Disentangling host-parasite and male-female coevolutionary effects on host fitness

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Table of contents

1 Summary

The rapid evolution of male and female reproductive traits has long been regarded as driven only by sexual selection. However, also natural selection, for instance mediated by parasites, can shape reproductive traits. Microbes are probably the most common parasite group and all animals are in constant contact with them. Besides microbes causing sexually transmitted diseases, opportunistic environmental bacteria seem to be sexually transmitted. Transmitted opportunistic microbes can eventually cause disease or death but they can also reduce reproductive success for example due to their spermicidal activity or indirectly by activating the female immune system resulting in tissue and sperm damage. In addition, transmitted opportunistic microbes might disturb the microbial communities inhabiting the reproductive organs, the genital microbiomes. Such disturbances could activate immune responses that are costly in terms of resources and might cause autoimmunity. Host populations are likely exposed to different environmental microbes or different microbe prevalences as well as differences in the composition of genital microbiomes. If hosts and environmental microbes and/or hosts and their genital microbiomes are locally adapted, host populations likely differ in the type of strength of immune defence. Unfortunately, to date little is known about the composition and mating-induced change of the genital microbiomes in insects and how the female immune system reacts to invading microbes. To characterise the genital microbiomes, I conducted two metagenomic studies based on the 16S rRNA gene of the bacteria present in and on the reproductive organs of the common bedbug (*Cimex lectularius* L.). By comparing the genital microbiomes of virgin and mated bedbugs of both sexes from four different populations, I found that genital microbiomes are organ-, sex-, and population-specific, indicating local adaptation. Differences in genital microbiomes might interfere with reproductive success if they lead to reproductive incompatibilities, and ultimately lead to speciation. Indeed, I found that mating-induced changes in the composition of the genital microbiomes are partly due to exchanges of bacterial strains during mating, indicating sexual transmission in both directions. Some of these sexually transmitted bacteria were

opportunistic bacteria, i.e. bacteria from the cuticle. This indication was supported by my third study that investigated the transmission rate of opportunistic microbes and the growth rate and effect of such bacteria on the female immune system. Bacteria applied to the copulatory organs of males and females were transmitted to the opposite sex during mating in at least ninety percent of the cases, indicating that females are regularly threatened by sexually transmitted opportunistic microbes. Bacteria injected into the female paragenital sperm-receiving organ grew slowly within the first six hours, followed by a rapid growth within the next eighteen hours. The injection of bacteria but also the injection of ejaculate induced lysozyme-like activity and the production of antimicrobial peptides that can lyse bacterial cell walls and regulate bacterial growth. This might be a mechanism to eliminate the residual bacteria within the sperm-receiving organ. Against my expectation, I did not find indications of population-specific immune responses to mating-associated bacteria, suggesting that either the host is not locally adapted or that microbe presence per se is costly, leading to immunity being induced regardless of the pathogenicity of specific microbes. This thesis provides important results for understanding reproduction and how reproduction is affected by natural selection via host-microbe interactions. My findings suggest that sexually reproducing organisms are regularly exposed to invading microbes and therefore, they should be under selection to defend themselves against infections. I have identified two possible substances used for the defence against mating-associated microbes, providing evidence for the interaction of mating and immunity in bedbugs. With this thesis, I have laid the foundations for a model system in which many aspects of reproduction can be manipulated and experimentally tested, including the genital microbiome as part of an interdependent metaorganism. Future studies should investigate whether genital microbiome colonisation depends on environmental microbes or host genotype and whether the genital microbiomes affect reproductive success. Furthermore, it would be interesting to investigate whether mating-induced changes are reversible and how the female immune response is triggered by mating.

2 Zusammenfassung

Als Hauptursache für die schnelle Evolution von Reproduktionsmerkmalen bei Männchen und Weibchen wurde lange die sexuelle Selektion angesehen. Allerdings kann auch natürliche Selektion die Reproduktionsmerkmale beeinflussen. Mikroben sind vermutlich die am weitesten verbreitete Parasitengruppe und alle Tiere sind in kontinuierlichem Kontakt mit ihnen. Neben Mikroben, die sexuell übertragbare Infektionen verursachen, scheinen auch opportunistische Bakterien sexuell übertragen zu werden. Übertragene opportunistische Mikroben können auf lange Sicht Infektionen verursachen und zum Tod führen, aber sie können auch den Reproduktionserfolg durch ihre spermizide Aktivität vermindern oder indirekt durch eine Aktivierung des weiblichen Immunsystems zu Schäden an Geweben, oder Spermien führen. Des Weiteren könnten opportunistische Mikroben die in den Reproduktionsorganen lebende mikrobielle Gemeinschaft, die genitalen Mikrobiome, stören. Solche Störungen könnten Immunantworten auslösen, die Kosten verursachen, z.B. in Hinsicht auf Ressourcen, oder durch Autoimmunität. Wirtspopulationen sind vermutlich verschiedenen Umweltmikroben, oder verschiedenen Prävalenzen dieser Mikroben ausgesetzt und verschiedenen Zusammensetzungen der genitalen Mikrobiome. Falls Wirt und Umweltmikroben und/oder Wirt und genitale Mikrobiome lokal angepasst sind, unterscheiden sich Wirtspopulationen vermutlich in der Art oder Stärke ihrer Immunabwehr. Leider ist bisher wenig darüber bekannt, wie die genitalen Mikrobiome von Insekten zusammengesetzt sind, wie sich diese Zusammensetzung durch die Paarung verändert und wie das weibliche Immunsystem auf eindringende Mikroben reagiert. Um die genitalen Mikrobiome zu untersuchen, führte ich mithilfe des 16S rRNA-Gens der Bakterien in und auf den Reproduktionsorganen von Bettwanzen (*Cimex lectularius* L.) zwei metagenomische Studien durch. Durch einen Vergleich der genitalen Mikrobiome von virginen und verpaarten Bettwanzen beider Geschlechter aus vier verschiedenen Populationen, fand ich heraus, dass genitale Mikrobiome organ-, sex-, und populationsspezifisch sind, was für lokale Adaptation spricht. Unterschiedliche genitale Mikrobiome könnten den Reproduktionserfolg stören, wenn

sie zu reproduktiven Inkompatibilitäten führen, und könnten schließlich zur Artbildung führen. Tatsächlich entdeckte ich, dass Änderungen in der Zusammensetzung der genitalen Mikrobiome nach der Paarung teilweise auf einen Austausch von Bakterienstämmen während der Paarung zurückzuführen sind, was auf eine sexuelle Übertragung in beide Richtungen hinweist. Einige dieser sexuell übertragenen Bakterien waren opportunistische Bakterien, also Bakterien, die von der Cuticula stammten. Diese Hinweise wurden durch meine dritte Studie unterstützt, die sich mit der Übertragungsrate opportunistischer Mikroben und dem Wachstum und Effekt dieser Bakterien auf das weibliche Immunsystem beschäftigte. Bakterien, die auf die Reproduktionsorgane von Männchen und Weibchen aufgetragen wurden, wurden während der Paarung in mindestens 90% der Fälle auf das andere Geschlecht übertragen, sodass angenommen werden kann, dass Weibchen regelmäßig mit sexuell übertragenen opportunistischen Mikroben konfrontiert werden. Bakterien, die in das paragenitale Organ des Weibchens injiziert wurden, das der Spermienaufnahme dient, wuchsen in den ersten sechs Stunden langsam und in den folgenden achtzehn Stunden wesentlich schneller. Die Injektion von Bakterien, aber auch die Injektion von Ejakulat induzierte lysozymähnliche Aktivität und die Produktion von antimikrobiellen Peptiden, die die Zellwände von Bakterien auflösen und das Bakterienwachstum regulieren können. Dies könnte ein Mechanismus sein, um die verbliebenen Bakterien im Organ des Weibchens, das der Spermienaufnahme dient, zu beseitigen. Im Gegensatz zu meinen Erwartungen fand ich keine Hinweise auf populationsspezifische Immunantworten auf mit der Paarung assoziierte Bakterien, was entweder so interpretiert werden kann, dass der Wirt nicht lokal angepasst ist, oder dass die Anwesenheit von Mikroben an sich große Kosten verursacht und daher zu einer Aktivierung des Immunsystems führt, egal wie pathogen die Mikroben sind. Diese Arbeit liefert wichtige Ergebnisse für das Verständnis der Reproduktion und wie diese durch natürliche Selektion über Wirt-Mikroben-Interaktionen beeinflusst wird. Die Ergebnisse deuten an, dass Organismen, die sich sexuell vermehren, regelmäßig mit eindringenden Mikroben in Berührung kommen und daher unter Selektion stehen sollten, sich gegen Infektionen zu

verteidigen. Ich habe zwei Substanzen identifiziert, die potenziell für die Verteidigung gegen mit der Paarung verbundenen Mikroben verwendet werden. Dies weist auf eine Interaktion von Reproduktion und Immunität in Bettwanzen hin. Mit dieser Arbeit habe ich das Fundament für ein Modellsystem gelegt, in dem viele Aspekte der Reproduktion manipuliert und experimentell getestet werden können, einschließlich des genitalen Mikrobioms als Teil eines voneinander abhängigen Metaorganismus. Künftige Studien sollten untersuchen, ob die Kolonisation der genitalen Mikrobiome von Umweltmikroben abhängt, oder vom Wirtsgenotyp und ob die genitalen Mikrobiome den Reproduktionserfolg beeinflussen. Des Weiteren wäre es interessant zu untersuchen, ob die Änderungen des Mikrobioms, die durch die Paarung verursacht wurden, umkehrbar sind und wie die weibliche Immunantwort durch eine Paarung ausgelöst wird.

3 Introduction

3.1 Conflicts drive the evolution of reproductive traits

Sexual selection can be divided into intrasexual and intersexual selection. The former is characterised by members of the same sex competing for access to members of the opposite sex while the latter can be described by members of one sex choosing mates of the other sex (Darwin, 1859). Intersexual selection can give rise to conflicts if the optima of the choosing and the chosen sex differ. Males and females were long perceived as cooperating mating partners. However, even Darwin discovered that some animals are polygamous as indicated by a letter to Charles Lyell that described barnacle females as having “two little pockets, in each of which she kept a little husband” (Burkhardt, 2008). The recent growing body of evidence suggests that genetic monogamy is actually extremely rare (Clutton-Brock & Isvaran, 2006; Griffith et al., 2002). Whenever males and females are not monogamous, there is potential for sexual conflict because the sexes differ in their optimal mating rates: in males, the number of offspring increases with the number of mates whereas females do not necessarily increase their reproductive success when mating with more than one male (Bateman, 1948). Different mating strategies can give rise to differences in physiology, morphology, and behaviour between the sexes, ultimately leading to sexually antagonistic selection on specific traits (Arnqvist & Rowe, 2005).

Reproductive traits of males and females are one of the most rapidly evolving traits (Swanson & Vacquier, 2002). In this context, sexual selection and sexual conflict intuitively seem to be the drivers of divergence. The well-known “good genes” hypothesis states that females select males based on traits that signal genetic advantages and will hence increase the fitness of her offspring (Hamilton & Zuk, 1982). One such trait assessed by females might be resistance against parasites signalled via ornaments (Milinski & Bakker, 1990). Hamilton later stated that both types of selection can interact: natural selection has the ability to change the strength of sexual selection (Hamilton, 1990). In accordance with this hypothesis, both natural and sexual selection seem to play a role in the evolution of cuticular hydrocarbons in

two *Drosophila* species that are sympatric in nature and use cuticular hydrocarbons to recognise potential mates (Blows, 2002). When only natural selection acts on males and females, the CHC content decreases, potentially due to the costly production. In comparison to that of females, male CHC composition reacts to sexual selection and the evolution is sped up by the interaction of sexual and natural selection (Blows, 2002).

Besides this example indicating the power of an interaction of natural and sexual selection, there is evidence for effects of natural selection on the evolution of reproductive traits without involving sexual selection. In addition to the external environment (Endler, 1986; Foster & Endler, 1999; Houde, 1997) and gamete ageing (Reinhardt, 2007; Siva-Jothy, 2000), parasites can affect reproductive traits directly. For instance, rat tapeworms inhibit the vitellogenesis in mealworm beetles, resulting in delayed egg laying and reduced egg viability (Hurd, 1998). Similarly, the parasitic mite *Coccipolipus hippodamiae* decreases egg production and egg viability in the two-spotted ladybird (Hurst et al., 1995). These examples clearly show the importance of natural selection in the context of reproductive trait evolution. They further suggest that natural selection shapes reproductive traits in a similar way and with a similar speed as sexual selection (Reinhardt, 2007; Sheldon, 1993). Unfortunately, we lack knowledge on whether and how other types of parasites, for instance microbes, shape reproductive traits via natural selection.

3.2 Sexually transmitted microbes affect reproductive traits

Parasites form a very diverse group of organisms spanning all kingdoms. Microbes, i.e. bacteria, viruses, fungi, and protozoans, are probably the most common group. Virtually all animals are surrounded by a rich community of microbes. They can be found on host surfaces and inside the host (Goodrich et al., 2016; Huttenhower et al., 2012; Kostic et al., 2013). Symbiotic associations reach from mutualistic to parasitic although for many species it is not clear which group they belong to because the type of association depends on the host and its environment (Dale & Moran, 2006). Many mutualistic microbes help the host with digestion

(Dale & Moran, 2006). For instance, the bacterial symbionts of aphids from the genus *Buchnera* have been shown to provide their hosts with essential amino acids (Douglas, 1998). Similarly, symbionts of blood-feeding insects seem to provide their host with B vitamins, for instance *Wigglesworthia* in tsetse flies (Akman et al., 2002) and *Wolbachia* in bedbugs (Hosokawa et al., 2010). In contrast, parasitic microbes, whether living on the host (ectoparasites) or inside the host (endoparasites) have negative effects, ranging from relatively mild damage of external tissue (Richardson, 1991) to multiple organ failure resulting in death (Matsuda & Hattori, 2006).

Microbes are transmitted horizontally between individuals from the same species or vertically from parent to offspring (Bright & Bulgheresi, 2010; Cory, 2015; Ebert, 2013; Perlmutter & Bordenstein, 2020) and even mixed modes of transmission exist (Cory, 2015; Ebert, 2013). In addition to obvious ingression routes like the skin or cuticle, the digestive, or the respiratory tract (Boucias & Pendland, 2012), microbes can be transmitted during mating (Knell & Webberley, 2004; Lockhart et al., 1996). In mammals, we have a large body of evidence for sexually transmitted microbes (STM), spanning 56 viruses like HIV and 51 bacteria causing infections such as gonorrhoea or chlamydia (Knell & Webberley, 2004). In contrast, according to Knell and Webberley (2004) 17 viruses but no bacteria are sexually transmitted in insects. They intentionally ignored microbes such as *Wolbachia* that manipulate reproduction because these are maternally inherited microbes that are mostly not transmitted during mating.

Instead of being sexually transmitted, reproductive manipulators use four different mechanisms to guarantee a more successful spread via vertical transmission (Engelstädter & Hurst, 2009). One mechanism used by these microbes is to feminise males, i.e. change the developmental pathway of an individual from male to female (Hiroki et al., 2002; Negri et al., 2006; Rigaud & Juchault, 1992; Terry et al., 1999; Weeks et al., 2001) to convert non-transmitting individuals to transmitting individuals. The same is achieved by inducing parthenogenesis (Huigens & Stouthamer, 2003; Kremer et al., 2009; Pannebakker et al., 2005;

Pijls et al., 1996). Furthermore, reproductive manipulators kill male embryos (Dyson & Hurst, 2004; Hurst et al., 2003; Zeh & Zeh, 2006) or larvae (Hurst et al., 2003; Nakanishi et al., 2008). Last but not least, cytoplasmic incompatibility (Bourtzis et al., 2003) between non-infected and infected mating partners causes offspring mortality and hence increases the relative fitness of infected females. Only in some cases, male offspring are killed in a later life stage and have the potential to be transmitted horizontally (Engelstädter & Hurst, 2009).

Typical diseases due to STM have been reported for humans or species with high public interest, i.e. hosts that are economically important (Lockhart et al., 1996). They span the continuum from being of relatively little effect to being highly virulent, for instance by causing high mortality or sterility. In insects, sexually transmitted viruses reduce fertility, offspring survival, hatch rate, adult survival and longevity, or damage sperm (Knell & Webberley, 2004).

3.3 Reproductive organs harbour microbiomes that are affected by mating

Microbes have been reported to colonise the reproductive organs (Hickey et al., 2012; Hirsh, 1999; Hupton et al., 2003; Ravel et al., 2011; White et al., 2011) and ejaculates (Baud et al., 2019; González-Marín et al., 2011; Lombardo & Thorpe, 2000; Skau & Folstad, 2003; Virecoulon et al., 2005), even those of healthy individuals. For instance, bacterial presence was detected in almost three quarters of human semen samples from infertile males (Virecoulon et al., 2005) and in half of the semen samples from bovine (González-Marín et al., 2011). At least a quarter of the ejaculate samples, male cloacal swabs, and female cloacal swabs from red-winged blackbirds (Hupton et al., 2003) and semen samples from tree swallows (Lombardo & Thorpe, 2000) contained bacteria.

Such genital microbiomes seem to be the rule rather than the exception and even insects harbour genitalia-associated microbes (Otti, 2015; Otti et al., 2017). The few studies in insects reported more than 10 bacteria species from the sperm storage organ of female Formosan subterranean termites (Raina et al., 2007) and from the testicles of wood-boring beetles (Rizzi et al., 2013). Eight bacteria species were found in the sperm-receiving organ of bedbug

females (Otti et al., 2017) and 7 microbe species occurred on the copulatory organ of bedbug males (Otti et al., 2017; Reinhardt et al., 2005), often represented by several different strains (Otti et al., 2017).

Microbiomes react to environmental changes, such as diet (David et al., 2014; Martínez et al., 2013), age (Odamaki et al., 2016), time of the day (Thaiss et al., 2014) and hormonal changes (Cauci et al., 2002; Nuriel-Ohayon et al., 2016). Mating is an event that is inevitable for sexually reproducing species and has been shown to change the genital microbiomes of vertebrates (Kulkarni & Heeb, 2007; Mändar et al., 2015; White et al., 2010, 2011) and invertebrates (Otti et al., 2017). For instance, sexual intercourse decreases the relative abundance of *Lactobacillus crispatus*, a dominant species of the vaginal microbiome (Mändar et al., 2015). In birds, bacterial numbers of both sexes are correlated after mating whereas there is no correlation when male and female are not mated (White et al., 2010). One of the rare studies in insects showed that mated bedbug females harbour different bacterial strains compared to virgin females (Otti et al., 2017). These mating-induced changes might increase with the number of mating partners, as suggested by an increase in species richness and compositional differences of genital microbiomes in polyandrous compared to monandrous lizard females (White et al., 2011).

All of the microbe species found on the copulatory organ of bedbug males were simultaneously present in the culture vials (Reinhardt et al., 2005), indicating that microbes in the reproductive organs could be environmental opportunistic microbes (OM) instead of STM or symbionts. In addition, bacteria applied to the copulatory organs of males can be transmitted to females (Miest & Bloch-Qazi, 2008), suggesting that not only STM but even OM have the potential to be sexually transmitted. Moreover, OM might invade the reproductive organs via copulatory wounds that frequently occur in a variety of insect species (Lange et al., 2013) and even humans (Reinhardt et al., 2015). To date, little is known about the composition of the genital microbiomes and how they are affected by mating, especially in insects.

3.4 Microbes invading the reproductive tract confer costs

Microbes entering the reproductive tract confer both direct and indirect costs (Fig.1). While STM usually cause disease, OM eventually become pathogenic when the immune system of the host is disturbed (Klainer & Beisel, 1969) and can increase mortality, for instance in bedbug females (Reinhardt et al., 2003).

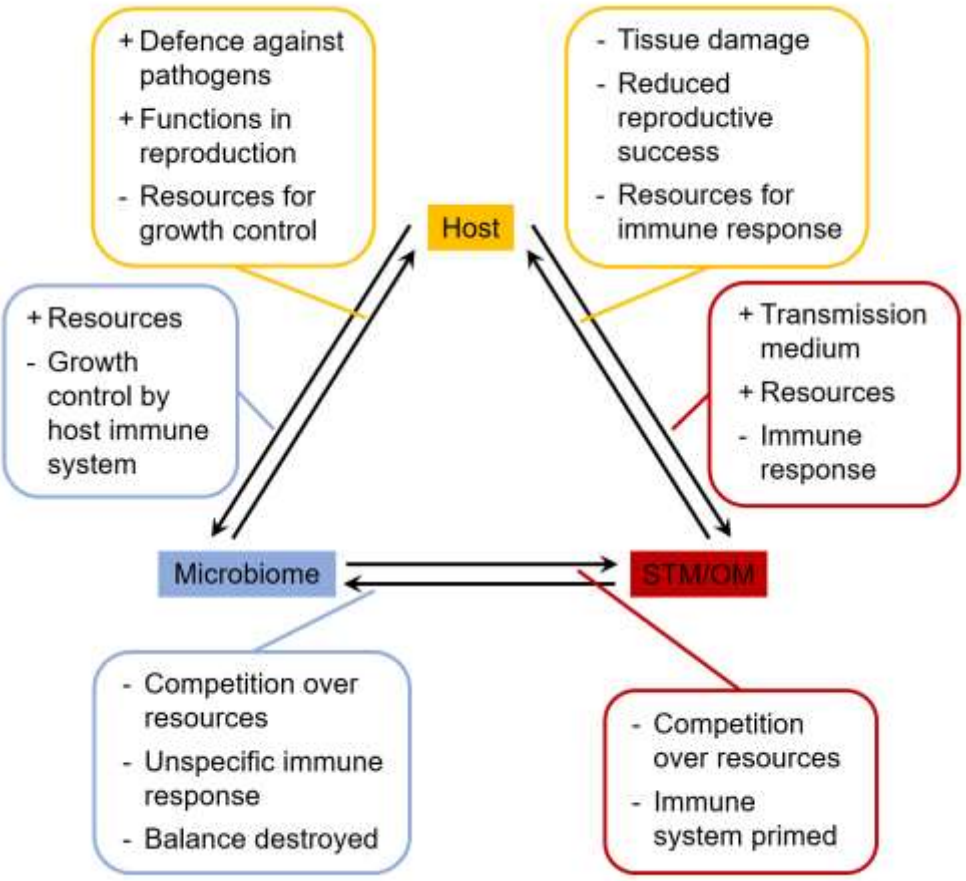

Figure 1 Costs and benefits arising via interactions of host, genital microbiome, and mating-associated microbes. The colour code indicates which consequences are expected for which interaction partner.

This direct cost of STM and OM might be accompanied by indirect costs. Infections threaten especially females because mating often compromises immunity due to the allocation of resources away from the immune system to reproduction (Sheldon & Verhulst, 1996; Zuk & Stoehr, 2002), potentially making the female more vulnerable to microbes after mating. But it

was also hypothesised that the opposite can happen, i.e. that females allocate more resources to immunity instead of reproduction, thereby reducing reproductive success (Sheldon & Verhulst, 1996; Zuk & Stoehr, 2002). In accordance with this hypothesis, the great pond snail has a lower reproductive output when held in microbe-enriched water (Rigby & Jokela, 2000). Furthermore, female pied flycatchers have less offspring and lower fledgling quality when immunised with non-pathogenic antigens from a diphtheria-tetanus vaccine (Ilmonen et al., 2000). More detail on female immune responses can be found in chapter 3.5.

Fitness could also be reduced by microbe-induced sperm damage which occurs in several mammal, avian and insect species (reviewed in Rowe et al., 2020). In humans, bacteria like *Escherichia coli* (Diemer et al., 1996, 2003; Prabha et al., 2010), *Pseudomonas aeruginosa* (Huwe et al., 1998), and *Staphylococcus aureus* (Kaur et al., 2010) decrease sperm motility and cause the agglutination of sperm *in vitro*. The same effect has been found for *Acinetobacter baumannii* on rabbit spermatozoa (Tvrdá et al., 2018). In insects, sperm motility was drastically reduced when males were infected with an iridovirus (Adamo et al., 2014) and environmental bacteria from the culturing vials increase sperm mortality *in vitro* (Otti et al., 2013). Besides sperm agglutination due to adhesion of microbes, sperm damage has been attributed to the release of toxic microbial lipopolysaccharides (Galdiero et al., 1988), the production of reactive oxygen species (Eley et al., 2005), and antibodies being active against both microbes and spermatozoa (Kurpisz & Alexander, 1995). Reduced sperm motility or survival should decrease reproductive success of both males and females. Indeed, the presence of *E. coli* in boar semen is positively correlated with the agglutination of sperm and both *E. coli* presence and sperm agglutination are positively correlated with reduced litter size (Maroto Martín et al., 2010). In chicken, *Lactobacillus acidophilus* decreases sperm motility and artificial insemination with ejaculates exposed to *L. acidophilus* results in complete infertility (Haines et al., 2015).

Furthermore, non-resident bacteria might invade the genital microbiomes that threaten the resident microbiomes by the competition over available resources (Li & Stevens, 2012; Mallon

et al., 2003). In humans, the composition and structure of the vaginal microbiome has been shown to change to a transition state after disturbances such as sexual activity but homoeostatic mechanisms can restore the initial state (Gajer et al., 2012). Besides the energy spent on these homoeostatic mechanisms, the disturbance or loss of mutualists might impair reproduction directly. In humans, the endometrial microbiome is normally characterised by a high abundance of *Lactobacillus*, which decreases after sexual intercourse (Mändar et al., 2015). Microbiomes not dominated by *Lactobacillus* are associated with low implantation and pregnancy rates and decreased live birth (Moreno et al., 2016). Therefore, a disturbance of the genital microbiome can have severe fitness consequences, especially if genital microbiomes are beneficial for reproduction. Hence, it is important to characterise the genital microbiomes and investigate potential interactions between sexually transmitted microbes and the resident microbiome.

3.5 Both sexes react to mating-associated microbes

Microbes invading a host will be attacked by immunological defence mechanisms. Invertebrate immune responses comprise a variety of constitutive (always active) and induced (elicited by an immune challenge) defences (Schmid-Hempel, 2005; Siva-Jothy et al., 2005). Although both sexes are threatened by mating-associated microbes, each sex might use different protective mechanisms or adjust the strength of an immune response depending on the costs conferred by microbes.

Females have evolved several mechanisms to regulate the number of STM or OM and thereby prevent fitness costs due to disease or sperm mortality. As part of the constitutive defence in humans, the female reproductive tract contains a family of antimicrobial peptides, so called defensins, that are thought to disrupt the membrane of microbes (Quayle et al., 1998; Valore et al., 1998). In bedbugs, the female sperm-receiving organ has evolved to protect the female from mating-associated microbes (Reinhardt et al., 2003) and is filled with haemocytes (Carayon, 1966) that are able to phagocytose bacteria (Siva-Jothy et al., 2005).

As a special form of induced defence, *Drosophila* females up-regulate immune genes that provide protection against a sexually transmitted fungus (Zhong et al., 2013) in response to male courtship song (Immonen & Ritchie, 2012) and bedbug females induce the production of lysozyme-like activity in anticipation of mating (Siva-Jothy et al., 2019). These pre-copulatory induced defences allow the female to react faster in comparison with other induced responses. At the same time, they save energy by inducing the mechanism only when necessary, i.e. when females are likely to mate within the next hours. Although knowledge on anticipatory immunity is scarce, it was suggested to be a common strategy among insects because mating should be highly predictable when females are in control over mating (Siva-Jothy et al., 2019).

Like females, males use antimicrobial substances to assure reproductive success. The ejaculates of humans (Edström et al., 2008), mallard ducks (Rowe et al., 2011), and *Drosophila melanogaster* (Lung et al., 2001; Samakovlis et al., 1991) contain antimicrobial peptides and the ejaculate of bedbugs has lysozyme-like activity (Otti et al., 2009) that can protect sperm from attacking bacteria (Otti et al., 2013). These constitutively expressed substances are likely produced to protect sperm in the female reproductive tract after ejaculate transfer rather than inside the male reproductive tract. But the expression of antimicrobial peptides inside the genital tract of *Drosophila melanogaster* males in response to bacteria applied to the genital plate (Gendrin et al., 2009) indicates that there are protective mechanisms that target microbes associated with the copulatory organs of males. Although it is known that females and males have evolved immune defences against mating-associated bacteria, we have little knowledge about their effectiveness.

In addition to immune responses, symbionts might provide their host with protection against STM and OM. Protection by symbionts against invading parasites has been reported for humans (Boris et al., 1998; Kamada et al., 2013; Reid et al., 1987), nematodes (King & Bonsall, 2017), and arthropods (Braquart-Varnier et al., 2015; Kaltenpoth & Engl, 2014; Koch & Schmid-Hempel, 2012; Mattoso et al., 2012; Oh et al., 2009). The results of these studies suggest that growth inhibition, competitive metabolic interactions, and immune priming are the

main mechanisms by which symbionts protect their hosts. Interestingly, even pathogenic symbionts have the potential to rapidly evolve to defend their host when parasites that are more virulent are present (King et al., 2016).

3.6 Immune responses are population-specific

If hosts and microbes encounter each other regularly, which is the case for sexually transmitted microbes and genital microbiomes, both host and microbes represent a constantly changing environment to which the rival has to adapt (Gandon & Michalakis, 2002). The pre-requisite for such local adaptation is a form of genotype X environment interaction for fitness out of which antagonistic pleiotropy (alleles have opposite effects on fitness in different habitats) is the most important for local adaptation (Kawecki & Ebert, 2004). Antagonistic pleiotropy causes trade-offs between adaptation to different habitats, resulting in specialist genotypes that are only superior in specific habitats, i.e. no single superior genotype in all habitats exists (Kawecki & Ebert, 2004).

The two pre-requisites for adaptation to a constantly changing environment are the strength of selection and the evolutionary potential which depends on mutation, migration, and recombination (Gandon & Michalakis, 2002). Parasites, including microbes, are often regarded as having the bigger evolutionary potential than their hosts because of their higher rates of mutation and migration, shorter generation times, and larger population sizes. In accordance with this view, a meta-analysis based on 22 reciprocal studies, i.e. studies of which the experimental design included at least one sympatric pairing and one allopatric pairing, found local adaptation characterised by better performances of parasites on sympatric than on allopatric hosts (Hoeksema & Forde, 2008). However, other studies did not find any evidence for parasite local adaptation (Dufva, 1996; Morand et al., 1996; Mutikainen et al., 2000) or reported parasite maladaptation (Imhoof & Schmid-Hempel, 1998; Kaltz et al., 1999; Oppliger et al., 1999), suggesting that the parasite is not always leading the arms race between parasite and host.

Since sexually reproducing animals are constantly exposed to sexually transmitted bacteria and their genital microbiomes, it is reasonable that local adaptation plays a role in this interaction. If hosts are locally adapted, they should show immune responses that are tailored to the microbes they frequently encounter. Factors influencing immunity might be the prevalence and pathogenicity of sexually transmitted bacteria or the diversity and composition of the genital microbiomes that might vary between host populations. Both types of microbes have the potential to shape their host's immune response because even microbes like OM or symbionts that do not directly cause disease have the potential to become pathogenic when the host's immune system is disturbed (Klainer & Beisel, 1969). It has been shown that even endosymbionts are prevented from uncontrolled growth by the host's immune system (Login et al., 2011). If both sexually transmitted microbes and genital microbiomes are controlled by the immune system of their host, it is likely that an immune response that protects the host against one type of microbes affects the opposite type of microbes as well. Since host populations are often exposed to different environmental microbes and their genital microbiomes might differ if both host genotype and microbe genotype have adaptively diversified, one would expect host immune defences to differ between populations, for instance in strength or type of defence.

There are several studies that support the idea of population-specific immune defences that might be caused by local adaptation. Freshwater shrimps exhibit strong between-population variation in the prophenoloxidase activity of the haemolymph, an important component of invertebrate immunity (Cornet et al., 2009). The examined shrimp populations are usually infected with three different species of acanthocephalan parasites and their richness and prevalence varies between populations. In accordance with the predicted link between immunity and parasite presence, prophenoloxidase was found to be negatively associated with prevalence of acanthocephalan parasites (Cornet et al., 2009). Similarly, the expression of antimicrobial peptides in the abdomen differs between bumblebee colonies and even collection sites when challenged with a trypanosome gut parasite (Brunner et al., 2013).

The authors suggested that variation between collection sites might be related to differences in trypanosome gut parasite prevalence between sites that could impose different selection regimes. Unfortunately, we lack knowledge on the effect of local adaptation to population-specific microbes on immunity.

As mentioned in chapter 3.5, symbionts might also provide their host with protection against STM and OM, for instance by growth inhibition, competitive metabolic interactions, and immune priming (Boris et al., 1998; Braquart-Varnier et al., 2015; Kaltenpoth & Engl, 2014; Kamada et al., 2013; King & Bonsall, 2017; Koch & Schmid-Hempel, 2012; Mattoso et al., 2012; Oh et al., 2009; Reid et al., 1987) and even pathogenic symbionts can defend their host when parasites that are more virulent are present (King et al., 2016). Such protective microbes provide a third level to the interaction between host and parasite. Antiviral protection by symbiotic bacteria from the genus *Wolbachia* is common across *Drosophila* species and depends on symbiont strain (Martinez et al., 2014, 2017) and density (Martinez et al., 2014) in host tissues. Protection has been shown to decrease resistance to a virus after nine generations of selection (Martinez et al., 2016), indicating that protective microbes can reduce selection for host immunity and hence be an important part of host-parasite coevolutionary interactions.

3.7 Bedbugs are an optimal system to study male-female and host-parasite interactions

The common bedbug, *Cimex lectularius* L., is one of the best-known pest insects, probably because of its close association and impacts on humans. The recent incidence of bedbugs returning to levels comparable to those before World War 2 have drawn the attention of the public towards the investigation of bedbugs as indicated by a 15-fold increase of global searches for the word “bedbugs” between 2004 and 2018 (Scarpino & Althouse, 2019). Besides the investigation of costs introduced by bedbugs and eradication methods, bedbugs are a great system for the investigation of sexual conflict due to different optima in the mating

rate of both sexes leading to antagonistic male traits (Stutt & Siva-Jothy, 2001) and for reproductive immunity (Siva-Jothy et al., 2019).

Males do not inseminate females via the genital tract in any bedbug species (Carayon, 1966). Instead, the male intromittent organ pierces the female abdomen through a groove covering the pleural membrane of the female abdominal wall, the ectospermalege, and injects sperm into the mesospermalege, a female immune organ situated underneath the groove. In addition to its function as a sperm-receiving organ, the mesospermalege also functions as an immune organ (Reinhardt et al., 2003) and is filled with haemocytes (Carayon, 1966), cells that can phagocytise bacteria (Siva-Jothy et al., 2005). From the mesospermalege, sperm migrate to the ovaries and sperm storage organs via the haemocoel (Carayon, 1966). This procedure of traumatic insemination involves copulatory wounding, which is costly for females (Stutt & Siva-Jothy, 2001) but potentially beneficial for males.

The arms race between males and females has resulted in males having the control over pre-copulatory choice. Fully-fed females cannot resist mating (Reinhardt et al., 2009) and the actual mating rate is higher than the optimal mating rate of females, apparently without any direct or indirect benefit of re-mating (Stutt & Siva-Jothy, 2001) but reduced lifespan (Morrow & Arnqvist, 2003; Reinhardt et al., 2003). Therefore, bedbug females might use post-copulatory mechanisms to select for high-quality males or good genes. Some invertebrate females eject or attack sperm via immunologically active substances (Firman et al., 2017) and bedbug females might possess similar post-copulatory mechanisms. Sexual selection and sexual conflict are therefore a potential driver for the evolution of copulatory traits in bedbugs.

Studies indicate that opportunistic microbes are sexually transmitted in bedbugs (Reinhardt et al., 2005) and that such microbes increase the mortality of bedbug females (Reinhardt et al., 2003) and sperm mortality, at least *in vitro* (Otti et al., 2013). This suggests that in addition to male-female coevolution, bedbugs are also subject to host-parasite coevolution and both types of coevolution might interact in shaping reproductive traits. To date,

little is known about the impact of mating-associated microbes invading the reproductive organs on the genital microbiomes and their bedbug host.

4 Objectives

I. Does mating change the genital microbiomes?

As a first step towards an understanding of the interaction between male and female bedbugs and bacteria, I will focus on the bacterial communities in and on the reproductive organs of bedbugs, the genital microbiomes. In comparison to the gut microbiome, little is known about the composition of the genital microbiomes and how they are affected by mating. Furthermore, most studies have been conducted in humans, ignoring the effects of mating on other organisms. Culture-dependent studies have shown that environmental microbes exist on the copulatory organ of bedbug males (Reinhardt et al., 2005) and that both virgin and mated bedbugs harbour bacteria (Otti et al., 2017). The fact that bacteria can be found in the sperm-receiving organ of virgin females indicates that these bacteria are part of the resident genital microbiome rather than originating from the environment. However, mated females also harboured several bacteria that are not present in virgin females (Otti et al., 2017), suggesting sexual transmission. None of these introduced species is a classical STM, suggesting that even OM are sexually transmitted in bedbugs.

To the best of my knowledge, no studies in insects have ever addressed a compositional change in terms of both present species and their abundance in the genital microbiomes of insects. I therefore analysed metagenomic data from the genital microbiomes of bedbugs and how they change shortly after mating (Manuscript 1, chapter 6.1). Metagenomic approaches have a large advantage because they track the majority of microorganisms within a sample in comparison to culture-based approaches that result in a limited number of microorganisms due to the difficulty to cultivate several species (Degnan & Ochman, 2011). This study provided me with a first insight into the composition of bedbug genital microbiomes and how they are affected by mating. Since bedbug populations caught on different continents likely differ in their genetic background it is conceivable that the populations harbour distinct genital microbiomes given the potential for local adaptation. If the resident microbes compete with invading microbes, we might find differences in mating-induced changes between bedbug populations.

Furthermore, the bedbug populations might be adapted to the invading microbes if the bedbugs are constantly exposed to the same microbes. Therefore, differences in frequency or pathogenicity of microbes between populations could be reflected in population-specific mating-induced changes. I addressed this idea with a second metagenomic study (Manuscript 2, chapter 6.2) using four different bedbug populations originating from infestations in the UK and Kenia and a long-term lab population.

II. How do females react to invading OM?

The first two studies (Manuscript 1, chapter 6.1; Manuscript 2, chapter 6.2) revealed that bedbug females are constantly confronted with mating-associated bacteria. As a defence against introduced pathogens, they have evolved a special immune organ that serves as the sperm-receiving organ (Reinhardt et al., 2003). The so-called mesospermalege is filled with haemocytes (Carayon, 1966), cells that can phagocytise bacteria (Siva-Jothy et al., 2005). Recently, bedbug females have also been shown to upregulate the production of lysozyme-like activity in anticipation of mating (Siva-Jothy et al., 2019), indicating that they possess constitutive and induced defence mechanisms.

Despite the fact that several immune defence mechanisms of bedbug females have been investigated, we have little knowledge about their effectiveness and whether they differ between populations. In the second part of my thesis, I therefore investigated how long OM survive in the mesospermalege of females after being introduced during mating (Manuscript 3, chapter 6.3). This study showed that after 24 hours, OM were still present in all populations, even in the presence of sperm. To clarify whether OM induce mechanisms that can reduce bacterial abundance, I decided to measure two immune traits, lysozyme-like activity and growth inhibition due to antibacterial peptide presence, after the injection of OM into the female mesospermalege (Manuscript 3, chapter 6.3). Injection of each mating-associated component separately, i.e. sperm, seminal fluid, and bacteria, before measuring the two immune traits

allowed me to disentangle the effect induced by male components and the effect induced by bacteria.

5 List of publications in peer-reviewed journals

Manuscript 1

Bellinvia, S., Johnston, P. R., Reinhardt, K. & Otti, O. (2020) Bacterial communities of the reproductive organs of virgin and mated common bedbugs, *Cimex lectularius*. *Ecological Entomology*, *45*(1), 142–154. https://doi.org/10.1111/een.12784.

Manuscript 2

Bellinvia, S., Johnston, P. R., Mbedi, S. & Otti, O. (2020) Mating changes the genital microbiome in both sexes of the common bedbug *Cimex lectularius* across populations. *Proceedings of the Royal Society B: Biological Sciences*, *287*(1926), 20200302. https://doi.org/10.1098/rspb.2020.0302

Manuscript 3

Bellinvia, S., Spachtholz, A., Borgwardt, I., Schauer, B. & Otti, O. (2020) Female immunity in response to sexually transmitted opportunistic bacteria in the common bedbug *Cimex lectularius*. *Journal of Insect Physiology*, *123*, 104048. https://doi.org/10.1016/j.jinsphys.2020.104048

6 Manuscripts and declaration of own contribution

6.1 Manuscript 1

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

Authors: Sara Bellinvia, Paul R. Johnston, Klaus Reinhardt, Oliver Otti

Journal and status: *Ecological Entomology, 45*(1), 142-154

Own contribution: concept and study design: 0%, data acquisition: 0%, data analysis and figures: 90%, interpretation of results: 80%, manuscript writing: 80%

O.O., P.R.J., and K.R. conceived the idea and designed the experiment. O.O., P.R.J., and K.R. carried out the experiment. S.B. and P.R.J. performed the bioinformatics and statistical analysis. S.B., O.O., and K.R. interpreted the results and wrote the manuscript.

