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Bacterial communities of the reproductive organs of virgin and mated common bedbugs, *Cimex lectularius*

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Abstract. 1. Microbes associated with reproductive organs of animals are either sexually transmitted or opportunistic. Both can affect host defence, immunity, and future colonisation with other microbes. There are only few studies on the microbiota of reproductive organs in insects and how they are affected by copulation.

2. This study examines the bacterial communities associated with reproductive organs in the common bedbug *Cimex lectularius*, a well-established insect model for the effects of microbes on male and female reproduction. Combining a metagenomic approach with a controlled mating scheme, we found 31 sequence variants (SVs) across 55 organ samples, with on average three SVs in each sample. Male and female reproductive organs harboured distinct bacterial communities in terms of present SVs.

3. Using a community ecology approach, we found three potential indications of sexual transmission of bacteria in the common bedbug: (i) copulation increased the similarity of the communities of male and female organs; (ii) mated individuals harboured bacteria that were found in non-mated individuals of the opposite sex but not in non-mated individuals of the same sex; and (iii) bacterial communities showed a high SV turnover between non-mated and mated individuals, suggesting a mating-induced replacement of bacteria.

4. Our findings show that the community ecology approach is useful to examine the bacterial dynamics on reproductive organs, especially when combined with studies that quantify the frequency of transmission and/or estimate the effect of the transmitted microbes on the host immune system and the host endosymbionts.

Key words. Microbiome, reproductive ecology, sexually transmitted diseases.

Introduction

The microbial community surrounding and inhabiting the organism is an important, and increasingly recognised, component of an organism's environment. This community may shape reproductive structures, physiology, reproductive behaviour, and, ultimately, fitness. These effects have been mainly explored for sexually transmitted microbes (STMs) (Afzelius *et al.*, 1989; Lockhart *et al.*, 1996; Shalika *et al.*, 1996; Knell & Webberley, 2004; Eley *et al.*, 2005; Puerta Suarez *et al.*, 2017). However, reproductive organs also harbour environmental contaminants (or opportunistic microbes, OMs) (Marius-Jestin, 1987; Reinhardt *et al.*, 2005; Otti *et al.*, 2017) and can receive them via copulation by spreading through the reproductive tract and through copulatory wounds (Lange *et al.*, 2013; Reinhardt *et al.*, 2015).

In vertebrates, microbes colonise various reproductive organs in a wealth of species (Hirsh, 1999; Lombardo & Thorpe, 2000; Hupton *et al.*, 2003; Virecoulon *et al.*, 2005; González-Marín *et al.*, 2011; White *et al.*, 2011). The few studies in insects found bacteria associated with the reproductive organs of female Formosan subterranean termites (Raina *et al.*, 2007), male wood-boring beetles (Rizzi *et al.*, 2013), and male and female common bedbugs (Reinhardt *et al.*, 2005, Otti *et al.*, 2017; see Table 1). Most of the bacteria in and on the copulatory organs of insects belong to the classes of Actinobacteria, Bacilli, or Gammaproteobacteria (Otti, 2015).

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Table 1. Presence of previously found bacterial genera in the reproductive organs of non-mated and mated bedbugs.

Genus	Present study	Reinhardt *et al.* (2005)	Otti *et al.* (2017)
Bacillus	–	I	–
Enterobacter	–	I	–
Micrococcus	–	–	C_M
Pseudomonas	S_{NM}, I_{NM}	–	C_M
Staphylococcus	S_{NM}, S_M, I_{NM}, I_M	–	I, C_{NM}, C_M
Stenotrophomonas	–	I	–
Streptococcus	I_M	–	C_M

Given are the results from Reinhardt *et al.* (2005) and Otti *et al.* (2017) and the present study for each organ (S, male sperm container; I, male intromittent organ; C, female copulatory organ) and mating status (NM, non-mated; M, mated). In contrast to our study, both previous studies are based on culture-dependent methods.

The effect of these microbes has been less considered even though OMs are ubiquitous and copulatory wounding is both common and widespread across taxa (Lange *et al.*, 2013; Reinhardt *et al.*, 2015). Despite causing infections (Klainer & Beisel, 1969), bacteria affect sperm function directly (Otti *et al.*, 2013; Reinhardt *et al.*, 2015), lower the proportion of viable sperm in the female storage organs (McNamara *et al.*, 2014), and cause fitness costs due to resource allocation to the immune system (Sheldon & Verhulst, 1996; Zuk & Stoehr, 2002). Such fitness costs due to immune challenge include reduced probability of reproduction (Rigby & Jokela, 2000) and reduced offspring quality (Ilmonen *et al.*, 2000).

Opportunistic microbes have the potential to become pathogenic when the immune system of the host is disturbed (Klainer & Beisel, 1969). They interact with the immune system of the host that regulates the growth of OMs, and even endosymbionts (Login *et al.*, 2011). If such an immune response does not target specific OMs, the immune response might affect symbionts at the same time. Mating therefore has the potential to change the microbial communities in the reproductive organs not only by transmitting microbes but also by eliciting immune responses that shape the resident microbial community. Even more complicated, symbionts probably compete with OMs or STMs because invasion into a bacterial community is limited by available resources (Li & Stevens, 2012; Mallon *et al.*, 2015). Symbionts have actually been shown to provide protection against invading microbes (Reid *et al.*, 1987; Boris *et al.*, 1998; Oh *et al.*, 2009; Koch & Schmid-Hempel, 2012; Mattoso *et al.*, 2012; Kamada *et al.*, 2013; Kaltenpoth & Engl, 2014; Braquart-Varnier *et al.*, 2015; King *et al.*, 2016). We suggest that this protection might also be directed against sexually transmitted microbes.

Some of the interactions among OMs, STMs, and symbionts and some of the fitness effects have been established, but it seems important to consider two basic but fundamental insights from community ecology – that species are not necessarily redundant and that ecosystem effects vary with species composition of the community (Allison & Martiny, 2008; Rillig *et al.*, 2015). In terms of species redundancy, it is clear that different microbe species, and even populations, have different effects on the host. For example, in humans, *Chlamydia trachomatis* (Eley *et al.*, 2005), *Escherichia coli* (Diemer *et al.*, 1996; Diemer *et al.*, 2003; Prabha *et al.*, 2010), *Pseudomonas aeruginosa* (Huwe *et al.*, 1998), and *Staphylococcus aureus* (Kaur *et al.*, 2010) were shown to cause agglutination and apoptosis of spermatozoa, whereas an *Enterococcus* species, *Staphylococcus saprophyticus* (Huwe *et al.*, 1998) and *Chlamydia trachomatis* (Puerta Suarez *et al.*, 2017) did not have such an effect. In terms of community effects, the interaction of invading bacteria with each other or with resident bacteria (Otti *et al.*, 2017) could affect the outcome of an infection. If genital-associated bacteria behave as a community, then changes in the species composition, and even in the abundance of individual species, will decisively affect the impact of this community on the host. As a minimal precaution and a first step towards this somewhat visionary notion of insect reproductive ecology, it will be important for descriptive and functional studies on the microbes' role in reproduction to consider the mixed-species nature of natural microbe associations with reproductive organs.

