filled with leaf material of one of the three
plant species cotton (G. hirsutum), chickpea (C. arietinum) and tobacco (N. tabacum).
After emergence, batches of 20-30 first instar larvae were transferred to whole plants,
resulting in 10 batches per host plant species. The plants were kept outdoors until
experiments started (i.e., when larvae were placed on them to feed). Each plant was
kept in an individual cage made of fine-meshed gauze (diameter: 60 cm, height: 1 m).
After larval had reached the fifth instar, one larva was randomly collected from each
plant batch and starved for 8 h. These 30 larvae were washed by submerging them for
10 s in 1% (wt/vol) SDS solution and for 10 s in sterile water. After the wash step, guts
were extracted by cutting the larva between the 1st and 2nd thoracic and between the
7th and 8th abdominal segments. Dissected guts were stored at -80 °C until DNA
extraction. The remaining larvae were left to pupate in the soil of the potted whole
plant and subsequently used in the bacterial transmission experiment (see below).

Diversity and composition of the bacterial community in adult females and their
eggs
To determine bacterial communities in females and their corresponding eggs, a
subset of the field larvae that was not used to determine the larval bacterial
community was allowed to pupate in the soil and develop into adults. Upon
emergence, males and females that had fed on the same plant species were mated in
carton cups (200 ml) in single pairs. The cups contained leaves of the same plant
species on which the mating pairs had been as larvae to stimulate, and provide a
substrate for, oviposition. After four days, we collected abdomens from three
females and 30 of their eggs per plant species group, and thus obtained nine female-
egg combinations in total. DNA from eggs was extracted in batches of 30 eggs per
female. Eggs were collected from the plant leaves with a brush, transferred to 1.5-ml
Eppendorf tubes and placed immediately on ice until storage at -80 °C. Female
abdomens were cut off and submerged in 1% (wt/vol) SDS solution for 10 s,
followed by a 10 s washing step in sterile water. Abdomens were then transferred to
1.5-ml Eppendorf tubes and placed immediately on ice until storage at -80 °C. All
instruments were rinsed with 1% (wt/vol) SDS solution and sterile water between
samples. All tissues were collected in 1.5-ml Eppendorf tubes and placed
immediately on ice to prevent changes in the bacterial community composition, after
which the samples were stored at -80 °C.

Effect of laboratory-rearing and antibiotics treatment on bacterial communities of
field larvae
To assess whether bacterial communities change after transfer from the field to the
laboratory and how the antibiotic tetracycline that is normally part of the laboratory
diet affects the bacterial gut community, moths that originated from eggs that were
collected in the field to assess microbial communities (see above) were kept in the
laboratory at the University of Amsterdam for three generations (in the following referred to as field-lab larvae). Larvae were kept on artificial pinto-bean diet as described above, but without the addition of antibiotics. Females of the third generation were mated in single pairs in empty 200-ml plastic cups. Eggs of the fourth generation hatched in these cups, and the resulting first instars were transferred to cotton leaves with (AB group) or without tetracycline coating (NoAB group). Every other day, cotton leaves were coated with tetracycline by pipetting 1 ml 0.1% (wt/vol) tetracycline onto the upper leaf surface. Control leaves were treated in the same way with only water. In addition, we wrapped the leaf stems with cotton soaked with 0.1% (wt/vol) tetracycline or water in the treatment or the control group, respectively. When the larvae reached the fifth instar, they were starved for 8 h, then washed by submerging them for 10 s in 1% SDS solution and for 10 s in sterile water, after which their gut was extracted as described above. The guts were stored at -80 °C until DNA extraction.

**DNA extraction**

DNA from the field and field-lab population was extracted by grinding samples in 500 µl TES buffer \[100 mM \text{tris(hydroxymethyl) aminomethane hydrochloride pH 8, 10 M ethylenediaminetetraacetic acid, 2% sodium dodecyl sulfate}\] and 4 µl lysozyme from chicken egg white (100 mg/ml) (Sigma-Aldrich), after which the samples were incubated at 37 °C for 30 min. Next we added 2.5 µl proteinase K (20 mg/ml) (Sigma-Aldrich) and incubated the samples for 56 °C overnight. The rest of the extraction procedure was conducted according to the standard CTAB/chloroform protocol as described in Unbehend et al. (2013). DNA from the lab-strain larvae was extracted with the Epicenter MasterPure kit (Epicentre Technologies, USA) according to manufacturer’s instructions. Additionally, a lysozyme step [4 µl lysozyme (100 mg/ml), incubated at 37 °C for 30 min] was added before the proteinase K step.

**Bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP)**

Samples were pooled for sequencing. For the larvae of the laboratory strain, we made three pools, one pool contained DNA of seven larvae that were reared on each of the three plant species (cotton, tobacco or chickpea). For the larvae of the field population, we made six pools of DNA, two pools (biological replicates) for each plant species, to assess the variation between biological replicates; each pool contained the DNA of five larvae. For the field-lab population, we made two pools, one pool of 45 NoAB larvae and one pool of 49 AB larvae. Pooled samples were sent to an external service provider (Molecular Research Lab, MR DNA, Shallowater, TX, USA) for bTEFAP, using the 16S rRNA primers Gray28F (5’-GAGTTTGATC NTGGCTCA-3’) and Gray519R (5’-GTNTTACNGCGCKGCTG-3’) (Ishak et al., 2011). A sequencing library was constructed via one-step PCR with 30 cycles, using a mixture of Hot Start and HotStar hi-fidelity polymerases (Qiagen). Sequencing
was performed on a Roche 454 FLX instrument with Titanium reagents and procedures and protocols of Molecular Research LP (http://www.mrdnalab.com/).

**Sequence analysis**
Analysis of the sequences was performed in QIIME (quantitative insights into microbial ecology), which is a standard pipeline to analyse microbial communities based on 16S rRNA amplicon sequencing (Caporaso et al., 2010a). Raw data were de-noised with denoise_wrapper (Reeder & Knight, 2010). Low-quality reads (quality cut-off = 25) and sequences that were shorter than 200 bp or longer than 600 bp were excluded from further analysis. The remaining sequences were clustered into operational taxonomic units (OTUs) with the open-reference OTU picking command, using uclust (version 1.2.22) (Edgar, 2010) and applying 97% similarity cut-offs. Sequences were first clustered against a reference data set (http://greengenes.lbl.gov/). The sequences that did not cluster with the reference sequences were clustered de novo and checked for chimeras with Chimera Slayer (Haas et al., 2011). Identified chimeras were removed from the dataset for downstream analysis.

From each OTU cluster, the most abundant sequence was taken as the representative sequence. Taxonomy was assigned to the representative sequences with the uclust consensus taxonomy classifier. The resulting OTU table was manually edited, and global singletons as well as chloroplasts, mitochondria and OTUs that were classified as moth genomic DNA were removed. Representative sequences of the remaining OTUs were aligned with PyNAST using the Greengenes core set (http://greengenes.lbl.gov/) as a template (Caporaso et al., 2010b). A phylogenetic tree was constructed with the open source software Fasttree 2.1.3, applying the generalized time-reversible (GTR) model of nucleotide evolution (Price et al., 2010). Local support values for tree splits were calculated with the Shimodaira-Hasegawa test (Shimodaira & Hasegawa, 1999). This phylogenetic tree, which included all identified OTUs, was used to calculate the weighted UniFrac metric (Lozupone & Knight, 2005) which was used as the basis for principal coordinates analysis (PCoA) (see below).

**Diversity and phylogenetic analysis**
Diversity analysis of our samples was done with the QIIME pipeline. Since our samples showed unequal sampling depth (see TABLES S2.1-2.3), we investigated alpha- and beta-diversity of the bacterial communities of our samples with rarefied OTU tables. The number of sequences used for rarefaction corresponded to the number of sequences present in the sample with the lowest number of sequences (339 sequences). To assess alpha-diversity, we calculated the Shannon index based on the rarefaction tables (Shannon, 1948). To identify possible clusters based on similarity of bacterial communities among our samples, we produced a PCoA plot on the basis of weighted non-normalized UniFrac distances and with rarefied OTU
Bacterial community in *Heliothis virescens*

tables (Lozupone & Knight, 2005). To assess the robustness of our results, we additionally performed a Jackknife analysis on weighted UniFrac distances, which is included in the QIIME pipeline (see Lozupone & Knight, 2005; Caporaso et al., 2010; Lozupone et al., 2011). Jackknife support is included in the PCoA plots as the size of the ellipsoid areas surrounding the data points.

**OTU patterns across samples**

None of the bacterial OTUs was present in all samples, i.e., present in samples of all origins, rearing plants, and life stages. Most common were three OTUs which were present in 65% of the samples. These OTUs were mainly present in females and eggs, which had a high sample size compared to the other groups. To also consider OTUs that were present in the larvae, we filtered for OTUs that were on average represented more than 1% across all samples, resulting in 18 OTUs (i.e., first the percentage of an OTU in a sample was calculated followed by the average of the percentage of the OTU in all samples, see TABLE 2.1). To classify and construct a phylogenetic tree of these 18 OTUs, we first aligned the sequences of our dataset and 16S rRNA reference sequences obtained from Genbank (Benson et al., 2011), using ClustalW (Thompson et al., 1994), after which we constructed a maximum likelihood tree in MEGA 6 (Tamura et al., 2013) with the Tamura-Nei model (Tamura & Nei, 1993), using uniform rate variation and 500 bootstrap replicates. To root the tree, we used three Archaea species as outgroups: *Halobacterium salinarum* (NR_113428.1), *Sulfolobus acidocaldarius* (NR_074267.1) and *Pyrolobus fumarii* (NR_102985.1). Outgroup taxa were excluded from the FIGURE for clarity (FIGURE 2.1).