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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

Ecological Entomology, doi: 10.1111/een.12784

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

Ecological Entomology, doi: 10.1111/een.12784

'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

Ecological Entomology, doi: 10.1111/een.12784

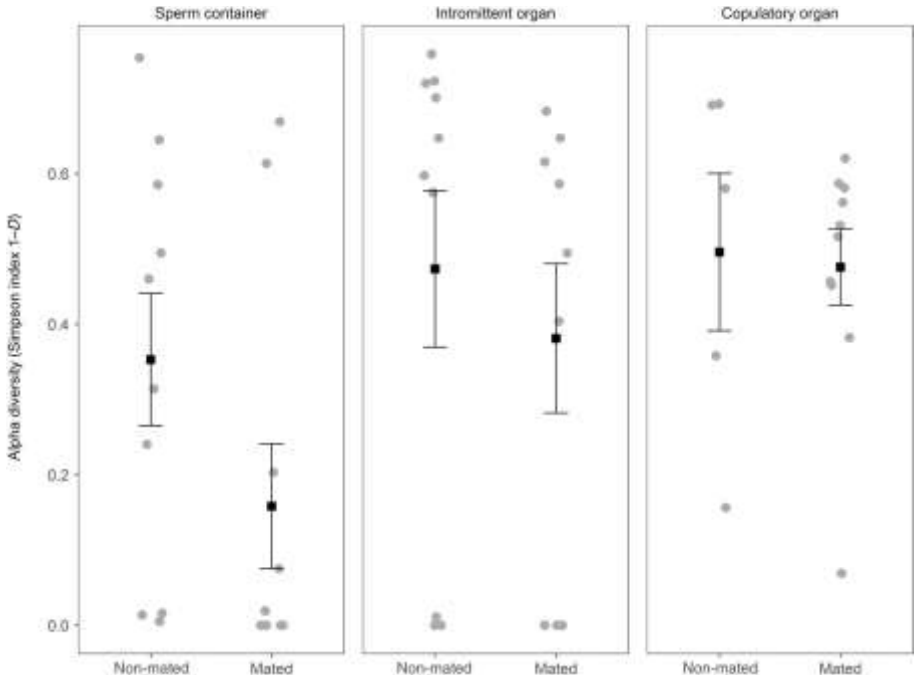

Fig. 1. Alpha diversity of bacteria from the reproductive organs of bedbugs. The Simpson index ($1 - D$) is shown. Each grey dot represents the diversity of one specific sample. The means and SEs for each organ and mating status are depicted in black. There were 10 samples per organ and mating status,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

Ecological Entomology, doi: 10.1111/een.12784

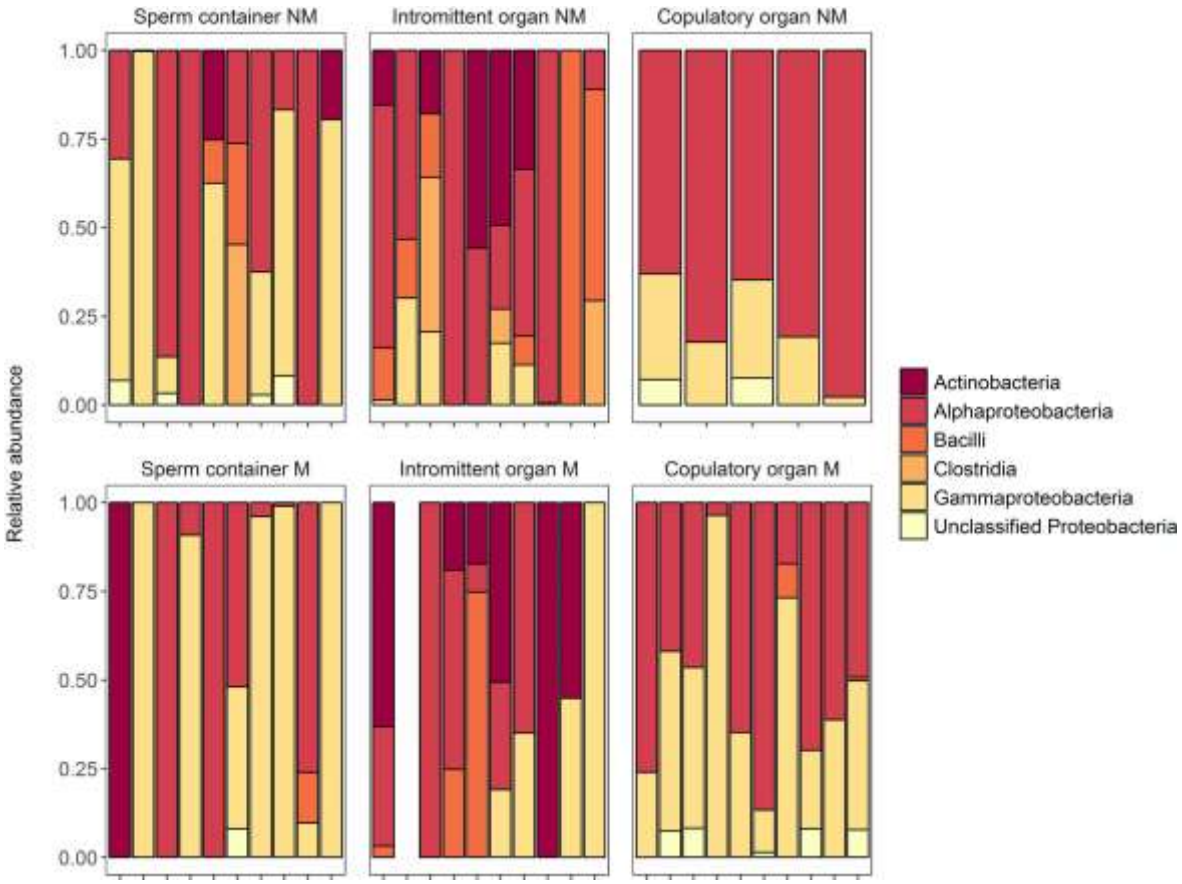

Fig. 2. Relative abundances of the six classes found in the reproductive organs of non-mated (NM) or mated (M) bedbugs. Relative abundances were calculated based on the number of reads of the same class within a given sample divided by the total number of reads within that sample. Each bar represents 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

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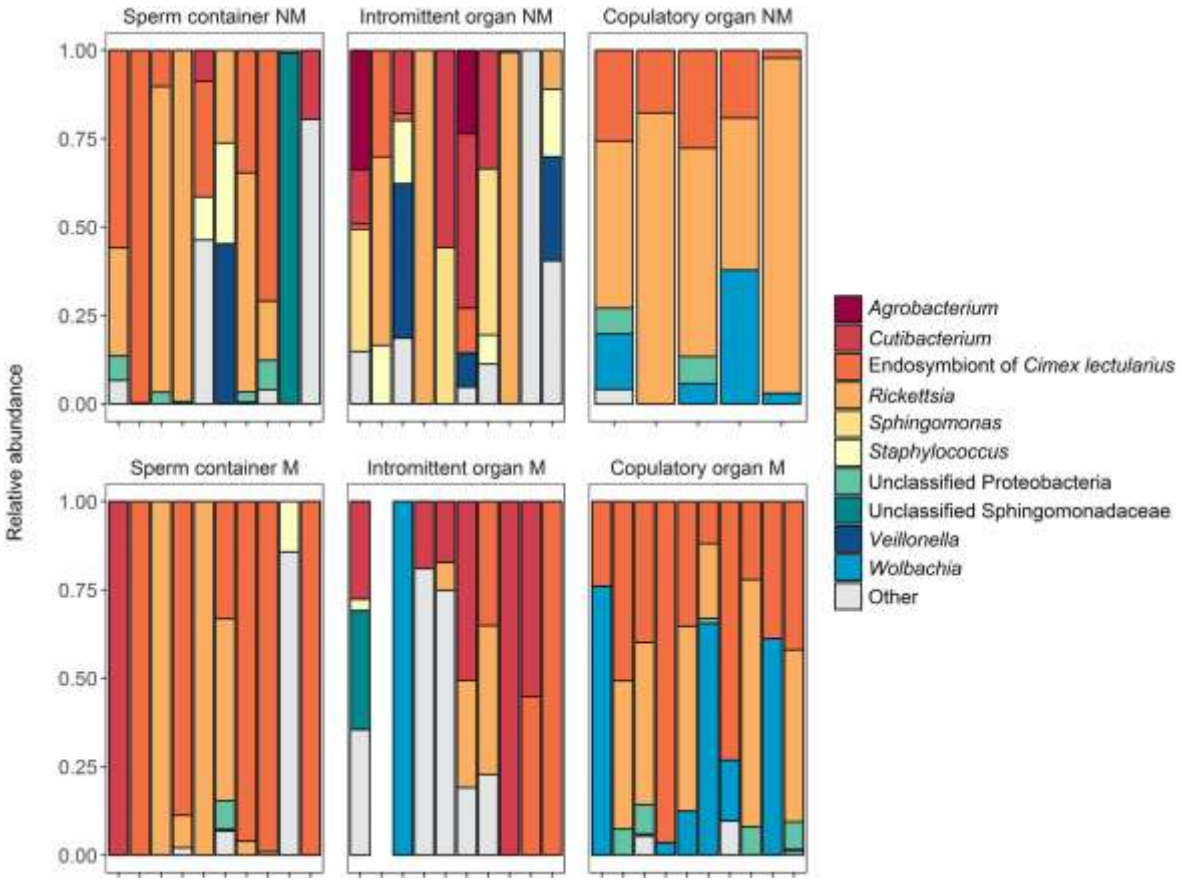

Fig. 3. Relative abundance of the 10 most abundant out of the 20 genera that were harboured by the reproductive organs of non-mated (NM) and mated (M) bedbugs. Relative abundances were calculated based on the number of reads of the same genus within a given sample divided by the total number of reads 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. [Colour figure can be viewed at wileyonlinelibrary.com].

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

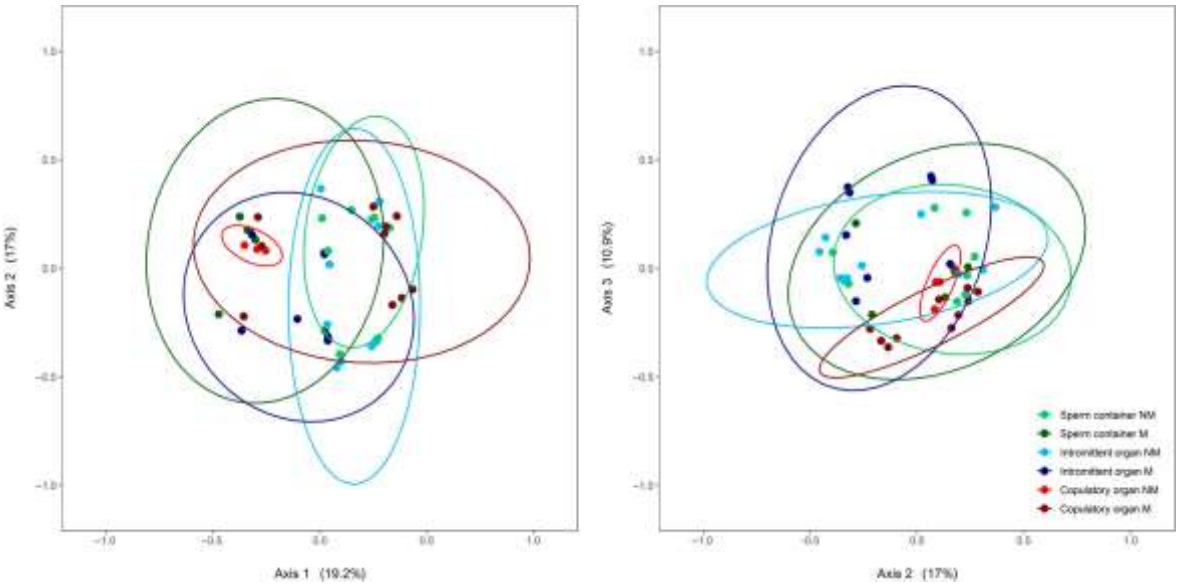

Fig. 4. Variation in the structure of bacterial communities in and on the reproductive organs of non-mated (NM) and mated (M) bedbugs. Shown are the first three axes of a principal coordinate analysis based on the Jaccard index. Each circle represents a sample with its bacterial community. Ellipses give 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). [Colour figure can be viewed at wileyonlinelibrary.com].

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

Ecological Entomology, doi: 10.1111/een.12784

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.

References

Afzelius, B.A., Alberti, G., Dallai, R., Godula, J. & Witalinski, W. (1989) Virus- and *Rickettsia*-infected sperm cells in arthropods. *Journal of Invertebrate Pathology*, **53**, 365–377.

Allison, S.D. & Martiny, J.B. (2008) Resistance, resilience, and redundancy in microbial communities. *Proceedings of the National Academy of Sciences of the United States of America*, **105**, 11512–11519.

Anahtar, M.N., Byrne, E.H., Doherty, K.E., Bowman, B.A., Yamamoto, H.S., Soumillon, M. *et al.* (2015) Cervicovaginal bacteria are a major modulator of host inflammatory responses in the female genital tract. *Immunity*, **42**, 965–976.

Anders, S. & Huber, W. (2010) Differential expression analysis for sequence count data. *Genome Biology*, **11**, R106.

Boris, S., Suárez, J.E., Vázquez, F. & Barbés, C. (1998) Adherence of human vaginal lactobacilli to vaginal epithelial cells and interaction with uropathogens. *Infection and Immunity*, **66**, 1985–1989.

Braquart-Varnier, C., Altinli, M., Pigeault, R., Chevalier, F.D., Grève, P., Bouchon, D. *et al.* (2015) The mutualistic side of *Wolbachia*-isopod interactions: *Wolbachia* mediated protection against pathogenic intracellular bacteria. *Frontiers in Microbiology*, **6**, 1388.

Broderick, N.A. & Lemaitre, B. (2012) Gut-associated microbes of *Drosophila melanogaster*. *Gut Microbes*, **3**, 307–321.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A. & Holmes, S.P. (2016) DADA2: high-resolution sample inference from Illumina amplicon data. *Nature Methods*, **13**, 581–583.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K. *et al.* (2010) QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*, **7**, 335–336 Version 1.9.1.

Carayon, J. (1966) Traumatic insemination and the paragenital system. *Monograph of Cimicidae (Hemiptera-Heteroptera)* (ed. by R. Usinger), pp. 81–167. Entomological Society of America, Philadelphia, Pennsylvania, College Park, MD.

Chaston, J.M., Dobson, A.J., Newell, P.D. & Douglas, A.E. (2015) Host genetic control of the microbiota mediates *Drosophila* nutritional phenotype. *Applied and Environmental Microbiology*, **82**, 671–679.

Costello, E.K., Lauber, C.L., Hamady, M., Fierer, N., Gordon, J.I. & Knight, R. (2009) Bacterial community variation in human body habitats across space and time. *Science*, **326**, 1694–1697.

De Santis, T.Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E.L., Keller, K. *et al.* (2006) Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Applied and Environmental Microbiology*, **72**, 5069–5072.

Diemer, T., Weidner, W., Michelmann, H.W., Schiefer, H.G., Rovan, E. & Mayer, F. (1996) Influence of *Escherichia coli* on motility parameters of human spermatozoa in vitro. *International Journal of Andrology*, **19**, 271–277.

Diemer, T., Huwe, P., Ludwig, M., Schroeder-Printzen, I., Michelmann, H.W., Schiefer, H.G. *et al.* (2003) Influence of autogenous leucocytes and *Escherichia coli* on sperm motility parameters in vitro. *Andrologia*, **35**, 100–105.

Eley, A., Hosseinzadeh, S., Hakimi, H., Geary, I. & Pacey, A.A. (2005) Apoptosis of ejaculated human sperm is induced by co-incubation with *Chlamydia trachomatis* lipopolysaccharide. *Human Reproduction*, **20**, 2601–2607.

Giorgini, M., Bernardo, U., Monti, M.M., Nappo, A.G. & Gebiola, M. (2010) *Rickettsia* symbionts cause parthenogenetic reproduction in the parasitoid wasp *Pnigalio soemius* (Hymenoptera: Eulophidae). *Applied and Environmental Microbiology*, **76**, 2589–2599.

González-Marín, C., Roy, R., López-Fernández, C., Diez, B., Carabaño, M.J., Fernández, J.L. *et al.* (2011) Bacteria in bovine semen can increase sperm DNA fragmentation rates: a kinetic experimental approach. *Animal Reproductive Science*, **123**, 139–148.

Hagimori, T., Abe, Y., Date, S. & Miura, K. (2006) The first finding of a *Rickettsia* bacterium associated with parthenogenesis induction among insects. *Current Microbiology*, **52**, 97–101.

Haro, C., Rangel-Zúñiga, O.A., Alcalá-Díaz, J.F., Gómez-Delgado, F., Pérez-Martínez, P., Delgado-Lista, J. *et al.* (2016) Intestinal microbiota is influenced by gender and body mass index. *PLoS One*, **11**, e0154090.

Hendry, T.A., Hunter, M.S. & Baltrus, D.A. (2014) The facultative symbiont *Rickettsia* protects an invasive whitefly against entomopathogenic *Pseudomonas syringae* strains. *Applied and Environmental Microbiology*, **80**, 7161–7168.

Hirsh, D.C. (1999) The genital tract as a microbial habitat. *Veterinary Microbiology* (ed. by D. C. Hirsh and Y. Zee), pp. 190–191. Blackwell Science, Oxford, U.K.

Hosokawa, T., Koga, R., Kikuchi, Y., Meng, X.Y. & Fukatsu, T. (2010) Wolbachia as a bacteriocyte-associated nutritional mutualist. *Proceedings of the National Academy of Sciences of the United States of America*, **107**, 769–774.

Hou, D., Zhou, X., Zhong, X., Settles, M.L., Herring, J., Wang, L. *et al.* (2013) Microbiota of the seminal fluid from healthy and infertile men. *Fertility and Sterility*, **100**, 1261–1269.

Hupton, G., Portocarrero, S., Newman, M., & Westneat, D.F. (2003) Bacteria in the reproductive tracts of red-winged blackbirds. *The Condor*, **105**, 453–464.

Human Microbiome Project Consortium (2012) Structure, function and diversity of the healthy human microbiome. *Nature*, **486**, 207–214.

Hurst, G.D.D., von der Schulenburg, J.H.G., Majerus, T.M.O., Bertrand, D., Zakharov, I.A., Baungaard, J. *et al.* (1999) Invasion of one insect species, *Adalia bipunctata*, by two different male-killing bacteria. *Insect Molecular Biology*, **8**, 133–139.

Huwe, P., Diemer, T., Ludwig, M., Liu, J., Schiefer, H.G. & Weidner, W. (1998) Influence of different uropathogenic microorganisms on human sperm motility parameters in an in vitro experiment. *Andrologia*, **30**(S1), 55–59.

Ilmonen, P., Taarna, T. & Hasselquist, D. (2000) Experimentally activated immune defence in female pied flycatchers results in reduced breeding success. *Proceedings of the Royal Society B*, **267**, 665–670.

Kaltenpoth, M. & Engl, T. (2014) Defensive microbial symbionts in Hymenoptera. *Functional Ecology*, **28**, 315–327.

Kamada, N., Chen, G.Y., Inohara, N. & Núñez, G. (2013) Control of pathogens and pathobionts by the gut microbiota. *Nature Immunology*, **14**, 685–690.

Ecological Entomology, doi: 10.1111/een.12784

Kaur, S., Prabha, V., Shukla, G. & Sarwal, A. (2010) Interference of human spermatozoa motility by live *Staphylococcus aureus*. *American Journal of Biomedical Sciences*, **2**, 91–97.

King, K.C., Brockhurst, M.A., Vasieva, O., Paterson, S., Betts, A., Ford, S.A. *et al.* (2016) Rapid evolution of microbe-mediated protection against pathogens in a worm host. *The ISME Journal*, **10**, 1915–1924.

Klainer, A.S. & Beisel, W.R. (1969) Opportunistic infection: a review. *The American Journal of the Medical Sciences*, **258**, 431–456.

Knell, R.J. & Webberley, K.M. (2004) Sexually transmitted diseases of insects: distribution, evolution, ecology and host behaviour. *Biological Reviews*, **79**, 557–581.

Koch, H. & Schmid-Hempel, P. (2012) Gut microbiota instead of host genotype drive the specificity in the interaction of a natural host-parasite system. *Ecology Letters*, **15**, 1095–1103.

Lange, R., Reinhardt, K., Michiels, N.K. & Anthes, N. (2013) Functions, diversity, and evolution of traumatic mating. *Biological Reviews*, **88**, 585–601.

Lavine, M.D. & Strand, M.R. (2002) Insect hemocytes and their role in immunity. *Insect Biochemistry and Molecular Biology*, **32**, 1295–1309.

Li, W. & Stevens, M.H.H. (2012) Fluctuating resource availability increases invasibility in microbial microcosms. *Oikos*, **121**, 435–441.

Liu, C.M., Osborne, B.J., Hungate, B.A., Shahabi, K., Huibner, S., Lester, R. *et al.* (2014) The semen microbiome and its relationship with local immunology and viral load in HIV infection. *PLoS Pathogens*, **10**, e1004262.

Lockhart, A.B., Thrall, P.H. & Antonovics, J. (1996) Sexually transmitted diseases in animals: ecological and evolutionary implications. *Biological Reviews*, **71**, 415–471.

Login, F.H., Balmand, S., Vallier, A., Vincent-Monégat, C., Vigneron, A., Weiss-Gayet, M. *et al.* (2011) Antimicrobial peptides keep insect endosymbionts under control. *Science*, **334**, 362–365.

Lombardo, M.P. & Thorpe, P.A. (2000) Microbes in tree swallow semen. *Journal of Wildlife Diseases*, **36**, 460–468.

Mallon, C.A., Poly, F., Le Roux, X., Marring, I., van Elsas, J.D. & Salles, J.F. (2015) Resource pulses can alleviate the biodiversity–invasion relationship in soil microbial communities. *Ecology*, **96**, 915–926.

Marius-Jestin, V. (1987) Normal phallus flora of the gander. *Zoonoses and Public Health*, **34**, 67–78.

Markle, J.G., Frank, D.N., Mortin-Toth, S., Robertson, C.E., Feazel, L.M., Rolle-Kampczyk, U. *et al.* (2013) Sex differences in the gut microbiome drive hormone-dependent regulation of autoimmunity. *Science*, **339**, 1084–1088.

Martinez Arbizu, P. (2017). *pairwiseAdonis: Pairwise Multilevel Comparison using Adonis*. R package, version 0.0.1.

Mattoso, T.C., Moreira, D.D.O. & Samuels, R.I. (2012) Symbiotic bacteria on the cuticle of the leaf-cutting ant *Acromyrmex subterraneus subterraneus* protect workers from attack by entomopathogenic fungi. *Biology Letters*, **8**, 461–464.

McCarthy, D.J., Chen, Y. & Smyth, G.K. (2012) Differential expression analysis of multifactor RNA-Seq experiments with respect to biological variation. *Nucleic Acids Research*, **40**, 4288–4297. R package version 3.24.0.

McMurdie, P.J. & Holmes, S. (2013) phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One*, **8**, e61217. R package version 1.26.0.

McNamara, K.B., Van Lieshout, E. & Simmons, L.W. (2014) Females suffer a reduction in the viability of stored sperm following an immune challenge. *Journal of Evolutionary Biology*, **27**, 133–140.

Michels, J., Gorb, S.N. & Reinhardt, K. (2015) Reduction of female copulatory damage by resilin represents evidence for tolerance in sexual conflict. *Journal of the Royal Society Interface*, **12**, 20141107.

Moran, N.A., Hansen, A.K., Powell, J.E. & Sabree, Z.L. (2012) Distinctive gut microbiota of honey bees assessed using deep sampling from individual worker bees. *PLoS One*, **7**, e36393.

Morrow, E.H. & Arnqvist, G. (2003) Costly traumatic insemination and a female counter-adaptation in bed bugs. *Proceedings of the Royal Society B*, **270**, 2377–2381.

Nasidze, I., Li, J., Quinque, D., Tang, K. & Stoneking, M. (2009) Global diversity in the human salivary microbiome. *Genome Research*, **19**, 636–643.

Oh, D.C., Poulsen, M., Currie, C.R., & Clardy, J. (2009) Dentigerumycin: a bacterial mediator of an ant-fungus symbiosis. *Nature Chemical Biology*, **5**, 391–393.

Oksanen, J., Blanchet, G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D. *et al.* (2018). *vegan: Community Ecology Package*. R package, version 2.5-3.

Osei-Poku, J., Mbogo, C.M., Palmer, W.J. & Jiggins, F.M. (2012) Deep sequencing reveals extensive variation in the gut microbiota of wild mosquitoes from Kenya. *Molecular Ecology*, **21**, 5138–5150.

Otti, O. (2015) Genitalia-associated microbes in insects. *Insect Science*, **22**, 325–339.

Otti, O., Naylor, R.A., Siva-Jothy, M.T. & Reinhardt, K. (2009) Bacteriolytic activity in the ejaculate of an insect. *The American Naturalist*, **174**, 292–295.

Otti, O., Mctighe, A.P. & Reinhardt, K. (2013) In vitro antimicrobial sperm protection by an ejaculate-like substance. *Functional Ecology*, **27**, 219–226.

Otti, O., Deines, P., Hammerschmidt, K. & Reinhardt, K. (2017) Regular wounding in a natural system: bacteria associated with reproductive organs of bedbugs and their quorum sensing abilities. *Frontiers in Immunology*, **8**, 1855.

Prabha, V., Sandhu, R., Kaur, S., Kaur, K., Sarwal, A., Mavuduru, R.S. *et al.* (2010) Mechanism of sperm immobilization by *Escherichia coli*. *Advances in Urology*, **2010**, 240268.

Puerta Suarez, J., Sanchez, L.R., Salazar, F.C., Saka, H.A., Molina, R., Tissera, A. *et al.* (2017) *Chlamydia trachomatis* neither exerts deleterious effects on spermatozoa nor impairs male fertility. *Scientific Reports*, **7**, 1126.

Raina, A., Murphy, C., Florane, C., Williams, K., Park, Y.I. & Ingber, B. (2007) Structure of spermatheca, sperm dynamics, and associated bacteria in Formosan subterranean termite (Isoptera: Rhinotermitidae). *Annals of the Entomological Society of America*, **100**, 418–424.

Ravel, J., Gajer, P., Abdo, Z., Schneider, G.M., Koenig, S.S., McCulle, S.L. *et al.* (2011) Vaginal microbiome of reproductive-age women. *Proceedings of the National Academy of Sciences of the United States of America*, **108**, 4680–4687.

R Core Team (2013). *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/ [accessed on 25 April 2019]. Version 3.5.1.

Reid, G., Cook, R.L. & Bruce, A.W. (1987) Examination of strains of lactobacilli for properties that may influence bacterial interference in the urinary tract. *The Journal of Urology*, **138**, 30–335.

Reinhardt, K., Naylor, R. & Siva-Jothy, M.T. (2003) Reducing a cost of traumatic insemination: female bedbugs evolve a unique organ. *Proceedings of the Royal Society B*, **270**, 2371–2375.

Reinhardt, K., Naylor, R. & Siva-Jothy, M.T. (2005) Potential sexual transmission of environmental microbes in a traumatically inseminating insect. *Ecological Entomology*, **30**, 607–611.

Reinhardt, K., Anthes, N. & Lange, R. (2015) Copulatory wounding and traumatic insemination. *Cold Spring Harbor Perspectives in Biology*, **7**, a017582.

Rigby, M.C. & Jokela, J. (2000) Predator avoidance and immune defence: costs and trade-offs in snails. *Proceedings of the Royal Society B*, **267**, 171–176.

Rillig, M.C., Antonovics, J., Caruso, T., Lehmann, A., Powell, J.R., Veresoglou, S.D. *et al.* (2015) Interchange of entire communities: microbial community coalescence. *Trends in Ecology & Evolution*, **30**, 470–476.

Rizzi, A., Crotti, E., Borruso, L., Jucker, C., Lupi, D., Colombo, M. *et al.* (2013) Characterization of the bacterial community associated with larvae and adults of *Anoplophora chinensis* collected in Italy by culture and culture-independent methods. *BioMed Research International*, **2013**, 420287.

Robinson, M.D., McCarthy, D.J. & Smyth, G.K. (2010) edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*, **26**, 139–140. R package version 3.24.0.

Shalika, S., Dugan, K., Smith, R.D. & Padilla, S.L. (1996) The effect of positive semen bacterial and *Ureaplasma* cultures on in-vitro fertilization success. *Human Reproduction*, **11**, 2789–2792.

Sheldon, B.C. & Verhulst, S. (1996) Ecological immunology: costly parasite defences and trade-offs in evolutionary ecology. *Trends in Ecology & Evolution*, **11**, 317–321.

Siddiqui, H., Nederbragt, A.J., Lagesen, K., Jeansson, S.L. & Jakobsen, K.S. (2011) Assessing diversity of the female urine microbiota by high throughput sequencing of 16S rDNA amplicons. *BMC Microbiology*, **11**, 244.

Valiente Moro, C.V., Tran, F.H., Raharimalala, F.N., Ravelonandro, P. & Mavingui, P. (2013) Diversity of culturable bacteria including *Pantoea* in wild mosquito *Aedes albopictus*. *BMC Microbiology*, **13**, 70.

Virecoulon, F., Wallet, F., Fruchart-Flamenbaum, A., Rigot, J.M., Peers, M.C., Mitchell, V. *et al.* (2005) Bacterial flora of the low male genital tract in patients consulting for infertility. *Andrologia*, **37**, 160–165.

von der Schulenburg, J.H.G., Habig, M., Sloggett, J.J., Webberley, K.M., Bertrand, D., Hurst, G.D. *et al.* (2001) Incidence of male-lilling *Rickettsia* spp.(α-Proteobacteria) in the ten-spot ladybird beetle *Adalia decempunctata* L.(Coleoptera: Coccinellidae). *Applied and Environmental Microbiology*, **67**, 270–277.

Werren, J.H., Hurst, G.D., Zhang, W., Breeuwer, J.A., Stouthamer, R. & Majerus, M.E. (1994) Rickettsial relative associated with male killing in the ladybird beetle (*Adalia bipunctata*). *Journal of Bacteriology*, **176**, 388–394.

White, J., Richard, M., Massot, M. & Meylan, S. (2011) Cloacal bacterial diversity increases with multiple mates: evidence of sexual transmission in female common lizards. *PLoS One*, **6**, e22339.

Zhou, Y., Gao, H., Mihindukulasuriya, K.A., La Rosa, P.S., Wylie, K.M., Vishnivetskaya, T. *et al.* (2013) Biogeography of the ecosystems of the healthy human body. *Genome Biology*, **14**, R1.

Zuk, M. & Stoehr, A.M. (2002) Immune defense and host life history. *The American Naturalist*, **160**, S9–S22.

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SUPPLEMENTARY INFORMATION FOR

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

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INDEX:

SUPPLEMENTARY FIGURES

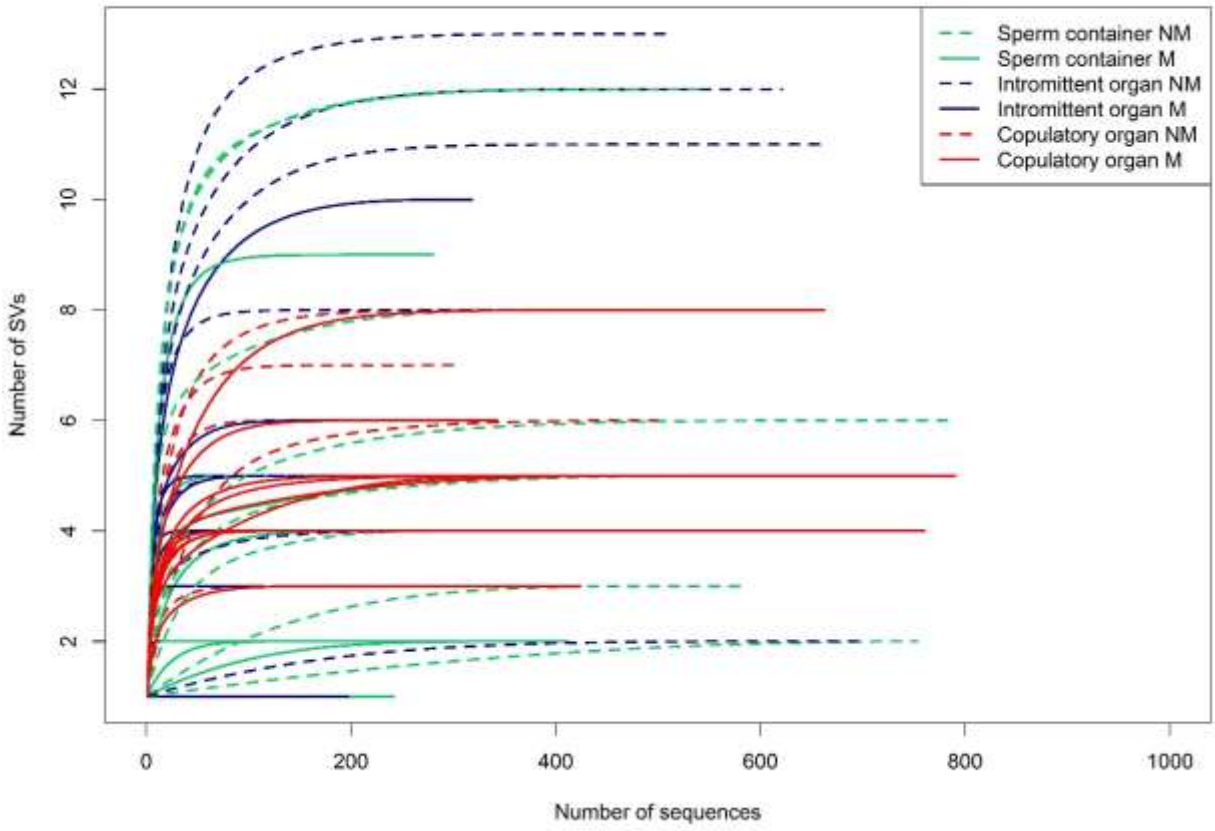

Fig. S1 Rarefaction curves for each sample from the reproductive organs of non-mated (NM) and mated (M) bedbugs.

SUPPLEMENTARY TABLES

The separate Excel files for Table S1-S3 containing the “raw” data after the pre-processing steps, such as denoising and filtering, can be accessed under:

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6.2 Manuscript 2

Title: Mating changes the genital microbiome in both sexes of the common bedbug *Cimex lectularius* across populations

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O.O., P.R.J., and S.B. conceived the idea and designed the experiment. S.B. and S.M. carried out the experiment. S.B. and P.R.J. performed the bioinformatics and statistical analysis. S.B., P.R.J., S.M. and O.O. interpreted the results and S.B. and O.O. wrote the manuscript.

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THE ROYAL SOCIETY PUBLISHING

Mating changes the genital microbiome in both sexes of the common bedbug *Cimex lectularius* across populations

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Many bacteria live on host surfaces, in cells and in specific organ systems. In comparison with gut microbiomes, the bacterial communities of reproductive organs (genital microbiomes) have received little attention. During mating, male and female genitalia interact and copulatory wounds occur, providing an entrance for sexually transmitted microbes. Besides being potentially harmful to the host, invading microbes might interact with resident genital microbes and affect immunity. Apart from the investigation of sexually transmitted symbionts, few studies have addressed how mating changes genital microbiomes. We dissected reproductive organs from virgin and mated common bedbugs, *Cimex lectularius* L., and sequenced their microbiomes to investigate composition and mating-induced changes. We show that mating changes the genital microbiomes, suggesting bacteria are sexually transmitted. Also, genital microbiomes varied between populations and the sexes. This provides evidence for local and sex-specific adaptation of bacteria and hosts, suggesting bacteria might play an important role in shaping the evolution of reproductive traits. Coadaptation of genital microbiomes and reproductive traits might further lead to reproductive isolation between populations, giving reproductive ecology an important role in speciation. Future studies should investigate the transmission dynamics between the sexes and populations to uncover potential reproductive barriers.

1. Introduction

Animals have intimate associations with bacteria. Even reproductive organs and the semen of a variety of animals often harbour several different microbe species [1–9]. In insects, many studies have investigated intracellular microbes that manipulate host reproduction, among others by male killing, or cytoplasmic incompatibility (reviewed in [10]). Besides these reproductive manipulators, extracellular microbes have been found on the male copulatory organ and within the female reproductive organs (reviewed in [11]; also see [12,13]). Interestingly, the microbiomes of whole body homogenates or the gut [14–16] and even the genital microbiomes [3,12,13] are sex-specific in a variety of species. The exact reasons for those differences as well as the role of genital microbes in reproduction and the exact composition of these genital microbiomes are unknown, especially for insects.

Microbiomes are dynamic and react to environmental change, such as diet [17,18], climate [19] and time of day [20]. In humans, life-history events such as pregnancy [21] and menopause [22] change the vaginal microbiome. Another life-history event with the potential to affect genital microbiomes is mating as

the microbiomes of both sexes encounter each other and potentially interact. Mating-induced alterations of the genital microbiome are documented in vertebrates, for instance in birds [23] and humans [24,25]. By contrast, little is known about the potential effects of mating on the composition of genital microbiomes in invertebrates.

Microbes are sexually transmitted in a large range of species (e.g. [26–28]). Not only microbes that cause sexually transmitted infections (STI) are transferred during mating. We assume also environmental bacteria in and on the genitalia [13,29,30] can be transferred or enter the reproductive organs via genital openings or copulatory wounds, which frequently occur in invertebrates [31]. The environmental bacteria with the potential to colonize the genitalia because they can tolerate the local abiotic factors, such as pH or temperature, will henceforth be called opportunistic microbes (OM). OM do not necessarily always cause an infection but become pathogenic when the host immune system is disturbed [32].

Once transferred into the female genital tract, sperm is exposed to a rich microbial flora [1,5]. In this context, OM might have a more direct effect compared to microbes causing STI: OM can decrease sperm motility and agglutinate spermatozoa in humans [33–37] and increase sperm mortality *in vitro* in insects [38]. Therefore, we predict male reproductive success to depend on the microbe communities within the female. In addition, invading OM might disturb the present genital microbiome. Because sexual intercourse decreases the relative abundance of one of the dominant species in the vaginal microbiome [24], similar mating-induced disturbances of insect genital microbiomes are conceivable. Copulatory wounding during mating further increases the risk of OM invasion in invertebrate species [39,40] and in humans [41]. Sexually transmitted bacteria disturbing the composition of the genital microbiome have been predicted to select not only for a host immune response to prevent uncontrolled growth [11], but also for defensive responses in the resident microbiota [12]. Therefore, the host and its endosymbionts have a mutual interest in keeping invading bacteria in check. In some organisms, the resident microbiota is part of the interaction with invading microbes [42–45].

If genital microbiomes are subject to OM, an adaptation to environmental microbes seems conceivable. North American women from four different ethnicities harboured distinct vaginal microbiomes [7], raising the question whether there are conserved differences in the community composition between populations and whether mating-induced changes are involved in this differentiation.

Bedbugs are an interesting system to study mating-induced changes of insect genital microbiomes because several organs are involved in reproduction. During mating and the ejaculate transfer, microbes potentially invade these reproductive organs. The male ejaculate consists of spermatozoa that are stored in the male sperm vesicles and seminal fluid from the seminal fluid vesicles. After mounting the female, the male transfers the ejaculate via its copulatory organ, the paramere, into the female paragenital copulatory organ, the mesospermalege [46]. After a few hours, sperm travel through the haemolymph towards the ovaries [46]. All tissues involved could be invaded by microbes that impose a risk of infection or sperm damage.

Microbial communities differ between reproductive organs in bedbugs [13]. Copulation increases the similarity of female and male organs, and bacteria present in mated but not virgin individuals of one sex are found in the opposite sex. Also, some of the resident bacteria are replaced with introduced bacteria [13]. Our aim is to investigate whether these findings within one bedbug population are a general pattern across bedbug populations and whether genital microbiomes differ between populations. Here, we use a community ecology approach based on 16S rRNA sequencing data from the genital microbiomes (i.e. the microbial communities of all external and internal organs involved in reproduction) of four bedbug populations. We focus on a potential difference in the microbial community between populations, between the two sexes, between organs, and between virgin and mated individuals. We thereby evaluate the effect of differences in environmental microbes and genotypes between populations on the genital microbiome and the risk of a change in composition via copulation.

2. Material and methods

(a) Bedbug culture

We used four large stock populations of the common bedbug (*Cimex lectularius* L.) out of which three populations were field caught from London, UK in 2006 (A), from Nairobi, Kenya, in 2008 (B), and from Watamu, Kenya, in 2010 (C). The fourth population (D) was a long-term laboratory stock originally obtained from the London School of Hygiene and Tropical Medicine over 20 years ago. All populations were held in separate 60 ml plastic pots containing filter paper in a climate chamber at 26 ± 1°C, 70% relative humidity, and a light cycle of 12 L : 12D at the University of Bayreuth and fed weekly with the same sterile food source using the protocol of Hase [47]. After eclosion, all individuals were kept in sex-specific groups of 20–30 individuals in 60 ml plastic pots containing filter paper. Males were fed twice. Females were fed three times, with the last feeding on the day of dissection because fully fed females cannot resist copulation [48].

(b) Mating and sample preparation

Dissections and DNA extractions were conducted at the University of Bayreuth in 2016 and 2017. We dissected 643 three-week-old males and females from four populations (population A: $n = 163$, population B: $n = 160$, population C: $n = 160$, population D: $n = 160$). Half of the individuals were randomly mated before dissection. For this, females were placed individually in a petri dish with a fresh filter paper. Then a male was added. Sixty seconds after insertion of the paramere, female and male were separated with forceps and transferred to separate containers. Both were dissected 1–2 h after successful mating, ensuring the sperm were still inside the mesospermalege of mated females at the time of dissection. The potential of contamination was minimized by a laboratory butane burner (Labogaz 206, Campingaz, Hattersheim, Germany) placed next to the dissection microscope. The dissection kit was autoclaved each day and all forceps and surgical scissors were dipped in ethanol (70%) and flame-sterilized before each dissection.

We collected different reproductive tissues and cuticle samples from both sexes ($n = 10 \pm 0$ per mating status, organ, sex and population; mean ± s.d.; see electronic supplementary material, table S1). Each sample was taken from a different bedbug since it is difficult to obtain all tissues from the same individual. From males we collected sperm vesicles, seminal fluid vesicles and paramere. In females, we investigated the mesospermalege, ovaries and haemolymph. Haemolymph was collected using a sterilized glass capillary pulled to a fine point. Each tissue was transferred into an Eppendorf tube containing 150 µl of phosphate-buffered saline. We sampled the cuticle by transferring whole females or males, whose paramere had been removed, into a tube. As a

contamination control, we put an open Eppendorf tube containing only phosphate-buffered saline next to the dissection microscope during dissections. This tube was processed in the same way as all tissue samples. All samples were frozen in liquid nitrogen and stored at −80°C.

(c) Molecular methods

Prior to DNA extraction, we homogenized the samples using pestles made from sterile pipette tips (200 µl). We followed the protocol of the MO BIO UltraClean Microbial DNA Isolation Kit (dianova GmbH, Hamburg, Germany), which includes a bead beating step, except that we dissolved the DNA in 30 µl elution buffer for higher yield. DNA was stored at −20°C. To control for contamination during DNA extraction, we performed one extraction without adding any tissue.