Common bedbugs, *Cimex lectularius*, have previously been used as a model to study the effects of microbes associated with reproduction. Male bedbugs traumatically inseminate females by piercing the female's abdominal wall with an intromittent organ (Carayon, 1966), called paramere. Sperm are injected from the sperm container (sperm vesicles) into a paragenital female copulatory organ, called the mesospermalege. The microbial species situated on the male intromittent organ consist of environmental bacteria and fungi (i.e. OMs) (Reinhardt *et al.*, 2005; Otti *et al.*, 2013) and can kill females (Reinhardt *et al.*, 2003). The female copulatory organ has evolved to reduce the mortality after infections derived from microbes on the male intromittent organ (Reinhardt *et al.*, 2003; but see Morrow & Arnqvist, 2003). Bacteria found on the male intromittent organ kill sperm *in vitro* (Otti *et al.*, 2013). Male responses include the presence of a constitutive immune effector (lysozyme-like activity) in the seminal fluid (Otti *et al.*, 2009) which can reduce sperm mortality (Otti *et al.*, 2013). A notable omission by many, if not most, of these studies is that the transmission is rarely examined, either directly by observation or indirectly by inference from comparing the microbiota of virgin and mated individuals.

Here we use a community ecology approach to describe the bacterial communities of different reproductive tissues in both sexes of the common bedbug and to examine the potential for sexual transmission of bacteria. We expected the communities to be shaped by the location of the given organ or its function. We assumed that the external intromittent organ of males harbours different communities, mostly consisting of environmental bacteria, as compared with internal organs of females and males which might harbour a core microbiome. We expected the communities in the female copulatory organ to be different from the communities in the male sperm container, because the sperm-receiving female copulatory organ is more likely to be invaded by bacteria during mating than is the male sperm container.

We mainly focused on analysing four assumptions of sexual transmission of bacterial communities. For this we analysed differences in bacterial diversity and abundance, represented

by the number of reads, between the organ communities of non-mated and mated bedbugs, and the prevalence of bacteria introduced during mating. For male-to-female transmission we expected that the copulatory organs of mated female bedbugs show increased diversity and abundance, compared with virgins. Furthermore, we expected the copulatory organ of mated females to harbour bacteria that are found in non-mated males but not in non-mated females. If transmission is quantitatively significant, we expected that bacterial diversity or abundance would decrease in the sperm container and/or intromittent organ of males after versus before mating. Although hardly considered in the literature, it may be that female-to-male transmission is significant, in which case we would expect that the sperm containers and/or intromittent organs of mated males show increased diversity and/or abundance compared with non-mated ones. We would also expect the copulatory organ of mated males to harbour bacteria that are found in non-mated females but not in non-mated males, and possibly that bacterial diversity and abundance would be lower in mated than in virgin females.

Materials and methods

Bedbug culture and reproductive biology

All bedbugs were maintained in an incubator at 26 ± 1 °C, at 70% RH with an LD 12:12 h photoperiod. After eclosion, we divided virgin males and females into sex-specific groups and fed them twice with an interval of 1 week. The feeding and maintenance protocol was as described by Reinhardt *et al.* (2003). We used individuals from one large stock population (> 1000 individuals) which had been collected from an infestation in London and started as laboratory culture in the laboratory in Sheffield in 2006. This population was transferred to the laboratory of Animal Population Ecology at the University of Bayreuth in 2011 and maintained under identical culture conditions.

Mating and sample preparation

We collected the reproductive organs of 35 individual bedbugs in May 2012 to analyse the bacterial communities of the bedbug reproductive system. Ten 3-week-old virgin females were mated for 60 s to the same number of 3-week-old virgin males. Within 1–2 h after mating, we dissected the mated bedbugs. We collected the copulatory organ (mesospermalege) from the mated females and both the intromittent organ (paramere) and sperm containers (sperm vesicles) from mated males by sampling both organs from the same male. Spermatozoa leave the female copulatory organ after 4 h to travel through the haemolymph to the sperm storage organ (Carayon, 1966). This means that the sperm were still inside the copulatory organ at the time of dissection. We also collected the reproductive organs of five virgin females and 10 males randomly drawn from the stock populations. These males of unknown age and unknown mating status were isolated for 2 weeks prior to dissection. Not allowing them to copulate with a female ensured that they were at their full reproductive potential. Hereafter, we refer to virgin females and these males collectively as 'non-mated'. Except for the copulatory organ of non-mated females ($n = 5$), we collected the organs of 10 individuals for each reproductive organ and mating status.

We used standard dissection techniques under sterile conditions using a laboratory butane burner (Labogaz 206; Campingaz, Hattersheim, Germany) to minimise the potential of aerial bacteria contamination. We checked for contamination by placing LB agar plates next to the dissection microscope. No colonies were observed on these plates. Prior to any dissections, we autoclaved the dissection kit and, after each dissection, forceps and surgical scissors were dipped in ethanol (70%) and flame-sterilised. To prevent contamination with bacteria from the integument, we rinsed the integument of females with 70% ethanol prior to dissection. To further reduce the risk of contamination, we used different forceps to hold the male bedbug and to collect the internal organs. Dissected organs were transferred directly into the MicroBead solution (MO BIO Ultra Clean Microbial DNA Isolation Kit, catalogue no. 12224-250, dianova GmbH, Hamburg, Germany) for DNA extraction.

DNA extraction, library preparation, and sequencing

The bacterial community in and on the reproductive organs of bedbugs was described by the sequences of the 16S V4 region of bacteria obtained from three organs. We followed the protocol from the MO BIO UltraClean Microbial DNA Isolation kit with some additional steps. Instead of the MO BIO Vortex Genie, we used the Vortex Disruptor Genie (vertical 12-sample vortex). Before vortexing the samples in MicroBead tubes, we homogenised the samples with sterile pipette tips (200 µl) melted at the tip to form a pestle. These samples were then incubated and shaken at 65 °C for 10 min. The kit uses microbeads and a lysing solution in combination to homogenise the tissue and extract the bacteria. We subjected the samples to PCR with barcoded versions of the universal primers 27f and 519r. Roche multiplex identifiers were incorporated between the sequences of adaptor A and 519r to give the structure: 5′-Adaptor_A-sequencing_key-multiplex_identifier-519r-3′. PCR consisted of an initial denaturation step of 2 min at 94 °C and 25 cycles of 30 s at 94 °C, 20 s at 52 °C, and 60 s at 65 °C. We checked the PCR products by gel electrophoresis, purified them with AMPure XP beads (catalogue no. A63881, Beckman Coulter GmbH, Krefeld, Germany), and sequenced them at the Earlham Institute (Norwich, U.K.) on a 454 titanium GS FLX (Roche, Basel, Switzerland) at 24-plex per quarter pico-titre plate.