**TABLE 2.1.** Bacterial OTUs with an average presence of more than 1% across all samples.

<table>
<thead>
<tr>
<th>#</th>
<th>Family (Genus)</th>
<th>Total Average (29)</th>
<th>Larvae Lab (3)</th>
<th>Larvae Field (6)</th>
<th>Larvae Field-lab (2)</th>
<th>Adults (9)</th>
<th>Eggs (9)</th>
<th>Overall presence &gt; 1%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Enterobacteriaceae (Klebsiella/Enterobacter)</td>
<td>27.63</td>
<td>0.00</td>
<td>0.00</td>
<td>35.62</td>
<td>72.34</td>
<td>35.95</td>
<td>68.97</td>
</tr>
<tr>
<td>2</td>
<td>Enterococcaceae (Enterococcus)</td>
<td>11.40</td>
<td>50.55</td>
<td>0.00</td>
<td>61.70</td>
<td>0.00</td>
<td>0.01</td>
<td>17.24</td>
</tr>
<tr>
<td>3</td>
<td>Enterobacteraeaceae (Serratia)</td>
<td>8.57</td>
<td>0.00</td>
<td>0.00</td>
<td>0.01</td>
<td>7.20</td>
<td>22.42</td>
<td>44.83</td>
</tr>
<tr>
<td>4</td>
<td>Pseudomonadaceae (Pseudomonas)</td>
<td>4.82</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>6.90</td>
</tr>
<tr>
<td>5</td>
<td>Enterobacteraeaceae (Enterobacter)</td>
<td>3.97</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>5.06</td>
<td>5.29</td>
<td>20.69</td>
</tr>
<tr>
<td>6</td>
<td>Acetobacteraeaceae (Asaia)</td>
<td>2.38</td>
<td>49.45</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>6.90</td>
</tr>
<tr>
<td>7</td>
<td>Enterobacteraeaceae (unclassified)</td>
<td>2.15</td>
<td>0.00</td>
<td>7.75</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>6.90</td>
</tr>
<tr>
<td>8</td>
<td>Mycobacteraeaceae (Mycobacterium)</td>
<td>2.15</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>4.13</td>
<td>27.59</td>
</tr>
<tr>
<td>9</td>
<td>Acidobacteraeaceae (unclassified)</td>
<td>2.04</td>
<td>0.00</td>
<td>8.42</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>3.45</td>
</tr>
<tr>
<td>10</td>
<td>Pseudomonadaceae (Pseudomonas)</td>
<td>1.87</td>
<td>0.00</td>
<td>0.00</td>
<td>0.04</td>
<td>5.32</td>
<td>0.02</td>
<td>3.45</td>
</tr>
</tbody>
</table>
Statistical analysis of similarities between bacterial communities of females and their eggs

To test whether egg bacterial communities resemble the bacterial community of their mother, we performed a linear mixed model in the package lme4 (Bates et al., 2014) and lmerTest (Kuznetsova et al., 2014) in the software R, version 3.0.2 (R Core Team, 2013). Weighted UniFrac distance values of all 81 female-egg combinations were used as response variable. We used two predictor variables: female-egg combination (eggs belonged to one female or not) and plant species (eggs belonged to the same plant species – cotton, chickpea, or tobacco – as a female or not). To account for repeatedly entering females and eggs into the model, we added egg and female as random factors into the model. Because there was no interaction effect between plant and female-egg combination, we excluded the interaction from the model. Degrees of freedom were approximated using the Satterthwaite method. As a post hoc test for the plant effect, we performed planned pairwise comparisons between female-eggs combinations of one plant species group and female-egg combinations in which females did, but eggs did not belong to that respective plant group, by using the multcomp package (Hothorn et al., 2008) in R, applying a Bonferroni correction for multiple comparisons.

RESULTS

Bacterial community composition of Heliothis virescens

We received a total of 312,324 sequences, of which 295,289 sequences remained after quality filtering. Other sequences were global singletons or chimeras, derived from chloroplasts and mitochondria or fragments that were identified as part of the moth genome. The bacterial sequences clustered into 566 OTUs, of which 42 were unassigned. An overview of numbers of sequences before and after quality filtering, as well as numbers of OTUs in the different samples is given in TABLES S2.1-2.3.
None of the OTUs in *H. virescens* was present in all samples (*n* = 29), and only three OTUs were found in more than 65% of our samples (65% was the highest percentage of samples in which OTUs were commonly present). When we assessed how many OTUs had an average percentage of more than 1% across all samples, this resulted in 18 OTUs, but only two of these were present with an average percentage of more than 10% across all samples (Table 2.1). The phylogenetic analysis revealed that these 18 OTUs belong to the following bacterial families (bacterial orders in parentheses): Acetobacteraceae (Rhodospirillales), Methylobacteriaceae (Rhizobiales), Sphingomonadaceae (Sphingomonadales), Comamonadaceae (Burkholderiales), Enterobacteriaceae (Enterobacteriales), Pseudomonadaceae, Moraxellaceae (both Pseudomonadales), Xanthomonadaceae (Xanthomonadales), Mycobacteriaceae (Actinomycetales), Paenibacillaceae (Bacillales), Enterococcaceae (Lactobacillales); and one OTU belonged to the phylum Acidobacteria (Table 2.1 and Figure 2.1).