The library preparation and sequencing were done in the laboratory of the Berlin Center for Genomics in Biodiversity Research. The samples were split up into four sequencing runs, each balanced in terms of population, sex, organ and mating status, resulting in four libraries with 128 ± 25 (mean ± s.d.) samples each, including controls (see below). Using the universal primers 515fB (5′-GTGYCAGCMGCCGCGGTAA-3′ [49]) and 806rB (5′-GGACTACNVGGGTWTCTAAT-3′ [50]), we amplified the variable V4 region from the bacterial 16S rRNA (denaturation: 94°C, 3 min; 30 cycles of denaturation: 94°C, 45 s, annealing: 50°C, 1 min, extension: 72°C, 90 s; extension: 72°C, 10 min). After amplicons had been purified using Agencourt AMPure XP beads (Beckmann Coulter GmbH, Krefeld, Germany), a unique combination of two eight nucleotide long index sequences were used for barcoding each sample in a second PCR (denaturation: 95°C, 2 min; 8 cycles of denaturation: 95°C, 20 s, annealing: 52°C, 30 s, extension: 72°C, 30 s; extension: 72°C, 3 min). After another purification step with AMPure beads, the DNA concentration of the PCR products was quantified with the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA). Samples that had a higher concentration than 5 ng μl^{-1} were diluted to this concentration; all other samples were left undiluted. The quality of the pooled amplicons was verified with a microgel electrophoresis system (Agilent 2100 Bioanalyzer, Agilent Technologies, Santa Clara, CA, USA). The resulting libraries were subjected to an Illumina MiSeq sequencer and paired-end reads were generated using PhiX. For each plate and PCR type, we had 1–2 negative controls containing only purified water instead of DNA, resulting in 16 controls for the target PCR and 13 controls for the indexing PCR across all sequencing runs. Four additional samples per sequencing run contained the bacterial DNA from a whole homogenized bedbug to increase sequencing depth.

(d) Bioinformatical analysis

(i) Data processing and check for contamination

All data processing and analyses were performed in R (version 5.3.1 [51]) with the packages *dada2* (version 1.10.1 [52]), *decontam* (version 1.2.0 [53]), *DECIPHER* (version 2.10.2 [54]), *phangorn* (version 2.4.0 [55,56]), *phyloseq* (version 1.19.1 [57]), *pairwiseAdonis* (version 0.0.1 [58]), *vegan* (version 2.4-5 [59]) and *edgeR* (version 3.22.1 [60,61]). We used the *dada2* [52] pipeline to filter and trim the sequences. The first 10 bp were removed and the sequences were truncated after 260 bp (forward reads) and 200 bp (reverse reads), or at the first instance of a quality score ≤2. Sequences with expected errors greater than 2 were discarded. The remaining sequences were dereplicated and denoised. We constructed a sequence variant (SV) table and removed chimeric sequences. We scored all controls with the *decontam* package [53] based on prevalence and removed all contaminants (electronic supplementary material, table S2). All SVs that occurred in only one sample and that had less than 0.01% of the total number of unfiltered reads were removed as suggested by Caporaso *et al*. [62]. Out of 21 478 209 reads, 13 129 154 remained in the final dataset. The highest proportion of reads was lost during the first quality filtering step (18%) and decontamination (15%). The taxonomy of the remaining taxa was assigned with the Greengenes database [63]. We verified the taxonomical assignment with Blast2Go [64]. If Blast2Go did not find a match, we used NCBI's BLASTn. In both cases, we excluded uncultured or environmental samples from the database used for taxonomic assignment. The taxonomic assignments of the different algorithms were in accordance for kingdom until genus level in 89 out of 126 SVs. In one of the mismatches, 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*. [65]. In five other mismatches, all BLAST hits agreed on one genus. We therefore changed the taxonomy assignment of these SVs. For 31 cases without a clear BLAST result, we kept the Greengenes assignment for the levels that were congruent with the BLAST results. We changed the assignment of all other levels to 'Unclassified'. After aligning sequences with the *DECIPHER* package [54], we used the *phangorn* package [55,56] to fit a maximum likelihood tree (GTR + G + I). We added all information regarding sample type, read numbers for samples and SVs, and taxonomic assignments to the electronic supplementary material, table S3–S5.

(ii) Statistical analysis

All analyses except for the differential abundance test were based on relative abundances. Comparisons of the microbiome composition were based on Bray–Curtis dissimilarities obtained with the *phyloseq* package [57] as well as the alpha diversity estimates (Simpson index ($1 - D$)). Additionally, we used weighted UniFrac distances to compare the genital microbiomes from virgin and mated bedbugs.

(iii) Microbiomes of virgin bedbugs

We analysed the differences in microbiome composition between internal reproductive organs (sperm and seminal fluid vesicles of males; mesospermalege, haemolymph and ovaries of females), external reproductive organs (male paramere) and cuticle with a PERMANOVA (999 permutations, *vegan* package [59]) followed by a multilevel pairwise comparison using pairwise PERMANOVAs (*pairwiseAdonis* package [58]) and Benjamini–Hochberg adjusted *p*-values. Between-individual differences across the three groups were analysed with a multivariate test for homogeneity of group dispersions with the *vegan* package [59]. We compared the genital microbiome composition between populations, sexes and organs using a PERMANOVA with the fixed effects population, sex and organ nested within sex. Between-individual variation was compared with three separate tests for homogeneity of group dispersions for populations, sexes and organs.

(iv) Mating-induced effects on genital microbiomes

Compositional differences of genital microbiomes from virgin and mated bedbugs were analysed with a PERMANOVA with the fixed effects population, organ, mating status and their interactions. Removing the non-significant interactions improved the AIC. We then compared between-individual variation with separate tests for homogeneity of group dispersions for population, organ and mating status.

We estimated the proportions of bacterial strains present in mated individuals potentially originating from virgin individuals of the opposite sex using the programme SourceTracker2 [66]. To generate the input files, we split the dataset by population and transformed the *phyloseq* data into biom format with the *biomformat* package [67].

With the quasi-likelihood tests in the *edgeR* package [60,61], we tested for differential abundance of SVs, here represented by read

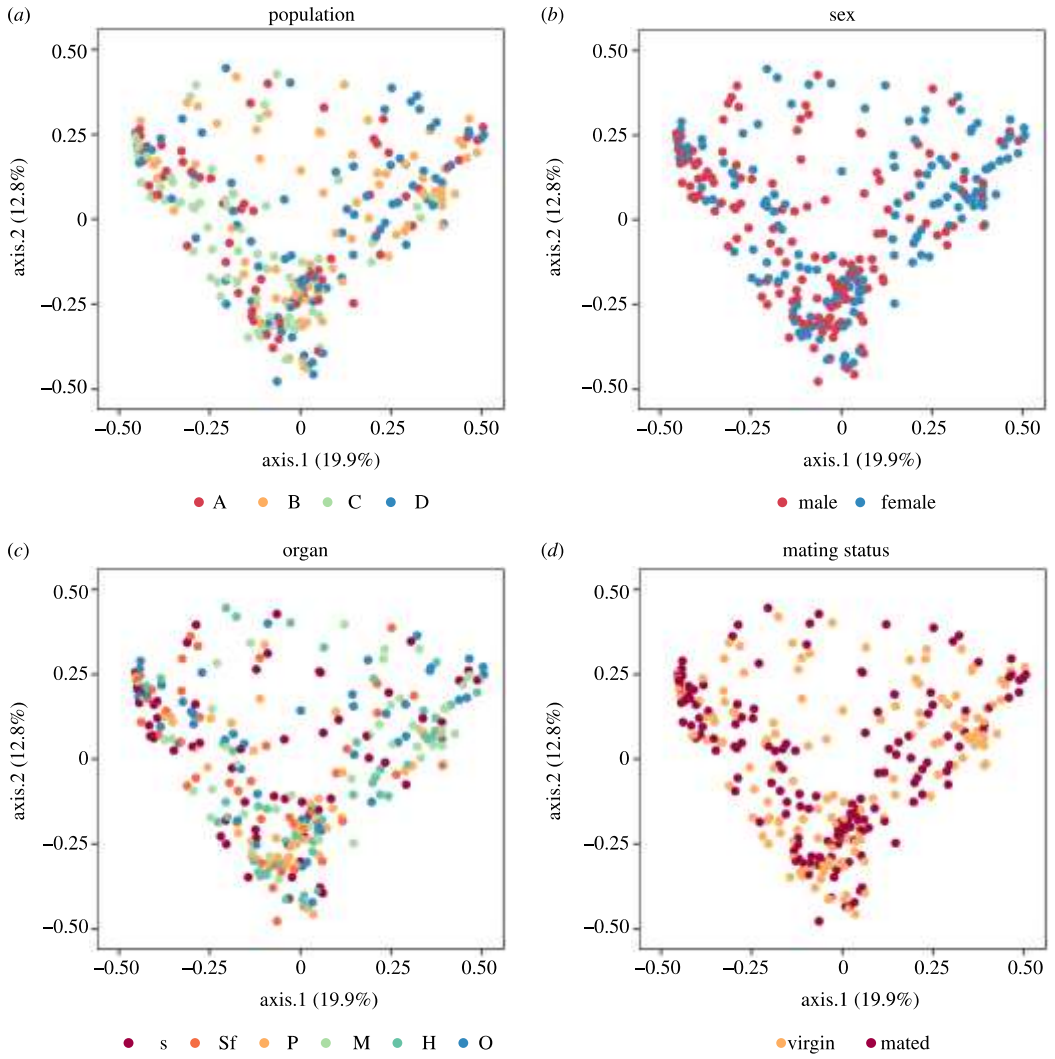

Figure 1. PCoA of all groups of genital microbiomes performed with the *phyloseq* package [57] on Bray–Curtis dissimilarities. Organs are sperm vesicle (S), seminal fluid vesicle (Sf), paramere (P), mesospermalege (M), haemolymph (H) and ovary (O). (Online version in colour.)

numbers, between the genital microbiomes of virgin and mated bedbugs. Normalization factors were calculated with the relative log expression [68] and applied to read counts with an added pseudocount of 1. Contrasts were built for every mating status within organ and population. *p*-values were adjusted with the Benjamini–Hochberg procedure (FDR = 0.05). The proportion of SVs with significant differential abundance was analysed with a logistic regression with quasibinomial error structure and the fixed effects population and sex, including their interaction. Groups were then compared with an ANOVA. Non-significant interactions were removed to analyse the main effects, which improved the AIC.

3. Results and discussion

We sequenced 643 samples from bedbug reproductive organs or cuticle via 16S rRNA amplicon sequencing to characterize the composition of the genital microbiomes and investigate the effect of mating. We sampled the cuticle from both sexes, the external intromittent organ (paramere) and the internal sperm vesicles and seminal fluid vesicles from males and the sperm-receiving organ (mesospermalege), the ovaries and the haemolymph, which are all internal female organs. After filtering, we obtained 126 sequence variants (SVs) from 495 samples ($n = 8 \pm 1$ per mating status, organ, sex and population; mean ± s.d.)(electronic supplementary material, table S1). On average, filtered samples yielded 23 867 (18673, 29 061; mean and CI) reads. Average alpha diversity was 0.59 (0.56, 0.61; Simpson index (1 – *D*); electronic supplementary material, figure S1).

(a) Microbiomes of virgin bedbugs

(i) Compositional differences between cuticular and genital microbiomes

Virgin bedbugs harboured distinct cuticular, external and internal genital microbiomes (PERMANOVA: Bray–Curtis: $F_{2,245} = 3.451$, $R^2 = 0.028$, $p = 0.001$; UniFrac: $F_{2,245} = 5.915$, $R^2 = 0.046$, $p = 0.001$). Multiple comparisons showed that the composition differed between the cuticle and the internal reproductive organs of both sexes (pairwise PERMANOVA with Benjamini–Hochberg correction: Bray–Curtis: $F_{1,219} = 5.264$, $R^2 = 0.001$, $q =$

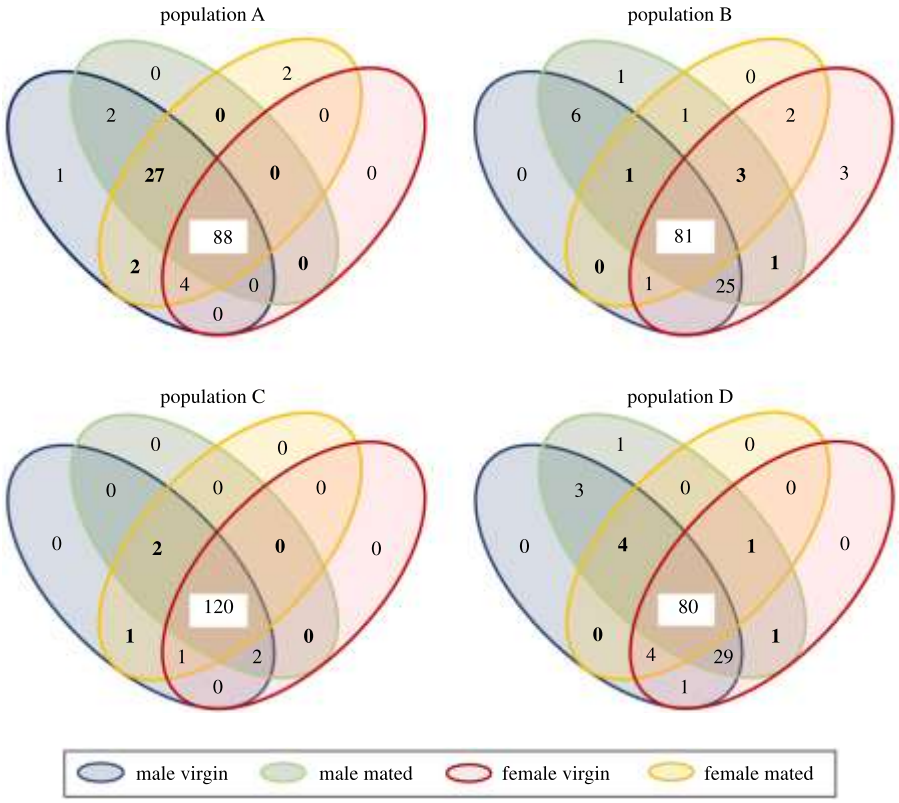

Figure 2. Occurrence of all SVs within the genital microbiomes split up by population, sex and mating status. Numbers on white background represent strains shared by both sexes and mating status, bold numbers strains that might have been sexually transmitted. The dataset consisted of 126 SVs, of which all were harboured by population A–C, and 124 by population D. (Online version in colour.)

0.003) and between the paramere and the internal reproductive organs of both sexes ($F_{1,178} = 2.351$, $R^2 = 0.013$, $q = 0.008$) but not between the cuticle and the paramere ($F_{1,92} = 1.404$, $R^2 = 0.118$, $q = 0.12$) (electronic supplementary material, figure S2). Cuticle, external and internal reproductive organs did not differ in between-individual difference (Multivariate test for homogeneity of variances: Bray–Curtis: $F_{2,243} = 1.467$, $p = 0.23$; UniFrac: $F_{2,243} = 0.887$, $p = 0.41$).

The compositional differences between microbiomes seem to be based on whether organs are internal or external. Environmental bacteria have been found on the external reproductive organ of bedbugs [30], suggesting cuticle and paramere are mostly colonized by environmental bacteria. By contrast, internal organs might be better protected from environmental bacteria and, therefore, are composed of a different set of bacteria. While it makes sense for internal organs to harbour symbionts that play a role in reproduction, the function of the paramere as a sperm-delivering organ is unlikely to require symbionts.

(ii) Composition of genital microbiomes

The genital microbiomes of virgin bedbugs harboured on average 20 (15, 25; mean and CI) SVs (female) or 30 (22, 37) SVs (male). The composition of the microbial communities differed between populations (PERMANOVA: Bray–Curtis: $F_{3,170} = 1.891$, $R^2 = 0.031$, $p = 0.007$; UniFrac: $F_{3,170} = 2.307$, $R^2 = 0.036$, $p = 0.008$) (figure 1*a*) and sexes (Bray–Curtis: $F_{1,170} = 3.780$, $R^2 = 0.020$, $p = 0.001$; UniFrac: $F_{1,170} = 7.248$, $R^2 = 0.038$, $p = 0.001$) (figure 1*b*). Even different organs from the same sex harboured distinct microbiomes (Bray–Curtis: $F_{4,170} = 1.579$, $R^2 = 0.034$, $p = 0.005$) but not when correcting for phylogeny (UniFrac: $F_{4,170} = 1.438$, $R^2 = 0.030$, $p = 0.110$) (figure 1*c*). Between-individual variation did not differ between populations (Multivariate test for homogeneity of variances: Bray–Curtis: $F_{3,175} = 1.420$, $p = 0.24$; UniFrac: $F_{3,175} = 1.664$, $p = 0.18$), sexes (Bray–Curtis: $F_{1,177} = 2.073$, $p = 0.15$) or organs (Bray–Curtis: $F_{5,173} = 1.462$, $p = 0.20$; UniFrac: $F_{5,173} = 1.350$, $p = 0.25$). Only when correcting for phylogeny, between-individual variation differed between sexes (UniFrac: $F_{1,177} = 4.793$, $p = 0.03$).

We did not find any SV present in all samples of a given sex, but the three most prevalent SVs occurred in at least half of all individuals within each sex. Males and females did not differ in the prevalence of a gammaproteobacterial endosymbiont of *C. lectularius* (males: 67%, females: 64%) and one *Rickettsia* strain (males: 58%, females: 59%), whereas more females than males harboured a second *Rickettsia* strain (males: 59%, females: 69%). The relative abundance of these bacteria varied tremendously from 0% to 50% (gammaproteobacterial endosymbiont) or from 0% to 100% (both *Rickettsia* strains) in individual samples. Virgin males and females shared 92 (population A), 108 (population B), 123 (population C) or 114 (population D) out of all SVs found in virgin bedbugs from the specific population (population A–C: 126, population D: 124) (figure 2).

The variation in genital microbiomes between populations indicates local adaptation of microbes and hosts and

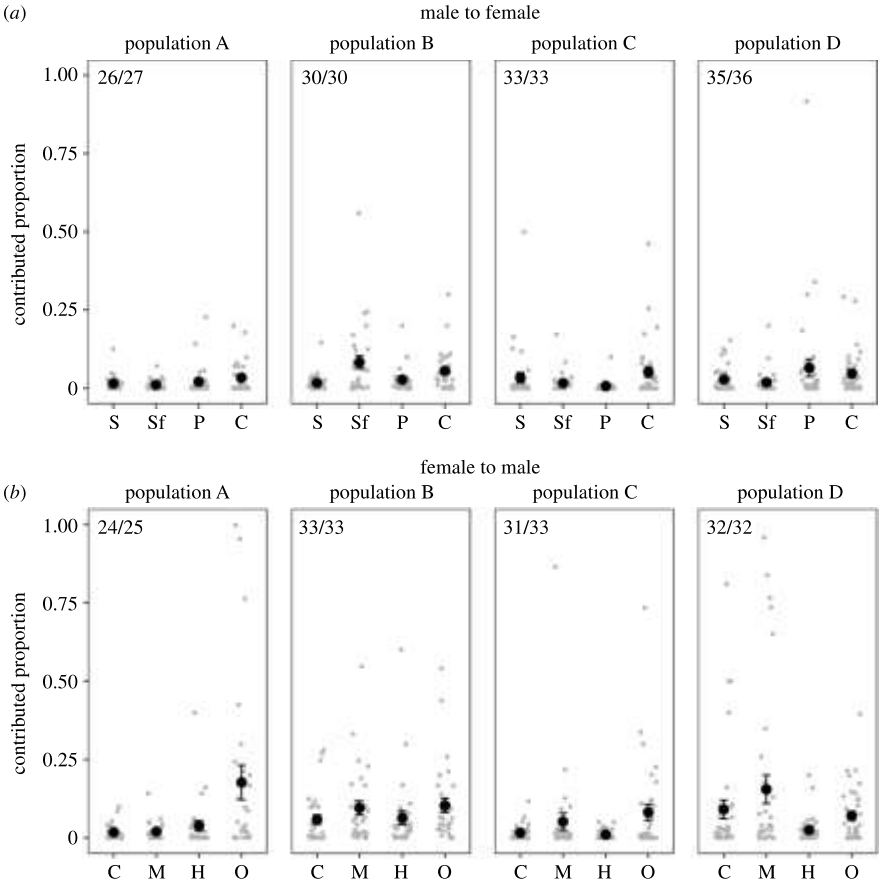

Figure 3. Potential for sexual transmission as estimated by SourceTracker2 [66]. Given are the proportions (*a*) that the sperm vesicle (S), the seminal fluid vesicle (Sf), the paramere (P) and the cuticle (C) of virgin males contribute to the microbiomes of mated females, and (*b*) that the cuticle (C), the mesospermalege (S), the haemolymph (H), and the ovaries (O) of virgin females contribute to the microbiomes of mated males. Depicted are means (black), standard errors of the mean, and individual data points (grey). The frequency of potential transmission for each population and transmission direction is given in the top left corner.

likely arises from host–microbe coevolution. While sexual conflict and genetic drift might affect this host–microbe interaction as well, we suspect bacteria to play an important role in shaping the evolution of reproductive traits and causing them to vary between populations. Ultimately, the coadaptation of genital microbiomes and reproductive traits might give rise to reproductive barriers between populations, leading to reproductive isolation and giving reproductive ecology an important role in speciation.

Despite of the controlled environmental factors in the laboratory, populations differed in their microbiome composition. Previous research has shown that bedbugs from infestations within the same city exhibit extreme levels of genetic differentiation [69] and that microbial communities of whole body homogenates are infestation specific [70]. In humans, vaginal microbiomes are ethnicity dependent [7], suggesting genital microbiomes might be adapted to host genotypes. In the laboratory setting, environmental bacteria are population specific in bedbugs [12]. These microbes probably originate from the faeces that are constantly deposited on the filter paper in the housing containers. Some of these bacteria might transfer to the paramere and the cuticle of both sexes and might finally be added to the genital microbiomes. Whether these differences in initial microbial communities are based on a founding effect or whether microbe colonization is host genotype specific remains unknown. Experimental evolution experiments combined with microbe exposure treatments could show whether genital microbiomes adapt to environmental microbes and/or whether the host genotype selects for adaptations.

Whole body homogenates or gut samples in a variety of species show sex-specific microbiome compositions [14–16]. These differences could be explained by different behaviours, feeding strategies, functions in the ecosystem or roles in reproduction. Despite a lack of studies investigating the origin of sexual dimorphism in the microbiome, pronounced differences exist between the genital microbiomes of female and male red-winged blackbirds [3] and bedbugs [12,13].

To our knowledge, we provide evidence for organ-specific genital microbiomes in female insects for the first time, a finding similar to the varying microbiome composition along the female reproductive tract in humans [71], which could be caused by the different accessibility for OM or the function of the organs. However, when correcting for phylogeny, genital microbiomes were not organ specific, suggesting that bacterial strains in different organs were related, possibly because the microbiomes of all organs within one sex originate from the same initial bacterial community.

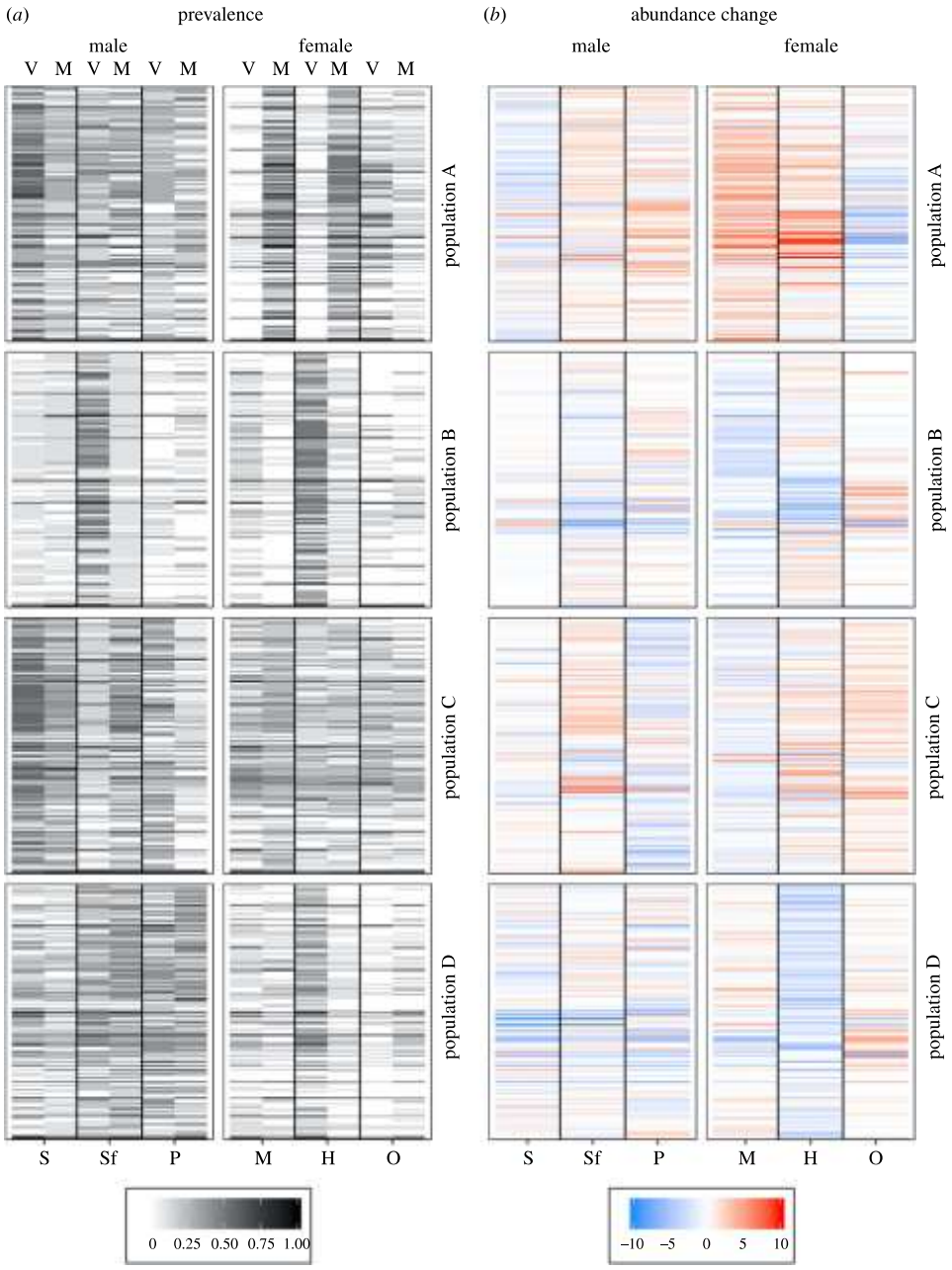

Figure 4. Changes in prevalence and abundance of SVs present in the microbiomes of the sperm vesicle (S), the seminal fluid vesicle (Sf), the paramere (P), the mesospermalege (M), the haemolymph (H) and the ovary (O) from virgin versus mated individuals. Given is (*a*) the prevalence of each SV before and after mating and (*b*) the log2-fold abundance change for each SV due to mating as estimated by GLM fits in the *edgeR* package [60,61]. (Online version in colour.)

(b) Mating-induced changes in genital microbiomes

(i) Structural changes

We found mating-induced changes in the genital microbiomes of females and males as virgin and mated individuals harboured distinct genital microbiomes (PERMANOVA: $F_{1,355} = 1.932$, $R^2 = 0.005$, $p = 0.04$) (figure 1*d*). We found no interaction of organ and mating status ($F_{5,317} = 1.003$, $R^2 = 0.013$, $p = 0.47$), population and mating status ($F_{3,317} = 0.762$, $R^2 = 0.006$, $p = 0.84$), or population, organ and mating status ($F_{15,317} = 0.777$, $R^2 = 0.031$, $p = 0.99$). Between-individual variation was similar between mating status (Multivariate test for homogeneity of variances: $F_{1,363} = 0.010$, $p = 0.92$), organs ($F_{5,359} = 1.440$, $p = 0.21$) and populations ($F_{3,361} = 0.280$, $p = 0.84$).

In all four populations, virgin males and mated females shared bacterial strains that were not present in virgin females (figure 2). In two populations (population B and D), mated males harboured bacteria that were present in virgin females but not in virgin males (figure 2). Out of the 32 bacteria that were potentially transmitted from males to females, one strain (unclassified Comamonadaceae) was shared by population A and B, two strains (both *Pseudomonas* sp.) by

population A and C, and another two strains (unclassified Lactobacillaceae and *Cloacibacterium* sp.) by population A and D (for a detailed list of transmitted bacteria species see electronic supplementary material, table S6). None of the bacterial strains were potentially transmitted from females to males in more than one population (for a detailed list of transmitted bacteria species see electronic supplementary material, table S7). All bacteria that were only found in the genital microbiomes of mated individuals occurred on the cuticle of virgin bedbugs. According to SourceTracker2, bacteria in mated individuals likely originated from at least one of the organs of the opposite sex in 96% to 100% (mated females) (figure 3*a*) and 94% to 100% (mated males) (figure 3*b*) of the cases.

In accordance with the previous studies in vertebrates [23,24,72,73] and invertebrates [12,13], we found mating-induced changes in the genital microbiomes of bedbugs. Changes in strain composition can be caused by immunological substances targeting members of the genital microbiome, by the sexual transmission of bacteria via the ejaculate or male genitalia, by strains transferred from the cuticle or by strains invading genital openings and copulatory wounds. Indeed, our results indicate that a part of the bacteria in mated individuals of one sex originate from the reproductive organs of the opposite sex as well as from the cuticle. Bacteria from the cuticle invading the genital microbiomes are highly likely to be OM rather than bacteria causing STI, which is in accordance with a previous study in bedbugs using culture-based methods [30].

Surprisingly, even males seemed to be subject to transmission, although males are less likely to face copulatory wounding, and the distance between the environment and the internal reproductive organs is larger compared with females. However, in case males do not apply pressure to transfer their ejaculate, bacteria might reach the internal organs through the ejaculatory duct via a capillary effect similar to the invasion of human testicles by urethral pathogens [74]. Antimicrobial peptide production in the genital tract of *Drosophila* males in response to bacteria deposited on the genital plate [75] suggests microbes regularly enter the genital microbiomes of male insects. Experimental manipulation of bacteria on the paramere might clarify whether and how bacteria can enter the paramere and move through the ejaculatory duct towards the internal organs.

(ii) Prevalence and abundance changes

The prevalence of many bacterial strains was changed by mating but there was no clear direction of change, i.e. decrease or increase in prevalence. However, the prevalence of several bacteria seemed to change simultaneously within the same organ, sex and population (figure 4*a*). Mating affected the abundance of several bacterial strains in all organs except for sperm vesicle, paramere and haemolymph samples from population B and mesospermalege samples from population D. The proportion of differentially abundant SVs did not differ between males and females ($F_{1,19} = 3.254$, $p = 0.09$) or between populations ($F_{3,19} = 1.56$, $p = 0.23$) and population did not interact with sex ($F_{3,16} = 0.367$, $p = 0.78$). No clear direction of abundance change for populations, sexes or organs was identified (figure 4*b*).

Genital microbiomes of females should be more affected by invading bacteria because bacteria could enter the immune organ via the ejaculate and via copulatory wounds. In accordance with this idea, sexual transmission of bacteria in birds is higher when males are the transmitting sex [72]. Since mating induced prevalence and abundance changes of several bacteria in both sexes of the common bedbug, even the microbiomes of males seem to be affected by mating. Bacteria can decrease survival [76] but also cause a trade-off between immunity and mating and hence decrease fecundity [77]. Moreover, bacteria could harm sperm within the male and female directly. OM decrease sperm motility [33–37] and incapacitate spermatozoa [36] in humans, at least *in vitro*, and environmental bacteria increase sperm mortality in bedbugs [38]. To reduce the costs of mating-associated infections, bedbugs have evolved the mesospermalege [76]. The high number of haemocytes [46] able to phagocytose bacteria [78] in this organ might stabilize its microbiome and protect against invading bacteria. In addition, lysozyme in the seminal fluid of males [79] and in the mesospermalege produced in anticipation of mating [80] could help to reduce invading bacteria. Furthermore, endosymbionts have been shown to interact with invading microbes [42–45] and might help to control non-resident bacteria in the genital microbiomes. Future studies should investigate the effect of the species with the largest prevalence and abundance changes on fecundity and survival and what adaptations have evolved to eliminate the possible threat to host integrity.

We have demonstrated that genital microbiomes of the common bedbug *C. lectularius* differ between populations, sexes and organs. Our findings show that genital microbiomes are sensitive to mating, an activity that every sexually reproducing animal experiences. Future studies should investigate sexual transmission dynamics of OM in combination with fitness effects on both sexes. Experimental manipulation of the female immune system could provide information about the importance of immunity in response to genitalia-associated bacteria. Finally, the coadaptation of genital microbiomes and reproductive traits might lead to reproductive isolation between populations, giving reproductive ecology an important role in speciation.

Data accessibility. Sequencing reads were deposited in NCBI's Sequence Read Archive with the accession number PRJNA560165.

Authors' contributions. O.O., P.R.J. and S.B. conceived the idea and designed the experiment. S.B. and S.M. carried out the experiment. S.B. and P.R.J. performed the bioinformatics and statistical analysis. S.B., P.R.J., S.M. and O.O. interpreted the results and S.B. and O.O. wrote the manuscript. All authors read and approved of the final manuscript.

Competing interests. The authors declare no competing interests.

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References

1. Hirsh DC. 1999 The genital tract as a microbial habitat. In *Veterinary microbiology* (eds DC Hirsh, YC Zee), pp. 190–191. Oxford, UK: Blackwell Science.

2. Lombardo MP, Thorpe PA. 2000 Microbes in tree swallow semen. *J. Wildl.*

Dis. **36**, 460–468. (doi:10.7589/0090-3558-36.3.460)

3. Hupton G, Portocarrero S, Newman M, Westneat DF. 2003 Bacteria in the reproductive tracts of red-winged blackbirds. *Condor* **105**, 453–464. (doi:10.1650/7246)
4. Skau PA, Folstad I. 2003 Do bacterial infections cause reduced ejaculate quality? A meta-analysis of antibiotic treatment of male infertility. *Behav. Ecol.* **14**, 40–47. (doi:10.1093/beheco/14.1.40)
5. Virecoulon F, Wallet F, Rigot J, Peers M, Mitchell V. 2005 Bacterial flora of the low male genital tract in patients consulting for infertility. *Andrologia* **37**, 160–165. (doi:10.1111/j.1439-0272.2005.00673.x)
6. González-Marín C, Roy R, López-Fernández C, Diez B, Carabaño MJ, Fernández JL, Kjelland ME, Moreno JF, Gosálvez J. 2011 Bacteria in bovine semen can increase sperm DNA fragmentation rates: a kinetic experimental approach. *Anim. Reprod. Sci.* **123**, 139–148. (doi:10.1016/j.anireprosci.2010.11.014)
7. Ravel J *et al*. 2011 Vaginal microbiome of reproductive-age women. *Proc. Natl Acad. Sci. USA* **108**, 4680–4687. (doi:10.1073/pnas.1002611107)
8. Hickey RJ, Zhou X, Pierson JD, Ravel J, Forney LJ. 2012 Understanding vaginal microbiome complexity from an ecological perspective. *Transl. Res.* **160**, 267–282. (doi:10.1016/j.trsl.2012.02.008)
9. Baud D, Pattaroni C, Vulliemoz N, Castella V, Marsland BJ, Stojanov M. 2019 Sperm microbiota and its impact on semen parameters. *Front. Microbiol.* **10**, 234. (doi:10.3389/fmicb.2019.00234)
10. Engelstädter J, Hurst GD. 2009 The ecology and evolution of microbes that manipulate host reproduction. *Annu. Rev. Ecol. Evol. Syst.* **40**, 127–149. (doi:10.1146/annurev.ecolsys.110308.120206)
11. Otti O. 2015 Genitalia-associated microbes in insects. *Insect Sci.* **22**, 325–339. (doi:10.1111/1744-7917.12183)
12. Otti O, Deines P, Hammerschmidt K, Reinhardt K. 2017 Regular wounding in a natural system: bacteria associated with reproductive organs of bedbugs and their quorum sensing abilities. *Front. Immunol.* **8**, 1855. (doi:10.3389/fimmu.2017.01855)
13. Bellinvia S, Johnston PR, Reinhardt K, Otti O. 2020 Bacterial communities of the reproductive organs of virgin and mated common bedbugs, Cimex lectularius. *Ecol. Entomol.* **45**, 142–154. (doi:10.1111/een.12784)
14. Markle JGM *et al*. 2013 Sex differences in the gut microbiome drive hormone-dependent regulation of autoimmunity. *Science* **339**, 1084–1088. (doi:10.1126/science.1233521)
15. Valiente Moro C, Tran FH, Nantenaina Raharimalala F, Ravelonandro P, Mavingui P. 2013 Diversity of culturable bacteria including *Pantoea* in wild mosquito *Aedes albopictus*. *BMC Microbiol.* **13**, 70. (doi:10.1186/1471-2180-13-70)
16. Haro C *et al*. 2016 Intestinal microbiota is influenced by gender and body mass index. *PLoS ONE* **11**, e0154090. (doi:10.1371/journal.pone.0154090)
17. Martínez I *et al*. 2013 Gut microbiome composition is linked to whole grain-induced immunological improvements. *ISME J.* **7**, 269–280. (doi:10.1038/ismej.2012.104)
18. David LA *et al*. 2014 Diet rapidly and reproducibly alters the human gut microbiome. *Nature* **505**, 559–563. (doi:10.1038/nature12820)
19. Bestion E, Jacob S, Zinger L, Di Gesu L, Richard M, White J, Cote J. 2017 Climate warming reduces gut microbiota diversity in a vertebrate ectotherm. *Nat. Ecol. Evol.* **1**, 0161. (doi:10.1038/s41559-017-0161)
20. Thaiss CA *et al*. 2014 Transkingdom control of microbiota diurnal oscillations promotes metabolic homeostasis. *Cell* **159**, 514–529. (doi:10.1016/j.cell.2014.09.048)
21. Nuriel-Ohayon M, Neuman H, Koren O. 2016 Microbial changes during pregnancy, birth, and infancy. *Front. Microbiol.* **7**, 1031. (doi:10.3389/fmicb.2016.01031)
22. Cauci S *et al*. 2002 Prevalence of bacterial vaginosis and vaginal flora changes in peri- and postmenopausal women. *J. Clin. Microbiol.* **40**, 2147–2152. (doi:10.1128/JCM.40.6.2147-2152.2002)
23. White J, Mirleau P, Danchin E, Mulard H, Hatch SA, Heeb P, Wagner RH. 2010 Sexually transmitted bacteria affect female cloacal assemblages in a wild bird. *Ecol. Lett.* **13**, 1515–1524. (doi:10.1111/j.1461-0248.2010.01542.x)
24. Mändar R *et al*. 2015 Complementary seminovaginal microbiome in couples. *Res. Microbiol.* **166**, 440–447. (doi:10.1016/j.resmic.2015.03.009)
25. Gajer P *et al*. 2012 Temporal dynamics of the human vaginal microbiota. *Sci. Transl. Med.* **4**, 132ra52. (doi:10.1126/scitranslmed.3003605)
26. Afzelius BA, Alberti G, Dallai R, Godula J, Witalinski W. 1989 Virus- and *Rickettsia*-infected sperm cells in arthropods. *J. Invertebr. Pathol.* **53**, 365–377. (doi:10.1016/0022-2011(89)90102-X)
27. Lockhart AB, Thrall PH, Antonovics J. 1996 Sexually transmitted diseases in animals: ecological and evolutionary Implications. *Biol. Rev.* **71**, 415–471. (doi:10.1111/j.1469-185X.1996.tb01281.x)
28. Eley A, Hosseinzadeh S, Hakimi H, Geary I, Pacey AA. 2005 Apoptosis of ejaculated human sperm is induced by co-incubation with *Chlamydia trachomatis* lipopolysaccharide. *Hum. Reprod.* **20**, 2601–2607. (doi:10.1093/humrep/dei082)
29. Marius-Jestin V, Menec ML, Thibault E, Mosan JC, L'Hospitalier R, Alexandre M, Coiffard J-M. 1987 Normal phallus flora of the gander. *Zoonoses Public Health* **34**, 67–78. (doi:10.1111/j.1439-0450.1987.tb00371.x)
30. Reinhardt K, Naylor R, Siva-Jothy MT. 2005 Potential sexual transmission of environmental microbes in a traumatically inseminating insect. *Ecol. Entomol.* **30**, 607–611. (doi:10.1111/j.0307-6946.2005.00730.x)
31. Lange R, Reinhardt K, Michiels NK, Anthes N. 2013 Functions, diversity, and evolution of traumatic mating. *Biol. Rev.* **88**, 585–601. (doi:10.1111/brv.12018)
32. Klainer AS, Beisel WR. 1969 Opportunistic infection: a review. *Am. J. Med. Sci.* **258**, 431–456. (doi:10.1097/00000441-196912000-00007)
33. Diemer T, Weidner W, Michelmann HW, Schiefer HG, Rovan E, Mayer F. 1996 Influence of *Escherichia coli* on motility parameters of human spermatozoa in vitro. *Int. J. Androl.* **19**, 271–277. (doi:10.1111/j.1365-2605.1996.tb00475.x)
34. Huwe P, Diemer T, Ludwig M, Liu J, Schiefer HG, Weidner W. 1998 Influence of different uropathogenic microorganisms on human sperm motility parameters in an *in vitro* experiment. *Andrologia* **30**, 55–59. (doi:10.1111/j.1439-0272.1998.tb02827.x)
35. Diemer T, Huwe P, Ludwig M, Schroeder-Printzen I, Michelmann HW, Schiefer HG, Weidner W. 2003 Influence of autogenous leucocytes and *Escherichia coli* on sperm motility parameters *in vitro*. *Andrologia* **35**, 100–105. (doi:10.1046/j.1439-0272.2003.00523.x)
36. Kaur S, Prabha V, Shukla G, Sarwal A. 2010 Interference of human spermatozoal motility by live *Staphylococcus aureus*. *Am. J. Biomed. Sci.* **2**, 91–97. (doi:10.5099/aj100100091)
37. Prabha V, Sandhu R, Kaur S, Kaur K, Sarwal A, Mavuduru RS, Singh SK. 2010 Mechanism of sperm immobilization by *Escherichia coli*. *Adv. Urol.* **2010**, 240268. (doi:10.1155/2010/240268)
38. Otti O, McTighe AP, Reinhardt K. 2013 *In vitro* antimicrobial sperm protection by an ejaculate-like substance. *Funct. Ecol.* **27**, 219–226. (doi:10.1111/1365-2435.12025)
39. Crudgington HS, Siva-Jothy MT. 2000 Genital damage, kicking and early death. *Nature* **407**, 855–856. (doi:10.1038/35038154)
40. Kamimura Y. 2012 Correlated evolutionary changes in *Drosophila* female genitalia reduce the possible infection risk caused by male copulatory wounding. *Behav. Ecol. Sociobiol.* **66**, 1107–1114. (doi:10.1007/s00265-012-1361-0)
41. Astrup BS, Ravn P, Lauritsen J, Thomsen JL. 2012 Nature, frequency and duration of genital lesions after consensual sexual intercourse—implications for legal proceedings. *Forensic Sci. Int.* **219**, 50–56. (doi:10.1016/j.forsciint.2011.11.028)
42. Dethlefsen L, McFall-Ngai M, Relman DA. 2007 An ecological and evolutionary perspective on human–microbe mutualism and disease. *Nature* **449**, 811–818. (doi:10.1038/nature06245)
43. Weiss B, Aksoy S. 2011 Microbiome influences on insect host vector competence. *Trends Parasitol.* **27**, 514–522. (doi:10.1016/j.pt.2011.05.001)
44. Kaltenpoth M, Engl T. 2014 Defensive microbial symbionts in Hymenoptera. *Funct. Ecol.* **28**, 315–327. (doi:10.1111/1365-2435.12089)
45. Rillig MC, Antonovics J, Caruso T, Lehmann A, Powell JR, Veresoglou SD, Verbruggen E. 2015 Interchange of entire communities: microbial community coalescence. *Trends Ecol. Evol.* **30**, 470–476. (doi:10.1016/j.tree.2015.06.004)
46. Carayon J. 1966 Traumatic insemination and the paragenital system. In *Monograph of Cimicidae (Hemiptera–Heteroptera)* (ed. R Usinger), pp. 81–166. College Park, MD: Entomological Society of America.
47. Hase A. 1930 Methoden der Züchtung von Wanzen, Läusen und Flöhen. In *Handbuch der pathogenen*

mikroorganismen (eds W Knolle, R Kraus, P Uhlenhut), pp. 591–616. Jena, Germany: Gustav Fischer.