Bioinformatic analysis

The data were demultiplexed with QIIME (Caporaso *et al.*, 2010). We removed sequences that did not match the default parameters of the 'split_libraries.py' script regarding quality score, sequence length and ambiguous bases. After this step, 68 513 out of 226 789 raw sequences remained in the dataset.

We subjected the remaining sequences to the DADA2 pipeline (Callahan *et al.*, 2016) in R (R Core Team, 2013). The sequences were filtered and trimmed with the default parameters of the

'fastqFilter' function. The first 15 bp were removed and the sequences were truncated after 300 bp to remove low-quality tails. Sequences with expected errors > 2 and a quality score < 2 were discarded. The remaining 23 695 sequences were dereplicated with the default parameters of the 'derepFastq' function and denoised with the 'DADA' function with the 'selfConsist' option enabled, a homopolymer gap penalty of −1 and a band size of 32. We then constructed a sequence variant (SV) table with the 'makeSequenceTable' function. The remaining 22 566 SVs were checked for chimeras with the 'removeBimeraDenovo' function and default parameters, resulting in 22 149 chimera-free sequences. The taxonomy of the SVs was assigned with the Greengenes database (De Santis *et al.*, 2006). We used NCBI's BLASTn with the default options to verify the taxonomical assignments. We excluded uncultured and environmental sample sequences. The taxonomy assignments of Greengenes and BLASTn were in accordance for kingdom, phylum, class, order, family, and genus level in 22 out of 31 SVs. In three of the cases that were not in accordance, the BLAST hit with the highest e-value and coverage belonged to an endosymbiont of *C. lectularius*, the unclassified gammaproteobacterium mentioned by Hosokawa *et al.* (2010). We therefore changed the taxonomy assignment of these SVs. Two out of the misassigned SVs had BLAST hits that all agreed on one genus and we therefore changed the assignment. In four other cases there was no clear BLAST result. Hence, we kept the Greengenes assignment for the levels that were congruent with the BLAST results and changed the assignment of the other levels to 'unclassified'. We compiled all sample descriptions, read numbers and assigned taxonomy for the SVs in the Supporting Information (Tables S1–S3). Sequences were deposited in NCBI's Sequence Read Archive with the accession number PRJNA534453. Rarefaction curves drawn with the 'rarecurve' function in the VEGAN package (Oksanen *et al.*, 2018) showed that our sampling captured most of the communities, as almost all curves reached a plateau (Fig. S1). We filtered out all SVs that belonged to chloroplasts or hosts and all SVs that occurred in less than two samples. The final SV table contained 31 SVs. There was one sample from the intromittent organ of a mated male which did not yield any SVs after the mentioned filtering. It was therefore excluded from the statistical analysis.

Statistical analysis of the bacterial communities in non-mated bedbugs

We focused on differences in bacterial diversity, prevalence, and abundance between the reproductive organs of non-mated bedbugs to describe the primary communities. We analysed the dissimilarity of bacterial communities between organs of non-mated bedbugs with a PERMANOVA ('adonis', 999 permutations, VEGAN package; Oksanen *et al.*, 2018). Distances were estimated with the Jaccard index with the 'distance' function in the PHYLOSEQ package (McMurdie & Holmes, 2013). We calculated pairwise contrasts between the organs with the function 'pairwise.adonis' from the PAIRWISEADONIS package (Martinez Arbizu, 2017) and corrected the *P*-values with the inbuilt Benjamini-Hochberg procedure. We used the function 'betadisper' (VEGAN package; Oksanen *et al.*, 2018) followed by an ANOVA to assess between-individual variation of bacterial communities across organs. To compare organs pairwise, we applied the 'TukeyHSD.betadisper' function in the VEGAN package (Oksanen *et al.*, 2018). We estimated alpha diversity (Simpson index, $1 - D$) with the 'estimate_richness' function in the PHYLOSEQ package (McMurdie & Holmes, 2013) and compared alpha diversity between organs of non-mated bedbugs with a generalised linear model followed by an ANOVA. We visually inspected residual versus fitted plots to verify that residuals followed a normal distribution. To calculate the relative abundances of classes and genera, we divided the number of reads for the specific class or genus within a given sample by dividing them by the total number of reads within that sample.

Statistical analysis of mating-induced changes in bacterial communities

We analysed the effect of mating status regarding a possible sexual transmission of bacteria, including samples from non-mated and mated bedbugs. To compare alpha diversity (Simpson index $1 - D$) between organs from non-mated and mated individuals, we fitted a generalised linear model followed by an ANOVA. Included as fixed effects were organ and mating status and their interaction term. We visually inspected residual versus fitted plots to verify that the residuals followed a normal distribution. We applied the function 'betadisper' (VEGAN package; Oksanen *et al.*, 2018) followed by an ANOVA to assess between-individual variation of bacterial communities between mating status. Distances were estimated based on the Jaccard index with the 'distance' function in the PHYLOSEQ package (McMurdie & Holmes, 2013). We compared the number of reads in samples from non-mated and mated bedbugs with exact tests in the EDGER package (Robinson *et al.*, 2010; McCarthy *et al.*, 2012) after normalising read numbers based on the median ratio of each sample to the median library as a scale factor (Anders & Huber, 2010). To evaluate the effect of mating on the normalised number of reads of each bacterial genus, organs were analysed separately. *P*-values were adjusted with the inbuilt Benjamini–Hochberg procedure and a false discovery rate of 1%, and SVs that occurred only in non-mated or mated individuals were discarded. We used a Principal Coordinates Analysis to analyse whether mating increases the similarity of the bacterial communities in the reproductive organs. This analysis was based on an ordination calculated with the 'ordinate' function in the PHYLOSEQ package (McMurdie & Holmes, 2013) and the Jaccard index. We then analysed the dissimilarity of bacterial communities between non-mated and mated individuals with a PERMANOVA ('adonis', 999 permutations, VEGAN package; Oksanen *et al.*, 2018), including the interaction of organ and mating status. To analyse which bacteria might be sexually transmitted, we extracted the SVs that are found in mated but not in non-mated individuals of one sex and in the organs of non-mated individuals of the opposite sex. Partitioning beta diversity (Sørensen index) into turnover and nestedness with the function 'nestedbetasor' in the VEGAN package (Oksanen *et al.*, 2018), we investigated the mechanism of the mating-induced change in bacterial communities. We therefore produced a presence–absence matrix for each