**Variability of bacterial communities between laboratory and field larvae**

We compared bacterial communities between larvae from a long-term laboratory colony and larvae that had been collected in the field as eggs. Laboratory and field larvae had no OTUs in common. The bacterial community of lab larvae was dominated by two OTUs that were classified as *Enterococcus* sp. (Lactobacillales) (#2) and *Asaia* sp. (Rhodospirillales) (#6), while larvae of the field population did not contain any OTUs affiliated with these genera. In fact, field larvae did not contain clearly dominant OTUs, but harboured Enterobacteriales, Burkholderiales or Rhizobiales in relatively high percentages (see below). Furthermore, bacterial communities in the field-lab larvae differed greatly from the bacterial communities of the field larvae: only 1.4% of the OTUs (*n* = 148) of the field larvae were detected in the field-lab larvae that were reared without antibiotics (NoAB). *Enterococcus* sp. (#2) was more abundant in the AB field-lab larvae than in NoAB larvae (Figure 2.2).

Larvae of the field population and the laboratory colony also differed in the diversity of their associated bacterial communities. Larvae from the laboratory colony harboured an average of 25 OTUs, while field larvae had an average of 35 OTUs. Additionally, communities of the field larvae showed a greater evenness than those of the laboratory larvae (Figure 2.2), which was reflected in the higher Shannon index of the field larvae (Figure 2.3).

**Host plant-associated variability of bacterial communities in larvae**

The influence of diet on the bacterial communities of laboratory and field larvae was investigated by comparing bacterial communities in larvae that fed on three different host plant species. In laboratory-reared larvae, we found one OTU (#2) from the family Enterococcaceae dominating the bacterial community in the guts of larvae fed chickpea or tobacco, i.e., 97.8 and 91.5% abundance, respectively (Figure 2.2). The
FIGURE 2.1. Maximum likelihood phylogenetic tree of bacterial OTUs with an average presence of more than 1% across all samples. OTUs that were identified in *Heliothis virescens* are indicated in bold font and labelled OTU #1 to OTU #18. Reference sequences were obtained from Genbank; strain names are given behind the species or genus name. Accession numbers are between brackets. Font colours correspond to different bacterial families, background colours indicate bacterial phyla (or class in the Proteobacteria). The tree was constructed on the basis of partial 16S rRNA gene sequences, applying 500 bootstrap replications. Bootstrap values are indicated above the branches.
**FIGURE 2.2.** Relative abundance of bacterial OTUs in *Heliothis virescens* larvae. Bacterial OTUs were combined on the family level. Numbers in brackets indicate how many OTUs one family contains. Rare OTUs that were represented less than 0.5% in at least one sample are not included. Larvae originated from a laboratory colony, from the field or from the field, after which they were reared in the laboratory for four generations (field-lab): AB: larvae received antibiotics treatment, NoAB: larvae received no antibiotics treatment. Field and laboratory larvae were grown on cotton (C), chickpea (Ch) or tobacco (T); square brackets around taxa indicate that the taxon name is not fully established yet.

**FIGURE 2.3.** Rarefaction curves of alpha diversity of the bacterial communities of *Heliothis virescens* larval guts. a) Number of observed bacterial OTUs. b) Shannon index of the bacterial communities. Laboratory (L) and field larvae (F) were fed on cotton (C), chickpea (Ch) or tobacco (T). AB: field larvae that were reared for four generations in the laboratory and received antibiotics during one generation; NoAB: field larvae that were reared for four generations in the laboratory and did not receive antibiotics treatment.
closest hits for OTU #2 were *Enterococcus faecium* (HG937762), *E. gallinarum* (HG937758) and *E. casseliflavus* (KJ528946) with 99% similarity. The larvae fed on cotton also contained OTU #2, but in much lower abundance, i.e., 31.8%, while an OTU of the family Acetobacteraceae (#6) had the highest abundance (67.1%). The closest hit for this OTU was *Asaia lannensis* (KP208318), with 99% similarity.