48. Reinhardt K, Naylor RA, Siva-Jothy MT. 2009 Situation exploitation: higher male mating success when female resistance is reduced by feeding. *Evolution* (*NY*) **63**, 29–39. (doi:10.1111/j.1558-5646.2008.00502.x)
49. Parada AE, Needham DM, Fuhrman JA. 2016 Every base matters: assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. *Environ. Microbiol.* **18**, 1403–1414. (doi:10.1111/1462-2920.13023)
50. Apprill A, McNally S, Parsons R, Weber L. 2015 Minor revision to V4 region SSU rRNA 806R gene primer greatly increases detection of SAR11 bacterioplankton. *Aquat. Microb. Ecol.* **75**, 129–137. (doi:10.3354/ame01753)
51. R Core Team. 2018 *R: a language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing.
52. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. 2016 DADA2: high resolution sample inference from Illumina amplicon data. *Nat. Methods* **13**, 581–583. (doi:10.1016/j.bbamem.2015.02.010.Cationic)
53. Davis NM, Proctor DM, Holmes SP, Relman DA, Callahan BJ. 2018 Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. *Microbiome* **6**, 226. (doi:10.1186/s40168-018-0605-2)
54. Wright ES. 2016 Using DECIPHER v2.0 to analyse big biological sequence data in R. *R J.* **8**, 352–359.
55. Schliep KP. 2011 phangorn: phylogenetic analysis in R. *Bioinformatics* **27**, 592–593. (doi:10.1093/bioinformatics/btq706)
56. Schliep K, Potts AJ, Morrison DA, Grimm GW. 2017 Intertwining phylogenetic trees and networks. *Methods Ecol. Evol.* **8**, 1212–1220. (doi:10.1111/2041-210X.12760)
57. McMurdie PJ, Holmes S. 2013 phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One* **8**, e61465. (doi:10.1371/journal.pone.0061217)
58. Martinez Arbizu P. 2019 pairwiseAdonis: Pairwise multilevel comparison using adonis. See https://github.com/pmartinezarbizu/pairwiseAdonis.
59. Oksanen J *et al.* 2017 vegan: community ecology package. See https://cran.r-project.org/package=vegan.
60. Robinson MD, McCarthy DJ, Smyth GK. 2010 edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* **26**, 139–140. (doi:10.1093/bioinformatics/btp616)
61. McCarthy DJ, Chen Y, Smyth GK. 2012 Differential expression analysis of multifactor RNA-Seq experiments with respect to biological variation. *Nucleic Acids Res.* **40**, 4288–4297. (doi:10.1093/nar/gks042)
62. Caporaso JG, Lauber CL, Walters WA, Berg-lyons D, Lozupone CA, Turnbaugh PJ, Fierer N, Knight R. 2011 Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc. Natl Acad. Sci. USA* **108**, 4516–4522. (doi:10.1073/pnas.1000080107)
63. DeSantis TZ *et al.* 2006 Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl. Environ. Microbiol.* **72**, 5069–5072. (doi:10.1128/AEM.03006-05)
64. Götz S *et al*. 2008 High-throughput functional annotation and data mining with the Blast2GO suite. *Nucleic Acids Res.* **36**, 3420–3435. (doi:10.1093/nar/gkn176)
65. Hosokawa T, Koga R, Kikuchi Y, Meng X-Y, Fukatsu T. 2010 *Wolbachia* as a bacteriocyte-associated nutritional mutualist. *Proc. Natl Acad. Sci. USA* **107**, 769–774. (doi:10.1073/pnas.0911476107)
66. Knights D, Kuczynski J, Charlson ES, Zaneveld J, Mozer MC, Collman RG, Bushman FD, Knight R, Kelley ST. 2011 Bayesian community-wide culture-independent microbial source tracking. *Nat. Methods* **8**, 761–765. (doi:10.1038/nmeth.1650)
67. McMurdie PJ, Paulson JN. 2019 biomformat: An interface package for the BIOM file format. See www.bioconductor.org/packages/release/bioc/html/biomformat.html.
68. Anders S, Huber W. 2010 Differential expression analysis for sequence count data. *Genome Biol.* **11**, R106. (doi:10.1186/gb-2010-11-10-r106)
69. Fountain T, Duvaux L, Horsburgh G, Reinhardt K, Butlin RK. 2014 Human-facilitated metapopulation dynamics in an emerging pest species, *Cimex lectularius*. *Mol. Ecol.* **23**, 1071–1084. (doi:10.1111/mec.12673)
70. Meriweather M, Matthews S, Rio R, Baucom RS. 2013 A 454 survey reveals the community composition and core microbiome of the common bed bug (*Cimex lectularius*) across an urban landscape. *PLoS ONE* **8**, e61465. (doi:10.1371/journal.pone.0061465)
71. Chen C *et al*. 2017 The microbiota continuum along the female reproductive tract and its relation to uterine-related diseases. *Nat. Commun.* **8**, 875. (doi:10.1038/s41467-017-00901-0)
72. Kulkarni S, Heeb P. 2007 Social and sexual behaviours aid transmission of bacteria in birds. *Behav. Processes* **74**, 88–92. (doi:10.1016/j.beproc.2006.10.005)
73. White J, Richard M, Massot M, Meylan S. 2011 Cloacal bacterial diversity increases with multiple mates: evidence of sexual transmission in female common lizards. *PLoS ONE* **6**, e22339. (doi:10.1371/journal.pone.0022339)
74. Bhushan S, Schuppe HC, Tchatalbachev S, Fijak M, Weidner W, Chakraborty T, Meinhardt A. 2009 Testicular innate immune defense against bacteria. *Mol. Cell. Endocrinol.* **306**, 37–44. (doi:10.1016/j.mce.2008.10.017)
75. Gendrin M, Welchman DP, Poidevin M, Hervé M, Lemaitre B. 2009 Long-range activation of systemic immunity through peptidoglycan diffusion in *Drosophila*. *PLoS Pathog.* **5**, e1000694. (doi:10.1371/journal.ppat.1000694)
76. Reinhardt K, Naylor R, Siva-Jothy MT. 2003 Reducing a cost of traumatic insemination: female bedbugs evolve a unique organ. *Proc. R. Soc. Lond. B Biol. Sci.* **270**, 2371–2375. (doi:10.1098/rspb.2003.2515)
77. Schwenke RA, Lazzaro BP, Wolfner MF. 2016 Reproduction–immunity trade-offs in insects. *Annu. Rev. Entomol.* **61**, 239–256. (doi:10.1146/annurev-ento-010715-023924)
78. Siva-Jothy MT, Moret Y, Rolff J. 2005 Insect immunity: an evolutionary ecology perspective. *Adv. Insect Phys.* **32**, 1–48. (doi:10.1016/S0065-2806(05)32001-7)
79. Otti O, Naylor RA, Siva-Jothy MT, Reinhardt K. 2009 Bacteriolytic activity in the ejaculate of an insect. *Am. Nat.* **174**, 292–295. (doi:10.1086/600099)
80. Siva-Jothy MT, Zhong W, Naylor R, Heaton L, Hentley W, Harney E. 2019 Female bed bugs (*Cimex lectularius* L) anticipate the immunological consequences of traumatic insemination via feeding cues. *Proc. Natl. Acad. Sci. USA.* **116**, 14 682–14 687. (doi:10.1073/pnas.1904539116)

SUPPLEMENTARY INFORMATION FOR

Mating changes the genital microbiome in both sexes of the common bedbug *Cimex lectularius* across populations

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INDEX:

SUPPLEMENTARY FIGURES

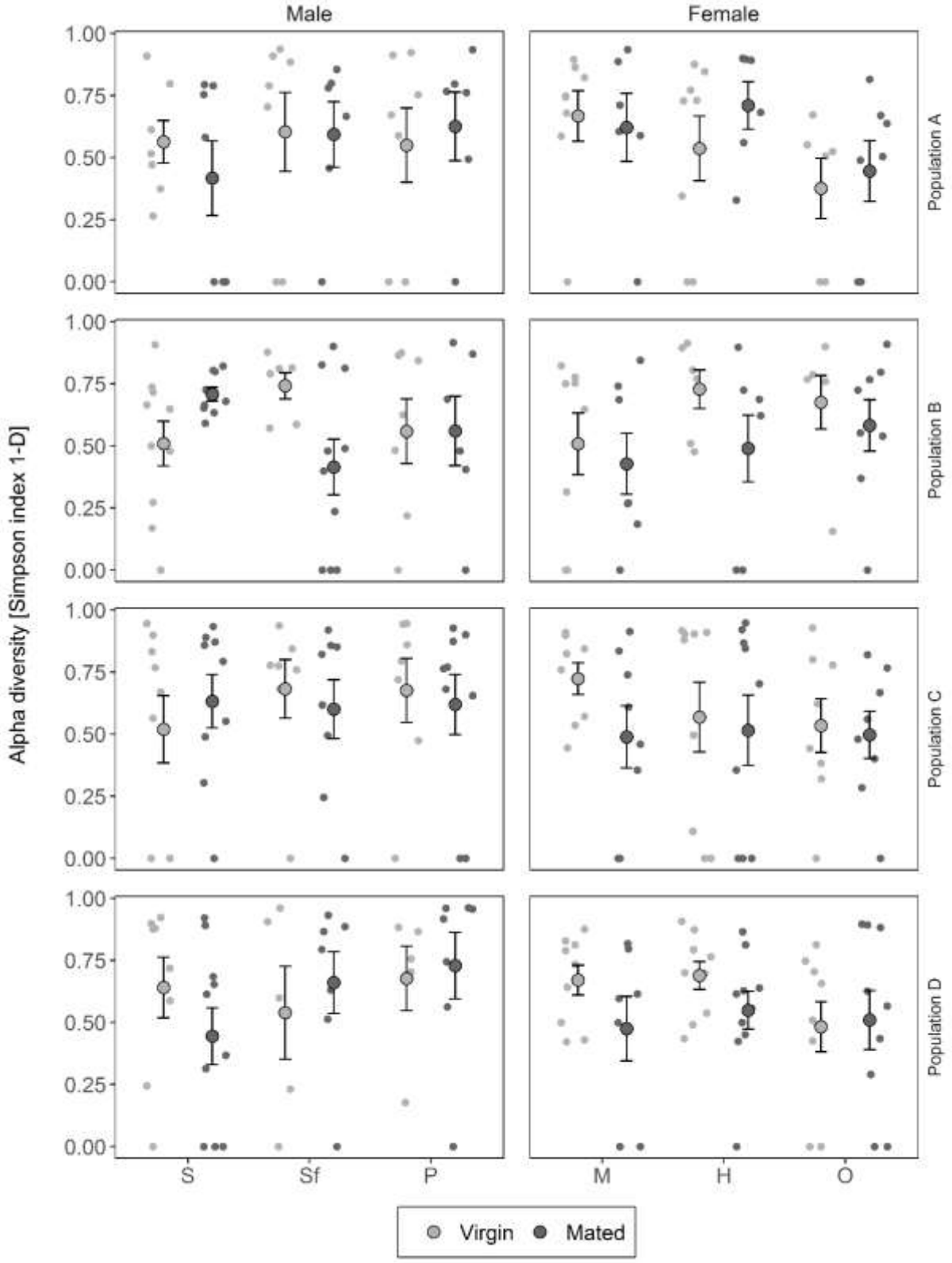

Figure S1 Alpha diversity of each sample in the sperm vesicle (S), the seminal fluid vesicle (Sf), on the paramere (P), in the mesospermalege (M), the haemolymph (H) and the ovary (O). Depicted are means, standard errors of the mean, and all individual data points.

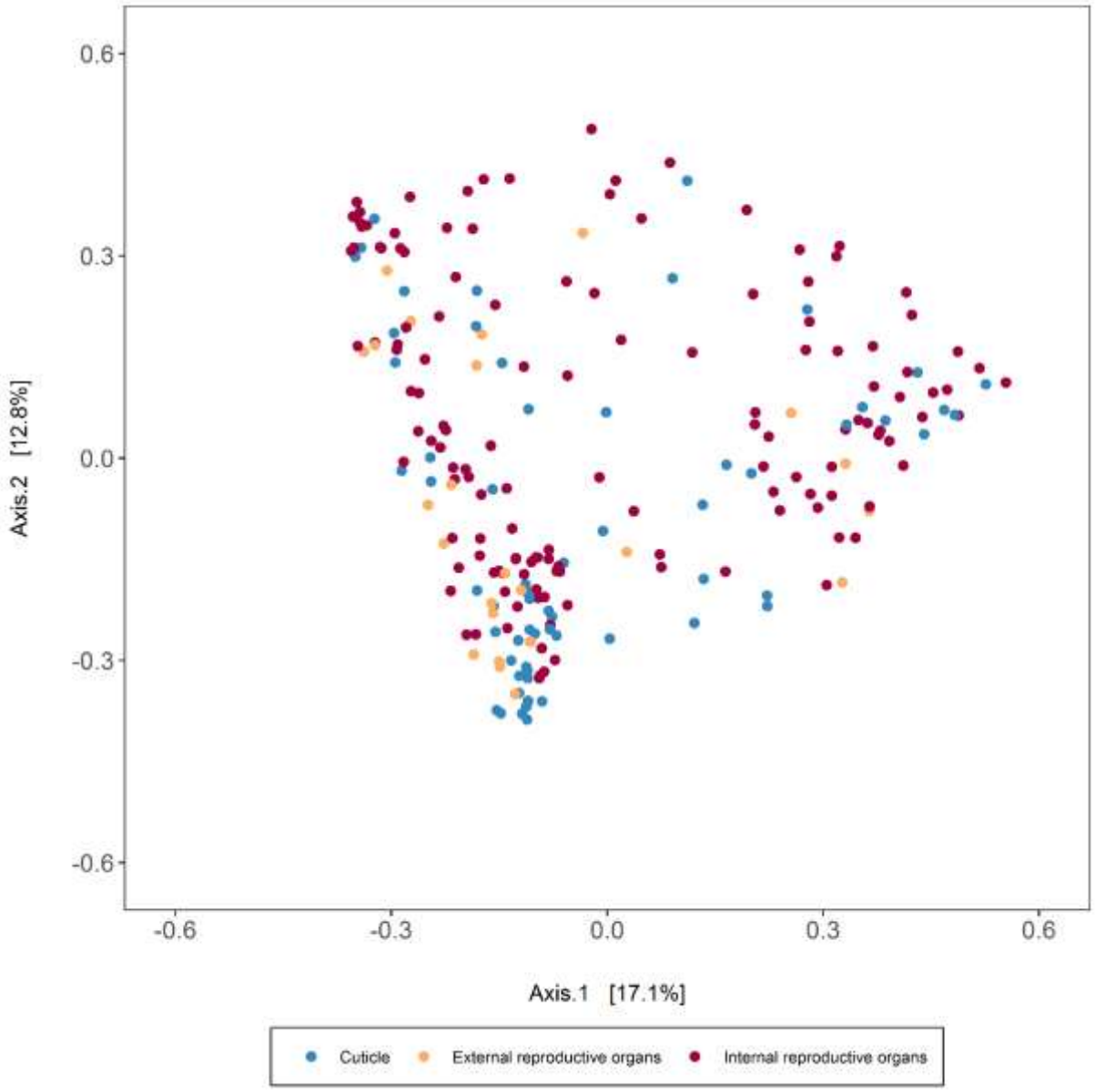

Figure S2 PCoA of microbiomes from cuticle in comparison to the external reproductive organ of males) and internal reproductive organs of both sexes based on Bray-Curtis dissimilarities.

SUPPLEMENTARY TABLES

Table S1 Sample sizes and number of bacterial communities that were successfully sequenced and endured quality filtering for each group of samples. Sampled organs were: cuticle (C), sperm vesicle (S), seminal fluid vesicle (Sf), paramere (P), mesospermalege (M), haemolymph (H) and ovary (O).

Population	Sex	Organ	Mating status	N total	N sequenced
A	Male	C	Virgin	10	7
A	Male	C	Mated	10	6
A	Male	S	Virgin	10	7
A	Male	S	Mated	11	7
A	Male	Sf	Virgin	10	7
A	Male	Sf	Mated	10	6
A	Male	P	Virgin	10	7
A	Male	P	Mated	10	6
A	Female	C	Virgin	11	7
A	Female	C	Mated	11	8
A	Female	M	Virgin	10	8
A	Female	M	Mated	10	6
A	Female	H	Virgin	11	8
A	Female	H	Mated	10	6
A	Female	O	Virgin	10	6
A	Female	O	Mated	9	7
B	Male	C	Virgin	10	9
B	Male	C	Mated	10	8
B	Male	S	Virgin	10	10
B	Male	S	Mated	10	9
B	Male	Sf	Virgin	10	6
B	Male	Sf	Mated	10	10
B	Male	P	Virgin	12	7
B	Male	P	Mated	8	6
B	Female	C	Virgin	10	10
B	Female	C	Mated	10	8
B	Female	M	Virgin	10	8
B	Female	M	Mated	10	7
B	Female	H	Virgin	10	6
B	Female	H	Mated	10	8
B	Female	O	Virgin	10	6
B	Female	O	Mated	10	8
C	Male	C	Virgin	10	9
C	Male	C	Mated	10	7
C	Male	S	Virgin	10	9
C	Male	S	Mated	10	9
C	Male	Sf	Virgin	10	7
C	Male	Sf	Mated	10	8

C	Male	P	Virgin	10	7
C	Male	P	Mated	10	9
C	Female	C	Virgin	10	9
C	Female	C	Mated	10	8
C	Female	M	Virgin	10	8
C	Female	M	Mated	10	8
C	Female	H	Virgin	10	9
C	Female	H	Mated	10	9
C	Female	O	Virgin	10	8
C	Female	O	Mated	10	8
D	Male	C	Virgin	10	8
D	Male	C	Mated	10	8
D	Male	S	Virgin	10	8
D	Male	S	Mated	10	10
D	Male	Sf	Virgin	10	5
D	Male	Sf	Mated	10	7
D	Male	P	Virgin	10	5
D	Male	P	Mated	10	7
D	Female	C	Virgin	10	8
D	Female	C	Mated	10	10
D	Female	M	Virgin	10	9
D	Female	M	Mated	10	7
D	Female	H	Virgin	10	9
D	Female	H	Mated	10	10
D	Female	O	Virgin	10	9
D	Female	O	Mated	10	9

Table S2 Contaminants found with the *decontam* package (Davis et al. 2018) based on prevalences in controls. Given are the contaminants and their prevalences within each type of control, i.e. control for dissection, control for DNA isolation, control for target PCR, and control for indexing PCR.

Contaminant	Dissection (N=1)	DNA isolation (N=1)	Target PCR (N=16)	Indexing PCR (N=13)
Unclassified Enterobacteriaceae	1	0	0.81	0.62
***Wolbachia* sp.**	0	0	0.75	0.77
***Bradyrhizobium* sp.**	0	0	0.44	0.08
***Stenotrophomonas* sp.**	0	0	0.25	0.08
***Myroides* sp.**	0	0	0.13	0.00
***Methylobacterium* sp.**	0	0	0.38	0.08
***Pseudomonas* sp.**	0	0	0.13	0.00
***Methylobacterium* sp.**	0	0	0.19	0.08
***Enterococcus*sp.**	0	0	0.13	0.00
***Sphingomonas* sp.**	0	0	0.25	0.00
Unclassified Streptophyta	0	0	0.13	0.00
***Curvibacter* sp.**	0	0	0.25	0.08
***Rickettsia* sp.**	0	0	0.06	0.08
***Sphingomonas* sp.**	0	0	0.19	0.00
***Curvibacter* sp.**	0	0	0.19	0.00
Unclassified Streptophyta	0	0	0.06	0.00
Unclassified Erythrobacteraceae	0	0	0.13	0.08
***Janthinobacterium* sp.**	0	0	0.13	0.00
Unclassified Erythrobacteraceae	0	0	0.13	0.00
Unclassified Lactobacillales	0	0	0.06	0.00
***Janthinobacterium* sp.**	0	0	0.13	0.00
***Pseudomonas* sp.**	0	0	0.06	0.08
Unclassified Lactobacillales	0	0	0.06	0.00
***Sphingomonas* sp.**	0	0	0.13	0.00
***Methylobacterium* sp.**	0	0	0.06	0.00
***Methylobacterium* sp.**	0	0	0.06	0.00
***Novosphingobium* sp.**	0	0	0.13	0.08
***Paracoccus* sp.**	0	0	0.13	0.00
***Renibacterium* sp.**	0	0	0.13	0.00
Unclassified Pseudomonadaceae	0	0	0.06	0.00
Unclassified Rhodospirillaceae	0	0	0.06	0.00
Bacillus thermoalkalophilus	0	0	0.06	0.00
***Prevotella* sp.**	0	0	0.13	0.00
Pseudoxanthomonas taiwanensis	0	0	0.13	0.00

Unclassified Rhodospirillaceae	0	0	0.06	0.00
***Rickettsia* sp.**	0	0	0.06	0.08
Unclassified Pseudomonadaceae	0	0	0.06	0.00
***Thermomonas* sp.**	0	0	0.06	0.00
Unclassified Erythrobacteraceae	0	0	0.06	0.00
Unclassified Rhodospirillaceae	0	0	0.06	0.00
Bacillus thermoalkalophilus	0	0	0.06	0.00
***Thermus* sp.**	0	0	0.06	0.00
***Rubrivivax* sp.**	0	0	0.06	0.00
***Bosea* genosp.**	0	0	0.06	0.00
Unclassified Pseudomonadaceae	0	0	0.06	0.00
Unclassified Phyllobacteriaceae	0	0	0.06	0.00
***Sphingobacterium* sp.**	0	0	0.06	0.00
Unclassified Comamonadaceae	0	0	0.06	0.00
***Marinobacter* sp.**	0	0	0.06	0.00
***Bosea* genosp.**	0	0	0.06	0.00
***Marinobacter* sp.**	0	0	0.06	0.00
***Nevskia* sp.**	0	0	0.06	0.00

Table S6 SVs potentially transmitted from males to females. Given are prevalence and relative abundance of all SVs that were found in mated females and in virgin males but not in virgin females.

ID	SV	Prevalence	Min. relative abundance	Max. relative abundance
Population A		*(N=19)*		
SV30	Unclassified Aeromonas	6	0.0009	0.0065
SV44	*Staphylococcus* sp.	2	0.0001	0.0003
SV51	*Bradyrhizobium* sp.	6	0.0006	0.0091
SV66	*Caulobacter* sp.	7	0.0001	0.0045
SV68	*Alcanivorax* sp.	4	0.0004	0.0024
SV69	*Brevibacterium* sp.	1	0.0004	0.0004
SV73	*Staphylococcus* sp.	2	0.0010	0.0018
SV77	*Alcanivorax* sp.	4	0.0003	0.0017
SV83	*Staphylococcus* sp.	1	0.0010	0.0010
SV86	Unclassified Comamonadaceae	3	0.0004	0.0020
SV94	*Bradyrhizobium* sp.	3	0.0003	0.0020
SV97	*Cutibacterium acnes*	5	0.0001	0.0029
SV98	*Acinetobacter* sp.	2	0.0003	0.0016
SV99	*Bradyrhizobium* sp.	4	0.0005	0.0009
SV100	Unclassified Gammaproteobacteria	1	0.0002	0.0002
SV106	*Corynebacterium* sp.	5	0.0002	0.0023
SV109	*Paracoccus* sp.	1	0.0014	0.0014
SV111	*Pseudomonas* sp.	3	0.0005	0.0008
SV114	*Cloacibacterium* sp.	2	0.0000	0.0007
SV115	Unclassified Alcaligenaceae	1	0.0003	0.0003
SV116	*Acinetobacter* sp.	5	0.0001	0.0018
SV117	Unclassified Lactobacillaceae	1	0.0004	0.0004
SV118	*Cloacibacterium* sp.	2	0.0002	0.0005
SV120	*Lactobacillus* sp.	1	0.0002	0.0002
SV122	Unclassified Gammaproteobacteria	2	0.0003	0.0018
SV124	*Pseudomonas* sp.	2	0.0006	0.0006
SV125	*Acinetobacter lwoffii*	4	0.0003	0.0021
SV126	*Comamonas* sp.	3	0.0004	0.0015
SV135	Unclassified Comamonadaceae	2	0.0004	0.0047
Population B		*(N=22)*		
SV135	Unclassified Comamonadaceae	1	0.0030	0.0030
Population C		*(N=25)*		
SV111	*Pseudomonas* sp.	4	0.0002	0.0063
SV124	*Pseudomonas* sp.	1	0.0004	0.0004

SV140	Unclassified Rhodobacteraceae	1	0.0001	0.0001
Population D		*(N=26)*		
SV33	*Stenotrophomonas* sp.	1	0.0003	0.0003
SV117	Unclassified Lactobacillaceae	3	0.0001	0.0023
SV118	*Cloacibacterium* sp.	2	0.0001	0.0009
SV138	*Corynebacterium* sp.	1	0.0003	0.0003

Table S7 SVs potentially transmitted from females to males. Given are prevalence and relative abundance of all SVs that were found in mated males and in virgin females but not in virgin males.

ID	SV	Prevalence	Min. relative abundance	Max. relative abundance
Population A			*(N=19)*	
-	-	-	-	-
Population B			*(N=25)*	
SV90	*Bradyrhizobium* sp.	2	0.0011	0.0033
SV93	*Halomonas* sp.	2	0.0015	0.0024
SV122	Unclassified Gammaproteobacteria	2	0.0004	0.0008
SV134	Unclassified Gammaproteobacteria	2	0.0004	0.0009
Population C			*(N=26)*	
-	-	-	-	-
Population D			*(N=24)*	
SV44	*Staphylococcus* sp.	1	0.0016	0.0016
SV108	*Lactobacillus* sp.	1	0.0002	0.0002

The separate Excel files for Table S3-S5 containing the “raw” data after the pre-processing steps, such as denoising and filtering, can be accessed under:

https://royalsocietypublishing.org/doi/suppl/10.1098/rspb.2020.0302

SUPPLEMENTARY REFERENCES

Davis, N. M., Proctor, D. M., Holmes, S. P., Relman, D. A., & Callahan, B. J. (2018). Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. *Microbiome*, *6*(1), 226. https://doi.org/10.1186/s40168-018-0605-2

6.3 Manuscript 3

Title: Female immunity in response to sexually transmitted opportunistic bacteria in the common bedbug *Cimex lectularius*

Authors: Sara Bellinvia, Andrea Spachtholz, Ina Borgwardt, Bastian Schauer, Oliver Otti

Journal and status: *Journal of Insect Physiology, 123*, 104048

Own contribution: concept and study design: 75%, data acquisition: 60%, data analysis and figures: 90%, interpretation of results: 90%, manuscript writing: 90%

O.O. and S.B. conceived the idea and designed the experiments. S.B., A.S., I.B., and B.S. carried out the experiments. S.B. and O.O. performed the statistical analysis. S.B. and O.O. interpreted the results and wrote the manuscript.

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Female immunity in response to sexually transmitted opportunistic bacteria in the common bedbug *Cimex lectularius*

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Reproductive immunity

ABSTRACT

Besides typical sexually transmitted microbes, even environmental, opportunistic microbes have been found in copulatory organs of insects and even humans. To date, only one study has experimentally investigated the sexual transmission of opportunistic microbes from male to female insects, whereas nothing is known about the transmission from females to males. Even if opportunistic microbes do not cause infection upon transmission, they might eventually become harmful if they multiply inside the female. While the immune system of females is often assumed to target sexually transmitted microbes, most studies ignore the role of mating-associated opportunistic microbes. Variation in immunity between populations has been linked to parasite or bacteria prevalence but no study has ever addressed between-population differences in immune responses to sexually transmitted opportunistic microbes. We here show that bacteria applied to the copulatory organs of common bedbugs, *Cimex lectularius*, are sexually transmitted to the opposite sex at a high rate, including the transmission from female to male. Bacterial growth in the female sperm-receiving organ was inhibited over the first hours after introduction, but after this initial inhibition bacterial numbers increased, suggesting a shift of investment from immune defence towards reproduction. However, 24 h after the injection of bacteria, male components, or saline as a control, the sperm-receiving organ showed lysozyme-like activity and inhibited the growth of Gram-negative and Gram-positive bacteria *in vitro*, potentially to mop up the remaining bacteria. Contrasting our prediction, neither bacterial growth nor immune responses differed between populations. Future studies should link transmission dynamics, immune responses and fitness effects in both sexes. Experimental manipulation of environmental bacteria could be used to investigate how transmission frequency and toxicity of sexually transmitted opportunistic microbes shapes bacteria clearance and immune responses across populations.

1. Introduction

Microbes are known to be able to colonise a large range of environments. In animals, bacteria are associated with host surfaces, host cells, or specific organ systems, such as the gut (Goodrich et al., 2016; Huttenhower et al., 2012; Kostic et al., 2013). Even the reproductive organs often harbour several different microbe species. Microbes live in female (Hickey et al., 2012; Hirsh, 1999; Hupton et al., 2003; Ravel et al., 2011; White et al., 2011) and male reproductive organs and semen (González-Marín et al., 2011; Hupton et al., 2003; Lombardo and Thorpe, 2000; Skau and Folstad, 2003; Virecoulon et al., 2005) of vertebrates, including humans. Because animals are constantly exposed to them, microbes play a crucial role in the biology and life cycle of many species, affecting development, immunity, reproduction and other life history traits (Dale and Moran, 2006). Symbiotic associations between microbes and their hosts range from mutualistic to parasitic (Dale and Moran, 2006). While mutualistic interactions are mainly based on nutrition (Dale and Moran, 2006), some microbes defend their host against invaders, i.e. other microbes (Dethlefsen et al., 2007; Kaltenpoth and Engl, 2014; Rillig et al., 2015; Weiss and Aksoy, 2011) or eukaryotic parasites (Oliver et al., 2003).

In contrast, parasitic symbionts impact the host negatively, for example by causing cuticular damage, reducing reproductive success, or killing the host (Knell and Webberley, 2004). For the horizontal transmission between individuals of the same generation (Bright and Bulgheresi, 2010), various sites of potential ingression exist, e.g. the cuticle, the digestive and the respiratory tract (Boucias and Pendland, 2012). One very common horizontal transmission mode in vertebrates as well as in invertebrates is sexual transmission (Knell and Webberley, 2004).

Sexually transmitted microbes are well studied in humans (Lockhart et al., 1996). While the majority of sexually transmitted microbes in

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vertebrates are viruses or bacteria, nearly all known sexually transmitted microbes in invertebrates are multicellular organisms (Knell and Webberley, 2004). Sexually transmitted microbes mostly affect fertility and fecundity but can also reduce lifespan (Knell and Webberley, 2004). One of the few studies on the sexual transmission of bacteria in insects revealed that *Serratia marcescens* can be transmitted from *Drosophila* males to females (Miest and Bloch-Qazi, 2008). Two other metagenomic studies found potential evidence for a transmission of bacteria from male to female and vice versa in bedbugs (Bellinvia et al., 2020a, 2020b). Apart from these three studies, we have little knowledge about the sexual transmission of bacteria in insects.

Not only typical sexually transmitted microbes are transmitted during mating, also opportunistic microbes that are associated with the genitalia (Bellinvia et al., 2020a, 2020b; Marius-Jestin et al., 1987; Otti et al., 2017; Reinhardt et al., 2005) might be transmitted via genital openings and copulatory wounds, which frequently occur in insects (Lange et al., 2013). Once transferred to the female genital tract, sperm will be exposed to a rich microbial flora (Hirsh, 1999; Virecoulon et al., 2005). In humans, several bacterial species have been shown to decrease sperm motility and lead to agglutination of spermatozoa (Diemer et al., 2003, 1996; Huwe et al., 1998; Kaur et al., 2010; Prabha et al., 2010) and environmental bacteria can harm the sperm of insects (Otti et al., 2013).

As the reproductive success of both sexes depends on vital sperm, males and females might be subject to a considerable cost of reproduction, even if opportunistic microbes do not always cause an infection. Animals should, therefore, invest in reducing or regulating the number of sexually transmitted bacteria to prevent uncontrolled growth. The ejaculate of males contains antimicrobial peptides (AMPs) (Lung et al., 2001; Poiani, 2006) or has lysozyme-like activity (LLA) (Otti et al., 2009) that can protect spermatozoa from bacterial attack (Otti et al., 2013) and AMPs have been found in the female reproductive tract of insects (Peng et al., 2005) and even humans (Quayle et al., 1998; Valore et al., 1998). LLA is produced in anticipation of mating in bedbug females (Siva-Jothy et al., 2019) and AMPs are expressed upon deposition of bacteria on the genital plate of *Drosophila* males (Gendrin et al., 2009). Probably also in response to genitalia-associated microbes, female bedbugs have evolved a new paragenital sperm-receiving immune organ, the mesospermalege (Reinhardt et al., 2003). This organ is filled with haemocytes (Carayon, 1966) that can phagocytose bacteria (Siva-Jothy et al., 2005). Although these antimicrobial mechanisms are widespread among insects, we have little knowledge of how they interact in fighting invading microbes.

Immune responses cost resources (Sheldon and Verhulst, 1996; Zuk and Stoehr, 2002), reduce reproduction (Rigby and Jokela, 2000) and offspring quality (Ilmonen et al., 2000). Differences in transmission risk and bacterial toxicity across populations should, therefore, be reflected in the variation in immune defence between populations. Bumblebees vary in the upregulation of an AMP in response to a gut parasite (Brunner et al., 2013) and freshwater shrimps differ in prophenoloxidase activity (Cornet et al., 2009), both potentially due to variation in parasite prevalence across populations. Unfortunately, we lack knowledge about between-population differences in counter adaptations to bacteria invading the reproductive organs.

We predict that sexual transmission of bacteria is relatively common in bedbugs. As a consequence, females should control invading bacteria with haemocytes, AMPs, and/or LLA. Since the transmission frequency might vary across populations, we expect differences in the strength of immune responses. To test our predictions, we use GFP-labelled bacteria to investigate the frequency of sexual transmission of bacteria in the common bedbug *Cimex lectularius*. We further assess the bacterial growth within the female sperm-receiving organ and test the effect of invading bacteria on two immune traits in four different populations.

2. Material and methods

2.1. Bedbug system and culture

Common bedbugs (*Cimex lectularius* L.) have been used as a model system for examining consequences of sexual selection and reproductive physiology (Reinhardt et al., 2003; Stutt and Siva-Jothy, 2001). Males transfer their ejaculates to females by traumatic insemination. Females have evolved a paragenital organ consisting of a groove in the cuticle, hereafter called “copulatory groove” (ectospermalege), that guides the male copulatory organ (paramere) to the female subcuticular, paragenital sperm-receiving organ (mesospermalege) (Stutt and Siva-Jothy, 2001). Approximately four hours after insemination, the transferred sperm leave the female sperm-receiving immune organ and travel through the haemolymph to the sperm storage organs (Carayon, 1966).

All bedbugs were maintained in a climate chamber at 26 ± 1 °C, 70% relative humidity, and a light cycle of 12L:12D and fed weekly using the protocol of Hase (Hase, 1930). Individuals originated from four large stock populations (> 1000 individuals) of different locations (arbitrarily called *A, B, C, D*) maintained in the laboratory for different amounts of time. *A* was collected in London in 2006, *B* in Nairobi, Kenya in 2008 and *C* in Watamu, Kenya in 2010. *D* is a long-term lab stock (> 50 years) obtained from the London School of Hygiene and Tropical Medicine over 20 years ago. All virgin individuals were kept in sex-specific groups in 60 ml plastic pots containing a folded piece of filter paper.

2.2. Controlled transmission of bacteria

2.2.1. Sample preparation and measurements

For the transmission experiments we used population *D* since the physical mechanism of transmission unlikely relies on the variation in immune responses between populations. We examined how often environmental bacteria are sexually transmitted from bedbug males to females and vice versa as indicated by Bellinvia et al. (2020a, 2020b). We investigated the transmission i) from the male copulatory organ to the female copulatory groove, ii) from the female copulatory groove to the male copulatory organ, iii) from the male copulatory organ to the female sperm-receiving organ and iv) from the female copulatory groove to the female sperm-receiving organ due to mating. Males were fed twice before the application of bacteria and mating. Females were fed three times, with the last feeding on the day of mating and dissection because fully fed females cannot resist mating (Reinhardt et al., 2009). Bacteria were applied to the male copulatory organ (transmission experiments i and iii) or the female copulatory groove (transmission experiments ii and iv) by gently touching the organs with forceps dipped in bacteria. For the transmission experiment iv, the male was left untreated. The treated individual was immediately transferred to a petri dish (55 mm) containing filter paper and an individual of the opposite sex. After a mating of 60 s, both sexes were separated with forceps. Pictures of the respective part or organ were taken with a camera (DFC 450C, Leica Microsystems, Germany) attached to a fluorescence microscope (DM 2000 LED, Leica Microsystems, Germany) under UV light (pE-300, CoolLED Limited, Andover, UK). To make its contents visible, the female sperm-receiving organ was dissected with surface-sterilised forceps and homogenised on the microscope slide. Each type of transmission was assessed in 30 individuals.

2.2.2. Bacteria culture

GFP-labelled bacteria are suitable for investigating sexual transmission in bedbugs since the female sperm-receiving organ does not harbour fluorescent bacteria (see *Supplementary methods*). We decided to use *Asaia* sp. as a representation of OM that do not necessarily cause an infection but could eventually become pathogenic when the host immune system is disturbed (Klainer and Beisel, 1969) because *Asaia*

sp. does not occur in the reproductive organs of virgin bedbugs (Bellinvia et al., 2020a, 2020b) but can colonise the male reproductive tract of another blood-feeding insect (Favia et al., 2007). To obtain the bacteria for transmission experiments, we inoculated glycerol agar plates (2% agar, 1% yeast extract, 2.25% glycerol) with GFP-labelled *Asaia* sp. (Favia et al., 2007) and incubated the plates at 30 °C for 48 h. We picked an individual colony and inoculated 5 ml of glycerol medium (1% yeast extract, 2.25% glycerol) in a 15 ml Falcon tube. The tubes were incubated in a shaking incubator at 30 °C and 200 rpm for 24 h. To produce a bacteria suspension, the incubated bacteria culture (overnight culture) (23900 ± 17184 colony forming units (CFUs) per µl, mean ± SD; see *Supplementary methods*), was centrifuged for 5 min at 2350 g. The bacteria pellet was transferred to a Petri dish and left to dry for 24 h at 30 °C to improve the adhesion of the bacteria to the male copulatory organ and the female copulatory groove, especially for the transmission tests between male copulatory organ and the female copulatory groove. For the transmission from the male copulatory organ and the female copulatory groove to the female sperm-receiving organ, we mixed the dried bacteria with fresh overnight culture (approximately 1000:1 CFUs, dried:fresh) to improve the contrast under the fluorescence microscope against the auto-fluorescence of the female sperm-receiving organ.

2.2.3. Data analysis

We used descriptive tools to analyse the different transmission routes and the location of transmitted bacteria. First, we designed templates of the female copulatory groove and of the male copulatory organ in PowerPoint (Microsoft, version 2016). We transferred the location of bacteria found on the microscope pictures for each individual to one template using natural landmarks, such as intersegmental membranes and the point where the male copulatory organ and the abdomen meet and superimposed all templates with an opacity of 90% to obtain one picture including all individuals. Like this we were able to illustrate the number of bacteria in a particular location on the male and female reproductive organs, i.e. the darker a certain area is the more bacteria were transferred during mating to this location on the reproductive organ.

