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

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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

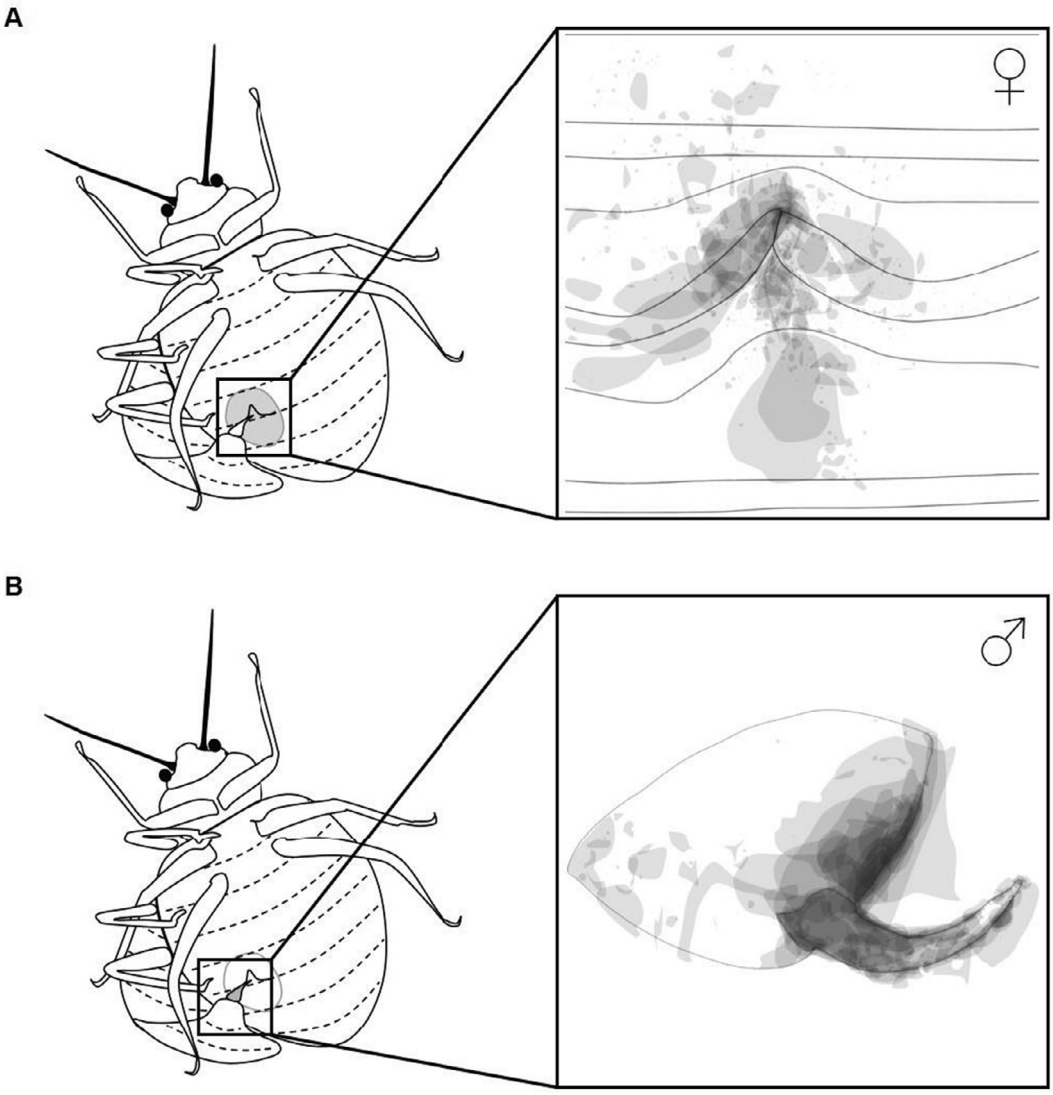

Fig. 1. Distribution of the sexually transmitted bacteria a) from the male copulatory organ to the female copulatory groove and b) from the female copulatory groove to the male copulatory organ. Templates of the organs indicating the location of bacteria detected with fluorescence microscopy were superimposed 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