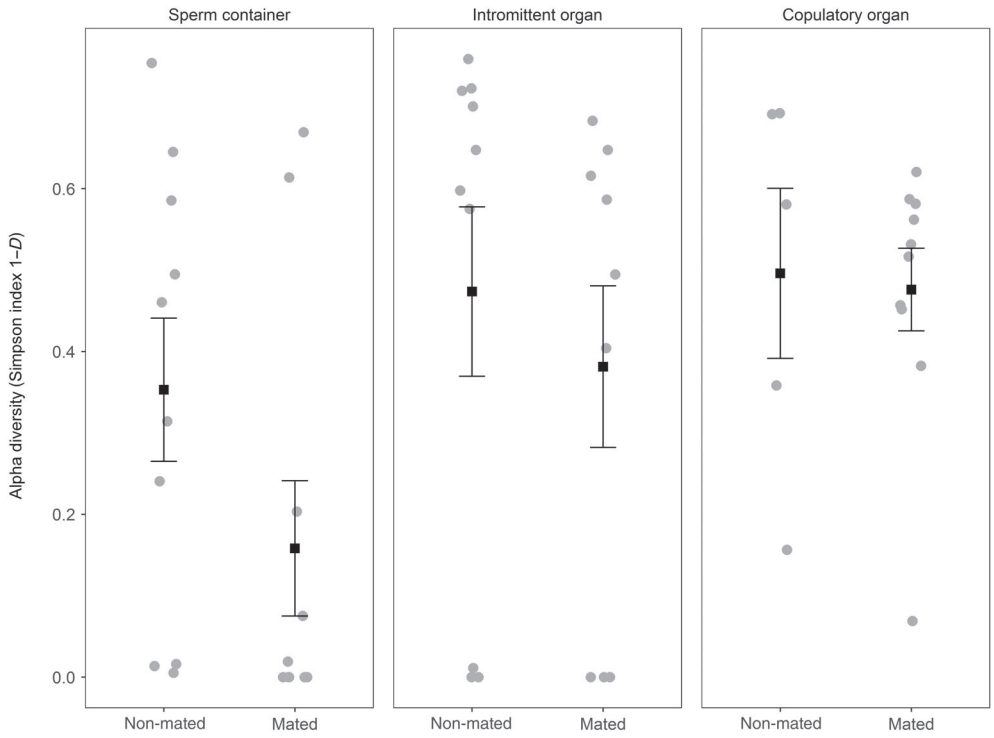

Fig. 1. Alpha diversity of bacteria from the reproductive organs of bedbugs. The Simpson index ($1 - D$) is shown. Each grey dot represents the diversity of one specific sample. The means and SEs for each organ and mating status are depicted in black. There were 10 samples per organ and mating status, with the exception of the copulatory organ of non-mated females ($n = 5$).

organ and mating status, which included all SVs present in the particular group of samples. We then calculated the proportion of beta diversity that was explained by turnover, i.e. a replacement of resident SVs with newly introduced SVs, and the proportion explained by nestedness, i.e. a loss or introduction of SVs.

Results

We sequenced the bacterial communities of three reproductive organs of the common bedbug, (i) the female copulatory organ, (ii) the male intromittent organ, and (iii) the male sperm container. Except for the copulatory organ of non-mated females ($n = 5$), we sequenced the communities of 10 individuals for each organ and mating status, resulting in a total of 55 samples. After filtering out chimeric sequences, chloroplast sequences, host sequences, and SVs that occurred in only one sample, we identified a total of 31 SVs. On average, each sample contained 3 ± 1 SVs (mean $\pm$ SD) and 340 ± 224 reads. Average alpha diversity was 0.38 ± 0.28 (Simpson index, $1 - D$), or 0.68 ± 0.52 (Shannon index). There was one sample that harboured only SVs that were filtered out. It was therefore excluded from the statistical analysis.

As expected, the communities were shaped by the location of the given organ or its function. The structure of bacterial communities of non-mated bedbugs differed between organs ($F_{2,22} = 3.031$, $R^2 = 0.216$, $P = 0.001$, based on the Jaccard index). The female copulatory organ harboured distinct communities in comparison to the male sperm container ($F_{1,13} = 3.203$, $R^2 = 0.198$, $P = 0.003$, $Q = 0.005$) and the male intromittent organ ($F_{1,13} = 4.128$, $R^2 = 0.241$, $P = 0.001$, $Q = 0.003$), whereas the sperm container communities were similar to those on the male intromittent organ ($F_{1,19} = 2.181$, $R^2 = 0.108$, $P = 0.020$, $Q = 0.02$). The between-individual variation in community structure differed between organs of non-mated bedbugs ($F_{2,22} = 5.064$, $P = 0.02$). The female copulatory organ had a lower between-individual variation than the male sperm container (Tukey honestly significant difference (HSD), $P = 0.01$). Between-individual variation did not differ between the female copulatory organ and the intromittent organ (Tukey HSD, $P = 0.08$) or between the male intromittent organ and the sperm container (Tukey HSD, $P = 0.53$). Against our predictions, alpha diversity was similar across organs of non-mated bedbugs ($F_{2,22} = 0.58$, $P = 0.567$) (Fig. 1).

In total, we detected 20 bacterial genera from six different classes in the reproductive organs of bedbugs. Most SVs belonged to the classes of Actinobacteria, Alphaproteobacteria, and Gammaproteobacteria. The relative abundance of these classes was highly variable between individual bedbugs (Fig. 2), even though they originated from the same population and environment. The female copulatory organ harboured almost only Alpha- and Gammaproteobacteria. In addition to Alpha- and Gammaproteobacteria, males harboured large proportions of Actinobacteria and a few males had Bacilli and Clostridia. Whereas females seem to have a core microbiome