2.3. Bacterial growth

2.3.1. Sample preparation and measurements

To examine how fast mating-associated bacteria are eliminated by the female immune system, we surface-sterilised the copulatory grooves of females from four different populations (N = 240) with 70% ethanol, which usually all females survive (Reinhardt et al., 2003). With a glass capillary (GB1000F-10, Science Products GmbH, Hofheim, Germany) pulled to a fine point we injected 0.5 µl bacteria (for bacteria preparation see following section) into the female sperm-receiving organ. Females were randomly chosen for dissection after four time points: 5 min, 1 h, 3 h, or 6 h (N = 15 females per population and time point). The sperm-receiving organs were dissected and transferred to a 0.5 ml Eppendorf tube containing 100 µl sterile PBS. For each dissection step we used different forceps to prevent contamination from the cuticle. After each individual, forceps were rinsed with 70% ethanol and flame-sterilised. We homogenised the organs using forceps and pestles made from melted 200 µl pipette tips. Samples were vortexed for 5 s and plated out on a glycerol agar plate using sterile glass beads. After incubating the plates for 48 h at 30 °C, we used a 395 nm UV LED torchlight (ePathChina Ltd, Kowloon, Hong Kong) to verify and identify fluorescent colonies. Plates were photographed with a Gel iX Imager (software: INTAS GDS, INTAS Science Imaging Instruments GmbH, Göttingen, Germany) and CFUs were counted with OpenCFU (version 3.8-BETA) (Geissmann, 2013).

Next, we analysed whether bacterial growth interacts with sperm. We injected 83 females with either 0.5 µl bacteria or a combination of 0.25 µl bacteria and 0.25 µl sperm and followed the procedure described above. Both solutions had the same bacteria concentration. To obtain sperm, we dissected males and transferred both sperm vesicles, the containers in which sperm are stored before copulation, into 10 µl sterile PBS. We ruptured the sperm vesicles and mixed sperm and PBS with forceps. We only injected females from population *D*, because bacterial growth did not differ between populations (ANOVA: interaction time point x population: $F_{9,224} = 1.381$, $p = 0.20$). Females were dissected after 5 min, 6 h, or 24 h (N = 14 per time point and injection type, except for bacteria-injected females at 24 h: N = 13, one female died) because we were interested in the growth rate after an initial plateau phase observed in the previous experiment.

Since it is more important for the host to stop bacterial growth if the bacterium has higher costs, for instance reduced fitness due to spermicidal activity, we tested how *Asaia* sp. affects sperm viability. We dissected 14 males per treatment combination from each population (N = 224) in random order and tested the decline in fluorescence of sperm stained with SYBR green live stain from the Live/Dead Sperm Viability Kit (L7011, Invitrogen, Carlsbad, USA) using a microplate reader (Synergy HT, BioTek Instruments, Winooski, VT, USA) (for the development of this protocol see *Supplementary methods*). We exposed stained sperm without or with seminal fluid to bacteria and compared both organ combinations to their respective control without bacteria. For this, we transferred both sperm vesicles or both sperm vesicles and both seminal fluid vesicles into a 0.5 ml Eppendorf tube containing 50 µl sterile PBS stored on ice. We ruptured the vesicles with forceps, mixed the solution using a pipette and transferred it to a FLUOTRAC 384 well plate (781076, Greiner, Frickenhausen, Germany). We then added 20 µl of SYBR green (first diluted 1:50 in DMSO and then 1:40 in sterile PBS) and incubated the plate in the dark at room temperature for 10 min. Subsequently, we added 10 µl bacteria solution to one half of the wells and 10 µl sterile PBS to the other half. The fluorescence of the bacteria and PBS, we calibrated by measuring 16 replicates of 10 µl of bacteria solution in 70 µl sterile PBS and 80 µl PBS, respectively. Fluorescence was measured every 5 min for 30 min in a microplate reader with an excitation at 485 nm, an emission at 528 and a gain of 75. The plate was shaken for 3 s before each measurement. We subtracted the mean of all measurements for bacteria or PBS controls at a given time point from the fluorescence of the samples.

2.3.2. Bacteria culture

Overnight cultures of *Asaia* sp. were prepared as for the transmission experiments and diluted to an $OD_{600} = 0.1$. To stop bacteria from growing further, we centrifuged the overnight culture for 5 min at 2350 g and replaced the supernatant with sterile PBS. The resulting solution was diluted 1:100 (221 ± 85 CFUs per 0.5 µl, mean ± SD; see *Supplementary methods*) or 1:50 (277 ± 127 CFUs per 0.25 µl) in case of additional injection of sperm to keep the injected volume constant. To determine the spermicidal potential of *Asaia* sp., we used the same procedure described above. After centrifugation, the bacteria-PBS solution was diluted 1:20, resulting in 1195 ± 859 CFUs per µl.

2.3.3. Data analysis

All statistical analyses were performed in R (version 3.5.1, R Core Team, 2018). The effect of time on the number of CFUs in the female sperm-receiving organ was evaluated with a generalised linear model (GLM) with quasi-Poisson error structure and the fixed effects population, time, and their interaction term. For the long-term experiment, we applied a GLM based on the fixed effects sperm presence, time, and their interaction term. We analysed the effect of bacteria on the number of viable sperm represented by fluorescence fitting a GLM with population, bacteria, and time as fixed effects including all interaction terms. If time was significant, we conducted pairwise comparisons (Tukey) and adjusted p-values after Benjamini-Hochberg (BH) (Benjamini and Hochberg, 1995). If residuals were not normally distributed, we used Box-Cox transformation after adding 0.00001 to the raw variables (long-term experiment: number of CFUs, sperm viability experiment:

fluorescence) to obtain positive values for all our fitted models. We only report significant interaction terms in the main text (see Tab. S1 for statistical details).

2.4. Female reproductive immunity in response to mating-associated substances

2.4.1. Sample preparation and measurements

We investigated immunity in response to mating-associated substances of 15 females per population and treatment (N = 360, including controls) that had been fed once, one week before treatment. We prepared the following five treatments: injection control (I), sperm (S), seminal fluid (Sf), sperm and seminal fluid (S + Sf), and bacteria (B). A control group of 15 females per population was injected with 0.5 µl sterile PBS. For treatments S, Sf, and S + Sf, individual males from the same population as the females were dissected to extract their reproductive organs (N = 180). We collected either the pair of sperm vesicles, the pair of seminal fluid vesicles, or both the pair of sperm vesicles and the pair of seminal fluid vesicles. Using forceps, the organs were gently homogenised in 10 µl sterile PBS. Bacteria were harvested with cuticle washes by vortexing 5 males and 5 females from a given population in 5 ml sterile PBS for 15 min after chilling on ice for 15 min. The resulting bacteria solution contained 64 ± 11 CFUs per µl (mean ± SD; see *Supplementary methods*). Sterilised glass capillaries pulled to a fine point were used to inject 0.5 µl of a given treatment solution into the sperm-receiving organ of females that had been anaesthetised on ice. 24 h after the injection, we dissected the sperm-receiving organs and transferred them to 30 µl sterile PBS in a 1.5 ml Eppendorf tube, which we put into liquid nitrogen until further processing for the lysozyme and the AMP assay (see *Immunoassays*). We used the same protocol to investigate if the injection of a liquid differed from the effect of wounding by the glass capillary. Therefore, we measured LLA in untreated virgin females (Untreated), females pricked with a glass capillary (Prick), and females injected with 0.5 µl sterile PBS (Injection) (15 females per population and treatment, N = 180).

2.4.2. Immunoassays

Lysozyme assay. We used the lysozyme assay described in Otti et al. (Otti et al., 2013) to measure lysozyme-like activity (LLA) in the sperm-receiving organs of the injected females. The frozen organs were homogenised with pestles made from melted 200 µl pipette tips and vortexed for 10 s. Two µl of the tissue mixture were then transferred to a 5 ml agar plate containing lyophilised *Micrococcus lysodeikticus* (ATCC No. 4698, Sigma-Aldrich, Hamburg, Germany). All plates were incubated at 30° C for 48 h and photographed. The diameter of the clearance zone was measured twice using ImageJ (version 1.51 k, Schneider, Rasband and Eliceiri, 2012) and converted into units of lysozyme, using a standard curve (Fig. S3) to make LLA comparable across studies. The measurements were conducted blind with regard to population and treatment.

2.4.2.1. AMP assay. We used the liquid growth inhibition assay described in Otti et al. (2013) to measure antimicrobial peptide (AMP) activity against the Gram-positive bacterium *Arthrobacter globiformis* and the Gram-negative bacterium *Escherichia coli*. The homogenised tissue mixture from the lysozyme assay was centrifuged for 5 min at 160 g. Ten µl of the supernatant were transferred to a 96-well microplate containing 30 µl of *E. coli* solution or *A. globiformis* solution, respectively. Both solutions had a starting OD_{600} = 0.5. Growth controls contained 30 µl of the given bacteria solution and 10 µl sterile PBS. Contamination controls contained 40 µl of LB medium and none of them showed any sign of growth. OD_{600} was determined in a microplate reader at 26 °C for 5 h, every 30 min. Before each measurement, the plate was shaken for 3 s. Maxima of the growth curves for each sample were determined and the area under the curve was calculated with the *AUC* function (spline method) in the *DescTools* package (version 0.99.28, Signorell, 2019). While Gram-positive bacteria are susceptible to lysozyme, Gram-negative bacteria are protected from a lysozyme attack by their outer membrane and are thus insensitive to lysozyme (Masschalck and Michiels, 2003). Using both Gram types, therefore, allowed us to distinguish between the inhibitory effect of lysozyme and other AMPs.

2.4.3. Data analysis

To analyse the effect of injected mating-associated substances and the effect of wounding on LLA in the sperm-receiving organ, we fitted two GLMs with the response variable LLA and treatment, population, and their interaction as fixed effects. If treatment was significant, we conducted pairwise comparisons (Tukey) and adjusted p-values (BH) (Benjamini and Hochberg, 1995). To analyse the effect on AMP activity against *A. globiformis* and *E. coli* in the sperm-receiving organ, we first calculated one of the two response variables, i.e. the area under the curve (AUC), to fit three GLMs. For the first GLM, we created a dummy variable with the two levels, i.e. growth control vs. all other treatments. Then, we fitted AUC as a response variable and the dummy variable, bacteria species and their interaction term as fixed effects. For the following GLMs we excluded the growth control from our data set. Then, we fitted two GLMs with the response variables AUC and maximum absorbance, respectively. For both models we included the fixed effects population, treatment, bacteria species and all interaction terms. If residuals were not normally distributed, we used Box-Cox transformation for all our fitted models. We only report significant interaction terms in the main text (see Tab. S2 and Tab. S3 for statistical details).

3. Results

3.1. Controlled transmission of bacteria

Bacteria applied to the male copulatory organ were transmitted to the female copulatory groove in 29 out of 30 cases. Most of the transmitted bacteria were located close to the entry point of the male copulatory organ (Fig. 1a). Bacteria applied to the female copulatory groove were transmitted to the male copulatory organ in 28 out of 30 cases. Most of the bacteria transmitted to the male were located at the proximal part of the copulatory organ (Fig. 1b). Only 2 out of 30 males whose copulatory organs had been treated with GFP-labelled bacteria successfully introduced their copulatory organ into the female sperm-receiving organ. Both males transferred sperm, but no bacteria were visible immediately after mating. All 30 males were able to extend their copulatory organ, and 27 out of 30 mounted a female, indicating their willingness to mate. When bacteria were applied to the female copulatory groove but not to the male (transmission experiment iv), 24 out of 30 sperm-receiving organs contained bacteria after females had been mated.

3.2. Bacterial growth

After the injection of bacteria, the number of CFUs in the female sperm-receiving organ changed significantly between time points (ANOVA: $F_{3,224} = 8.972$, $p < 0.001$). Within the first hour, there was a significant 5-fold increase in the number of CFUs (pairwise comparison with BH correction: $p < 0.001$), followed by a stationary phase after one to three hours (Fig. 2a). This growth pattern did not differ between populations (ANOVA: interaction time point x population: $F_{9,224} = 1.381$, $p = 0.20$) and CFU counts of *Asaia* sp. did not differ between populations (ANOVA: $F_{3,224} = 0.376$, $p = 0.77$).

After the injection of either bacteria or bacteria and sperm, the number of CFUs in the female sperm-receiving organ differed significantly between time points (ANOVA: $F_{2,77} = 21.393$, $p < 0.001$). A non-significant 3-fold increase within the first 6 h (pairwise comparison with BH correction: $p = 0.86$) was followed by a significant 16-fold increase until 24 h post injection (pairwise comparison with BH

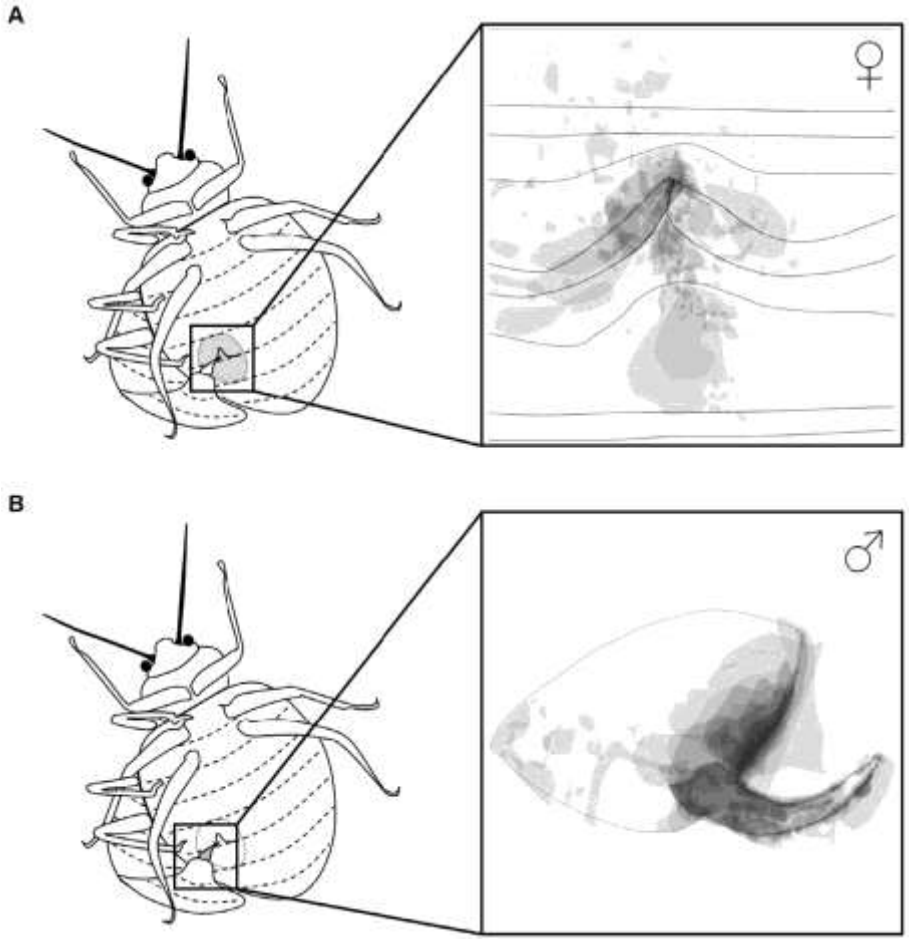

Fig. 1. Distribution of the sexually transmitted bacteria a) from the male copulatory organ to the female copulatory groove and b) from the female copulatory groove to the male copulatory organ. Templates of the organs indicating the location of bacteria detected with fluorescence microscopy were superimposed in PowerPoint. Darker areas indicate a higher rate of transmission.

correction: $p < 0.001$) (Fig. 2b). This bacteria growth pattern did not depend on the presence of sperm (ANOVA: interaction time point x treatment: $F_{2,77} = 0.153$, $p = 0.86$).

Sperm viability in the controls and in the bacteria exposure treatments decreased over time in a very similar way and samples with and without seminal fluid did not show differences in sperm viability due to bacteria over time (Table S1) (Fig. S2). Populations did not differ in the decrease in sperm viability over time, nor was there an interaction of population, bacteria, and time (Table S1) (Fig. S2).

3.3. Female reproductive immunity in response to mating-associated substances

3.3.1. Lysozyme assay

The injection of cuticular bacteria resulted in similar levels of LLA in the female sperm-receiving organ as the injection of PBS (injection control), sperm, seminal fluid and the combination of sperm and seminal fluid (ANOVA: $F_{4,300} = 2.209$, $p = 0.07$) (Fig. 3a). Overall LLA did not differ between populations (ANOVA: $F_{3,300} = 1.122$, $p = 0.34$) and there was no interaction of treatment and population (ANOVA: $F_{12,300} = 0.639$, $p = 0.81$).

In the control experiment testing for the effect of injecting a liquid into the female sperm-receiving organ, LLA differed significantly between treatments ($F_{2,168} = 7.007$, $p = 0.001$) (Fig. 3b). The sperm-receiving organs of females injected with PBS showed significantly higher LLA than the ones from females pricked with a capillary (pairwise comparison with BH correction, $p < 0.001$) or left untreated (pairwise comparison with BH correction, $p < 0.001$). Populations showed similar LLA (ANOVA: $F_{3,168} = 1.371$, $p = 0.25$) and the treatment effect did not depend on population (ANOVA: $F_{6,168} = 1.522$, $p = 0.17$).

3.3.2. AMP assay

After an initial growth phase of *Arthrobacter globiformis* and *Escherichia coli* over the first hour, the growth was inhibited in all treatments except for the growth control as indicated by significant differences in the area under the curve (AUC) between growth control and treatments (ANOVA: $F_{1,700} = 67.926$, $p < 0.001$). AMPs did not only induce growth inhibition, but also killed bacteria as the OD curves started to decline after the initial peak in the injection treatments (Fig. 4). This pattern did not depend on bacteria species, i.e. the Gram type (ANOVA: interaction bacteria species x sample type: $F_{1,700} = 0.037$, $p = 0.85$).

The bacteria species and hence the Gram type, populations, and treatments did not vary in the maximum absorbance, nor did they

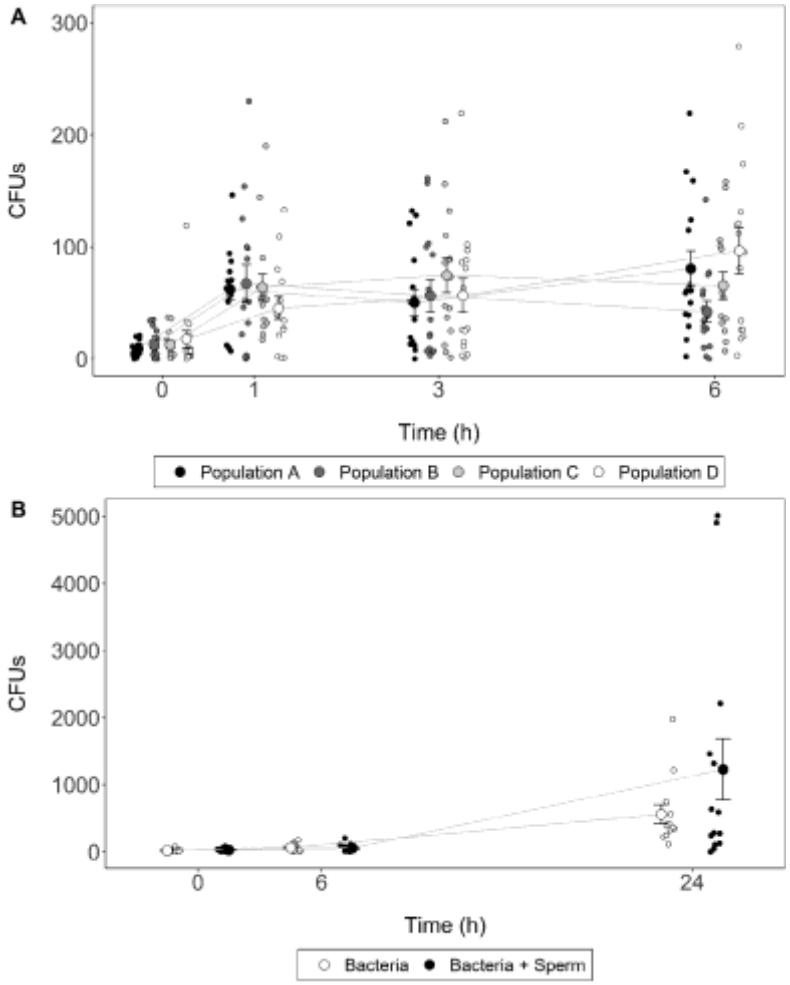

Fig. 2. Bacterial growth in the female sperm-receiving organ after the injection of a) bacteria or b) bacteria or both bacteria and sperm. The number of fluorescent CFUs after injection of GFP-labelled *Asaia* sp. was counted under UV light. Given are mean and standard error of the mean and all individual data points for a) each bedbug population (*A*, *B*, *C*, and *D*) or b) each treatment.

depend on each other (Table S2). The AUC did not differ between bacteria species (Gram types), populations, and treatments, nor was any of the interaction terms significant (Table S3).

4. Discussion

4.1. Controlled transmission of bacteria

We could show that in bedbugs, bacteria applied to the copulatory organs of males and females were transmitted to the external copulatory organs of the opposite sex at a high rate and in both directions. The only other study providing evidence that opportunistic microbes can be sexually transmitted in insects was conducted with *Drosophila* (Miest and Bloch-Qazi, 2008). However, this study ignored the sexual transmission to males. We here provide first evidence that males are subject to sexually transmitted opportunistic microbes. Bacteria deposited on the genital plate of immune deficient *Drosophila* males have been shown to invade the haemolymph (Gendrin et al., 2009), suggesting that the genital plate can provide an entry for bacteria. This makes the invasion of the internal reproductive organs of bedbug males by opportunistic microbes highly likely.

On average, untreated bedbug males copulate 8 times per hour when presented with a fed female (Kaldun and Otti, 2016). In our transmission experiment, 2 out of 30 males with bacteria on their copulatory organs pierced the female copulatory groove, hindering us to determine the transmission frequency to the female sperm-receiving organ. Since 90% of the males mounted a female, we concluded that this is not due to an adaptive strategy to prevent the spread of bacteria in the population but rather due to the bacteria layer interfering with tactile receptors on the copulatory organ. Chemoreceptors of bedbug males can detect the presence of ejaculate in the female sperm-receiving organ (Siva-Jothy and Stutt, 2003) and potentially guide the male to the female copulatory organ, as suggested by males probing the cuticle near the female copulatory groove before inserting their copulatory organ (Reinhardt and Siva-Jothy, 2007). The application of a bacteria layer might impair the function of tactile receptors and make it impossible to find the right spot for insertion. Nevertheless, metagenomic studies indicate that there is sexual transmission to the internal copulatory organs of bedbug females (Bellinvia et al., 2020a, 2020b).

Under natural conditions, bacteria transmitted to the copulatory

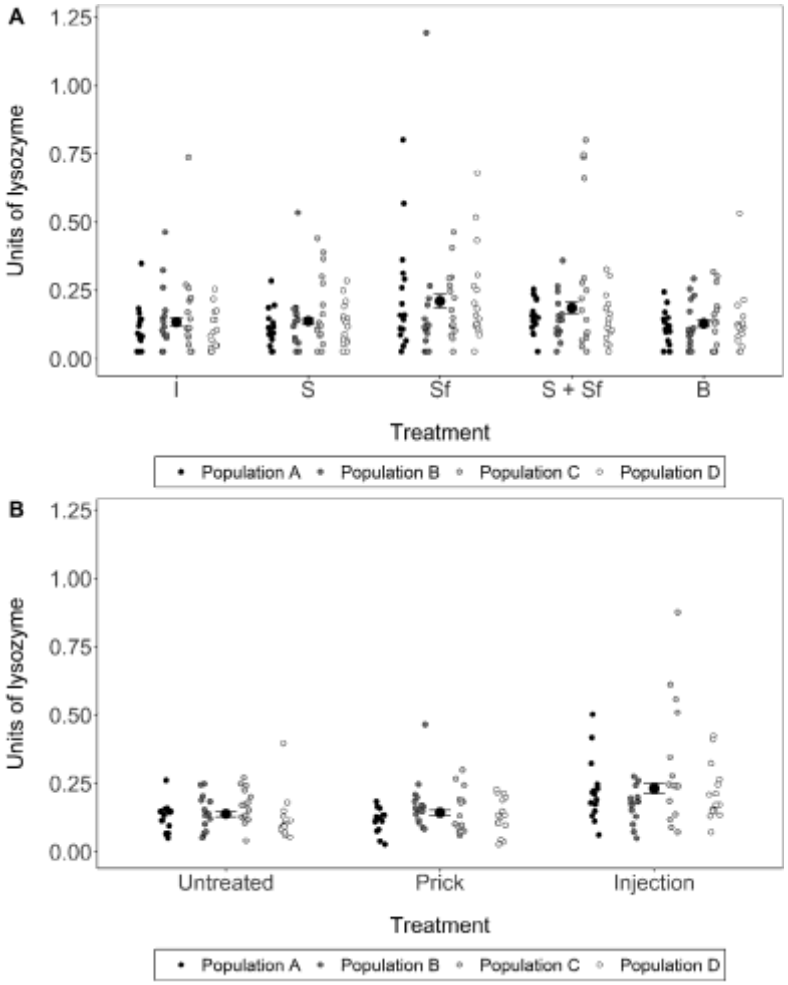

Fig. 3. Lysozyme-like activity in the female sperm-receiving organ in response to a) the injection of various mating-associated substances or b) wounding type. Given are mean and standard error of the mean for a) each treatment (I: Injection control, S: Sperm, Sf: Seminal fluid, S + Sf: Sperm + Seminal fluid, B: Bacteria) or b) each wounding type and all individual data points for each bedbug population (*A*, *B*, *C*, and *D*).

groove might enter the female during following matings since the bacteria on the copulatory groove were transferred to the sperm-receiving organ in 80% of the cases in our controlled study (transmission experiment iv). Bacteria on the female cuticle are therefore highly likely to enter the sperm-receiving organ regularly given the high mating rate observed in bedbugs (Stutt and Siva-Jothy, 2001) and via copulatory wounds, which frequently occur in insects (Lange et al., 2013). In all cases, females would be confronted with foreign microbes invading their sperm-receiving organs and therefore will be selected to invest in protection against opportunistic pathogens (Reinhardt et al., 2003; Siva-Jothy et al., 2019).

4.2. Bacterial growth

Every mating imposes the risk to be associated with opportunistic microbes and bacteria have been shown to cause infections and harm sperm (Diemer et al., 2003, 1996; Huwe et al., 1998; Kaur et al., 2010; Otti et al., 2013; Prabha et al., 2010). Therefore, females should reduce or regulate the number of sexually transmitted opportunistic microbes. In insects, possible mechanisms comprise the production of antimicrobial peptides (AMPs) (Peng et al., 2005) and lysozyme-like activity (LLA) (Siva-Jothy et al., 2019). In addition, bedbug females have evolved a sperm-receiving immune organ, the mesospermalege (Reinhardt et al., 2003). It is filled with haemocytes (Carayon, 1966) that can phagocytose bacteria (Siva-Jothy et al., 2005) and potentially protect the female from the uncontrolled growth of invading bacteria.

In accordance with the expected protection, the growth rate of bacteria (*Asaia* sp.) injected into the sperm-receiving organ of bedbug females was slowed down after one hour although *Asaia* sp. is a bacterium that is not associated with bedbugs (Bellinvia et al., 2020a, 2020b), according to our results does not have the ability to harm sperm, and hence might not signal any danger in the female reproductive organs. Sperm start to travel from the sperm-receiving organ towards the ovaries and storage organs approximately 4 h after insemination (Carayon, 1966). This makes growth control within the first hours during sperm travel especially important. The stationary phase within the first hours was followed by another phase of bacterial growth. It remains to be investigated whether this is due to a shift from investment in immunity to investment in reproduction when sperm reach the ovaries. The sperm-receiving organ has evolved as a

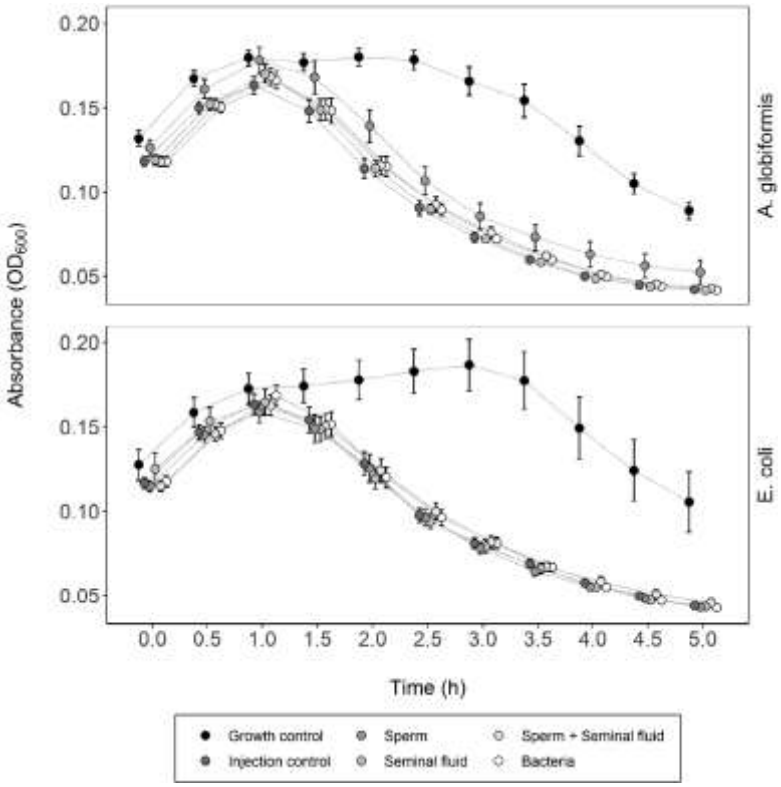

Fig. 4. Liquid growth inhibition of *Arthrobacter globiformis* and *Escherichia coli* by antimicrobial peptides produced in the female sperm-receiving organ in response to the injection of various mating-associated substances. Bacteria were incubated with the supernatant from centrifuged homogenised tissue whereas the growth control contained the same volume of PBS instead of supernatant.

protection from mating-associated bacteria (Reinhardt et al., 2003) and might be helpful not only to control bacterial growth via haemocytes, AMPs, and LLA, but also as a physical barrier towards the haemolymph when the female cannot invest in immunity, for instance due to a trade-off between immunity and reproduction.

4.3. Female reproductive immunity in response to mating-associated substances

To our knowledge, this is the first study measuring female immunity in response to an immune challenge in female bedbugs. Contrasting our predictions, all substances induced similar levels of LLA, even injection controls. Not the prick but the injection of a fluid seemed to be responsible for the production of LLA although wounding itself is a very reliable signal for immune investment and repair cascades as well as wound closure are common among invertebrates (Theopold et al., 2004). Instead, the introduction of liquid rather than specific pathogen-associated molecular patterns, seems to trigger the production of LLA. If females associate the transfer of liquid with the transfer of ejaculate, they should upregulate LLA because of the infection risk conferred by sexually transmitted bacteria. Future studies should investigate whether the frequency of sexually transmitted opportunistic microbes depends on the transfer of ejaculate to clarify why the injection of liquid elicits a stronger immune response compared to wounding.

Two other hemipterans possess an abdominal stretch receptor that is sensitive to internal pressure due to an abdominal distension (Chiang et al., 1990; Chiang and Davey, 1988), for instance caused by blood-feeding, and stretch receptors are attached to intersegmental muscles of other blood-feeding insects (Anwyl, 1972; Klowden and Lea, 1979). The female sperm-receiving organ lies ventral underneath the copulatory groove in the intersegmental membrane (Carayon, 1966) and could be connected to similar sensors. Supporting this idea, abdominal distension can inhibit feeding in bedbugs (Wintle and Reinhardt, 2008). It remains to be shown how such a signal can trigger an immune response.

As predicted, we found growth inhibition of a Gram-negative and a Gram-positive bacterium due to antimicrobial peptides (AMPs) produced in the female sperm-receiving organ in response to mating-associated substances. As for the bacterial growth experiment, bacterial growth was controlled one hour after exposure, suggesting that immunological reagents are very time-efficient. Curves did not differ between treatments, suggesting that even the injection control triggered the production of AMPs. Again, this might be explained by an immune response induced by a stretch sensor.

4.4. Population effects on bacterial growth and female reproductive immunity

Surprisingly, female reproductive immunity did not differ between populations although populations likely differ in the frequency of sexually transmitted opportunistic microbes and the toxicity of transmitted bacterial strains. In other insects, populations differ in responses to parasites or bacteria, potentially because they are adapted to different levels of parasite prevalence (Brunner et al., 2013; Cornet et al., 2009) and immune responses confer multiple costs (Ilmonen et al., 2000; Rigby and Jokela, 2000; Sheldon and Verhulst, 1996; Zuk and Stoehr, 2002). The lack of between-population differences might partly be because *Asaia* and bedbugs do not share any co-evolutionary history. However, the bacteria used for the immunoassays should have been co-evolved with the bedbugs since they were harvested from the bedbug cuticle. Therefore, missing between-population differences might indicate that the response of the host is similar across populations. Inducing a strong immune response regardless of prevalence and toxicity

might be less costly than the uncontrolled growth of the transmitted opportunistic microbes.

5. Conclusion

We have demonstrated that opportunistic bacteria have the potential to be sexually transmitted at a high rate in the common bedbug *Cimex lectularius*. To our knowledge, this is the first record of opportunistic bacteria being sexually transmitted from female to male in insects. We here provide the first evidence of female immunity, measured by antimicrobial and lysozyme-like activity, in response to mating-associated substances in bedbugs. Our findings suggest that female immune responses are induced by receiving a liquid. Bacteria usually not occurring in the environment of bedbugs were inhibited within the first hours after injection with sperm. However, after 6 h, bacterial numbers increased again. Against our expectations, populations did not differ in the measured immune traits or bacterial growth. Future studies should investigate how sexual transmission dynamics and immune responses affect fitness in both sexes. Experimental evolution with different environmental bacteria might clarify whether sexually transmitted opportunistic bacteria can shape the immune response in females.

Author contributions

S.B. and O.O. conceived the idea and designed the experiments. S.B., A.S., I.B., and B.S. carried out the experiments. S.B. and O.O. performed the statistical analysis. S.B. and O.O. interpreted the results and wrote the manuscript.

CRediT authorship contribution statement

Sara Bellinvia: Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Writing - original draft, Writing - review & editing, Project administration. **Andrea Spachtholz:** Methodology, Investigation, Visualization. **Ina Borgwardt:** Methodology, Investigation, Visualization. **Bastian Schauer:** Methodology, Investigation, Visualization. **Oliver Otti:** Conceptualization, Methodology, Writing - original draft, Writing - review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jinsphys.2020.104048.

REFERENCES

Anwyl, R., 1972. The structure and properties of an abdominal stretch receptor in *Rhodnius prolixus*. J. Insect Physiol. 18, 2143–2153. https://doi.org/10.1016/0022-1910(72)90245-4.

Bellinvia, S., Johnston, P.R., Mbedi, S., Otti, O., 2020a. Mating changes the genital microbiome in both sexes of the common bedbug Cimex lectularius across populations. Proc. R. Soc. B Biol. Sci, submitted. Preprint available at: https://www.biorxiv.org/content/10.1101/819300v1.abstract. https://doi.org/10.1101/819300.

Bellinvia, S., Johnston, P.R., Reinhardt, K., Otti, O., 2020b. Bacterial communities of the reproductive organs of virgin and mated common bedbugs, *Cimex lectularius*. Ecol. Entomol. 45, 142–154. https://doi.org/10.1111/een.12784.

Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. R. Stat. Soc. Ser. B 57, 289–300. https://doi.org/10.1111/j.2517-6161.1995.tb02031.x.

Boucias, D.G., Pendland, J.C., 2012. Principles of Insect Pathology. Springer Science & Business Media, Luxemburg.

Bright, M., Bulgheresi, S., 2010. A complex journey: transmission of microbial symbionts. Nat. Rev. Microbiol. 8, 218–230. https://doi.org/10.1038/nrmicro2262.

Brunner, F.S., Schmid-Hempel, P., Barribeau, S.M., 2013. Immune gene expression in *Bombus terrestris*: signatures of infection despite strong variation among populations, colonies, and sister workers. PLoS One 8, e68181. https://doi.org/10.1371/journal.pone.0068181.

Carayon, J., 1966. Traumatic insemination and the paragenital system. In: Usinger, R. (Ed.), Monograph of Cimicidae (Hemiptera-Heteroptera). Entomological Society of America, College Park, MD, pp. 81–166.

Chiang, R.G., Chiang, J.A., Davey, K.G., 1990. Structure of the abdominal receptor responsive to internally applied pressure in the blood-feeding insect, *Rhodnius prolixus*. Cell Tissue Res. 261, 583–587. https://doi.org/10.1007/BF00313538.

Chiang, R.G., Davey, K.G., 1988. A novel receptor capable of monitoring applied pressure in the abdomen of an insect. Science 241, 1665–1667. https://doi.org/10.1126/science.241.4873.1665.

Cornet, S., Biard, C., Moret, Y., 2009. Variation in immune defence among populations of *Gammarus pulex* (Crustacea: Amphipoda). Oecologia 159, 257–269. https://doi.org/10.1007/s00442-008-1211-y.

Dale, C., Moran, N.A., 2006. Molecular interactions between bacterial symbionts and their hosts. Cell 126, 453–465. https://doi.org/10.1016/j.cell.2006.07.014.

Dethlefsen, L., McFall-Ngai, M., Relman, D.A., 2007. An ecological and evolutionary perspective on human-microbe mutualism and disease. Nature 449, 811–818. https://doi.org/10.1038/nature06245.

Diemer, T., Huwe, P., Ludwig, M., Schroeder-Printzen, I., Michelmann, H.W., Schiefer, H.G., Weidner, W., 2003. Influence of autogenous leucocytes and *Escherichia coli* on sperm motility parameters *in vitro*. Andrologia 35, 100–105. https://doi.org/10.1046/j.1439-0272.2003.00523.x.

Diemer, T., Weidner, W., Michelmann, H.W., Schiefer, H.G., Rovan, E., Mayer, F., 1996. Influence of *Escherichia coli* on motility parameters of human spermatozoa in vitro. Int. J. Androl. 19, 271–277. https://doi.org/10.1111/j.1365-2605.1996.tb00475.x.

Favia, G., Ricci, I., Damiani, C., Raddadi, N., Crotti, E., Marzorati, M., Rizzi, A., Urso, R., Brusetti, L., Borin, S., Mora, D., Scuppa, P., Pasqualini, L., Clementi, E., Genchi, M., Corona, S., Negri, M., Grandi, G., Alma, A., Kramer, L., Esposito, F., Bandi, C., Sacchi, L., Daffonchio, D., 2007. Bacteria of the genus Asaia stably associate with Anopheles stephensi, an Asian malarial mosquito vector. Proc. Natl. Acad. Sci. USA 104, 9047–9051. https://doi.org/10.1073/pnas.0610451104.

Geissmann, Q., 2013. OpenCFU, a new free and open-source software to count cell colonies and other circular objects. PLoS One 8, e54072.

Gendrin, M., Welchman, D.P., Poidevin, M., Hervé, M., Lemaitre, B., 2009. Long-range activation of systemic immunity through peptidoglycan diffusion in *Drosophila*. PLoS Pathog. 5, e1000694. https://doi.org/10.1371/journal.ppat.1000694.

González-Marín, C., Roy, R., López-Fernández, C., Diez, B., Carabaño, M.J., Fernández, J.L., Kjelland, M.E., Moreno, J.F., Gosálvez, J., 2011. Bacteria in bovine semen can increase sperm DNA fragmentation rates: a kinetic experimental approach. Anim. Reprod. Sci. 123, 139–148. https://doi.org/10.1016/j.anireprosci.2010.11.014.

Goodrich, J.K., Davenport, E.R., Waters, J.L., Clark, A.G., Ley, R.E., 2016. Cross-species comparisons of host genetic associations with the microbiome. Science 352, 532–535. https://doi.org/10.1126/science.aad9379.

Hase, A., 1930. Methoden der Züchtung von Wanzen, Läusen und Flöhen. In: Knolle, W., Kraus, R., Uhlenhut, P. (Eds.), Handbuch Der Pathogenen Mikroorganismen. Gustav Fischer, Jena, pp. 591–616.

Hickey, R.J., Zhou, X., Pierson, J.D., Ravel, J., Forney, L.J., 2012. Understanding vaginal microbiome complexity from an ecological perspective. Transl. Res. 160, 267–282. https://doi.org/10.1016/j.trsl.2012.02.008.

Hirsh, D.C., 1999. The genital tract as a microbial habitat. In: Hirsh, D.C., Zee, Y.C. (Eds.), Veterinary Microbiology. Blackwell Science, Oxford, pp. 190–191.

Hupton, G., Portocarrero, S., Newman, M., Westneat, D.F., 2003. Bacteria in the reproductive tracts of red-winged blackbirds. Condor 105, 453–464. https://doi.org/10.1650/7246.

Huttenhower, C., Gevers, D., Knight, R., Abubucker, S., Badger, J.H., Chinwalla, A.T., Creasy, H.H., Earl, A.M., FitzGeral, M.G., 2012. Structure, function and diversity of the healthy human microbiome. Nature 486, 207–214. https://doi.org/10.1038/nature11234.

Huwe, P., Diemer, T., Ludwig, M., Liu, J., Schiefer, H.G., Weidner, W., 1998. Influence of different uropathogenic microorganisms on human sperm motility parameters in an *in vitro* experiment. Andrologia 30, 55–59. https://doi.org/10.1111/j.1439-0272.1998.tb02827.x.

Ilmonen, P., Taarna, T., Hasselquist, D., 2000. Experimentally activated immune defence in female pied flycatchers results in reduced breeding success. Proc. R. Soc. B Biol. Sci. 267, 665–670. https://doi.org/10.1098/rspb.2000.1053.

Kaldun, B., Otti, O., 2016. Condition-dependent ejaculate production affects male mating behavior in the common bedbug *Cimex lectularius*. Ecol. Evol. 6, 2548–2558. https://doi.org/10.1002/ece3.2073.

Kaltenpoth, M., Engl, T., 2014. Defensive microbial symbionts in Hymenoptera. Funct. Ecol. 28, 315–327. https://doi.org/10.1111/1365-2435.12089.

Kaur, S., Prabha, V., Shukla, G., Sarwal, A., 2010. Interference of human spermatozoal motility by live *Staphylococcus aureus*. Am. J. Biomed. Sci. 2, 91–97. https://doi.org/10.5099/aj100100091.

Klainer, A.S., Beisel, W.R., 1969. Opportunistic infection: a review. Am. J. Med. Sci. https://doi.org/10.1097/00000441-196912000-00007.