In the field larvae, we also found differences in bacterial communities depending on the host plant species they fed on. Enterobacteriaceae were the dominant family in the larvae that fed on chickpea and also occurred in larvae that fed on tobacco, while these were rare (<1%) in the larvae fed on cotton. The larvae fed on tobacco and cotton contained Burkholderiales (Comamonadaceae, e.g., #18, and Oxalobacteraceae), which were rare (<1%) in larvae fed on chickpea (Figure 2.2). Methylobacteriaceae (e.g., #11) were among the most abundant families in the larvae that fed on cotton, while this family was hardly detected in the other two groups. On the genus (and OTU) level, the two biological replicates from each plant group differed greatly from each other (Figure S2.1).

**Variability of bacterial communities across different life stages of the field population**

We assessed the temporal persistence of bacterial communities in *H. virescens* by comparing bacterial communities of different life stages of the field population (larvae, adults and eggs). Bacterial communities differed greatly across life stages. Females shared 76% of their OTUs (n = 200) with eggs, and eggs shared 52.1% of their OTUs (n = 292) with females. In contrast, larvae only shared 2.7% of their OTUs (n = 148) with females and 6.1% OTUs with eggs. Accordingly, in a PCoA plot, females and eggs partly clustered together, while five of the six larval samples were located in a different area of the plot (Figure 2.4). Females of the field population shared 22% of their OTUs (n = 200) with the field-lab larvae that were reared in the laboratory for four generations (the NoAB group), while the eggs had 18.8% of their OTUs (n = 292) in common with the field-lab larvae.

One OTU (#1) from the family Enterobacteriaceae had the highest relative abundance in both females and eggs (Figure 2.5). The closest hits for this OTU in NCBI were *Enterobacter ludwigi* (KJ767368), *E. cloacae* (KM817773) and *Klebsiella oxytoca* (KM408615), all with 97% similarity; this was confirmed by its position in the phylogeny (Figure 2.1). In the field larvae, OTU #1 was not present, but two other Enterobacteriaceae OTUs were found (#7 and #12), which clustered with OTU #1 in the phylogenetic tree (Figures 2.1 and 2.2).

**Vertical transmission of bacterial communities**

Based on weighted UniFrac distance values, bacterial communities present in females were not more similar to the bacterial communities of their own eggs than to the bacterial communities of eggs that were not their own ($F_{1,65.74} = 1.28$, $P = 0.26$). However, rearing on different plant species had a significant effect on bacterial com-
munities in female-egg combinations (F_{5,66} = 3.17, P = 0.013). None of the comparisons for the variable plant species were significant in planned pairwise comparisons with Bonferroni corrections. However, cotton-reared females tended to be more similar to bacterial communities of eggs of their own plant group than to those of a different plant group (z = -2.31, P = 0.062). This marginal effect was not found for tobacco females and eggs (z = 1.46, P = 0.43) and was even reversed – bacterial communities of females were more similar to bacterial communities of other eggs than to eggs of their own plant group – for chickpea-reared females (z = -2.33, P = 0.060). The principal coordinates analysis plot supports these results (see Figure 2.4).

**DISCUSSION**

In this study we assessed the variability of bacterial communities in the agricultural pest *H. virescens*. We observed that the bacterial communities of different life stages, of different populations (field population or laboratory colony) as well as of larvae that fed on different host plant species, and even biological replicates, were very different.

*Abundant bacterial strains in Heliothis virescens*

Most of the dominant bacterial families in our study, i.e., Acetobacteraceae, Methylobacteriaceae, Sphingomonadaceae, Comamonadaceae, Enterobacteriaceae, Pseudomonadaceae, Moraxellaceae Xanthomonadaceae, Paenibacillaceae and Enterococcaceae, have been identified in Lepidoptera before (Broderick *et al.*, 2004;
This suggests that representatives of these bacterial families may be commonly present in many Lepidoptera and the environments they live in.

Variability of bacterial communities between laboratory and field larvae

Bacterial communities differed greatly between laboratory and field larvae, in fact no OTUs were shared between the two groups. These large differences in bacterial communities underline the importance of including field populations of an organism in studies of the functional role of its bacterial communities. Bacterial communities of field and laboratory insect populations have for instance been studied in the moths *Helicoverpa armigera* (Xiang et al., 2006) and *Ostrinia nubilalis* (Belda et al., 2011; Pinto-Tomás et al., 2011; Gayatri Priya et al., 2012; Tang et al., 2012; Hammer et al., 2014). This suggests that representatives of these bacterial families may be commonly present in many Lepidoptera and the environments they live in.