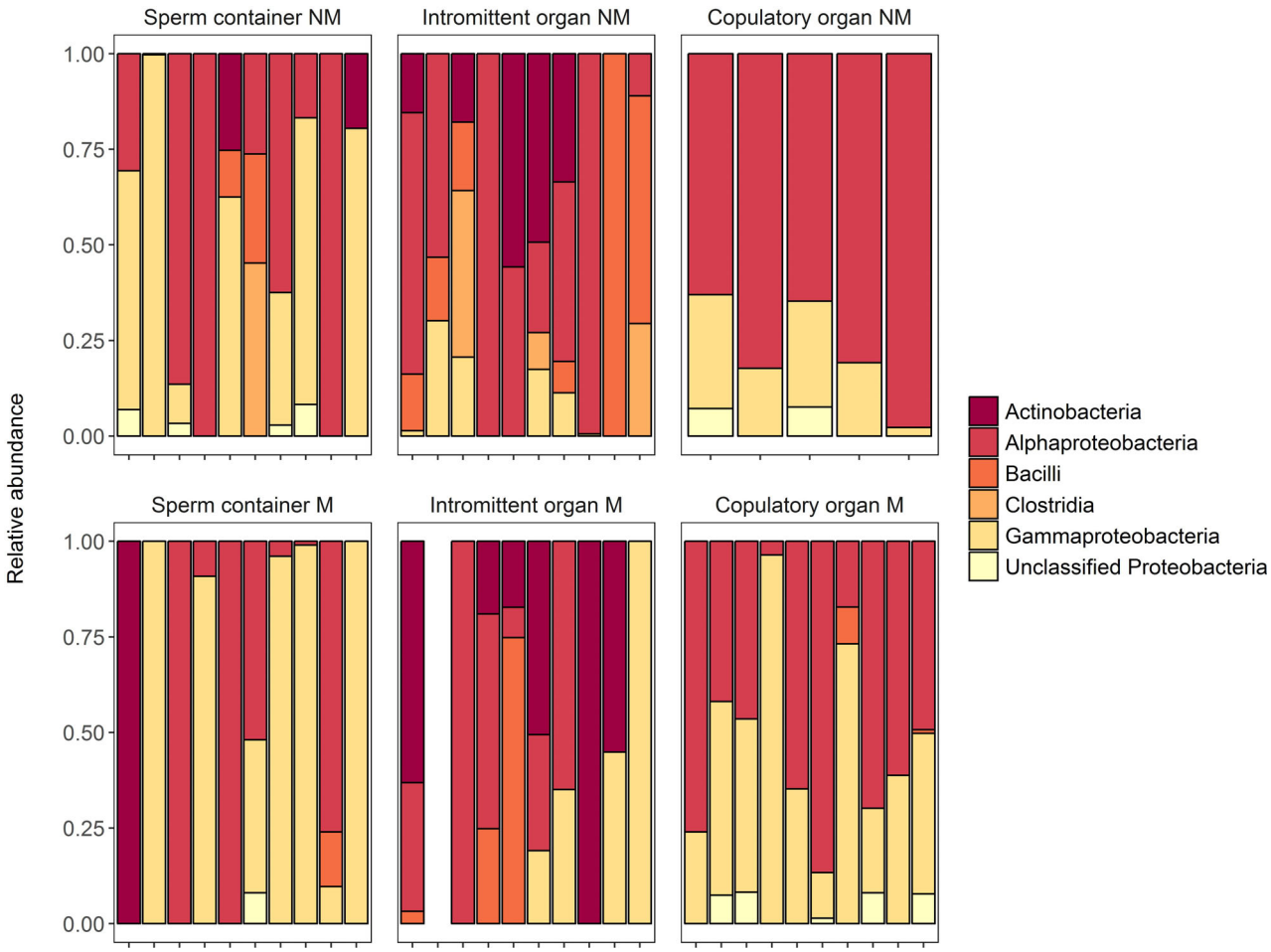

Fig. 2. Relative abundances of the six classes found in the reproductive organs of non-mated (NM) or mated (M) bedbugs. Relative abundances were calculated based on the number of reads of the same class within a given sample divided by the total number of reads within that sample. Each bar represents one individual bedbug. Bars across male organs correspond to the same individual and bars across organs of mated bedbugs are ordered by mating pair. If the sequence variant was not assigned any class, we report the lowest assigned taxon instead. [Colour figure can be viewed at wileyonlinelibrary.com].

with two SVs shared by all females (Table 2), male reproductive organs did not consistently share the same SVs (Table 2). Also, the relative abundances of the genera in male organs varied tremendously across individuals (Fig. 3). Compared with the other SVs, both shared SVs in females, *Rickettsia* and the gammaproteobacterial endosymbiont discovered by Hosokawa *et al.* (2010), had high relative abundances of 43–95% and 2–28%, respectively (Fig. 3).

As a pattern of sexual transmission of bacteria, we expected changes in diversity and abundance of bacteria in the reproductive organs. Newly introduced bacteria in mated individuals of one sex that can also be found in non-mated individuals of the opposite sex would further indicate sexual transmission. We also expected that mating would homogenise the communities. In contrast to our predictions, there were no mating-induced changes in alpha diversity ($F_{1,50} = 2.324$, $P = 0.13$) (Fig. 1). Alpha diversity was not affected by an organ-specific effect of mating status ($F_{2,48} = 0.448$, $P = 0.64$). Mating did not change between-individual variation ($F_{1,52} = 0.141$, $P = 0.70$) or the abundance of specific SVs in any of the organs ($-2 < \log_2$-fold change < 2, $Q > 0.01$).

However, as predicted for sexual transmission of bacteria, mating led to a larger overlap between the bacterial communities of females and males (Fig. 4). The community structure within organs (Jaccard index) was changed by mating status ($F_{1,48} = 2.793$, $R^2 = 0.044$, $P = 0.003$) and the effect was dependent on organs ($F_{2,48} = 3.515$, $R^2 = 0.111$, $P = 0.001$). As predicted by a transmission from the male to the female, mating introduced four new SVs to the copulatory organ (Table 3a), out of which one SV was harboured by both organs of non-mated males. Out of the two remaining SVs, three were found in the sperm container of non-mated males, and one on the intromittent organ of non-mated males. Out of the SVs that appeared on the intromittent organ after mating, two were found in the copulatory organ of non-mated females (Table 3b), suggesting a transmission from the female to the male. Mating introduced three SVs to the male sperm container that were harboured by non-mated females (Table 3c).

Table 2. Prevalence of sequence variants (SVs) in the reproductive organs of non-mated bedbugs.

SV ID	Class	Genus	Male sperm container ($n = 10$)	Male intromittent organ ($n = 10$)	Female copulatory organ ($n = 5$)
ASV23	Actinobacteria	*Corynebacterium*	0.1	0	0
ASV5	Actinobacteria	*Cutibacterium*	0.2	0.5	0
ASV12	Actinobacteria	*Cutibacterium*	0	0	0
ASV14	Actinobacteria	*Cutibacterium*	0	0.2	0
ASV13	Alphaproteobacteria	*Agrobacterium*	0	0.2	0
ASV15	Alphaproteobacteria	*Rhizobium*	0	0	0
ASV1	Alphaproteobacteria	*Rickettsia*	0.8	0.4	1
ASV65	Alphaproteobacteria	*Rickettsia*	0	0	0.4
ASV81	Alphaproteobacteria	*Rickettsia*	0	0	0.4
ASV6	Alphaproteobacteria	*Sphingomonas*	0	0.3	0
ASV4	Alphaproteobacteria	*Wolbachia*	0.2	0	0
ASV9	Alphaproteobacteria	*Wolbachia*	0	0	0.8
ASV27	Alphaproteobacteria	Unclassified Rhizobiaceae	0	0	0
ASV11	Alphaproteobacteria	Unclassified Sphingomonadaceae	0.1	0	0
ASV48	Bacilli	*Enterococcus*	0	0.1	0
ASV18	Bacilli	*Staphylococcus*	0.1	0.2	0
ASV21	Bacilli	*Staphylococcus*	0.1	0.2	0
ASV19	Bacilli	*Streptococcus*	0	0	0
ASV17	Bacilli	Unclassified Bacilli	0	0.2	0
ASV7	Clostridia	*Veillonella*	0.1	0.3	0
ASV20	Gammaproteobacteria	*Acinetobacter*	0.1	0.1	0
ASV66	Gammaproteobacteria	*Acinetobacter*	0	0.2	0
ASV2	Gammaproteobacteria	Endosymbiont of *Cimex lectularius*	0.6	0.5	0
ASV3	Gammaproteobacteria	Endosymbiont of *Cimex lectularius*	0	0	1
ASV30	Gammaproteobacteria	Endosymbiont of *Cimex lectularius*	0	0	0.2
ASV22	Gammaproteobacteria	*Pseudomonas*	0.1	0.1	0
ASV28	Gammaproteobacteria	*Serratia*	0.2	0	0
ASV46	Gammaproteobacteria	*Serratia*	0	0	0.2
ASV58	Gammaproteobacteria	Unclassified Xanthomonadales	0	0	0
ASV10	Unclassified Proteobacteria	Unclassified Proteobacteria	0.2	0	0.2
ASV39	Unclassified Proteobacteria	Unclassified Proteobacteria	0.2	0	0.2