Klowden, M.J., Lea, A.O., 1979. Abdominal distention terminates subsequent host-seeking behaviour of *Aedes aegypti* following a blood meal. J. Insect Physiol. 25, 583–585. https://doi.org/10.1016/0022-1910(79)90073-8.

Knell, R.J., Webberley, K.M., 2004. Sexually transmitted diseases of insects: distribution, evolution, ecology and host behaviour. Biol. Rev. 79, 557–581. https://doi.org/10.1017/S1464793103006365.

Kostic, A.D., Howitt, M.R., Garrett, W.S., 2013. Exploring host-microbiota interactions in animal models and humans. Genes Dev. 27, 701–718. https://doi.org/10.1101/gad.212522.112.of.

Lange, R., Reinhardt, K., Michiels, N.K., Anthes, N., 2013. Functions, diversity, and evolution of traumatic mating. Biol. Rev. 88, 585–601. https://doi.org/10.1111/brv.12018.

Lockhart, A.B., Thrall, P.H., Antonovics, J., 1996. Sexually transmitted diseases in animals: ecological and evolutionary Implications. Biol. Rev. 71, 415–471. https://doi.org/10.1111/j.1469-185X.1996.tb01281.x.

Lombardo, M.P., Thorpe, P.A., 2000. Microbes in tree swallow semen. J. Wildl. Dis. 36, 460–468. https://doi.org/10.7589/0090-3558-36.3.460.

Lung, O., Kuo, L., Wolfner, M.F., 2001. *Drosophila* males transfer antibacterial proteins from their accessory gland and ejaculatory duct to their mates. J. Insect Physiol. 47, 617–622. https://doi.org/10.1016/S0022-1910(00)00151-7.

Marius-Jestin, V., Menec, M.L., Thibault, E., Mosan, J.C., ĹHospitalier, R., Alexandre, M., Coiffard, J.-M., 1987. Normal phallus flora of the gander. Zoonoses Public Health 34, 67–78. https://doi.org/10.1111/j.1439-0450.1987.tb00371.x.

Masschalck, B., Michiels, C.W., 2003. Antimicrobial properties of lysozyme in relation to foodborne vegetative bacteria. Crit. Rev. Microbiol. 29, 191–214. https://doi.org/10.1080/713610448.

Miest, T.S., Bloch-Qazi, M.C., 2008. Sick of mating: sexual transmission of a pathogenic bacterium in *Drosophila melanogaster*. Fly 2, 215–219. https://doi.org/10.4161/fly.6726.

Oliver, K.M., Russell, J.A., Morant, N.A., Hunter, M.S., 2003. Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. Proc. Natl. Acad. Sci. 100, 1803–1807. https://doi.org/10.1073/pnas.0335320100.

Otti, O., Deines, P., Hammerschmidt, K., Reinhardt, K., 2017. Regular wounding in a natural system: bacteria associated with reproductive organs of bedbugs and their quorum sensing abilities. Front. Immunol. 8, 1855. https://doi.org/10.3389/fimmu.2017.01855.

Otti, O., McTighe, A.P., Reinhardt, K., 2013. *In vitro* antimicrobial sperm protection by an ejaculate-like substance. Funct. Ecol. 27, 219–226. https://doi.org/10.1111/1365-2435.12025.

Otti, O., Naylor, R.A., Siva-Jothy, M.T., Reinhardt, K., 2009. Bacteriolytic activity in the ejaculate of an insect. Am. Nat. 174, 292–295. https://doi.org/10.1086/600099.

Peng, J., Zipperlen, P., Kubli, E., 2005. *Drosophila* sex-peptide stimulates female innate immune system after mating via the Toll and Imd pathways. Curr. Biol. 15, 1690–1694. https://doi.org/10.1016/j.cub.2005.08.048.

Poiani, A., 2006. Complexity of seminal fluid: a review. Behav. Ecol. Sociobiol. 60, 289–310. https://doi.org/10.1007/s00265-006-0178-0.

Prabha, V., Sandhu, R., Kaur, S., Kaur, K., Sarwal, A., Mavuduru, R.S., Singh, S.K., 2010. Mechanism of sperm immobilization by *Escherichia coli*. Adv. Urol. 2010, 240268. https://doi.org/10.1155/2010/240268.

Quayle, A.J., Nussbaum, A.A., Wang, Y.M., Brabec, C., Mok, S.C., Porter, E., Yip, K.-P., 1998. Gene expression, immunolocalization, and secretion of human defensin-5 in human female reproductive tract. Am. J. Pathol. 152, 1247–1258.

R Core Team, 2018. R: A Language and Environment for Statistical Computing.

Ravel, J., Gajer, P., Abdo, Z., Schneider, G.M., Koenig, S.S.K., McCulle, S.L., Karlebach, S., Gorle, R., Russell, J., Tacket, C.O., Brotman, R.M., Davis, C.C., Ault, K., Peralta, L., Forney, L.J., 2011. Vaginal microbiome of reproductive-age women. Proc. Natl. Acad. Sci. 108, 4680–4687. https://doi.org/10.1073/pnas.1002611107.

Reinhardt, K., Naylor, R., Siva-Jothy, M.T., 2005. Potential sexual transmission of environmental microbes in a traumatically inseminating insect. Ecol. Entomol. 30, 607–611. https://doi.org/10.1111/j.0307-6946.2005.00730.x.

Reinhardt, K., Naylor, R., Siva-Jothy, M.T., 2003. Reducing a cost of traumatic insemination: female bedbugs evolve a unique organ. Proc. R. Soc. London B Biol. Sci. 270, 2371–2375. https://doi.org/10.1098/rspb.2003.2515.

Reinhardt, K., Naylor, R.A., Siva-Jothy, M.T., 2009. Situation exploitation: higher male mating success when female resistance is reduced by feeding. Evolution (N. Y) 63, 29–39. https://doi.org/10.1111/j.1558-5646.2008.00502.x.

Reinhardt, K., Siva-Jothy, M.T., 2007. Biology of the bed bugs (Cimicidae). Annu. Rev. Entomol. 52, 351–374. https://doi.org/10.1146/annurev.ento.52.040306.133913.

Rigby, M.C., Jokela, J., 2000. Predator avoidance and immune defence: costs and trade-offs in snails. Proc. R. Soc. London B Biol. Sci. 267, 171–176. https://doi.org/10.1098/rspb.2000.0983.

Rillig, M.C., Antonovics, J., Caruso, T., Lehmann, A., Powell, J.R., Veresoglou, S.D., Verbruggen, E., 2015. Interchange of entire communities: microbial community coalescence. Trends Ecol. Evol. 30, 470–476. https://doi.org/10.1016/j.tree.2015.06.004.

Schneider, C.A., Rasband, W.S., Eliceiri, K.W., 2012. NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9, 671–675. https://doi.org/10.1038/nmeth.2089.

Sheldon, B.C., Verhulst, S., 1996. Ecological immunology: costly parasite defences and trade-offs in evolutionary ecology. Trends Ecol. Evol. 11, 317–321. https://doi.org/10.1016/0169-5347(96)10039-2.

Signorell, A., 2019. DescTools: tools for descriptive statistics.

Siva-Jothy, M.T., Moret, Y., Rolff, J., 2005. Insect immunity: an evolutionary ecology perspective. Adv. Insect Phys. 32, 1–48. https://doi.org/10.1016/S0065-2806(05)32001-7.

Siva-Jothy, M.T., Stutt, A.D., 2003. A matter of taste: direct detection of female mating status in the bedbug. Proc. R. Soc. London B Biol. Sci. 270, 649–652. https://doi.org/10.1098/rspb.2002.2260.

Siva-Jothy, M.T., Zhong, W., Naylor, R., Heaton, L., Hentley, W., Harney, E., 2019. Female bed bugs (*Cimex lectularius* L) anticipate the immunological consequences of traumatic insemination via feeding cues. Proc. Natl. Acad. Sci. USA 116, 14682–14687. https://doi.org/10.1073/pnas.1904539116.

Skau, P.A., Folstad, I., 2003. Do bacterial infections cause reduced ejaculate quality? A meta-analysis of antibiotic treatment of male infertility. Behav. Ecol. 14, 40–47. https://doi.org/10.1093/beheco/14.1.40.

Stutt, A.D., Siva-Jothy, M.T., 2001. Traumatic insemination and sexual conflict in the bed bug *Cimex lectularius*. Proc. Natl. Acad. Sci. USA 98, 5683–5687. https://doi.org/10.1073/pnas.101440698.

Theopold, U., Schmidt, O., Söderhäll, K., Dushay, M.S., 2004. Coagulation in arthropods: defence, wound closure and healing. Trends Immunol. 25, 289–294. https://doi.org/10.1016/j.it.2004.03.004.

Valore, E.V., Park, C.H., Quayle, A.J., Wiles, K.R., McCray, P.B., Ganz, T., 1998. Human β-defensin-1: an antimicrobial peptide of urogenital tissues. J. Clin. Invest. 101, 1633–1642. https://doi.org/10.1172/JCI1861.

Virecoulon, F., Wallet, F., Rigot, J., Peers, M., Mitchell, V., 2005. Bacterial flora of the low male genital tract in patients consulting for infertility. Andrologia 37, 160–165. https://doi.org/10.1111/j.1439-0272.2005.00673.x.

Weiss, B., Aksoy, S., 2011. Microbiome influences on insect host vector competence. Trends Parasitol. 27, 514–522. https://doi.org/10.1016/j.pt.2011.05.001.

White, J., Richard, M., Massot, M., Meylan, S., 2011. Cloacal bacterial diversity increases with multiple mates: evidence of sexual transmission in female common lizards. PLoS One 6, e22339. https://doi.org/10.1371/journal.pone.0022339.

Wintle, K., Reinhardt, K., 2008. Temporary feeding inhibition caused by artificial abdominal distension in the bedbug, *Cimex lectularius*. J. Insect Physiol. 54, 1200–1204. https://doi.org/10.1016/j.jinsphys.2008.05.005.

Zuk, M., Stoehr, A.M., 2002. Immune defense and host life history. Am. Nat. 160, S9–S22. https://doi.org/10.1086/342131.

SUPPLEMENTARY INFORMATION FOR

Female immunity in response to sexually transmitted opportunistic bacteria in the common bedbug *Cimex lectularius*

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INDEX:

SUPPLEMENTARY METHODS

Suitability of GFP-labelled bacteria

We investigated whether green fluorescent bacteria naturally occur in the sperm-receiving organs of virgin females to verify the suitability of GFP-labelled bacteria for the investigation of sexual transmission. We therefore dissected 40 females (N=10 per bedbug population) and homogenised their sperm-receiving organs in 50 µl of sterile phosphate buffered saline (PBS) with a pestle made from a melted 200 µl pipette tip. The homogenate was plated out on glycerol agar (2% agar, 1% yeast extract, 2.25% glycerol) and incubated for 48 hours at 30°C. Fluorescence was examined with a 395nm UV LED torchlight (ePathChina Ltd, Kowloon, Hong Kong). We found no fluorescent bacteria in the dissected organs, suggesting that GFP-labelled bacteria are suitable for investigating sexual transmission in bedbugs.

Concentration of bacteria solutions

To determine the concentration of bacteria cultures for the transmission experiments, we inoculated glycerol agar plates (2% agar, 1% yeast extract, 2.25% glycerol) with GFP-labelled *Asaia* sp. and incubated the plates at 30°C for 48 hours. We picked an individual colony and inoculated 5 ml of glycerol medium (1% yeast extract, 2.25% glycerol) in a 15 ml Falcon tube. The tubes were incubated in a shaking incubator at 30°C and 200 rpm for 24 hours. Using sterile glass beads, we plated 100 µl of each bacteria culture (N=10) on glycerol agar after diluting them 1:1000000.

To determine the concentration of bacteria solutions for the bacterial growth experiments, we cultured the bacteria as described above and diluted the bacteria culture to an OD_{600}=0.1. We centrifuged the overnight culture for 5 minutes at 2350 g and replaced the supernatant with sterile PBS. We diluted the resulting solution 1:100 or 1:50 and with a glass capillary (GB1000F-10, Science Products GmbH, Hofheim, Germany) pulled to a fine point, we transferred 0.5 µl (N=10) or 0.25 µl (N=10) to an Eppendorf tube containing 100 µl sterile PBS. Using sterile glass beads, we plated 100 µl of each tube on glycerol agar.

To determine the concentration of bacteria solutions for the reproductive immunity experiments, we harvested cuticular bacteria with cuticle washes by vortexing 5 males and 5 females from population A in 5 ml sterile PBS for 15 minutes after chilling on ice for 15 minutes. We plated 100 µl of each bacteria solution (N=5) on LB agar after diluting them 1:100.

All plates were incubated for 48 hours at 30°C and photographed with a Gel iX Imager (software: INTAS GDS, INTAS Science Imaging Instruments GmbH, Göttingen, Germany) and the colony forming units were counted with OpenCFU (version 3.8-BETA).

Suitability of the sperm viability protocol

To analyse whether a decrease in sperm numbers is reflected by a decrease in fluorescence induced by a SYBR green stain (Live/Dead Sperm Viability Kit, L7011, Invitrogen, Carlsbad, USA), we dissected 12 males in two experiment blocks. Both sperm containers of all 12 males were pooled in an Eppendorf tube containing 960 µl Grace's Insect Medium (G8142, Sigma Aldrich, Hamburg, Germany), homogenised, and serially diluted to an end concentration of 0.0625. To measure the fluorescence, 20 µl of SYBR green (first diluted 1:50 in DMSO and then 1:40 in sterile Grace's Insect Medium) were added to each sample containing 60 µl of a given concentration and mixed with a pipette. After incubating the samples in the dark for 10 minutes, we transferred them to a FLUOTRAC 384 well plate (781076, Greiner, Frickenhausen, Germany) and measured every 5 minutes for 60 minutes in a microplate reader (Synergy HT, BioTek Instruments, Winooski, VT, USA) with an excitation at 485 nm, an emission at 528 and a gain of 75. The dilution of sperm resulted in lower fluorescence (Fig. S1), showing that a decrease in sperm numbers can be inferred from a decrease in fluorescence.

Lysozyme standard curve

We produced a standard curve for measuring the lysozyme units in each sample by serially diluting chicken lysozyme (from chicken egg white; A4972, PanReac AppliChem, Darmstadt,

Germany) in PBS to produce the following concentrations in units of lysozyme/ml: 40, 24, 16, 8, 4, 2.4, 1.6, 0.8, 0.4, 0.24, 0.16, 0.08, 0.04. We transferred 1 µl of each concentration to a 5 ml agar plate containing lyophilised *Micrococcus lysodeikticus* (ATCC No. 4698, Sigma-Aldrich, Hamburg, Germany) and incubated the plate at 30°C for 48 hours. In total, we had 19 replicates per concentration. We then measured the area of clear zones in ImageJ (version 1.51k) and plotted them against lysozyme concentration (Fig. S3).

SUPPLEMENTARY FIGURES

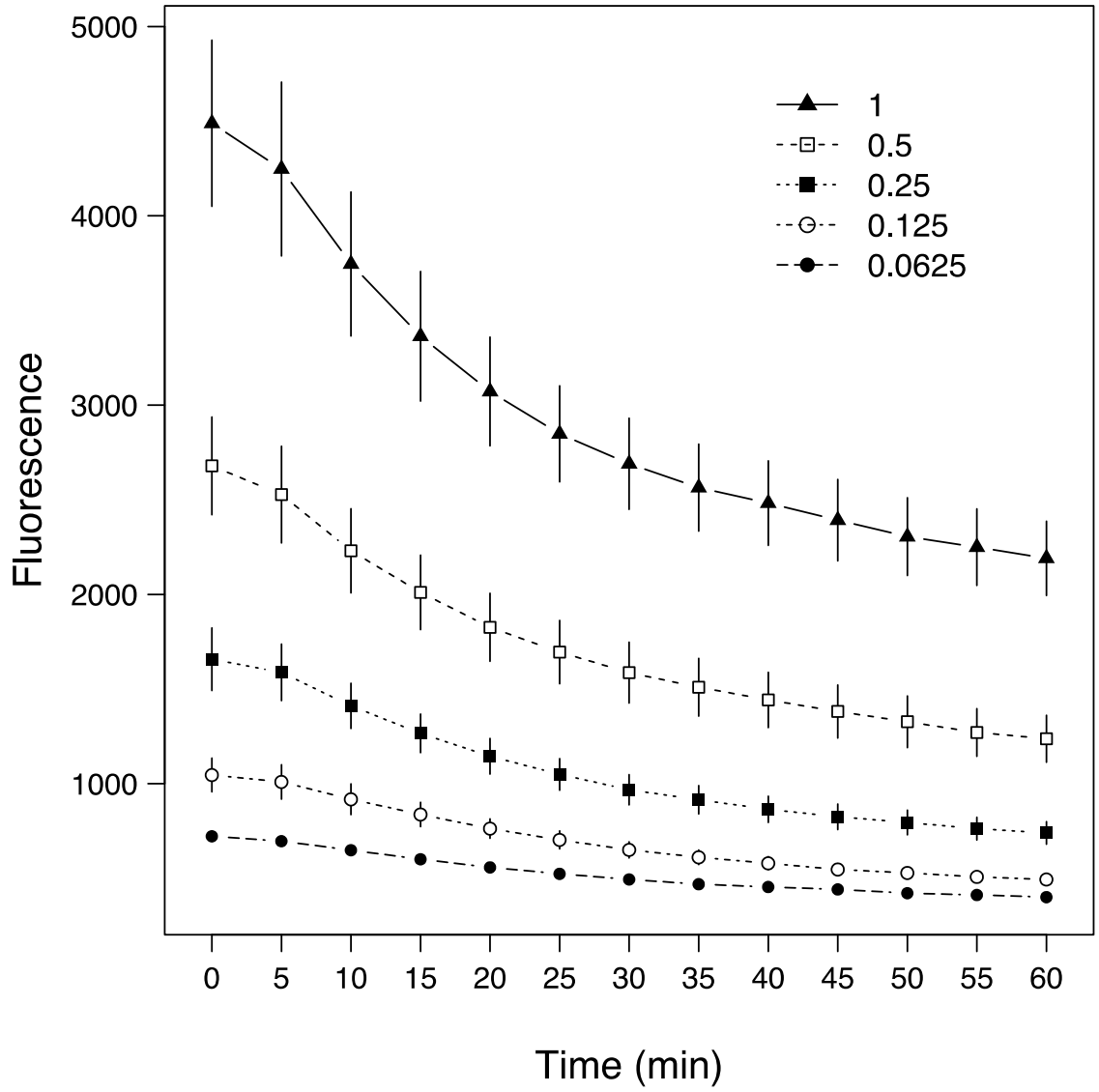

Figure S1 Suitability of the microplate reader to detect a decrease in sperm numbers with the Live/Dead Sperm Viability Kit (SYBR green). Given are mean and standard error of the mean for the fluorescence detected after diluting both sperm containers serially from a start concentration of 1 to an end concentration of 0.0625.

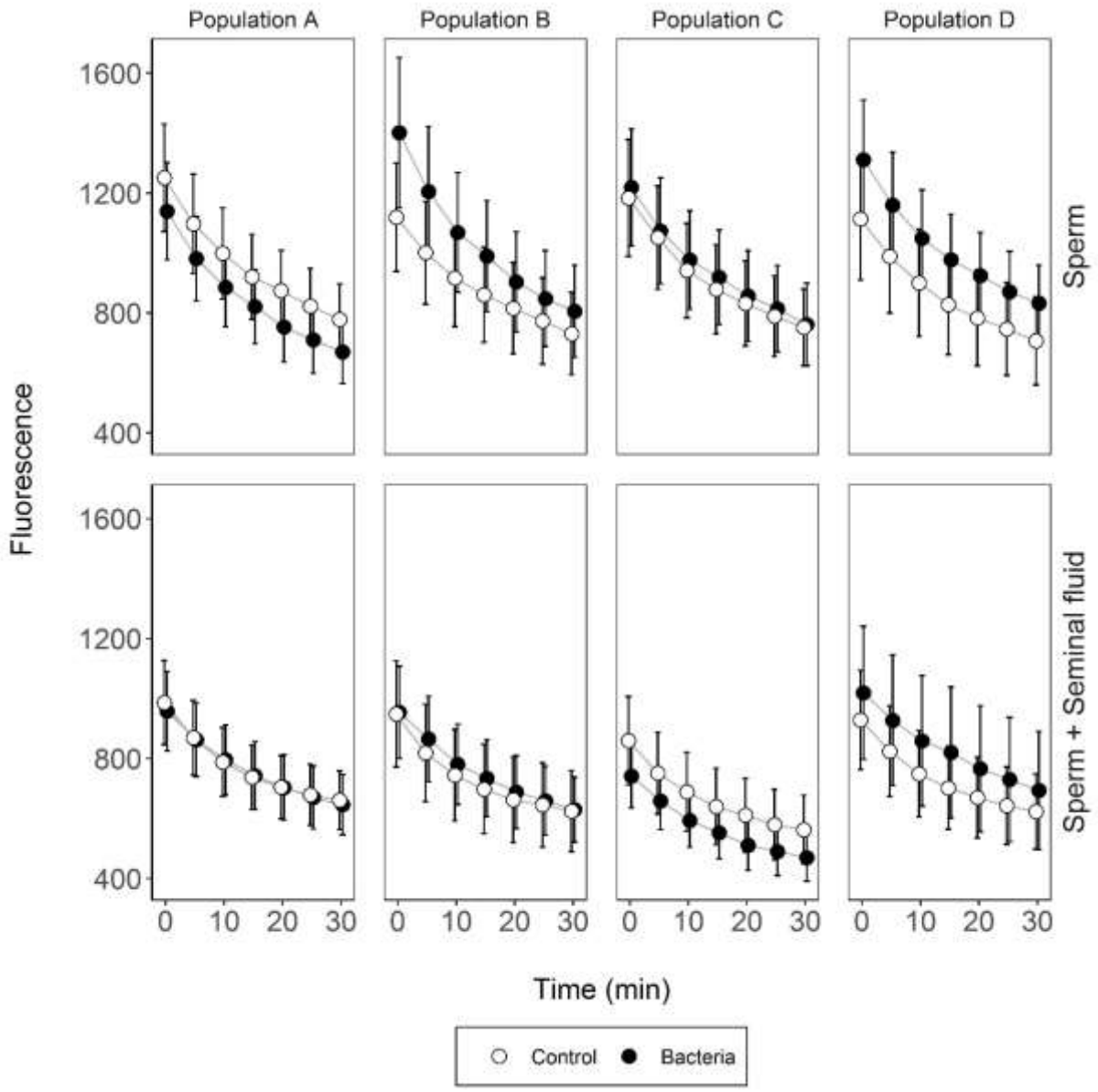

Figure S2 Potential spermicidal ability of *Asaia* sp. in vitro indicated by a decrease in fluorescence of spermatozoa labelled with SYBR 14. Given are mean and standard error of the mean for each treatment across populations (*A*, *B*, *C*, *D*).

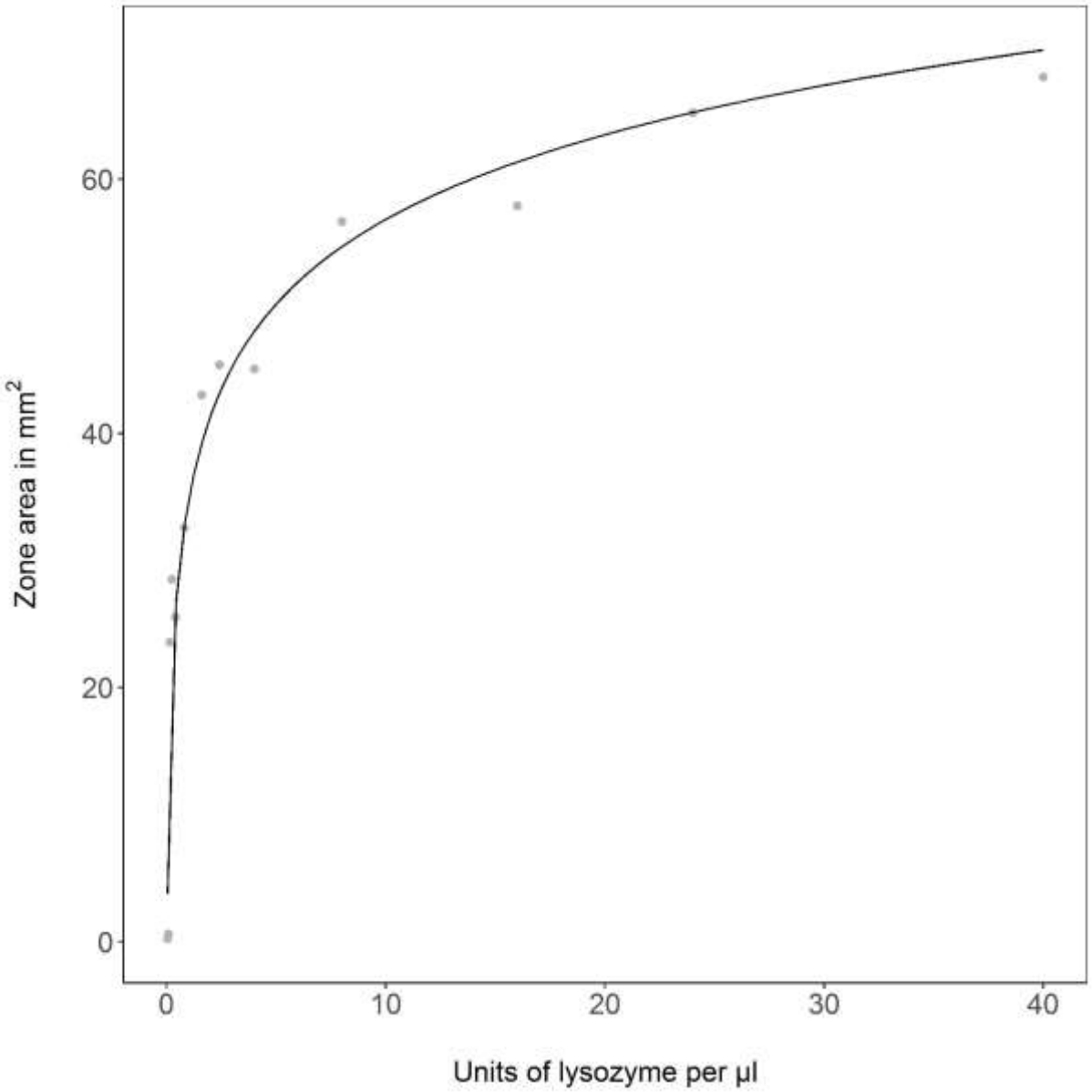

Figure S3 Standard curve for measuring the lysozyme units in tissue samples. Given are the means for each concentration and area.

SUPPLEMENTARY TABLES

Table S1 ANOVA results showing the effect of the given fixed effects on sperm viability determined by fluorescence measurements.

Fixed effect	Sum Sq	Df	F values	Pr(>F)
Population	11.017	3	0.205	0.893
Seminal fluid presence	15.771	1	0.879	0.349
Bacteria treatment	2.306	1	0.129	0.720
Time	96.107	6	0.892	0.500
Population x seminal fluid presence	4.047	3	0.075	0.973
Population x bacteria treatment	19.557	3	0.363	0.780
Seminal fluid presence x bacteria treatment	0.438	1	0.024	0.876
Population x time	0.717	18	0.002	1.000
Seminal fluid presence x time	1.996	6	0.019	1.000
Bacteria treatment x time	0.497	6	0.005	1.000
Population x seminal fluid presence x bacteria treatment	5.505	3	0.102	0.959
Population x seminal fluid presence x time	0.976	18	0.003	1.000
Population x bacteria treatment x time	2.917	18	0.009	1.000
Seminal fluid presence x bacteria treatment x time	0.373	6	0.003	1.000
Population x seminal fluid presence x bacteria treatment x time	2.468	18	0.008	1.000
Residuals	26133.519	1456		

Table S2 ANOVA results showing the effect of the given fixed effects on the maximum absorbance determined from the liquid growth inhibition induced by the injection of mating-associated substances into the female sperm-receiving organ.

Fixed effect	Sum Sq	Df	F values	Pr(>F)

Bacteria species	0.00002	1	0.006	0.939
Population	0.00215	3	0.169	0.917
Treatment	0.00570	4	0.336	0.854
Bacteria species x population	0.00084	3	0.066	0.978
Bacteria species x treatment	0.00336	4	0.198	0.939
Population x treatment	0.02419	12	0.475	0.929
Bacteria species x population x treatment	0.02971	12	0.584	0.856
Residuals	2.54507	600		

Table S3 ANOVA results showing the effect of the given fixed effects on the area under the curve determined from the liquid growth inhibition induced by the injection of mating-associated substances into the female sperm-receiving organ.

Fixed effect	Sum Sq	Df	F values	Pr(>F)
Bacteria species	0.00027	1	0.283	0.595
Population	0.00095	3	0.328	0.805
Treatment	0.00270	4	0.701	0.592
Bacteria species x population	0.00045	3	0.154	0.927
Bacteria species x treatment	0.00123	4	0.320	0.865
Population x treatment	0.00805	12	0.696	0.756
Bacteria species x population x treatment	0.00547	12	0.473	0.931
Residuals	0.57864	600		

7 Discussion

With this thesis I contribute to the understanding of reproduction and ecological factors affecting it. By conducting two metagenomic studies based on the 16S rRNA gene of the genital microbiomes of the common bedbug (*Cimex lectularius* L.), I characterised the structure and composition of the genital microbiomes that were organ-, sex- and population-specific. Mating changed the structure and composition of the genital microbiomes and these changes were partly due to exchanges of bacterial strains between the sexes, suggesting sexual transmission, and due to invading opportunistic bacteria from the cuticle. In a third study, I therefore investigated the transmission rate of opportunistic bacteria and their effects on the female immune system. In at least ninety percent of the cases, bacteria that had been applied to the copulatory organs of the opposite sex were transmitted during mating, indicating that females regularly face sexually transmitted opportunistic microbes and the related consequences. Bacteria injected into the female paragenital sperm-receiving organ, the mesospermalege, were able to survive for at least 24 hours although the growth was slower within the first six hours. The injection of bacteria but also the injection of ejaculate induced lysozyme-like activity and bacterial growth inhibition in vitro. Against my expectations, these immune responses were not population-specific.

7.1 Genital microbiomes seem to be locally adapted

Reproductive traits are more rapidly evolving than most other traits (Swanson & Vacquier, 2002) and sexual selection has been discussed as the driver of the rapid evolution of reproductive traits. In the last decades, it has been debated that natural and sexual selection can interact (Hamilton, 1990) and that natural selection on its own has the ability to affect reproductive traits (Endler, 1986; Foster & Endler, 1999; Houde, 1997; Hurd, 1998; Hurst et al., 1995; Reinhardt, 2007; Siva-Jothy, 2000).

Discussion

All animals are in constant contact with microbes. Even the reproductive organs (Hickey et al., 2012; Hirsh, 1999; Hupton et al., 2003; Ravel et al., 2011) and ejaculates (Baud et al., 2019; González-Marín et al., 2011; Lombardo & Thorpe, 2000; Skau & Folstad, 2003; Virecoulon et al., 2005) of healthy individuals harbour microbes that might shape reproductive traits via natural selection. In arthropods, most of the bacteria associated with mating belong to the classes Actinobacteria, Alphaproteobacteria, Bacilli, or Gammaproteobacteria (Bellinvia et al., 2020b; Otti, 2015; Perlmutter & Bordenstein, 2020). However, between-individual variation in genital microbiomes is very high, at least in bedbugs (Bellinvia et al., 2020b, 2020a), suggesting that genital microbiomes do not only harbour obligate mutualists.

Genital microbiomes differ between organs even if they are in close proximity as it is the case for the human female reproductive system (Chen et al., 2017) and the reproductive organs of bedbugs (Bellinvia et al., 2020a). Reproductive organs might differ in the accessibility for OM that colonise the organs, for instance due to different openings and organ shapes. Alternatively, the function of the organ might require specific symbionts. In addition to differences between organs, microbiomes are sex-specific as has been shown for whole body homogenates (Valiente Moro et al., 2013), intestinal samples (Haro et al., 2016; Markle et al., 2013), and organs involved in reproduction (Bellinvia et al., 2020b, 2020a; Hupton et al., 2003; Otti et al., 2017). Whether sex-specific microbiomes arise due to different behaviours, functions in the ecosystem, or roles in reproduction remains to be investigated.

Bacterial communities associated with insects vary between collection sites (Adams et al., 2010). This could be explained by environmental bacteria regularly colonising the microbiomes or by conserved differences. Symbionts living in the reproductive organs are potentially constantly exposed to the immune system of their host and therefore under selection to adapt to this special environment as even endosymbionts are prevented from uncontrolled growth by the host's immune system (Login et al., 2011). Furthermore, they should have a high evolutionary potential because of their likely higher rates of mutation, shorter generation times, and larger population sizes compared to their hosts. Therefore, interactions between genital

microbes and their hosts should fulfil both pre-requisites of local adaptation, i.e. selection pressure and evolutionary potential (Gandon & Michalakis, 2002). Indeed, genital microbiomes seem to be locally adapted as shown by ethnicity-dependent vaginal microbiomes (Ravel et al., 2011) and population-specific genital microbiomes of bedbugs (Bellinvia et al., 2020a).

Such differences between populations might be involved in speciation processes if mating with a partner who has a differently composed genital microbiome leads to incompatibilities or reduced reproductive success. Possible reasons for reduced fitness might be stronger immune responses due to different microbiomes (Rowe et al., 2020) or interference of non-adapted microbes with symbionts that help in reproduction. Furthermore, microbes have been shown to modify host signals, produce metabolites and odours (reviewed in Shropshire and Bordenstein, 2016). Furthermore, they induce cytoplasmic incompatibility (Bourtzis et al., 2003), i.e. incompatibility between the gametes of hosts infected with different microbe strains, male killing (Dyson & Hurst, 2004; Hurst et al., 2003; Nakanishi et al., 2008; Zeh & Zeh, 2006), feminisation (Hiroki et al., 2002; Negri et al., 2006; Rigaud & Juchault, 1992; Terry et al., 1999; Weeks et al., 2001), and parthenogenesis (Huigens & Stouthamer, 2003; Kremer et al., 2009; Pannebakker et al., 2005; Pijls et al., 1996). They can also affect mate choice and assortative mating likely results in reproductive isolation and, ultimately, in speciation (Perlmutter & Bordenstein, 2020).

Bacteria in the microbiomes of *Drosophila melanogaster* females interact with their hosts to affect attractiveness as indicated by a line-specific increase or decrease after antibiotic treatment (Arbuthnott et al., 2016). In laboratory mice, bacteria helping the host's digestion increase the attractiveness while antibiotic treatment and the knockout of host receptors for a specific nutrient cause decreased attractiveness (Li et al., 2013). Both studies indicate that bacteria have the ability to modify mating signals. Microbes can produce signals, such as odours, that are used for mate choice (Shropshire & Bordenstein, 2016). For instance, a correlation exists between variations in human skin microbiomes and underarm odour (James et al., 2013), which women use to rate male attractiveness (Havlicek et al., 2005; Saxton et

al., 2008). Arthropods seem to use cuticular compounds to evaluate the infection status of their potential mating partner and choose an uninfected partner (Gilbert & Uetz, 2019).

Additionally, microbes causing cytoplasmic incompatibility might select for avoidance of potential mates that are not compatible based on their microbiome (Perlmutter & Bordenstein, 2020; Shropshire & Bordenstein, 2016). This is the case for two *Drosophila* species which occur sympatrically in a region in Canada. Over 98% of *Drosophila recens* are infected with *Wolbachia* whereas *Drosophila subquinaria* is not infected (Shoemaker et al., 1999). Hybrids of the uninfected *D. subquinaria* females and infected *D. recens* males are inviable but reciprocal crosses produce viable offspring (Shoemaker et al., 1999). Hybrid inviability is mirrored by sympatric *D. subquinaria* females showing higher discrimination against *D. recens* males compared to allopatric females and no discrimination by *D. recens* females (Jaenike et al., 2006). Moreover, spider mite females that are not infected with *Wolbachia* prefer to mate with uninfected males whereas infected females aggregate their offspring (Vala et al., 2004). Despite of being population-specific, genital microbiomes do not seem to cause assortative mating in bedbugs as indicated by similar mating behaviours between coevolved and non-coevolved crosses (see chapter 8.1) but studies on the effect of reproductive success could clarify whether reproductive success depends on the genital microbiomes of both mating partners.

7.2 Mating changes the genital microbiomes

Microbiomes are affected by changes in the environment (Cauci et al., 2002; David et al., 2014; Martínez et al., 2013; Nuriel-Ohayon et al., 2016; Odamaki et al., 2016; Thaiss et al., 2014). Mating is a life-history event that affects all sexually reproducing animals and has been shown to alter vertebrate (Kulkarni & Heeb, 2007; Mändar et al., 2015; White et al., 2010, 2011) and invertebrate (Bellinvia et al., 2020b, 2020a; Otti et al., 2017) genital microbiomes. Interestingly, mating-induced changes seem to be a general pattern across populations (Bellinvia et al., 2020a). Compositional changes can be caused by the invasion of new

microbes and/or the loss of resident microbes. New microbial strains could be found due to three different scenarios which are not mutually exclusive: I) the organ recruits microbes from other organs to fulfil a different function after mating; II) microbes are sexually transmitted or transferred from the cuticle; III) microbes enter genital openings and copulatory wounds. Resident microbes could be lost if they are targeted by an immune response actually intended against microbes or if invading microbes compete for resources. In both cases, the host has to deal with the consequences of a disturbed genital microbiome.

Microbes are sexually transmitted in both vertebrates and invertebrates (reviewed in Knell & Webberley, 2004). In addition to microbes causing sexually transmitted diseases (STM), opportunistic microbes (OM) might be transmitted that can become pathogenic when the host immune system is disturbed (Klainer & Beisel, 1969). Previous studies in insects have reported that bacteria can be sexually transmitted (Miest & Bloch-Qazi, 2008) and that OM colonise the paramere of bedbug males and might be transmitted to females (Reinhardt et al., 2005). Indeed, bacteria are sexually transmitted in both sexes of the common bedbug (Bellinvia et al., 2020b, 2020a, 2020c). Bellinvia et al. (2020b, 2020a) found bacteria in mated individuals that did not occur in the genital microbiomes of virgin individuals of the same sex or the opposite sex, indicating that these bacteria must have been from a different source. Indeed, these bacteria originated from the cuticle (Bellinvia et al., 2020b, 2020a) and should, therefore, be OM instead of bacteria causing sexually transmitted diseases. Furthermore, most transmitted strains varied between populations (Bellinvia et al., 2020a), suggesting that environmental microbes are population-specific. This is in accordance with previous findings that microbes on the filter papers in the housing containers vary between bedbug populations (Otti et al., 2017).

The high transmission rate of bacteria applied to the copulatory organs of males and females (Bellinvia et al., 2020c) suggests that OM are regularly transmitted during mating under natural conditions. OM eventually become pathogenic (Klainer & Beisel, 1969) and increase mortality (Reinhardt et al., 2003). One other major problem caused by OM is

decreased reproductive success. Females might allocate resources to immunity instead of reproduction (Sheldon & Verhulst, 1996; Zuk & Stoehr, 2002), which has been shown to be the case for the great pond snail (Rigby & Jokela, 2000) and the pied flycatcher (Ilmonen et al., 2000). Furthermore, OM decrease sperm motility (Diemer et al., 1996, 2003; Kaur et al., 2010; Prabha et al., 2010), agglutinate sperm (Kaur et al., 2010), and increase sperm mortality (Otti et al., 2013) *in vitro*, consequently lowering reproductive success. In addition, mutualistic microbes in the genital microbiomes might compete with OM over available resources (Li & Stevens, 2012; Mallon et al., 2003). If the abundance of symbionts essential for specific functions of the organ decreases, this might have severe consequences for the host's reproductive success. For instance, in humans, mating decreases the abundance of *Lactobacillus* in the endometrium (Mändar et al., 2015) and lower *Lactobacillus* abundances have been associated with low implantation, pregnancy, and birth rates (Moreno et al., 2016). These threats should impose selection pressure on females to protect themselves and the received sperm from OM. Some of the observed increased immunity in females either prior to or after mating might be a response to such a threat (Harney et al. 2019, Oku et al. 2019, Zhong et al. 2013).

7.3 OM and ejaculate components elicit immune responses in females

Females possess various immunological defence mechanisms, both constitutive and induced (Rolff & Reynolds, 2009; Schmid-Hempel, 2005; Siva-Jothy et al., 2005) that might be used to fight invading STM and OM. In humans, antimicrobial peptides that lyse the membrane of microbes have been found in the reproductive tract (Quayle et al., 1998; Valore et al., 1998). In *Drosophila* females, male courtship song induces the production of immune genes (Immonen & Ritchie, 2012) that protect against a sexually transmitted fungus (Zhong et al., 2013). Even special organs have evolved to protect females from microbes invading during mating, for instance the mesospermalege in bedbugs (Morrow & Arnqvist, 2003; Reinhardt et al., 2003), a paragential immune organ that forms a barrier towards the haemolymph.

Additionally, it shows lysozyme-like activity in anticipation of mating (Siva-Jothy et al., 2019) and in response to mating-associated substances (Bellinvia et al., 2020c), suggesting that the selection pressure for protection from microbes can lead to the evolution of localised immunity and specialised organs.

One key aspect of ecological immunology is that immunity is costly, leading to trade-offs with traits like reproduction or survival due to a restricted resource budget (Boots et al., 2013; Sheldon & Verhulst, 1996). Often, mating is associated with reduced immunity due to the allocation of resources to reproduction instead of immune responses (Sheldon & Verhulst, 1996; Zuk & Stoehr, 2002). Thus, physical barriers preventing the host from a systemic infection might be helpful to optimise the benefits at a relatively low cost. Hence, the physical barrier function of the mesospermalege becomes especially important when sperm have started migrating through the haemolymph towards the ovaries and the resources need to be allocated to reproduction instead of immunity. Such a shift in resources is indicated by the slow growth rate of bacteria inside the mesospermalege within the first six hours, i.e. when sperm are still inside the mesospermalege or travelling to the ovaries and sperm storage organs, followed by the fast growth within the next 18 hours, i.e. when sperm have reached the ovaries (Bellinvia et al., 2020c). Potential mechanisms to mop up the bacteria seem to be lysozyme-like and antimicrobial activity, which are induced by any liquid injected into the mesospermalege (Bellinvia et al., 2020c).