**FIGURE 2.5.** Relative abundance of bacterial OTUs in the abdomen of *Heliothis virescens* females (F) and their eggs (E). Females (F1-F9) and their eggs (E1-E9) are shown next to each other. Females had fed on three different plants as larvae: cotton (C), chickpea (Ch), tobacco (T). Rare OTUs that were represented less than 0.5% in at least one sample are not shown. Brackets around genera signify that there were bacterial candidates with ≥ 97% identity, but support in the phylogenetic tree was weak [i.e., nodes that divide branches that contain different genera have bootstrap values equal to or below 10 (see FIGURE 2.1)].
In our study, Enterococcus sp. was the dominant bacterial strain in the laboratory colony and the AB group of the field-lab larvae, and it was the second most abundant strain in the NoAB group of the field-lab larvae. However, this strain was completely absent in the field larvae, suggesting that Enterococcus sp. was introduced to the insects in the laboratory. Enterococcus sp. has been identified as a dominant microbe in several Lepidopteran gut communities, including Galleria mellonella, Lymantria dispar, Helicoverpa armigera, Manduca sexta, Spodoptera littoralis and Heliconius erato (Jarosz, 1975; Broderick et al., 2004; Xiang et al., 2006; Brinkmann et al., 2008; Tang et al., 2012; Hammer et al., 2014). Interestingly, most of these studies used only laboratory colonies (Jarosz, 1975; Brinkmann et al., 2008; Tang et al., 2012). In Helicoverpa armigera and Ostrinia nubilalis, where both laboratory and
field populations were investigated (Xiang et al., 2006; Belda et al., 2011), single bacterial strains such as Enterococcus sp. and other gram-positive cocci (e.g., Staphylococcus sp. in O. nubilalis) were found to become dominant in laboratory populations, while they were less abundant in field populations. In H. erato, enterococci became more abundant in adults after this species had been in captivity for one generation (from adult to adult) compared to field-collected adults (Hammer et al., 2014). Possibly, enterococci are outcompeted in the field by other bacteria (e.g., Proteobacteria such as Enterobacter, Klebsiella and Pseudomonas species) in the insect gut and/or on the plants while competition changes in favour of enterococci under (more stable) laboratory conditions.

Treating H. virescens larvae with tetracycline in the laboratory probably selected for enterococci, as the relative abundance of enterococci was higher in larvae treated with antibiotics than in untreated larvae. This is in line with the finding that many Enterococcus strains have developed resistance against tetracycline (Huys et al., 2004; Wilcks et al., 2005; Cauwerts et al., 2007). It is interesting to note, however, that enterococci also became abundant in laboratory-reared larvae in the absence of antibiotics, indicating that antibiotic treatment alone does not account for the shift in microbial communities towards high abundance of Enterococcus spp.

**Host plant-associated variability of bacterial communities in larvae**

In both field and laboratory H. virescens larvae, bacterial gut communities varied depending on host plant. Diet has been found to influence bacterial gut community composition in larvae of many insect species, e.g., Drosophila (Chandler et al., 2011) and several lepidopteran species (Broderick et al., 2004; Gayatri Priya et al., 2012; Tang et al., 2012). Diet might influence bacterial communities in various ways. For example, different bacteria may be present in or on different host plants, the resources provided by the different plants may promote differential bacterial growth and/or secondary plant metabolites may have a selective effect on bacterial communities (Yang et al., 2001; Vorholt, 2012). Additionally, host plants can influence the physiochemical conditions of the larval gut, which possibly results in differential bacterial growth (Schultz & Lechowicz, 1986; Appel & Maines, 1995).

Although we did not sample bacteria from plants on which the larvae had fed, earlier studies have assessed bacterial communities of cotton and tobacco plants, and identified several bacterial families that we also detected in our samples (McInroy & Kloepper, 1995; Brinkmann et al., 2008), suggesting that the experimental H. virescens larvae had ingested bacteria from their food plants. For instance, Methylbacteriaceae was the most abundant bacterial family in the field larvae fed on cotton in our study, and this bacterial family has been isolated from different tissues of cotton (McInroy & Kloepper, 1995; Madhaiyan et al., 2012). Also, bacteria from the order Burkholderiales (Comamonadaceae and Oxalobacteraceae), which were mainly present in the field larvae fed on tobacco in our study, were previously isolated from
tobacco leaves (Brinkmann et al., 2008). Furthermore, Enterobacter, Pseudomonas and Serratia were the most abundant genera in females and eggs in our study, and have been isolated from, among others, cotton tissues and tobacco leaves (McInroy & Kloeper, 1995; Junker et al., 2011; Lv et al., 2012; Ma et al., 2013).

Interestingly, in L. dispar, bacteria that were isolated from leaves of its host plant aspen, have recently been shown to enhance larval growth of this moth in the presence of phenolic glycosides, a defence metabolite of aspen (Mason et al., 2014). Whether H. virescens can profit from bacteria that are transient or reside on its host plants still needs to be investigated.

Notably, there were differences in bacterial community composition between the two biological replicates per plant species in the field larvae, particularly at low taxonomic levels (i.e., genus or OTU). This indicates large individual variation in the bacterial gut community of H. virescens larvae in the field, and further suggests that the bacterial community is only loosely associated with this moth.