Shown are the proportions of samples from non-mated bedbugs harbouring the given SV.

Moreover, there was a large SV turnover of non-mated and mated bedbugs (male sperm container, 93%; male intromittent organ, 98%, female copulatory organ, 100% of the Sørensen index), suggesting a replacement of resident with newly introduced SVs in all organs.

Discussion

Using an ecological community approach, we found 31 SVs associated with the reproductive organs of the common bedbug, *C. lectularius*. The size of this bacterial community might be underestimated given that microbiomes of laboratory animals often seem to exhibit lower diversity compared with wild-caught individuals. For example, the gut microbiome of laboratory-reared *Drosophila melanogaster* consists of fewer taxa than the gut microbiome of wild-caught individuals (Broderick & Lemaitre, 2012).

We observed differences in bacterial community composition between individuals. These differences suggest either a strong host genotypic contribution or other properties of individual bedbugs that were not measured. Although large variation between closely related species (Chaston *et al.*, 2015) or individuals of the same species are known (Costello *et al.*, 2009; Nasidze *et al.*, 2009; Ravel *et al.*, 2011; Siddiqui *et al.*, 2011; Human Microbiome Project Consortium, 2012; Moran *et al.*, 2012; Osei-Poku *et al.*, 2012; Hou *et al.*, 2013; Zhou *et al.*, 2013; Liu *et al.*, 2014), these differences at the same time were likely to have hampered our organ- and mating-status-specific approach. Nevertheless, we found potential evidence of sexual transmission of bacteria because bacterial communities were more similar after mating, there was a high turnover of SVs in both sexes, and mating introduced new bacteria to the organs of mated females and males. Our results confirm that a community ecology approach can actually be used to analyse microbial transmission in insects.

Previous studies revealed that most of the bacteria associated with the reproductive system of insects belonged to the classes of Actinobacteria, Bacilli, or Gammaproteobacteria (Otti, 2015). In our study, a large proportion of individuals harboured Actinobacteria and Gammaproteobacteria. Bacilli were only present in a few individuals, whereas many individuals harboured Alphaproteobacteria. In the reproductive organs we found two genera that have been reported as endosymbionts of the common bedbug, *Wolbachia* and an unclassified gammaproteobacterium (Hosokawa *et al.*, 2010). Both symbionts can be