That sexually reproducing organisms constantly face microbes, be it their genital microbiomes or sexually transmitted microbes, provides the basis for adaptations to evolve. If microbes vary between populations, local adaptation of the host to the genital microbiomes and sexually transmitted microbes might lead to population-specific immune responses For instance, immunity could be affected by the prevalence and pathogenicity of STM and OM and by the composition of the genital microbiome. Such population-specific immune responses might manifest in different levels of immunity or different immunological substances used.

Empirical studies have shown that immune activity indeed varies between populations, for instance in freshwater shrimps (Cornet et al., 2009) and bumblebees (Brunner et al., 2013) and might be correlated with parasite prevalence. In contrast to these studies, Bellinvia et al. (2020c) did not find evidence for population-specific levels of lysozyme-like activity or growth inhibition by antimicrobial peptides in bedbugs although the genital microbiomes differ between populations (Bellinvia et al., 2020a), providing the basis for local adaptation of immunity.

Immune responses after mating are potentially not solely caused by sexually transmitted bacteria: in addition, they could be used by the female to exert post-copulatory selection. Polyandry has direct and indirect benefits for the female, the latter being higher offspring diversity, attractiveness, and viability (Birkhead & Pizzari, 2002). If females can choose gametes based on specific characteristics this benefit might be even higher. In accordance with the idea of post-copulatory selection, females have been shown to induce mechanisms that affect sperm physiology and motility (Kekäläinen & Evans, 2018; Yoshida et al., 2008). However, research on cryptic female choice has largely been neglected so far although it might be older than pre-copulatory selection (Parker, 2014).

Bedbug females cannot exert pre-copulatory mate choice since fully-fed females cannot prevent mating (Reinhardt et al., 2009). Therefore, post-copulatory cryptic female choice is a potential mechanism that enables the female to maximise fitness by choosing the sperm from males of better quality over the sperm from low quality males or even to choose spermatozoa of better quality (Eberhard, 1996). One mechanism that could be used by the female to exert cryptic female choice might be immune responses that target non-desired sperm. The pre-requisites of such choice mechanisms are that spermatozoa vary in resistance against this choice mechanism and that variation correlates with male quality, good genes, or compatibility between male and female.

In accordance with the idea of immune responses functioning as cryptic female choice mechanisms, Bellinvia et al. (2020c) found a higher lysozyme-like activity when females were injected with a liquid, likely representing ejaculate transfer, compared to when females were

pricked with a sterile needle. Whether the induction of lysozyme-like activity has evolved because of mating-associated bacteria or cryptic female choice is difficult to distinguish. The higher activity when females were injected with seminal fluid from males indicates that females react to male components. However, further studies are required to investigate whether lysozyme is indeed used as a cryptic choice mechanism since we have no evidence for cryptic female choice in the mesospermalege of bedbugs so far. If lysozyme is used for cryptic female choice, Bellinvia et al. (2020c) provide another example of a potential interaction between sexual and natural selection. On the other hand, commercially available lysozyme has been shown to protect bedbug sperm from environmental microbes *in vitro* (Otti et al., 2013), indicating that lysozyme-like activity is unlikely to be part of the female's repertoire of cryptic female choice mechanisms.

7.4 Conclusions

This thesis provides important new results for understanding reproduction and environmental factors affecting it. Apart from human studies, this is the first observation of genital microbiomes potentially being locally adapted and indicating the potential of these microbes to be involved in speciation, for instance via cytoplasmic incompatibility. Characterising the genital microbiomes has shown that the bacterial communities are subject to mating-induced changes and that opportunistic microbes seem to be transmitted during mating. This finding suggests that sexually reproducing organisms are regularly exposed to invading microbes and therefore, they should be under natural selection to defend themselves against infections. That is especially important given the large growth rate of opportunistic microbes inside the female sperm receiving organ that I found. This thesis has identified two possible immunologically active substances that might be the main actors in the defence against mating-associated microbes, providing evidence for the interaction of mating and immunity in bedbugs. Since these defences do not seem to be population-specific although OM likely differ between populations, I conclude that either the hosts are not locally adapted

or that the costs of an infection are more likely related to invading microbes per se than to the pathogenicity of specific microbes. In conclusion, this thesis gives an overview of the potential of host-microbe interactions to affect reproduction. I have laid the foundations for a model system in which many aspects of reproduction can be manipulated and experimentally tested, including the genital microbiome as part of an interdependent metaorganism.

7.5 Future directions

The findings of this thesis raise several interesting research questions that should be investigated further:

I. Does the genital microbiome colonisation depend on environmental microbes or host genotype?

My second metagenomic study revealed that the genital microbiomes are population-specific (Bellinvia et al., 2020a). Abiotic factors, such as pH or temperature will likely determine which part of the environmental microbe community can colonise the reproductive organs. Furthermore, colonisation by similar microbes can be caused by monogamy (reviewed in Rowe et al., 2020), similarities in behaviour, ecological niches, and diets, by a shared environment, and, last but not least, by host-genotype effects (reviewed in Shropshire and Bordenstein, 2016). To investigate whether genital microbiomes adapt to environmental microbiomes and whether the host selects for adaptations in the microbes that colonise the host, I would recommend conducting a study that combines experimental evolution with microbe exposure. Isogenic lines of different genotypes could be exposed to different communities of environmental microbes. After every third generation, their genital microbiomes would be sequenced because arthropods have the potential to adapt to symbionts within less than ten generations (Martinez et al., 2016). If genital microbiomes differ between isogenic lines exposed to different microbes, it is likely that environmental microbes shape genital microbiomes. If genital microbiomes exposed to the same environmental bacteria differ

between genotypes but not within genotypes, this would suggest that microbiomes are adapted to the genotype of their host. However, the resident microbiome likely determines which invading microbes can successfully colonise the genital microbiomes (Perlmutter & Bordenstein, 2020). Therefore, differences in the genital microbiomes investigated after experimental evolution with the same environmental bacteria do not necessarily indicate host genotype effects. To disentangle the effects of host genotype and microbiome, it would be necessary to include a group in the described experiment that is treated with antibiotics before the bacterial exposure.

II. Do genital microbiomes affect reproductive success?

Population-specific genital microbiomes could be a first step in the direction of speciation since microbes have the potential to cause cytoplasmic incompatibility and assortative mating, for instance by modifying or producing signals that are used for mate choice or by inducing cytoplasmic incompatibility (reviewed in Shropshire and Bordenstein, 2016). Although bedbugs do not seem to reject mates from other populations, mating partners from different populations might be incompatible due to differences in the genital microbiomes, or post-copulatory choice in the form of cryptic female choice might be used by females to choose sperm from compatible males. As a first step, the effect of mating with coevolved vs non-coevolved males on offspring numbers and hatching success should be investigated under natural conditions and after antibiotic treatment. If microbe-free bedbugs differ in reproductive success, this would hint at host genotype-effects. If this difference is altered in non-treated bedbugs, this would indicate that microbes can affect reproductive traits. With the experimental evolution approach described in question I., it would also be possible to compare the reproductive success between coevolved and non-coevolved lines to determine whether adaptation of the genital microbiome can affect reproductive success.

III. Are mating-induced changes reversible?

Genital microbiomes are changed by mating (Bellinvia et al., 2020b, 2020a; Kulkarni & Heeb, 2007; Mändar et al., 2015; Otti et al., 2017; White et al., 2010, 2011), which might be critical for reproductive success. It would be interesting to investigate whether females can restore the original microbiome and how long this takes. To do so, another metagenomic study could be conducted and the genital microbiomes before mating and at different time points after mating compared. For this, I would suggest the following time points: I) within one hour after mating, II) after six hours, III) after 12 hours, IV) after 24 hours, V) after 48 hours, VI) after 72 hours. With this sampling procedure it is possible to capture the changes I) immediately after mating, II) after the slow growth phase of invading bacteria inside the mesospermalege, III) after the sperm have reached the ovaries and invading bacteria have grown faster, IV) after antimicrobial substances are produced, and V) and VI) after the immune system might have restored the initial microbiome composition. In case that the restoration of the microbiome takes several days, it would be interesting to compare offspring numbers and hatching success between the time before and after normalisation of the genital microbiome to find out whether a disturbance has an effect on reproductive success.

IV. How does mating trigger immune responses of the female?

The injection of a liquid into the mesospermalege induced lysozyme-like activity (Bellinvia et al., 2020c). In contrast, there was no difference in lysozyme-like activity between females that had received a sterile prick and untreated females. To investigate whether a stretch receptor triggers immunity, one would have to artificially stretch the mesospermalege, for instance with air and compare the lysozyme-like activity induced by different volumes. A similar method has previously been used to mimic the feeding-induced abdomen distension (Wintle & Reinhardt, 2008).

8 Supplement

8.1 Influence of coevolution with environmental bacteria on mating behaviour

8.1.1 Introduction

To determine whether population-specific genital microbiomes (Bellinvia et al., 2020a) can affect pre-copulatory (as reviewed in Shropshire and Bordenstein, 2016) and copulatory behaviour, I analysed the mating behaviour of coevolved and non-coevolved bedbugs (*Cimex lectularius* L.). Bedbug males determine the mating rate because fed females cannot resist mating (Reinhardt et al., 2009). Males have been reported to mount females without showing any courtship behaviour (Carayon, 1966). However, they do not necessarily mate immediately after discovering a female. I have observed many males that initiated mating after having touched or walked over the female several times. After mounting, the male expands its needle-like intromittent organ and inserts it through the cuticle into the female paragenital sperm-receiving organ, the mesospermalege, to transfer the ejaculate. Before the male inserts his intromittent organ, females usually shake their body vigorously, potentially to resist mating (Reinhardt et al., 2009). This shaking stops after the male has successfully introduced its intromittent organ. I therefore considered the number of contacts and the time until mounting as pre-copulatory behaviour of the male and the time between mounting and successful intromission as pre-copulatory behaviour of the female. In addition, I measured copulatory behaviour via copulation duration, which should be rather determined by the male.

8.1.2 Methods

8.1.2.1 Mating scheme

Bedbugs were taken from 4 different populations, resulting in 8 mating pairs: AxA (male x female), CxC, AxC, CxA, DxD, BxB, DxB, BxD. Mating pairs from the same population were referred to as “coevolved”, pairs with male and female originating from different populations as “non-coevolved”. All bedbugs were kept in a climate chamber at 26±1°C, at a humidity of 70% and a light cycle of 12L:12D. After eclosion, all individuals were kept in sex-specific groups.

They were fed weekly four times with human blood before the start of the experiment. The fourth blood meal was given on the specific day of the experiment, because freshly-fed females cannot resist mating (Reinhardt et al., 2009). To start with, I placed virgin males and females from the given populations (N=30 for each mating combination), in a cleaned 70mm glass petri dish with new filter paper. To separate male and female, I isolated them with *Drosophila* vials. The bedbugs were left to acclimatise to the new environment for 2 minutes, after which I removed the vials.

8.1.2.2 Measurements

I measured the number of contacts before mounting (contacts), the time until mounting (latency) the time between mounting and insertion of the paramere (struggle), and the time from insertion until removal of the paramere (copulation duration). The number of contacts was counted as the number of times the two sexes had contact with any body part except for the legs until the male mounted the female. A new contact was only counted if the individuals had not touched each other after the previous contact. For the analysis the number of contacts was divided by the time until mounting. Latency was defined as the time from the release of the male and female from their *Drosophila* vials until the male mounted the female. I measured struggle as the time between the male mounts the female and the time when both sexes did not shake anymore, which served as a good proxy for the intromission of the paramere (Reinhardt et al., 2009). To determine copulation duration, I took the time between the insertion of the paramere and the time when the abdomen of the male was not attached to the female anymore. Times were measured to the nearest millisecond.

8.1.2.3 Statistical analysis

All statistical analyses were performed in R (version 3.4.0, R Core Team, 2017). The effect of coevolutionary status on the number of contacts was analysed with a generalised linear model with quasi-Poisson error structure and the fixed effects male population and coevolutionary

status and their interaction term. Three outliers were removed before fitting the model. To analyse the effect of coevolutionary status on the latency and copulation duration, I fitted a generalised linear model with gaussian error structure and the fixed effects male population and coevolutionary status and their interaction term. Since the residuals were not normally distributed, I used Box-Cox transformation. For the analysis of latency, I excluded one outlier. The effect of coevolutionary status on struggle was determined with a generalised linear model with gaussian error structure and the fixed effects female population and coevolutionary status and their interaction term. Since the residuals were not normally distributed, I used Box-Cox transformation.

8.1.3 Results and discussion

The number of contacts did not differ between coevolved and non-coevolved crosses ($F_{1,229}$=1.380, p=0.24) or between male populations ($F_{3,229}$=2.560, p=0.05) but there was a significant interaction between male population and coevolutionary status ($F_{3,229}$=2.859, p=0.04)(Fig. S1), suggesting that males from different populations reacted differently to the coevolutionary status of their mate. However, the number of contacts differed only slightly, indicating that this measure is probably not suitable for detecting effects on pre-copulatory behaviour.

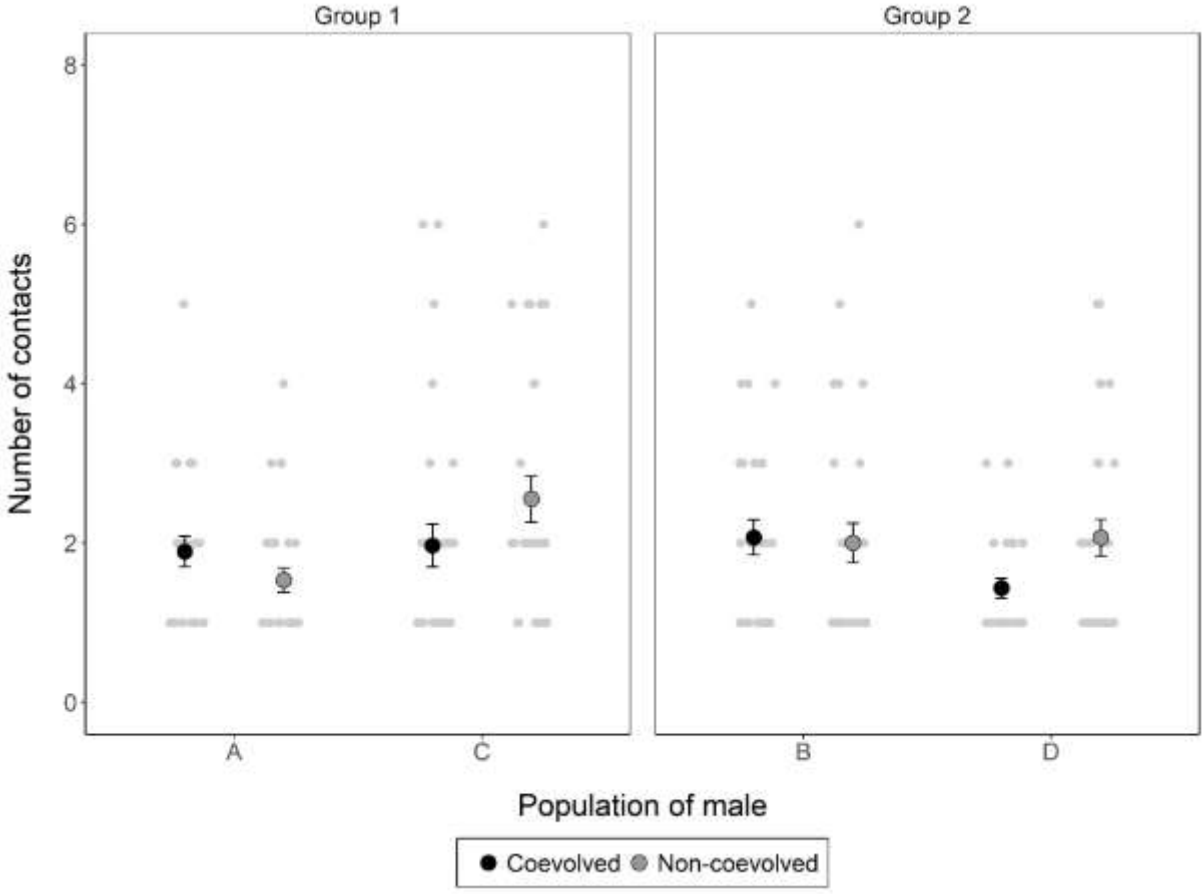

Figure S1 Number of contacts between male and female before the male mounted the female. Given are mean and standard error of the mean for both coevolutionary status and all individual data points.

There was no difference in the latency between coevolved and non-coevolved crosses ($F_{1,231}$=3.324, p=0.07) or between male population (Fig. S2). However, I found a significant interaction between male population and coevolutionary status ($F_{3,231}$=3.430, p=0.02) and male population significantly affected latency ($F_{3,231}$=4.132, p=0.01)(Fig. S2). This result indicates that pre-copulatory behaviour can be affected by coevolutionary status of the mate in some populations, potentially via cuticular compounds altered by microbes (Gilbert & Uetz, 2019).

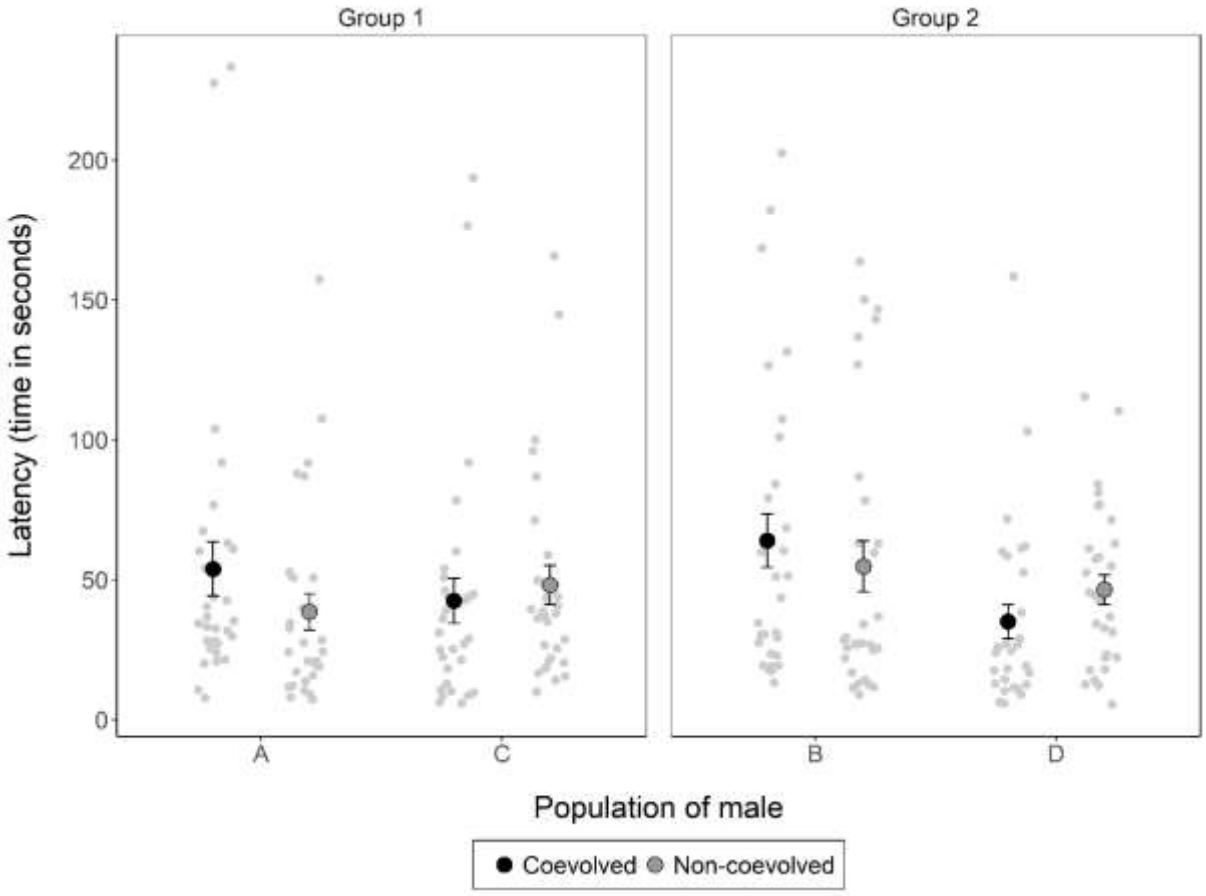

Figure S2 Latency, i.e. time until the male mounted the female. Given are mean and standard error of the mean for both coevolutionary status and all individual data points.

Struggle, i.e. the time between mounting and insertion of the male intromittent organ, was not affected by coevolutionary status ($F_{1,232}$=0.0002, p=0.99), female population ($F_{3,232}$=0.469, p=0.70) and there was no interaction between female population and coevolutionary status ($F_{3,232}$=0.305, p=0.82)(Fig. S3). Therefore, genital microbiomes do not seem to alter the pre-copulatory behaviour of bedbug females.

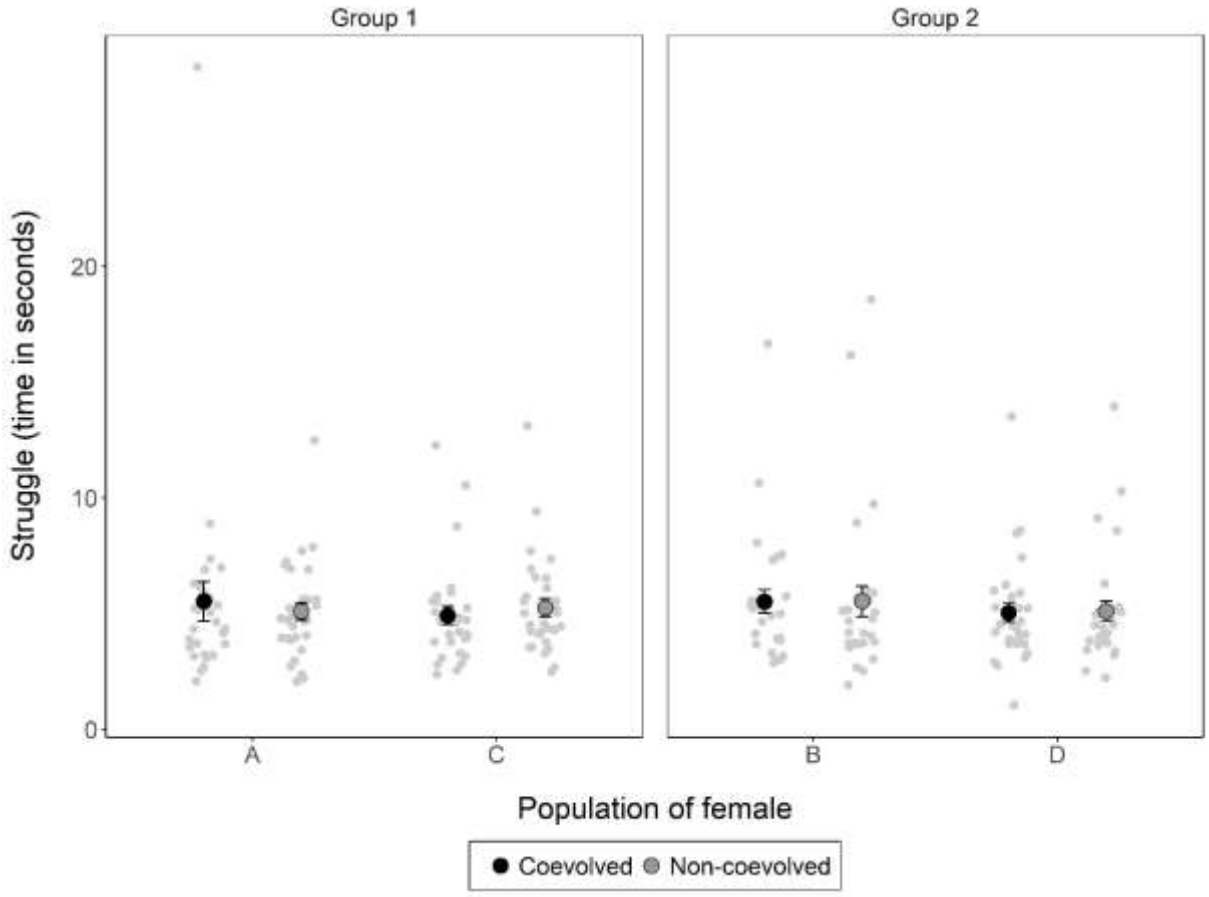

Figure S3 Struggle, i.e. time between the male mounted the female and the successful insertion of the male intromittent organ. Given are mean and standard error of the mean for both coevolutionary status and all individual data points.

Coevolutionary status did not affect mating duration ($F_{1,232}$=0.001, p=0.98) and coevolutionary status did not interact with male population ($F_{3,232}$=0.111, p=0.95)(Fig. S4). However, mating durations differed significantly between male populations ($F_{3,232}$=14.684, p<0.001)(Fig. S4). Our results show that it unlikely that males adjust their reproductive investment to the coevolutionary status of their mating partner as copulation duration is positively correlated with ejaculate transfer (Siva-Jothy & Stutt, 2003).

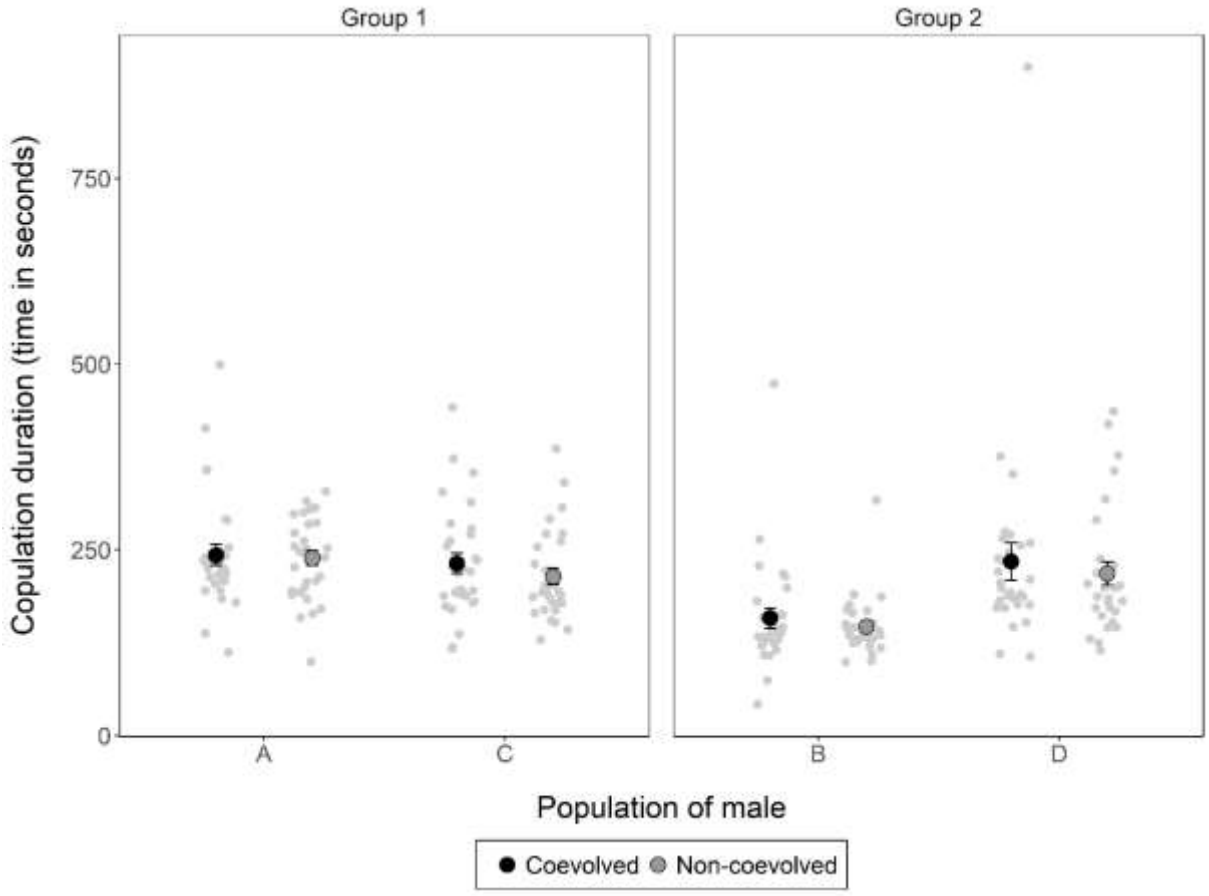

Figure S4 Copulation duration of bedbugs from coevolved or non-coevolved populations. Given are mean and standard error of the mean for both coevolutionary status and all individual data points.

8.2 Influence of bacteria concentration on female immunity

8.2.1 Introduction

Each mating is most likely associated with varying numbers of invading microbes if these are opportunistic and bacteria concentration can affect virulence, at least in the case of entomopathogenic bacteria (Pietri & Liang, 2018). Since immunity is associated with costs like autoimmunity (Schmid-Hempel, 2005) or reduced reproductive success due to allocation of resources away from reproduction (Sheldon & Verhulst, 1996; Zuk & Stoehr, 2002), it might be beneficial for the female to react to mating-associated microbes in a dose-dependent way. Furthermore, immunity to protect sperm might be related to the microbe concentration because *Escherichia coli* has been shown to reduce sperm motility only at high concentrations (Diemer et al., 1996). To determine whether the immune response depends on the number of bacteria entering the female body during mating, I measured the lysozyme-like activity in the mesospermalege of female bedbugs (*Cimex lectularius* L.) after mating and a bacteria prick with bacteria solutions of different concentrations.

8.2.2 Methods

8.2.2.1 Bedbugs

All bedbugs from population A were kept in a climate chamber at 26±1°C, at a humidity of 70% and a light cycle of 12L:12D. After eclosion, all virgin individuals were kept in sex-specific groups. They were fed weekly two times with human blood before the start of the experiment. Females were fed another time on the day of mating, because freshly-fed females cannot resist mating (Reinhardt et al., 2009).

8.2.2.2 Mating and bacteria prick

To surface-sterilise the males, their last abdominal segment of males was dipped into Kohrsolin (20%) for 20 seconds. The males were transferred to a petri dish with filter paper and a female was placed in the petri dish. The bedbugs were allowed to mate for 60 seconds,

to standardise the transfer of sperm and seminal fluid. Male and female were separated with forceps and the female was pricked with a minutia, which had been dipped into a bacteria solution of a specific concentration. The female was then transferred to a single Eppendorf tube (1.5 ml) containing a filter paper disc.

8.2.2.3 Treatments

Arthrobacter globiformis were plated out on LB agar plates and incubated at 30°C for 24h. One single colony was picked with an inoculation loop and transferred to a Falcon tube filled with 5ml LB medium. The tube was incubated at 30°C and shaken at 200 rpm for 24h. The overnight culture was vortexed and 1ml of the culture was transferred to an Eppendorf tube. The tubes were centrifuged at 2350g for 5 minutes. The supernatant was discarded, and the bacterial pellet was resuspended in 1000 µl. I serially diluted 100 µl of the solution with 900 µl PBS until a dilution of 1:1000. After mating, the prick was applied following a pseudo-random design. I dipped the minutia in the desired solution and pierced the ectospermalege with a slight angle. Only the tip of the minutia entered the mesospermalege. Each treatment was applied to 22 ± 1 (mean ± SD) females.

8.2.2.4 Sample collection and LLA measurement

24h and 48h after the bacteria prick, respectively, the females were dissected and the mesospermalege was photographed with a camera (LEICA DFC 290) attached to the dissection microscope (LEICA M165 C). Using forceps, the mesospermalege was homogenised in a 1.5 mm hole on a 5 ml agar plate containing lyophilised *Micrococcus lysodeikticus* (ATCC No. 4698, Sigma-Aldrich, Hamburg, Germany). Lysozyme assay plates were incubated at 30° C for 48 h and photographed with a Gel iX Imager (software: INTAS GDS, INTAS Science Imaging Instruments GmbH, Göttingen, Germany). The clearance zone was measured in ImageJ (version 1.51k) and converted into units of LLA, using a calibration

curve (see Supplement of Bellinvia et al., 2020c). Total LLA represents the activity per sample, while specific LLA refers to the activity per microliter.

8.2.2.5 Statistical analysis

The statistical analysis was performed in R (version 3.4.0, R Core Team, 2017). The effect of the bacteria concentration used to stab the female mesospermalege on lysozyme-like activity was analysed with a generalised linear model with gaussian error structure and the fixed effects time after dissection and treatment and their interaction term. Since the residuals were not normally distributed, I used Box-Cox transformation.

8.2.3 Results and discussion

LLA did not differ between the mesospermaleges of females stabbed with different bacteria concentrations ($F_{4,209}$=1.342, p=0.26)(Fig. S5). However, the time between treatment and dissection significantly interacted with the treatment ($F_{4,209}$=3.179, p=0.01). This indicates that females do not adjust LLA to the number of bacteria invading their mesospermalege during mating. The similar LLA level between sterile stab and bacteria after 24 hours suggests that copulatory wounding might trigger LLA instead of the invading bacteria. Since every copulatory wound increases the risk of females to be infected, it might be less costly and faster to induce an immune response upon wounding instead of assessing the number of bacteria before inducing a reaction.

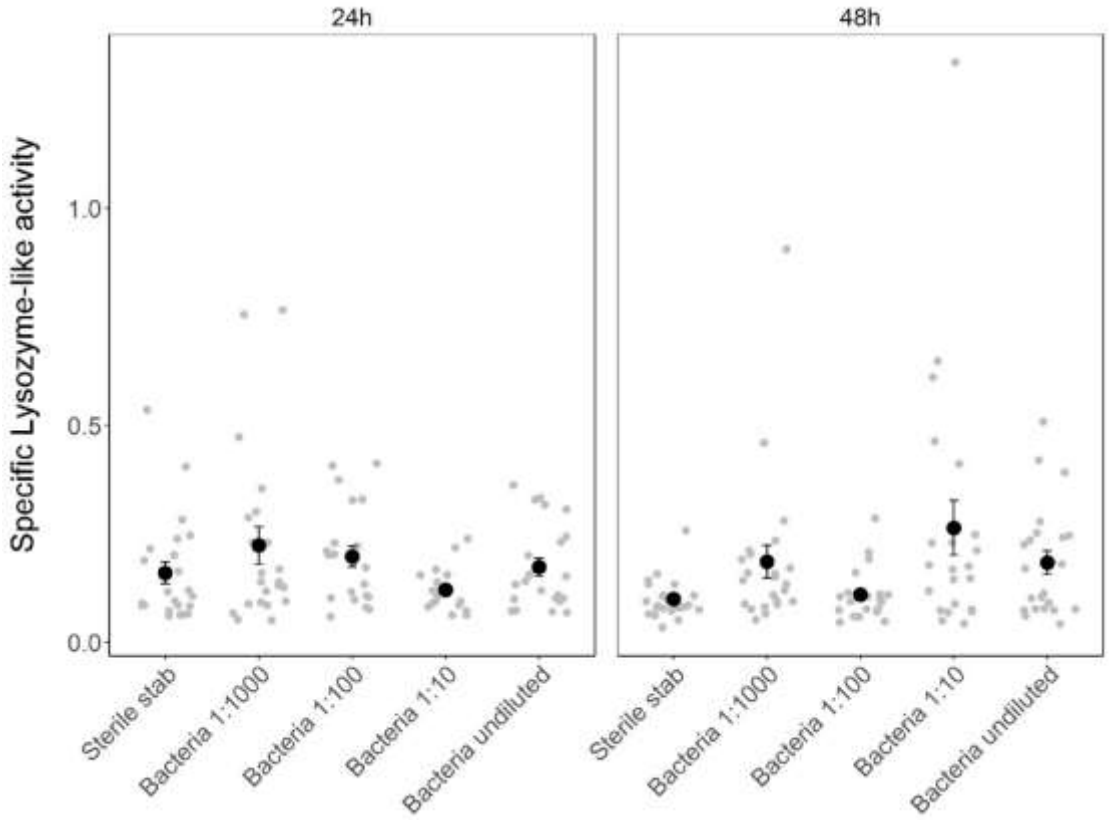

Figure S5 Lysozyme-like activity in the female mesospermalege in response to a sterile stab or stabs with different concentrations of bacteria (*Arthrobacter globiformis*) corrected for the volume of the mesospermalege. Given are mean and standard error of the mean (black points) and all individual data points (grey points).

9 References

Adamo, S. A., Kovalko, I., Easy, R. H., & Stoltz, D. (2014). A viral aphrodisiac in the cricket Gryllus texensis. *Journal of Experimental Biology*, *217*(11), 1970–1976. https://doi.org/10.1242/jeb.103408

Adams, A. S., Adams, S. M., Currie, C. R., Gillette, N. E., & Raffa, K. F. (2010). Geographic variation in bacterial communities associated with the red turpentine beetle (Coleoptera: Curculionidae). *Environmental Entomology*, *39*(2), 406–414. https://doi.org/10.1603/en09221

Akman, L., Yamashita, A., Watanabe, H., Oshima, K., Shiba, T., Hattori, M., & Aksoy, S. (2002). Genome sequence of the endocellular obligate symbiont of tsetse flies, *Wigglesworthia glossinidia*. *Nature Genetics*, *32*(3), 402–407. https://doi.org/10.1038/ng986

Arbuthnott, D., Levin, T. C., & Promislow, D. E. L. (2016). The impacts of *Wolbachia* and the microbiome on mate choice in *Drosophila melanogaster*. *Journal of Evolutionary Biology*, *29*(2), 461–468. https://doi.org/10.1111/jeb.12788

Arnqvist, G., & Rowe, L. (2005). *Sexual conflict*. Princeton, NJ: Princeton University Press.

Bateman, A. J. (1948). Intra-sexual selection in *Drosophila*. *Heredity*, *2*(3), 349–368. https://doi.org/10.1038/hdy.1948.21

Baud, D., Pattaroni, C., Vulliemoz, N., Castella, V., Marsland, B. J., & Stojanov, M. (2019). Sperm microbiota and its impact on semen parameters. *Frontiers in Microbiology*, *10*, 234. https://doi.org/10.3389/fmicb.2019.00234

Bellinvia, S., Johnston, P. R., Mbedi, S., & Otti, O. (2020a). Mating changes the genital microbiome in both sexes of the common bedbug *Cimex lectularius* across populations. *Proceedings of the Royal Society B: Biological Sciences*, *287*(1926), 20200302. https://doi.org/10.1098/rspb.2020.0302

Bellinvia, S., Johnston, P. R., Reinhardt, K., & Otti, O. (2020b). Bacterial communities of the reproductive organs of virgin and mated common bedbugs, *Cimex lectularius*. *Ecological Entomology*, *45*(1), 142–154. https://doi.org/10.1111/een.12784

Bellinvia, S., Spachtholz, A., Borgwardt, I., Schauer, B., & Otti, O. (2020c). Female immunity in response to sexually transmitted opportunistic bacteria in the common bedbug Cimex lectularius. *Journal of Insect Physiology*, *123*, 104048. https://doi.org/10.1016/j.jinsphys.2020.104048

Birkhead, T. R., & Pizzari, T. (2002). Postcopulatory sexual selection. *Nature Reviews Genetics*, *3*(4), 262–273. https://doi.org/10.1038/nrg774

References

Blows, M. W. (2002). Interaction between natural and sexual selection during the evolution of mate recognition. *Proceedings of the Royal Society B: Biological Sciences*, *269*(1496), 1113–1118. https://doi.org/10.1098/rspb.2002.2002

Boots, M., Donnelly, R., & White, A. (2013). Optimal immune defence in the light of variation in lifespan. *Parasite Immunology*, *35*(11), 331–338. https://doi.org/10.1111/pim.12055

Boris, S., Suárez, J. E., Vázquez, F., & Barbés, C. (1998). Adherence of human vaginal lactobacilli to vaginal epithelial cells and interaction with uropathogens. *Infection and Immunity*, *66*(5), 1985–1989. https://doi.org/0.1128/IAI.66.5.1985-1989.1998

Boucias, D. G., & Pendland, J. C. (2012). *Principles of insect pathology*. Boston, MA: Springer.

Bourtzis, K., Braig, H. R., & Karr, T. L. (2003). Cytoplasmic incompatibility. In K. Bourtzis & T. Miller (Eds.), *Insect Symbiosis* (pp. 217–246). Boca Raton, FL: CRC Press.

Braquart-Varnier, C., Altinli, M., Pigeault, R., Chevalier, F. D., Grève, P., Bouchon, D., & Sicard, M. (2015). The mutualistic side of *Wolbachia*-isopod interactions: *Wolbachia* mediated protection against pathogenic intracellular bacteria. *Frontiers in Microbiology*, *6*, 1388. https://doi.org/10.3389/fmicb.2015.01388

Bright, M., & Bulgheresi, S. (2010). A complex journey: transmission of microbial symbionts. *Nature Reviews Microbiology*, *8*(3), 218–230. https://doi.org/10.1038/nrmicro2262

Brunner, F. S., Schmid-Hempel, P., & Barribeau, S. M. (2013). Immune gene expression in *Bombus terrestris*: signatures of infection despite strong variation among populations, colonies, and sister workers. *PLoS ONE*, *8*(7), e68181. https://doi.org/10.1371/journal.pone.0068181

Burkhardt, F. (Ed.). (2008). *Origins: selected letters of Charles Darwin, 1822-1859* (Anniversary Edition). Cambridge University Press.

Carayon, J. (1966). Traumatic insemination and the paragenital system. In R. Usinger (Ed.), *Monograph of Cimicidae (Hemiptera-Heteroptera)* (pp. 81–166). Entomological Society of America.