When investigating microbial communities using bacterial tag-encoded FLX pyrosequencing of 16S rRNA amplicons, it is important to keep in mind that this method can lead to incorrect estimates of OTU numbers (Kunin et al., 2010; Schloss et al., 2011; Pinto & Raskin, 2012). Errors that have been described for this method include PCR bias, sequencing errors (particularly in homopolymeric regions) and the production of chimeric sequences during PCR reactions (Chandler et al., 1997; Margulies et al., 2005; Haas et al., 2011; Schloss et al., 2011). Even though we have implemented in our analyses a number of measures to decrease methodological errors (de-noising raw data, quality cut-off, length limitation of sequences, identification and removal of possible chimeric sequences, removal of global singletons), it is possible that the number of OTUs may still be overestimated and that the diversity patterns that we show on the family level for larvae is actually more realistic than the patterns we encountered on the OTU level (compare FIGURES 2.2 and S2.1).

**Variability of bacterial communities across different life stages of the field population**

In H. virescens, it seems that bacterial communities are completely restructured or lost during the metamorphosis from crawling larvae to winged adult, because bacterial communities in larvae of the field population differed strongly from those in adult females. One of the few Lepidoptera where bacterial persistence across metamorphosis has been studied is the butterfly H. erato (Hammer et al., 2014). In this species, bacterial communities between larvae and adults also differed greatly but more bacterial strains (13%) overlapped between larvae and adults in their study than in our study, in which only 2.7% of OTUs overlapped between larvae and adult females. A similar pattern was found in mosquitoes, in which bacterial composition partly changed during metamorphosis, but many strains were retained (Rani et al., 2009). One reason for the difference in bacterial communities between larvae and adults in Lepidoptera might be that larvae and adults feed on different diets: while
larvae usually feed on the foliage of their host plants, adults often feed only on nectar and in this study on honey water. The gut of lepidopteran larvae is less compartmentalized than the gut of many other insect species, mostly has a very alkaline pH, and is purged before metamorphosis (Nijhout & Williams, 1974; Dow, 1992; Appel & Maines, 1995; Chapman et al., 2013; Engel & Moran, 2013). Together, these characteristics might make it difficult for bacteria to successfully colonize Lepidopteran guts. Moreover, in moths an increase in lysozyme production before and during metamorphosis has been reported, which likely reduces bacterial abundance and diversity especially immediately before and after the pupal stage, i.e., pupating larvae and newly eclosed adults (Russell & Dunn, 1996; Zhang et al., 2009). Upon adult eclosion, bacterial titers may therefore be low and bacterial strains that are different from the larval bacterial community might be able colonize the adults (see also Hammer et al., 2014).

Vertical transmission of bacterial communities
We investigated whether bacteria in *H. virescens* are vertically transmitted by comparing bacterial communities of females and their eggs and tested if bacterial communities of eggs were more similar to bacterial communities of their own mother than to any other female. The fact that we did not find this effect suggests that vertical transmission likely does not occur in *H. virescens* at a significant rate. However, since bacterial communities were at least partly similar in females and eggs, we cannot completely exclude the occurrence of vertical transmission. A study using fluorescently-labelled *E. coli* was able to detect a certain amount of mother-offspring transmission in *Galleria mellonella*, although the viability of the bacteria was not assessed (Freitak et al., 2014). Also, a previous study about vertical transmission of *Serratia marcescens* in a laboratory population of *H. virescens* indicated that bacteria can be vertically transmitted in this moth (Sikorowski & Lawrence, 1998).

Conclusions
The huge variability of bacterial communities that we found between life stages, diets, biological replicates and field and lab populations indicates that the major part of the bacterial communities that we identified in the gut of *H. virescens* is of a transient nature and only loosely associated with its host. In fact, the bacterial communities seem to be entirely restructured during metamorphosis. Our results further suggest that bacterial communities are not transmitted at a significant rate from mothers to eggs in *H. virescens*. Based on these results, it is doubtful that particular bacterial strains that we have identified form a unit of selection with this moth. It is further unlikely that a long-term mutualistic symbiosis between *H. virescens* and bacteria that could facilitate host plant use and adaptation has evolved. Importantly, we found that enterococci are most probably introduced to *H. virescens* larvae in the laboratory. This finding stresses the importance of including field populations when bacterial communities of an organism are to be characterized.
Acknowledgements

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Bacterial community in *Heliothis virescens*