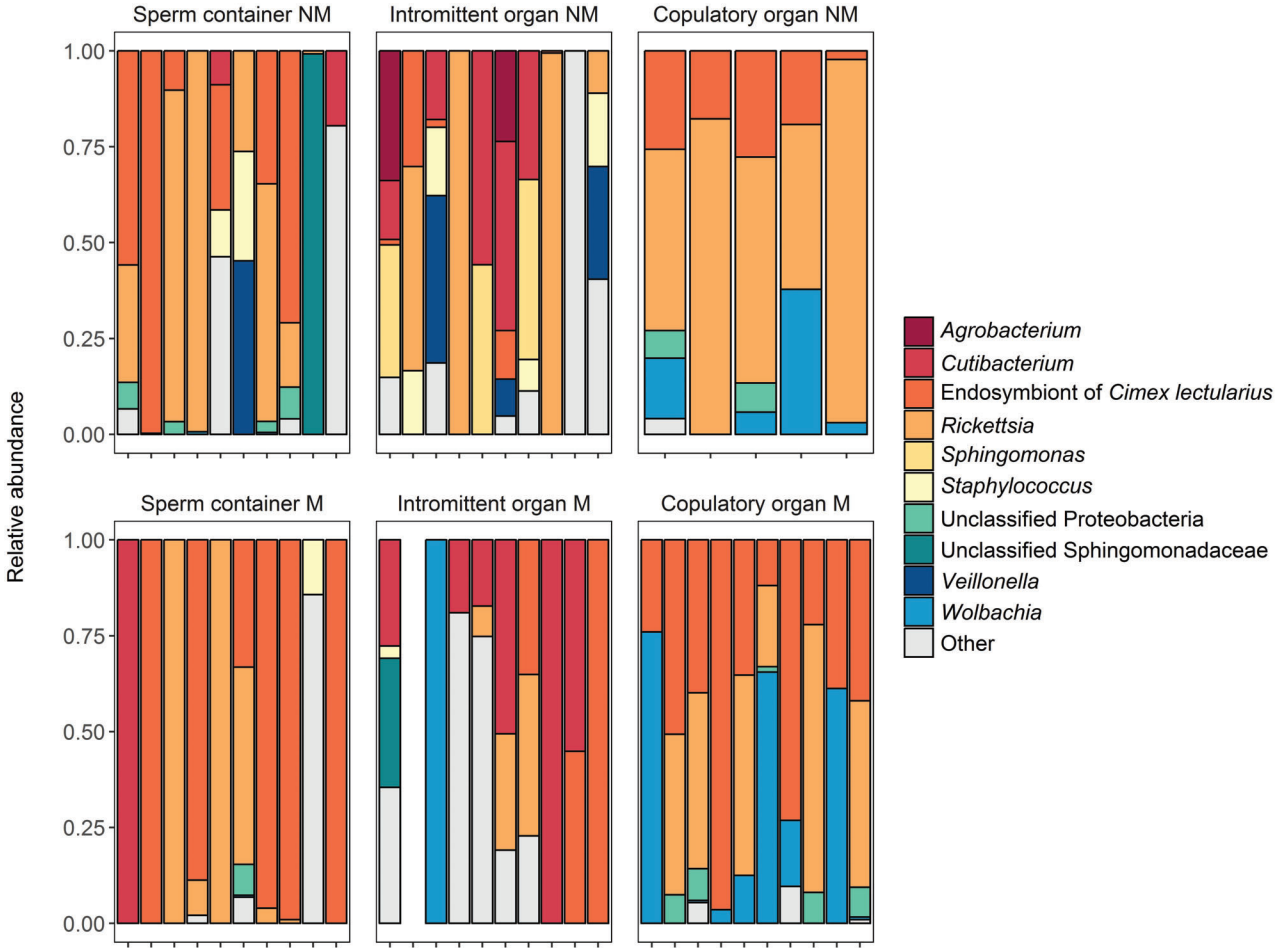

Fig. 3. Relative abundance of the 10 most abundant out of the 20 genera that were harboured by the reproductive organs of non-mated (NM) and mated (M) bedbugs. Relative abundances were calculated based on the number of reads of the same genus within a given sample divided by the total number of reads within that sample. Each bar represents one individual bedbug. Bars across male organs correspond to the same individual and bars across organs of mated bedbugs are ordered by mating pair. If the sequence variant was not assigned any genus, we report the lowest assigned taxon instead.

found in the bacteriomes that are attached or in close proximity to the reproductive organs. *Wolbachia* provides the host with B vitamins (Hosokawa *et al.*, 2010), whereas to date the function of the gammaproteobacterium is unknown.

Our results are in line with the findings of culture-dependent studies that common bedbugs (*C. lectularius*) carry opportunistic bacteria (Reinhardt *et al.*, 2005; Otti *et al.*, 2013; Otti *et al.*, 2017). Three genera previously found on the intromittent organ were not present in our data (Table 1). *Staphylococcus* was the only genus that was repeatedly found and that was associated with the same organ in more than one study (present study; Otti *et al.*, 2017). It is not clear whether this is due to differences in the study design or the consequence of the large differences in bacterial composition across individuals that our study reports. Such between-individual differences in the microbiome have been reported in a variety of human tissues, including saliva, urine, skin, nares and stool (e.g. Costello *et al.*, 2009; Nasidze *et al.*, 2009; Siddiqui *et al.*, 2011; Human Microbiome Project Consortium, 2012), and even in reproductive organs (Ravel *et al.*, 2011; Hou *et al.*, 2013; Zhou *et al.*, 2013; Liu *et al.*, 2014). Studies reporting on compositional between-individual differences in the microbiome of insects comprise, for instance, the gut of mosquitoes (Osei-Poku *et al.*, 2012) and honey bees (Moran *et al.*, 2012). To our knowledge, this is the first study to report on compositional between-individual differences in the bacterial community of the insect reproductive tract. Such microbial differences between individuals in a similar environment may be important when interpreting aspects of sexual behaviour in the context of animal 'personalities'.

The male intromittent organ and the female copulatory organ of non-mated bedbugs harboured distinct bacterial communities. In addition, the copulatory organ of non-mated females had a lower between-individual variation compared with the organs of non-mated males. Differences in community composition are unlikely to be caused by organ location as the intromittent organ and the sperm containers of bedbug males harboured

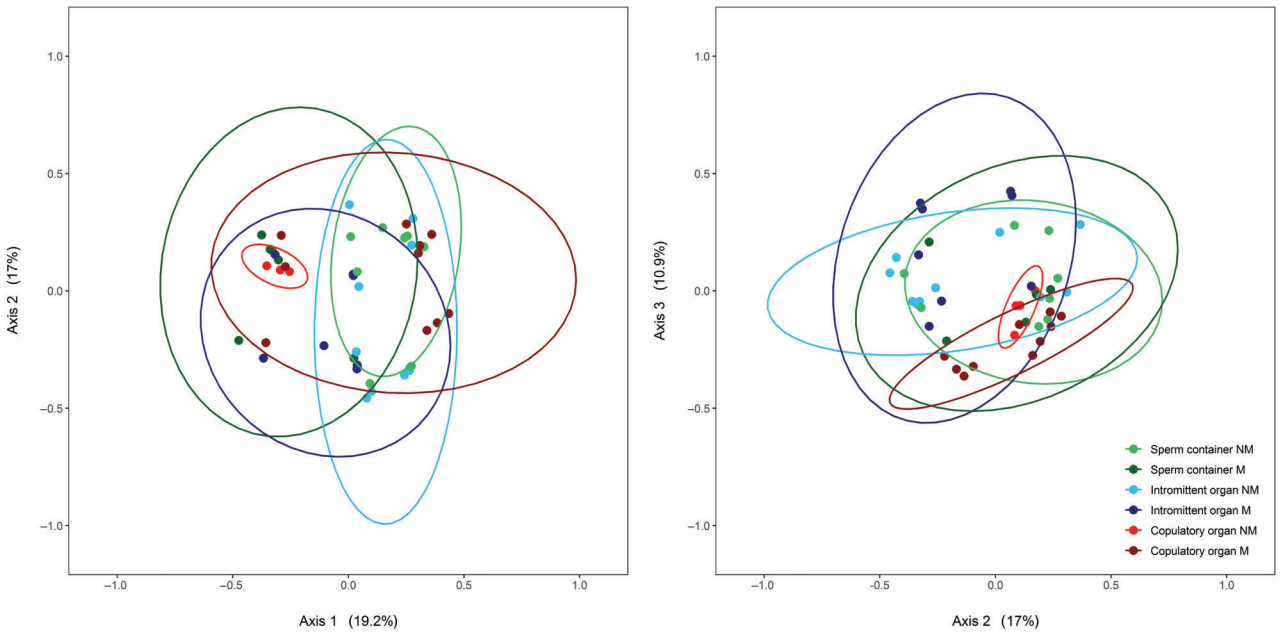

Fig. 4. Variation in the structure of bacterial communities in and on the reproductive organs of non-mated (NM) and mated (M) bedbugs. Shown are the first three axes of a principal coordinate analysis based on the Jaccard index. Each circle represents a sample with its bacterial community. Ellipses give the 95% confidence interval. There were 10 samples for each organ and mating status, with the exception of the intromittent organ of mated males (n=9) and the copulatory organ of non-mated females (n = 5).

bacterial communities with similar composition. Sex differences in diversity or composition of bacterial communities extracted from whole-body homogenates or from the gut are common across animals (Markle *et al.*, 2013; Valiente Moro *et al.*, 2013; Haro *et al.*, 2016). Some of these differences may arise from the different niche that the sexes and even organs may occupy; others, however, are likely to be closely linked to the most pronounced difference found between the sexes – reproduction. Hupton *et al.* (2003) and Otti *et al.* (2017) found pronounced differences in the microbiome of reproductive organs of female and male red-winged blackbirds and bedbugs, respectively.