Cauci, S., Driussi, S., De Santo, D., Penacchioni, P., Iannicelli, T., Lanzafame, P., De Seta, F., Quadrifoglio, F., De Aloysio, D., & Guaschino, S. (2002). Prevalence of bacterial vaginosis and vaginal flora changes in peri- and postmenopausal women. *Journal of Clinical Microbiology*, *40*(6), 2147–2152. https://doi.org/10.1128/JCM.40.6.2147-2152.2002

Chen, C., Song, X., Wei, W., Zhong, H., Dai, J., Lan, Z., Li, F., Yu, X., Feng, Q., Wang, Z., Xie, H., Chen, X., Zeng, C., Wen, B., Zeng, L., Du, H., Tang, H., Xu, C., Xia, Y., Xia, H., Yang, H., Wang, J., Wang, J., Madsen, L., Brix, S., Kristiansen, K., Xu, X., Li, J., Wu, R., & Jia, H. (2017). The microbiota continuum along the female reproductive tract and its relation to uterine-related diseases. *Nature Communications*, *8*, 875.

https://doi.org/10.1038/s41467-017-00901-0

Clutton-Brock, T. H., & Isvaran, K. (2006). Paternity loss in contrasting mammalian societies. *Biology Letters*, *2*(4), 513–516. https://doi.org/10.1098/rsbl.2006.0531

Cornet, S., Biard, C., & Moret, Y. (2009). Variation in immune defence among populations of *Gammarus pulex* (Crustacea: Amphipoda). *Oecologia*, *159*(2), 257–269. https://doi.org/10.1007/s00442-008-1211-y

Cory, J. S. (2015). Insect virus transmission: different routes to persistence. *Current Opinion in Insect Science*, *8*, 130–135. https://doi.org/10.1016/j.cois.2015.01.007

Dale, C., & Moran, N. A. (2006). Molecular interactions between bacterial symbionts and their hosts. *Cell*, *126*, 453–465. https://doi.org/10.1016/j.cell.2006.07.014

Darwin, C. (1859). *On the origin of species by means of natural selection*. London, UK: John Murray.

David, L. A., Maurice, C. F., Carmody, R. N., Gootenberg, D. B., Button, J. E., Wolfe, B. E., Ling, A. V., Devlin, A. S., Varma, Y., Fischbach, M. A., Biddinger, S. B., Dutton, R. J., & Turnbaugh, P. J. (2014). Diet rapidly and reproducibly alters the human gut microbiome. *Nature*, *505*(7484), 559–563. https://doi.org/10.1038/nature12820

Degnan, P. H., & Ochman, H. (2011). Illumina-based analysis of microbial community diversity. *The ISME Journal*, *6*(1), 183–194. https://doi.org/10.1038/ismej.2011.74

Diemer, T., Huwe, P., Ludwig, M., Schroeder-Printzen, I., Michelmann, H. W., Schiefer, H. G., & Weidner, W. (2003). Influence of autogenous leucocytes and *Escherichia coli* on sperm motility parameters *in vitro*. *Andrologia*, *35*(2), 100–105. https://doi.org/10.1046/j.1439-0272.2003.00523.x

Diemer, T., Weidner, W., Michelmann, H. W., Schiefer, H. G., Rovan, E., & Mayer, F. (1996). Influence of *Escherichia coli* on motility parameters of human spermatozoa in vitro. *International Journal of Andrology*, *19*(5), 271–277. https://doi.org/10.1111/j.1365-2605.1996.tb00475.x

Douglas, A. E. (1998). Nutritional interactions in insect-microbial symbioses: aphids and their symbiotic bacteria *Buchnera*. *Annual Review of Entomology*, *43*(1), 17–37. https://doi.org/10.1146/annurev.ento.43.1.17

Dufva, R. (1996). Sympatric and allopatric combinations of hen fleas and great tits: a test of the local adaptation hypothesis. *Journal of Evolutionary Biology*, *9*(4), 505–510. https://doi.org/10.1046/j.1420-9101.1996.9040505.x

Dyson, E. A., & Hurst, G. D. D. (2004). Persistence of an extreme sex-ratio bias in a natural population. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(17), 6520–6523. https://doi.org/10.1073/pnas.0304068101

References

Eberhard, W. G. (1996). *Female control: sexual selection by cryptic female choice*. Princeton, NJ: Princeton University Press.

Ebert, D. (2013). The epidemiology and evolution of symbionts with mixed-mode transmission. *Annual Review of Ecology, Evolution, and Systematics*, *44*(1), 623–643. https://doi.org/10.1146/annurev-ecolsys-032513-100555

Edström, A. M. L., Malm, J., Frohm, B., Martellini, J. A., Giwercman, A., Mörgelin, M., Cole, A. M., & Sørensen, O. E. (2008). The major bactericidal activity of human seminal plasma is zinc-dependent and derived from fragmentation of the semenogelins. *The Journal of Immunology*, *181*(5), 3413–3421. https://doi.org/10.4049/jimmunol.181.5.3413

Eley, A., Pacey, A. A., Galdiero, M., Galdiero, M., & Galdiero, F. (2005). Can *Chlamydia trachomatis* directly damage your sperm ? *The Lancet Infectious Diseases*, *5*(1), 53–57. https://doi.org/10.1016/S1473-3099(04)01254-X

Endler, J. A. (1986). *Natural selection in the wild* (21st ed.). Princeton, NJ: Princeton University Press.

Engelstädter, J., & Hurst, G. D. (2009). The ecology and evolution of microbes that manipulate host reproduction. *Annual Review of Ecology, Evolution, and Systematics*, *40*, 127–149. https://doi.org/10.1146/annurev.ecolsys.110308.120206

Firman, R. C., Gasparini, C., Manier, M. K., & Pizzari, T. (2017). Postmating female control: 20 years of cryptic female choice. *Trends in Ecology and Evolution*, *32*(5), 368–382. https://doi.org/10.1016/j.tree.2017.02.010

Foster, S. A., & Endler, J. A. (Eds.). (1999). *Geographic variation in behaviour*. New York, NY: Oxford University Press.

Gajer, P., Brotman, R. M., Bai, G., Sakamoto, J., Schütte, U. M. E., Zhong, X., Koenig, S. S. K., Fu, L., Ma, Z., Zhou, X., Abdo, Z., Forney, L. J., & Ravel, J. (2012). Temporal dynamics of the human vaginal microbiota. *Science Translational Medicine*, *4*(132), 132ra52. https://doi.org/10.1126/scitranslmed.3003605

Galdiero, F., Gorga, F., Bentivoglio, C., Mancuso, R., Galdiero, E., & Tufano, M. A. (1988). The action of LPS porins and peptidoglycan fragments on human spermatozoa. *Infection*, *16*(6), 349–353.

Gandon, S., & Michalakis, Y. (2002). Local adaptation, evolutionary potential and host-parasite coevolution: interactions between migration, mutation, population size and generation time. *Journal of Evolutionary Biology*, *15*(3), 451–462. https://doi.org/10.1046/j.1420-9101.2002.00402.x

Gendrin, M., Welchman, D. P., Poidevin, M., Hervé, M., & Lemaitre, B. (2009). Long-range activation of systemic immunity through peptidoglycan diffusion in *Drosophila*. *PLoS Pathogens*, *5*(12), e1000694. https://doi.org/10.1371/journal.ppat.1000694

Gilbert, R., & Uetz, G. W. (2019). Male chemical cues as reliable indicators of infection in the wolf spider *Schizocosa ocreata*. *Ethology*, *125*(4), 177–183. https://doi.org/10.1111/eth.12841

González-Marín, C., Roy, R., López-Fernández, C., Diez, B., Carabaño, M. J., Fernández, J. L., Kjelland, M. E., Moreno, J. F., & Gosálvez, J. (2011). Bacteria in bovine semen can increase sperm DNA fragmentation rates: a kinetic experimental approach. *Animal Reproduction Science*, *123*(3–4), 139–148. https://doi.org/10.1016/j.anireprosci.2010.11.014

Goodrich, J. K., Davenport, E. R., Waters, J. L., Clark, A. G., & Ley, R. E. (2016). Cross-species comparisons of host genetic associations with the microbiome. *Science*, *352*(6285), 532–535. https://doi.org/10.1126/science.aad9379

Griffith, S. C., Owens, I. P. F., & Thuman, K. A. (2002). Extra pair paternity in birds: A review of interspecific variation and adaptive function. *Molecular Ecology*, *11*(11), 2195–2212. https://doi.org/10.1046/j.1365-294X.2002.01613.x

Haines, M. D., Parker, H. M., McDaniel, C. D., & Kiess, A. S. (2015). When rooster semen is exposed to *Lactobacillus* fertility is reduced. *International Journal of Poultry Science*, *14*(9), 541–547. https://doi.org/10.3923/ijps.2015.541.547

Hamilton, W. D. (1990). Mate choice near or far. *American Zoologist*, *30*(2), 341–352. https://doi.org/10.1093/icb/30.2.341

Hamilton, W. D., & Zuk, M. (1982). Heritable true fitness and bright birds: a role for parasites? *Science*, *218*(4570), 384–387. https://doi.org/10.1126/science.7123238

Haro, C., Rangel-Zúñiga, O. A., Alcalá-Díaz, J. F., Gómez-Delgado, F., Pérez-Martínez, P., Delgado-Lista, J., Quintana-Navarro, G. M., Landa, B. B., Navas-Cortés, J. A., Tena-Sempere, M., Clemente, J. C., López-Miranda, J., Pérez-Jiménez, F., & Camargo, A. (2016). Intestinal microbiota is influenced by gender and body mass index. *PLoS ONE*, *11*(5), e0154090. https://doi.org/10.1371/journal.pone.0154090

Havlicek, J., Roberts, S. C., & Flegr, J. (2005). Women's preference for dominant male odour: effects of menstrual cycle and relationship status. *Biology Letters*, *1*(3), 256–259. https://doi.org/10.1098/rsbl.2005.0332

Hickey, R. J., Zhou, X., Pierson, J. D., Ravel, J., & Forney, L. J. (2012). Understanding vaginal microbiome complexity from an ecological perspective. *Translational Research*, *160*(4), 267–282. https://doi.org/10.1016/j.trsl.2012.02.008

Hiroki, M., Kato, Y., Kamito, T., & Miura, K. (2002). Feminization of genetic males by a symbiotic bacterium in a butterfly, *Eurema hecabe* (Lepidoptera: Pieridae). *Naturwissenschaften*, *89*(4), 167–170. https://doi.org/10.1007/s00114-002-0303-5

Hirsh, D. C. (1999). The genital tract as a microbial habitat. In D. C. Hirsh & Y. C. Zee (Eds.),

Veterinary Microbiology (pp. 190–191). Malden, MA: Blackwell Science.

Hoeksema, J. D., & Forde, S. E. (2008). A meta-analysis of factors affecting local adaptation between interacting species. *American Naturalist*, *171*(3), 275–290. https://doi.org/10.1086/527496

Hosokawa, T., Koga, R., Kikuchi, Y., Meng, X.-Y., & Fukatsu, T. (2010). *Wolbachia* as a bacteriocyte-associated nutritional mutualist. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(2), 769–774. https://doi.org/10.1073/pnas.0911476107

Houde, A. E. (1997). *Sex, color, and mate choice in guppies*. Princeton, NJ: Princeton University Press.

Huigens, M. E., & Stouthamer, R. (2003). Parthenogenesis associated with *Wolbachia*. In K. Bourtzis & T. A. Miller (Eds.), *Insect Symbiosis* (pp. 247–266). CRC Press.

Hupton, G., Portocarrero, S., Newman, M., & Westneat, D. F. (2003). Bacteria in the reproductive tracts of red-winged blackbirds. *The Condor*, *105*(3), 453–464. https://doi.org/10.1650/7246

Hurd, H. (1998). Parasite manipulation of insect reproduction: who benefits? *Parasitology*, *116*, S13–S21. https://doi.org/10.1017/S0031182000084900

Hurst, G. D. D., Sharpe, R. G., Broomfield, A. H., Walker, L. E., Majerus, T. M. O., Zakharov, I. A., & Majerus, M. E. N. (1995). Sexually transmitted disease in a promiscuous insect, *Adalia bipunctata*. *Ecological Entomology*, *20*(3), 230–236. https://doi.org/10.1111/j.1365-2311.1995.tb00452.x

Hurst, G. D., Jiggins, F. M., & Majerus, M. E. N. (2003). Inherited microorganisms that selectively kill male hosts: the hidden players of insect evolution? In K. Bourtzis & T. Miller (Eds.), *Insect Symbiosis* (pp. 177–198). Boca Raton, FL: CRC Press.

Huttenhower, C., Gevers, D., Knight, R., Abubucker, S., Badger, J. H., Chinwalla, A. T., Creasy, H. H., Earl, A. M., & FitzGeral, M. G. (2012). Structure, function and diversity of the healthy human microbiome. *Nature*, *486*(7402), 207–214. https://doi.org/10.1038/nature11234

Huwe, P., Diemer, T., Ludwig, M., Liu, J., Schiefer, H. G., & Weidner, W. (1998). Influence of different uropathogenic microorganisms on human sperm motility parameters in an *in vitro* experiment. *Andrologia*, *30*(Supplement 1), 55–59. https://doi.org/10.1111/j.1439-0272.1998.tb02827.x

Ilmonen, P., Taarna, T., & Hasselquist, D. (2000). Experimentally activated immune defence in female pied flycatchers results in reduced breeding success. *Proceedings of the Royal Society B: Biological Sciences*, *267*(1444), 665–670. https://doi.org/10.1098/rspb.2000.1053

Imhoof, B., & Schmid-Hempel, P. (1998). Patterns of local adaptation of a protozoan parasite to its bumblebee host. *Oikos*, *82*(1), 59–65. https://doi.org/10.2307/3546917

Immonen, E., & Ritchie, M. G. (2012). The genomic response to courtship song stimulation in female *Drosophila melanogaster*. *Proceedings of the Royal Society B: Biological Sciences*, *279*(1732), 1359–1365. https://doi.org/10.1098/rspb.2011.1644

Jaenike, J., Dyer, K. A., Cornish, C., & Minhas, M. S. (2006). Asymmetrical reinforcement and *Wolbachia* infection in *Drosophila*. *PLoS Biology*, *4*(10), 1852–1862. https://doi.org/10.1371/journal.pbio.0040325

James, A. G., Austin, C. J., Cox, D. S., Taylor, D., & Calvert, R. (2013). Microbiological and biochemical origins of human axillary odour. *FEMS Microbiology Ecology*, *83*(3), 527–540. https://doi.org/10.1111/1574-6941.12054

Kaltenpoth, M., & Engl, T. (2014). Defensive microbial symbionts in Hymenoptera. *Functional Ecology*, *28*(2), 315–327. https://doi.org/10.1111/1365-2435.12089

Kaltz, O., Gandon, S., Michalakis, Y., & Shykoff, J. A. (1999). Local maladaptation in the anther-smut fungus *Microbotryum violaceum* to its host plant *Silene latifolia*: evidence from a cross-inoculation experiment. *Evolution*, *53*(2), 395. https://doi.org/10.2307/2640776

Kamada, N., Chen, G. Y., Inohara, N., & Núñez, G. (2013). Control of pathogens and pathobionts by the gut microbiota. *Nature Immunology*, *14*(7), 685–690. https://doi.org/10.1038/ni.2608.Control

Kaur, S., Prabha, V., Shukla, G., & Sarwal, A. (2010). Interference of human spermatozoal motility by live *Staphylococcus aureus*. *American Journal of Biomedical Sciences*, *2*(1), 91–97. https://doi.org/10.5099/aj100100091

Kawecki, T. J., & Ebert, D. (2004). Conceptual issues in local adaptation. *Ecology Letters*, *7*(12), 1225–1241. https://doi.org/10.1111/j.1461-0248.2004.00684.x

Kekäläinen, J., & Evans, J. P. (2018). Gamete-mediated mate choice: towards a more inclusive view of sexual selection. *Proceedings of the Royal Society B: Biological Sciences*, *285*(1883), 20180836. https://doi.org/10.1098/rspb.2018.0836

King, K. C., & Bonsall, M. B. (2017). The evolutionary and coevolutionary consequences of defensive microbes for host-parasite interactions. *BMC Evolutionary Biology*, *17*, 190. https://doi.org/10.1186/s12862-017-1030-z

King, K. C., Brockhurst, M. A., Vasieva, O., Paterson, S., Betts, A., Ford, S. A., Frost, C. L., Horsburgh, M. J., Haldenby, S., & Hurst, G. D. (2016). Rapid evolution of microbe-mediated protection against pathogens in a worm host. *The ISME Journal*, *10*(8), 1915–1924. https://doi.org/10.1038/ismej.2015.259

Klainer, A. S., & Beisel, W. R. (1969). Opportunistic infection: a review. *The American Journal of the Medical Sciences*, *258*(6), 431–456. https://doi.org/10.1097/00000441-196912000-00007

Knell, R. J., & Webberley, K. M. (2004). Sexually transmitted diseases of insects: distribution, evolution, ecology and host behaviour. *Biological Reviews*, *79*, 557–581. https://doi.org/10.1017/S1464793103006365

Koch, H., & Schmid-Hempel, P. (2012). Gut microbiota instead of host genotype drive the specificity in the interaction of a natural host-parasite system. *Ecology Letters*, *15*(10), 1095–1103. https://doi.org/10.1111/j.1461-0248.2012.01831.x

Kostic, A. D., Howitt, M. R., & Garrett, W. S. (2013). Exploring host-microbiota interactions in animal models and humans. *Genes & Development*, *27*(7), 701–718. https://doi.org/10.1101/gad.212522.112.of

Kremer, N., Charif, D., Henri, H., Bataille, M., Prévost, G., Kraaijeveld, K., & Vavre, F. (2009). A new case of *Wolbachia* dependence in the genus *Asobara*: evidence for parthenogenesis induction in *Asobara japonica*. *Heredity*, *103*(3), 248–256. https://doi.org/10.1038/hdy.2009.63

Kulkarni, S., & Heeb, P. (2007). Social and sexual behaviours aid transmission of bacteria in birds. *Behavioural Processes*, *74*(1), 88–92. https://doi.org/10.1016/j.beproc.2006.10.005

Kurpisz, M., & Alexander, N. J. (1995). Carbohydrate moieties on sperm surface: physiological relevance. *Fertility and Sterility*, *63*(1), 158–165. https://doi.org/10.1016/S0015-0282(16)57312-9

Lange, R., Reinhardt, K., Michiels, N. K., & Anthes, N. (2013). Functions, diversity, and evolution of traumatic mating. *Biological Reviews*, *88*(3), 585–601. https://doi.org/10.1111/brv.12018

Li, Q., Korzan, W. J., Ferrero, D. M., Chang, R. B., Roy, D. S., Buchi, M., Lemon, J. K., Kaur, A. W., Stowers, L., Fendt, M., & Liberles, S. D. (2013). Synchronous evolution of an odor biosynthesis pathway and behavioral response. *Current Biology*, *23*(1), 11–20. https://doi.org/10.1016/j.cub.2012.10.047

Li, W., & Stevens, M. H. H. (2012). Fluctuating resource availability increases invasibility in microbial microcosms. *Oikos*, *121*(3), 435–441. https://doi.org/10.1111/j.1600-0706.2011.19762.x

Lockhart, A. B., Thrall, P. H., & Antonovics, J. (1996). Sexually transmitted diseases in animals: ecological and evolutionary Implications. *Biological Reviews*, *71*(3), 415–471. https://doi.org/10.1111/j.1469-185X.1996.tb01281.x

Login, F. H., Balmand, S., Vallier, A., Vincent-Monégat, C., Vigneron, A., Weiss-Gayet, M., Rochat, D., & Heddi, A. (2011). Antimicrobial peptides keep insect endosymbionts under

control. *Science*, *334*(6054), 362–365.

Lombardo, M. P., & Thorpe, P. A. (2000). Microbes in tree swallow semen. *Journal of Wildlife Diseases*, *36*(3), 460–468. https://doi.org/10.7589/0090-3558-36.3.460

Lung, O., Kuo, L., & Wolfner, M. F. (2001). *Drosophila* males transfer antibacterial proteins from their accessory gland and ejaculatory duct to their mates. *Journal of Insect Physiology*, *47*(6), 617–622. https://doi.org/10.1016/S0022-1910(00)00151-7

Mallon, E. B., Brockmann, A., & Schmid-Hempel, P. (2003). Immune response inhibits associative learning in insects. *Proceedings. Biological Sciences / The Royal Society*, *270*(1532), 2471–2473. https://doi.org/10.1098/rspb.2003.2456

Mändar, R., Punab, M., Borovkova, N., Lapp, E., Kiiker, R., Korrovits, P., Metspalu, A., Krjutškov, K., Nlvak, H., Preem, J. K., Oopkaup, K., Salumets, A., & Truu, J. (2015). Complementary seminovaginal microbiome in couples. *Research in Microbiology*, *166*(5), 440–447. https://doi.org/10.1016/j.resmic.2015.03.009

Markle, J. G. M., Frank, D. N., Mortin-Toth, S., Robertson, C. E., Feazel, L. M., Rolle-Kampczyk, U., von Bergen, M., McCoy, K. D., Macpherson, A. J., & Danska, J. S. (2013). Sex differences in the gut microbiome drive hormone-dependent regulation of autoimmunity. *Science*, *339*(6123), 1084–1088. https://doi.org/10.1126/science.1233521

Maroto Martín, L. O., Muñoz, E. C., De Cupere, F., Van Driessche, E., Echemendia-Blanco, D., Rodríguez, J. M. M., & Beeckmans, S. (2010). Bacterial contamination of boar semen affects the litter size. *Animal Reproduction Science*, *120*(1–4), 95–104. https://doi.org/10.1016/j.anireprosci.2010.03.008

Martínez, I., Lattimer, J. M., Hubach, K. L., Case, J. A., Yang, J., Weber, C. G., Louk, J. A., Rose, D. J., Kyureghian, G., Peterson, D. A., Haub, M. D., & Walter, J. (2013). Gut microbiome composition is linked to whole grain-induced immunological improvements. *The ISME Journal*, *7*(2), 269–280. https://doi.org/10.1038/ismej.2012.104

Martinez, J., Cogni, R., Cao, C., Smith, S., Illingworth, C. J. R., & Jiggins, F. M. (2016). Addicted? Reduced host resistance in populations with defensive symbionts. *Proceedings of the Royal Society B: Biological Sciences*, *283*, 20160778. https://doi.org/10.1098/rspb.2016.0778

Martinez, J., Longdon, B., Bauer, S., Chan, Y. S., Miller, W. J., Bourtzis, K., Teixeira, L., & Jiggins, F. M. (2014). Symbionts commonly provide broad spectrum resistance to viruses in insects: a comparative analysis of *Wolbachia* strains. *PLoS Pathogens*, *10*(9). https://doi.org/10.1371/journal.ppat.1004369

Martinez, J., Tolosana, I., Ok, S., Smith, S., Snoeck, K., Day, J. P., & Jiggins, F. M. (2017). Symbiont strain is the main determinant of variation in *Wolbachia*-mediated protection against viruses across *Drosophila* species. *Molecular Ecology*, *26*(15), 4072–4084. https://doi.org/10.1111/mec.14164

Matsuda, N., & Hattori, Y. (2006). Systemic inflammatory response syndrome (SIRS): molecular pathophysiology and gene therapy. *Journal of Pharmacological Sciences*, *101*(3), 189–198. https://doi.org/10.1254/jphs.CRJ06010X

Mattoso, T. C., Moreira, D. D. O., & Samuels, R. I. (2012). Symbiotic bacteria on the cuticle of the leaf-cutting ant *Acromyrmex subterraneus subterraneus* protect workers from attack by entomopathogenic fungi. *Biology Letters*, *8*(3), 461–464. https://doi.org/10.1098/rsbl.2011.0963

Miest, T. S., & Bloch-Qazi, M. C. (2008). Sick of mating: sexual transmission of a pathogenic bacterium in *Drosophila melanogaster*. *Fly*, *2*(4), 215–219. https://doi.org/10.4161/fly.6726

Milinski, M., & Bakker, T. C. M. (1990). Female sticklebacks use male coloration in mate choice and hence avoid parasitized males. *Nature*, *344*(6264), 330–333.

Morand, S., Manning, S. D., & Woolhouse, M. E. J. (1996). Parasite-host coevolution and geographic patterns of parasite infectivity and host susceptibility. *Proceedings of the Royal Society B: Biological Sciences*, *263*(1366), 119–128. https://doi.org/10.1098/rspb.1996.0019

Moreno, I., Codoñer, F. M., Vilella, F., Valbuena, D., Martinez-Blanch, J. F., Jimenez-Almazán, J., Alonso, R., Alamá, P., Remohí, J., Pellicer, A., Ramon, D., & Simon, C. (2016). Evidence that the endometrial microbiota has an effect on implantation success or failure. *American Journal of Obstetrics and Gynecology*, *215*(6), 684–703. https://doi.org/10.1016/j.ajog.2016.09.075

Morrow, E. H., & Arnqvist, G. (2003). Costly traumatic insemination and a female counter-adaptation in bed bugs. *Proceedings of the Royal Society B: Biological Sciences*, *270*(1531), 2377–2381. https://doi.org/10.1098/rspb.2003.2514

Mutikainen, P., Salonen, V., Puustinen, S., & Koskela, T. (2000). Local adaptation, resistance, and virulence in a hemiparasitic plant-host plant interaction. *Evolution*, *54*(2), 433–440. https://doi.org/10.1111/j.0014-3820.2000.tb00046.x

Nakanishi, K., Hoshino, M., Nakai, M., & Kunimi, Y. (2008). Novel RNA sequences associated with late male killing in *Homona magnanima*. *Proceedings of the Royal Society B: Biological Sciences*, *275*(1640), 1249–1254. https://doi.org/10.1098/rspb.2008.0013

Negri, I., Pellecchia, M., Mazzoglio, P. J., Patetta, A., & Alma, A. (2006). Feminizing *Wolbachia* in *Zyginidia pullula* (Insecta, Hemiptera), a leafhopper with an XX/XO sex-determination system. *Proceedings of the Royal Society B: Biological Sciences*, *273*(1599), 2409–2416. https://doi.org/10.1098/rspb.2006.3592

Nuriel-Ohayon, M., Neuman, H., & Koren, O. (2016). Microbial changes during pregnancy, birth, and infancy. *Frontiers in Microbiology*, *7*, 1031. https://doi.org/10.3389/fmicb.2016.01031

References

Odamaki, T., Kato, K., Sugahara, H., Hashikura, N., Takahashi, S., Xiao, J. Z., Abe, F., & Osawa, R. (2016). Age-related changes in gut microbiota composition from newborn to centenarian: a cross-sectional study. *BMC Microbiology*, *16*, 90. https://doi.org/10.1186/s12866-016-0708-5

Oh, D.-C., Poulsen, M., Currie, C. R., & Clardy, J. (2009). Dentigerumycin: a bacterial mediator of an ant-fungus symbiosis. *Nature Chemical Biology*, *5*(6), 391–393. https://doi.org/10.1038/nchembio.159

Oppliger, A., Vernet, R., & Baez, M. (1999). Parasite local maladaptation in the Canarian lizard *Gallotia galloti* (Reptilia: Lacertidae) parasitized by haemogregarian blood parasite. *Journal of Evolutionary Biology*, *12*(5), 951–955. https://doi.org/10.1046/j.1420-9101.1999.00101.x

Otti, O. (2015). Genitalia-associated microbes in insects. *Insect Science*, *22*(3), 325–339. https://doi.org/10.1111/1744-7917.12183

Otti, O., Deines, P., Hammerschmidt, K., & Reinhardt, K. (2017). Regular wounding in a natural system: bacteria associated with reproductive organs of bedbugs and their quorum sensing abilities. *Frontiers in Immunology*, *8*, 1855. https://doi.org/10.3389/fimmu.2017.01855

Otti, O., McTighe, A. P., & Reinhardt, K. (2013). *In vitro* antimicrobial sperm protection by an ejaculate-like substance. *Functional Ecology*, *27*(1), 219–226. https://doi.org/10.1111/1365-2435.12025

Otti, O., Naylor, R. A., Siva-Jothy, M. T., & Reinhardt, K. (2009). Bacteriolytic activity in the ejaculate of an insect. *The American Naturalist*, *174*(2), 292–295. https://doi.org/10.1086/600099

Pannebakker, B. A., Schidlo, N. S., Boskamp, G. J. F., Dekker, L., Van Dooren, T. J. M., Beukeboom, L. W., Zwaan, B. J., Brakefield, P. M., & Van Alphen, J. J. M. (2005). Sexual functionality of *Leptopilina clavipes* (Hymenoptera: Figitidae) after reversing *Wolbachia*-induced parthenogenesis. *Journal of Evolutionary Biology*, *18*(4), 1019–1028. https://doi.org/10.1111/j.1420-9101.2005.00898.x

Parker, G. A. (2014). The sexual cascade and the rise of pre-ejaculatory (Darwinian) sexual selection, sex roles, and sexual conflict. *Cold Spring Harbor Perspectives in Biology*, *6*(10), a017509. https://doi.org/10.1101/cshperspect.a017509

Perlmutter, J. I., & Bordenstein, S. R. (2020). Microorganisms in the reproductive tissues of arthropods. *Nature Reviews Microbiology*, *18*, 97–111. https://doi.org/10.1038/s41579-019-0309-z

Pietri, J. E., & Liang, D. (2018). Virulence of entomopathogenic bacteria in the bed bug, *Cimex lectularius*. *Journal of Invertebrate Pathology*, *151*, 1–6. https://doi.org/10.1016/j.jip.2017.10.004

Pijls, J. W. A. M., Van Steenbergen, H. J., & Van Alphen, J. J. M. (1996). Asexuality cured: the relations and differences between sexual and asexual *Apoanagyrus diversicornis*. *Heredity*, *76*(5), 506–513. https://doi.org/10.1038/hdy.1996.73

Prabha, V., Sandhu, R., Kaur, S., Kaur, K., Sarwal, A., Mavuduru, R. S., & Singh, S. K. (2010). Mechanism of sperm immobilization by *Escherichia coli*. *Advances in Urology*, *2010*, 240268. https://doi.org/10.1155/2010/240268

Quayle, A. J., Nussbaum, A. A., Wang, Y. M., Brabec, C., Mok, S. C., Porter, E., & Yip, K.-P. (1998). Gene expression, immunolocalization, and secretion of human defensin-5 in human female reproductive tract. *The American Journal of Pathology*, *152*(5), 1247–1258.

R Core Team. (2017). *R: a language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing.

Raina, A., Murphy, C., Florane, C., Williams, K., Park, Y. I., & Ingbers, B. (2007). Structure of spermatheca, sperm dynamics, and associated bacteria in Formosan subterranean termite (Isoptera : Rhinotermitidae). *Annals of the Entomological Society of America*, *100*(3), 418–424. https://doi.org/Doi 10.1603/0013-8746(2007)100[418:Sossda]2.0.Co;2

Ravel, J., Gajer, P., Abdo, Z., Schneider, G. M., Koenig, S. S. K., McCulle, S. L., Karlebach, S., Gorle, R., Russell, J., Tacket, C. O., Brotman, R. M., Davis, C. C., Ault, K., Peralta, L., & Forney, L. J. (2011). Vaginal microbiome of reproductive-age women. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(Supplement 1), 4680–4687. https://doi.org/10.1073/pnas.1002611107

Reid, G., Cook, R. L., & Bruce, A. W. (1987). Examination of strains of lactobacilli for properties that may influence bacterial interference in the urinary tract. *The Journal of Urology*, *138*, 330–335. https://doi.org/10.1016/S0022-5347(17)43137-5

Reinhardt, K. (2007). Evolutionary consequences of sperm cell aging. *The Quarterly Review of Biology*, *82*(4), 375–393.

Reinhardt, K., Anthes, N., & Lange, R. (2015). Copulatory wounding and traumatic insemination. *Cold Spring Harbor Laboratory Press*, *7*(5), a017582.

Reinhardt, K., Naylor, R. A., & Siva-Jothy, M. T. (2009). Situation exploitation: higher male mating success when female resistance is reduced by feeding. *Evolution*, *63*(1), 29–39. https://doi.org/10.1111/j.1558-5646.2008.00502.x

Reinhardt, K., Naylor, R., & Siva-Jothy, M. T. (2005). Potential sexual transmission of environmental microbes in a traumatically inseminating insect. *Ecological Entomology*, *30*(5), 607–611. https://doi.org/10.1111/j.0307-6946.2005.00730.x

Reinhardt, K., Naylor, R., & Siva-Jothy, M. T. (2003). Reducing a cost of traumatic insemination: female bedbugs evolve a unique organ. *Proceedings of the Royal Society*

of London B: Biological Sciences, *270*(1531), 2371–2375. https://doi.org/10.1098/rspb.2003.2515

Richardson, M. D. (1991). Opportunistic and pathogenic fungi. *Journal of Antimicrobial Chemotherapy*, *28*(Supplement A), 1–11. https://doi.org/10.1093/jac/28.suppl_a.1

Rigaud, T., & Juchault, P. (1992). Genetic control of the vertical transmission of a cytoplasmic sex factor in Armadillidium vulgare Latr. (Crustacea, Oniscidea). *Heredity*, *68*(1), 47–52. https://doi.org/10.1038/hdy.1992.6

Rigby, M. C., & Jokela, J. (2000). Predator avoidance and immune defence: costs and trade-offs in snails. *Proceedings of the Royal Society of London B: Biological Sciences*, *267*(1439), 171–176. https://doi.org/10.1098/rspb.2000.0983

Rizzi, A., Crotti, E., Borruso, L., Jucker, C., Lupi, D., Colombo, M., & Daffonchio, D. (2013). Characterization of the bacterial community associated with larvae and adults of *Anoplophora chinensis* collected in Italy by culture and culture-independent methods. *BioMed Research International*, *2013*, 420287. https://doi.org/10.1155/2013/420287

Rolff, J., & Reynolds, S. E. (Eds.). (2009). *Insect infection and immunity: evolution, ecology, and mechanisms*. Oxford, UK: Oxford University Press.

Rowe, M., Gábor Á rpád Czirják, McGraw, K. J., & Giraudeau, M. (2011). Sexual ornamentation reflects antibacterial activity of ejaculates in mallards. *Biology Letters*, *7*(5), 740–742. https://doi.org/10.1098/rsbl.2011.0276

Rowe, M., Veerus, L., Trosvik, P., Buckling, A., & Pizzari, T. (2020). The reproductive microbiome: an emerging driver of sexual selection, sexual conflict, mating systems, and reproductive isolation. *Trends in Ecology and Evolution*, *35*(3), 220–234. https://doi.org/10.1016/j.tree.2019.11.004

Samakovlis, C., Kylsten, P., Kimbrell, D. A., Engström, A., & Hultmark, D. (1991). The *Andropin* gene and its product, a male-specific antibacterial peptide in *Drosophila melanogaster*. *The EMBO Journal*, *10*(1), 163–169. https://doi.org/10.1002/j.1460-2075.1991.tb07932.x

Saxton, T. K., Lyndon, A., Little, A. C., & Roberts, S. C. (2008). Evidence that androstadienone, a putative human chemosignal, modulates women's attributions of men's attractiveness. *Hormones and Behavior*, *54*(5), 597–601. https://doi.org/10.1016/j.yhbeh.2008.06.001

Scarpino, S. V., & Althouse, B. M. (2019). Uncovering the hidden cost of bed bugs. *Proceedings of the National Academy of Sciences of the United States of America*, *116*(15), 7160–7162. https://doi.org/10.1073/pnas.1902404116

Schmid-Hempel, P. (2005). Evolutionary ecology of insect immune defenses. *Annual Review of Entomology*, *50*(1), 529–551. https://doi.org/10.1146/annurev.ento.50.071803.130420

Schneider, C. A., Rasband, W. S., & Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of

image analysis. *Nature Methods*, *9*(7), 671–675. https://doi.org/10.1038/nmeth.2089

Sheldon, B. C. (1993). Sexually transmitted disease in birds: occurrence and evolutionary significance. *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, *339*(1290), 491–497. https://doi.org/10.1098/rstb.1993.0044

Sheldon, B. C., & Verhulst, S. (1996). Ecological immunology: costly parasite defences and trade-offs in evolutionary ecology. *Trends in Ecology & Evolution*, *11*(8), 317–321. https://doi.org/10.1016/0169-5347(96)10039-2

Shoemaker, D. D., Katju, V., & Jaenike, J. (1999). Wolbachia and the evolution of reproductive isolation between *Drosophila recens* and *Drosophila subquinaria*. *Evolution*, *53*(4), 1157. https://doi.org/10.2307/2640819

Shropshire, J. D., & Bordenstein, S. R. (2016). Speciation by symbiosis: the microbiome and behavior. *MBio*, *7*(2), 1–11. https://doi.org/10.1128/mBio.01785-15

Siva-Jothy, M. T. (2000). The young sperm gambit. *Ecology Letters*, *3*(3), 172–174. https://doi.org/10.1046/j.1461-0248.2000.00146.x

Siva-Jothy, M. T., Moret, Y., & Rolff, J. (2005). Insect immunity: an evolutionary ecology perspective. *Advances in Insect Physiology*, *32*, 1–48. https://doi.org/10.1016/S0065-2806(05)32001-7

Siva-Jothy, M. T., & Stutt, A. D. (2003). A matter of taste: direct detection of female mating status in the bedbug. *Proceedings of the Royal Society of London B: Biological Sciences*, *270*(1515), 649–652. https://doi.org/10.1098/rspb.2002.2260

Siva-Jothy, M. T., Zhong, W., Naylor, R., Heaton, L., Hentley, W., & Harney, E. (2019). Female bed bugs (*Cimex lectularius* L) anticipate the immunological consequences of traumatic insemination via feeding cues. *Proceedings of the National Academy of Sciences of the United States of America*, *116*(29), 14682–14687. https://doi.org/10.1073/pnas.1904539116

Skau, P. A., & Folstad, I. (2003). Do bacterial infections cause reduced ejaculate quality? A meta-analysis of antibiotic treatment of male infertility. *Behavioral Ecology*, *14*(1), 40–47. https://doi.org/10.1093/beheco/14.1.40

Stutt, A. D., & Siva-Jothy, M. T. (2001). Traumatic insemination and sexual conflict in the bed bug *Cimex lectularius*. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(10), 5683–5687. https://doi.org/10.1073/pnas.101440698

Swanson, W. J., & Vacquier, V. D. (2002). The rapid evolution of reproductive proteins. *Genetics*, *3*(2), 137–144. https://doi.org/10.1038/nrg/733

Terry, R. S., Smith, J. E., Bouchon, D., Rigaud, T., Duncanson, P., Sharpe, R. G., & Dunn, A. M. (1999). Ultrastructural characterisation and molecular taxonomic identification of

Nosema granulosis n. sp., a transovarially transmitted feminising (TTF) microsporidium. *Journal of Eukaryotic Microbiology*, *46*(5), 492–499. https://doi.org/10.1111/j.1550-7408.1999.tb06066.x

Thaiss, C. A., Zeevi, D., Levy, M., Zilberman-Schapira, G., Suez, J., Tengeler, A. C., Abramson, L., Katz, M. N., Korem, T., Zmora, N., Kuperman, Y., Biton, I., Gilad, S., Harmelin, A., Shapiro, H., Halpern, Z., Segal, E., & Elinav, E. (2014). Transkingdom control of microbiota diurnal oscillations promotes metabolic homeostasis. *Cell*, *159*(3), 514–529. https://doi.org/10.1016/j.cell.2014.09.048

Tvrdá, E., Ďuračka, M., Kántor, A., Halenár, M., & Hleba, L. (2018). *In vitro* effects of *Acinetobacter baumannii* and selected natural biomolecules on rabbit spermatozoa motility. *Agrofor*, *3*(1), 54–63. https://doi.org/10.7251/agreng1801054t

Vala, F., Egas, M., Breeuwer, J. A. J., & Sabelis, M. W. (2004). *Wolbachia* affects oviposition and mating behaviour of its spider mite host. *Journal of Evolutionary Biology*, *17*(3), 692–700. https://doi.org/10.1046/j.1420-9101.2003.00679.x

Valiente Moro, C., Tran, F. H., Nantenaina Raharimalala, F., Ravelonandro, P., & Mavingui, P. (2013). Diversity of culturable bacteria including *Pantoea* in wild mosquito *Aedes albopictus*. *BMC Microbiology*, *13*, 70. https://doi.org/10.1186/1471-2180-13-70

Valore, E. V., Park, C. H., Quayle, A. J., Wiles, K. R., McCray, P. B., & Ganz, T. (1998). Human β-defensin-1: an antimicrobial peptide of urogenital tissues. *Journal of Clinical Investigation*, *101*(8), 1633–1642. https://doi.org/10.1172/JCI1861

Virecoulon, F., Wallet, F., Rigot, J., Peers, M., & Mitchell, V. (2005). Bacterial flora of the low male genital tract in patients consulting for infertility. *Andrologia*, *37*(5), 160–165. https://doi.org/10.1111/j.1439-0272.2005.00673.x

Weeks, A. R., Marec, F., & Breeuwer, J. A. J. (2001). A mite species that consists entirely of haploid females. *Science*, *292*(5526), 2479–2482. https://doi.org/10.1126/science.1060411

White, J., Mirleau, P., Danchin, E., Mulard, H., Hatch, S. A., Heeb, P., & Wagner, R. H. (2010). Sexually transmitted bacteria affect female cloacal assemblages in a wild bird. *Ecology Letters*, *13*(12), 1515–1524. https://doi.org/10.1111/j.1461-0248.2010.01542.x

White, J., Richard, M., Massot, M., & Meylan, S. (2011). Cloacal bacterial diversity increases with multiple mates: evidence of sexual transmission in female common lizards. *PLoS ONE*, *6*(7), e22339. https://doi.org/10.1371/journal.pone.0022339

Wintle, K., & Reinhardt, K. (2008). Temporary feeding inhibition caused by artificial abdominal distension in the bedbug, *Cimex lectularius*. *Journal of Insect Physiology*, *54*(7), 1200–1204. https://doi.org/10.1016/j.jinsphys.2008.05.005

Yoshida, M., Kawano, N., & Yoshida, K. (2008). Control of sperm motility and fertility: diverse

factors and common mechanisms. *Cellular and Molecular Life Sciences*, *65*(21), 3446–3457. https://doi.org/10.1007/s00018-008-8230-z

Zeh, J. A., & Zeh, D. W. (2006). Male-killing Wolbachia in a live-bearing arthropod: brood abortion as a constraint on the spread of a selfish microbe. *Journal of Invertebrate Pathology*, *92*(1), 33–38. https://doi.org/10.1016/j.jip.2006.02.004

Zhong, W., McClure, C. D., Evans, C. R., Mlynski, D. T., Immonen, E., Ritchie, M. G., & Priest, N. K. (2013). Immune anticipation of mating in *Drosophila*: *Turandot M* promotes immunity against sexually transmitted fungal infections. *Proceedings. Biological Sciences / The Royal Society*, *280*(1773), 20132018. https://doi.org/10.1098/rspb.2013.2018

Zuk, M., & Stoehr, A. M. (2002). Immune defense and host life history. *The American Naturalist*, *160*(S4), S9–S22. https://doi.org/10.1086/342131

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