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**SUPPORTING INFORMATION**

**Figure S2.1.** Relative abundance of bacterial OTUs in *Heliothis virescens* larvae. OTUs are identified at the genus level. Rare OTUs that were represented less than 0.5% in at least one sample are not included. Larvae originated from a laboratory colony, from the field or were collected from the field as eggs, after which they were reared in the laboratory for four generations (field-lab): AB: larvae received antibiotics treatment, NoAB: larvae received no antibiotics treatment. Field and laboratory larvae were grown on cotton (C), chickpea (Ch) or tobacco (T). Brackets around genera signify that there were bacterial candidates with ≥ 97% identity, but support in the phylogenetic tree was weak [i.e., nodes that divide branches that contain different genera have bootstrap values equal to or below 10 (see Figure 2.1)].
### TABLE S2.1. Statistics of bacterial tag-encoded FLX amplicon sequencing and number of OTUs in *Heliothis virescens* larvae. The bacterial communities of field larvae (F), laboratory larvae (L) and field-lab (AB/NoAB) larvae were characterized. The latter was reared in the laboratory for four generations after collection in the field. AB: treated with antibiotics (tetracycline); NoAB: not treated with antibiotics. Larvae of the field and laboratory strain were fed on three different plant species: cotton (C), chickpea (Ch), and tobacco (T). Field larvae: two sequencing pools per plant species; laboratory and field-lab larvae: one pool per plant species; # = number, Qual. seqs = quality filtered sequences.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Laboratory larvae</th>
<th>Field larvae</th>
<th>Field-lab larvae</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CL ChL TL</td>
<td>CF1 CF2 ChF1 ChF2 TF1 TF2</td>
<td>AB NoAB</td>
</tr>
<tr>
<td># Reads</td>
<td>30475 6917 5614</td>
<td>33880 2226 2590 2835 5356 3189</td>
<td>11464 10344</td>
</tr>
<tr>
<td># Qual. seqs.</td>
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<td>20963 1520 2318 2791 5333 3189</td>
<td>11443 10341</td>
</tr>
<tr>
<td># OTUs</td>
<td>27 21 27</td>
<td>55 23 53 23 16</td>
<td>64 90</td>
</tr>
</tbody>
</table>

### TABLE S2.2. Statistics of bacterial tag-encoded FLX amplicon sequencing and number of OTUs in individual *Heliothis virescens* females. Females fed on three plant species as larvae: cotton (Cot), chickpea (Chi), tobacco (Tob); # = number, Qual. seqs = quality filtered sequences.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cot fem1 Cot fem2 Cot fem3Chi fem4 Chi fem5 Chi fem6 Chi fem7 Chi fem8 Chi fem9</th>
<th>Cot fem1 Cot fem2 Cot fem3Chi fem4 Chi fem5 Chi fem6 Chi fem7 Chi fem8 Chi fem9</th>
<th>Cot fem1 Cot fem2 Cot fem3Chi fem4 Chi fem5 Chi fem6 Chi fem7 Chi fem8 Chi fem9</th>
<th>Cot fem1 Cot fem2 Cot fem3Chi fem4 Chi fem5 Chi fem6 Chi fem7 Chi fem8 Chi fem9</th>
</tr>
</thead>
<tbody>
<tr>
<td># Reads</td>
<td>7586 16398 7374 15853 13047 9509 13254 2895 16990</td>
<td>7586 16398 7374 15853 13047 9509 13254 2895 16990</td>
<td>7586 16398 7374 15853 13047 9509 13254 2895 16990</td>
<td>7586 16398 7374 15853 13047 9509 13254 2895 16990</td>
</tr>
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<td># Qual. seqs.</td>
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<td>7586 16398 7374 15853 13047 9509 13254 2895 16990</td>
<td>7586 16398 7374 15853 13047 9509 13254 2895 16990</td>
<td>7586 16398 7374 15853 13047 9509 13254 2895 16990</td>
</tr>
<tr>
<td># OTUs</td>
<td>33 44 34 65 72 65 54 38 57</td>
<td>33 44 34 65 72 65 54 38 57</td>
<td>33 44 34 65 72 65 54 38 57</td>
<td>33 44 34 65 72 65 54 38 57</td>
</tr>
</tbody>
</table>

### TABLE S2.3. Statistics of bacterial tag-encoded FLX amplicon sequencing and number of OTUs in *Heliothis virescens* eggs. Mothers of these eggs had fed on three different plant species as larvae: cotton (Cot), chickpea (Chi), tobacco (Tob); # = number, Qual. seqs = quality filtered sequences.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cot egg1 Cot egg2 Cot egg3 Cot egg4 Cot egg5 Cot egg6 Cot egg7 Cot egg8 Cot egg9</th>
<th>Cot egg1 Cot egg2 Cot egg3 Cot egg4 Cot egg5 Cot egg6 Cot egg7 Cot egg8 Cot egg9</th>
<th>Cot egg1 Cot egg2 Cot egg3 Cot egg4 Cot egg5 Cot egg6 Cot egg7 Cot egg8 Cot egg9</th>
<th>Cot egg1 Cot egg2 Cot egg3 Cot egg4 Cot egg5 Cot egg6 Cot egg7 Cot egg8 Cot egg9</th>
</tr>
</thead>
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<tr>
<td># Reads</td>
<td>1546 18062 8461 1724 8033 466 27786 8890 19760</td>
<td>1546 18062 8461 1724 8033 466 27786 8890 19760</td>
<td>1546 18062 8461 1724 8033 466 27786 8890 19760</td>
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<tr>
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