For the first time, we showed that mating changes the bacterial communities of the reproductive organs in insects. We even found indications of a sexual transmission of bacteria in insects. We predicted that, in the case of a transmission, some of the bacteria found in one sex might be transmitted to the opposite sex. This should be reflected in a decrease in diversity or abundance in the organs of one sex and an increase in the other. Furthermore, mating should homogenise the communities of both sexes. We showed that copulation increased the similarity of the bacterial communities of male and female organs. A high turnover of SVs between the organs of non-mated and mated males was found, suggesting a replacement of resident with newly introduced bacteria.

Indeed, there were newly introduced bacteria in the copulatory organs after mating that were also present in the organs of non-mated individuals of the opposite sex. Taken together, these observations suggest sexual transmission of bacteria in the common bedbug. Even more interesting, the transmission seems to be two-sided. We found shared bacteria between non-mated females and mated males, indicating a transmission from the female to the male.

Contradicting our expectations about sexual transmission, mating did not induce changes in bacterial abundance, as shown by similar read numbers in organs from non-mated and mated individuals. OMs might cause an infection, or lower reproductive success by increasing sperm mortality (Otti *et al.*, 2013). Therefore, females, and potentially even males, should have evolved mechanisms to protect themselves from these effects of OMs. Haemocytes are constantly present in the copulatory organ of female bedbugs (Carayon, 1966) and they can readily phagocytose bacteria as part of insect immune defence (Lavine & Strand, 2002). Even if bacteria are transmitted to the female, these could be eliminated by haemocytes without a costly systemic immune response. Physical barriers may also reduce the receipt of bacteria by females and therefore bacterial abundance. In the case of the bedbug, one may speculate that the highly elastic membrane in the bedbug copulatory organ of females (Michels *et al.*, 2015), which the male penetrates during copulation, may function like a boot scraper.

The microbiome of the reproductive tract might protect its host from invading microbes during copulation. In humans, the female genital tract is inhabited by high proportions of lactobacilli (Ravel *et al.*, 2011; but see Anahtar *et al.*, 2015), which were reported to inhibit the growth of uropathogenic bacteria and their adhesion to epithelial vaginal cells (Reid *et al.*, 1987; Boris *et al.*, 1998). *Rickettsia* and the gammaproteobacterial endosymbiont reported by Hosokawa *et al.* (2010) were the only genera that were commonly found in all non-mated females and had high relative abundances. The relationship

Table 3. Sequence variants (SVs) that were introduced to the reproductive organs of mated bedbugs: (a) the female copulatory organ; (b) the male intromittent organ; and (c) the male sperm container. It is indicated whether these SVs were present in the organs of non-mated bedbugs from the opposite sex (S, male sperm container: $n = 10$; I, male intromittent organ: $n = 10$; female copulatory organ: $n = 5$). A sexual transmission would be indicated by shared SVs between mated individuals of one sex and non-mated individuals of the opposite sex.

SV ID	Class	Genus	Presence in non-mated individuals of the opposite sex
(a)			
ASV4	Alphaproteobacteria	*Wolbachia*	S
ASV48	Bacilli	*Enterococcus*	I
ASV2	Gammaproteobacteria	Endosymbiont of *Cimex lectularius*	S,I
ASV28	Gammaproteobacteria	*Serratia*	S
(b)			
ASV23	Actinobacteria	*Corynebacterium*	No
ASV12	Actinobacteria	*Cutibacterium*	No
ASV15	Alphaproteobacteria	*Rhizobium*	No
ASV9	Alphaproteobacteria	*Wolbachia*	Yes
ASV27	Alphaproteobacteria	Unclassified Rhizobiaceae	No
ASV11	Alphaproteobacteria	Unclassified Sphingomonadaceae	No
ASV19	Bacilli	*Streptococcus*	No
ASV3	Gammaproteobacteria	Endosymbiont of *Cimex lectularius*	Yes
ASV58	Gammaproteobacteria	Unclassified Xanthomonadales	No
(c)			
ASV12	Actinobacteria	*Cutibacterium*	No
ASV15	Alphaproteobacteria	*Rhizobium*	No
ASV9	Alphaproteobacteria	*Wolbachia*	Yes
ASV27	Alphaproteobacteria	Unclassified Rhizobiaceae	No
ASV3	Gammaproteobacteria	Endosymbiont of *Cimex lectularius*	Yes
ASV46	Gammaproteobacteria	*Serratia*	Yes
ASV58	Gammaproteobacteria	Unclassified Xanthomonadales	No

between *Rickettsia* and its host is not essentially mutualistic as it has the ability to manipulate the reproduction of ladybird beetles (Werren *et al.*, 1994; Hurst *et al.*, 1999; von der Schulenburg *et al.*, 2001) and parasitoid wasps (Hagimori *et al.*, 2006; Giorgini *et al.*, 2010). However, *Rickettsia* has been shown to protect its whitefly host against a challenge with *Pseudomonas syringae* (Hendry *et al.*, 2014). Unfortunately, the function of the gammaproteobacterial endosymbiont of *C. lectularius* is still unknown. If symbionts provide protection against transmitted bacteria in *C. lectularius*, *Rickettsia* and the gammaproteobacterial endosymbiont might be the genera involved in a protection against invading bacteria. To date, nothing is known about potential protection mechanisms of symbionts in the reproductive organs of insects.

We found distinct bacterial communities in and on the mating-associated organs of *C. lectularius*. These communities were composed of species that are known to be endosymbiont of the common bedbug but also species that are thought to be OMs. Future research should investigate their role in reproduction in more detail and whether they can provide protection against bacteria invading the reproductive organs. Taken together, our results suggest that mating has an effect on the bacterial flora of organs involved in mating and that there might be sexual transmission of bacteria. The identification of the bacteria of reproductive organs using a community approach is an important first step to study the transmission of bacteria between the sexes. Our study highlights the need to consider the role of the entire microbial community when examining the impact of sexually transmitted bacteria on reproduction, both generally and, in insects, specifically. This notion includes traditional single-species transmission assays that quantify how often bacteria are actually transmitted during copulation because the results of these assays may depend on the microbial community present in the organ(s) considered, and whether this transfer is one-sided or reciprocal. Assuming that transmitted microbes also perturb the microbiome of the reproductive organs in species other than bedbugs, it would be interesting, too, to consider whether the immune responses in females and males also differ with respect to which microbe species enter which particular microbial communities of reproductive organs.

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Author contributions

OO, PRJ, and KR conceived the idea and designed the experiment. OO, PRJ, and KR carried out the experiment. SB and

PRJ performed the bioinformatics and statistical analysis. SB, OO, and KR interpreted the results and wrote the manuscript. All authors read and approved of the final manuscript.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Rarefaction curves for each sample from the reproductive organs of non-mated (NM) and mated (M) bedbugs.

Table S1. Sample descriptions regarding sex, organ, mating status, and mating pair.

Table S2. Read numbers for each sample and SV.

Table S3. Assigned taxonomy for each SV.

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