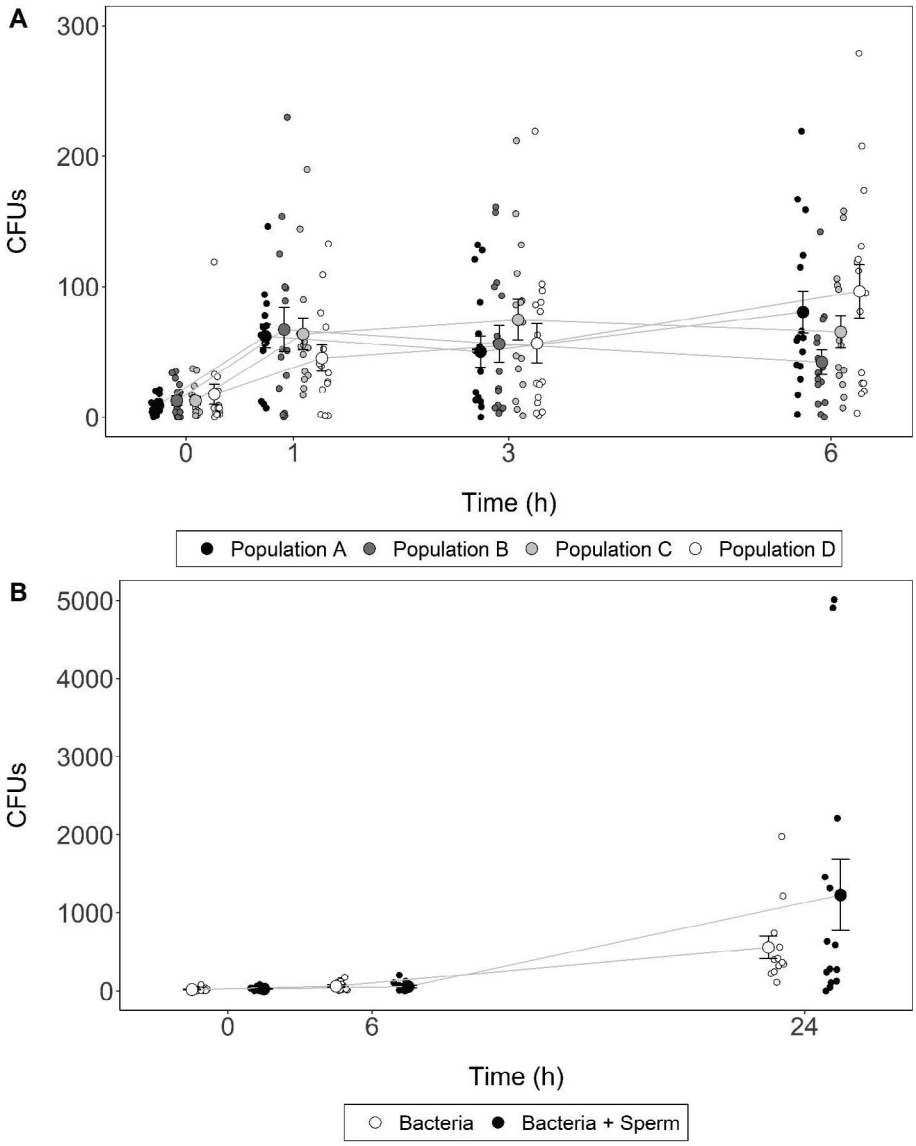

Fig. 2. Bacterial growth in the female sperm-receiving organ after the injection of a) bacteria or b) bacteria or both bacteria and sperm. The number of fluorescent CFUs after injection of GFP-labelled *Asaia* sp. was counted under UV light. Given are mean and standard error of the mean and all individual 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

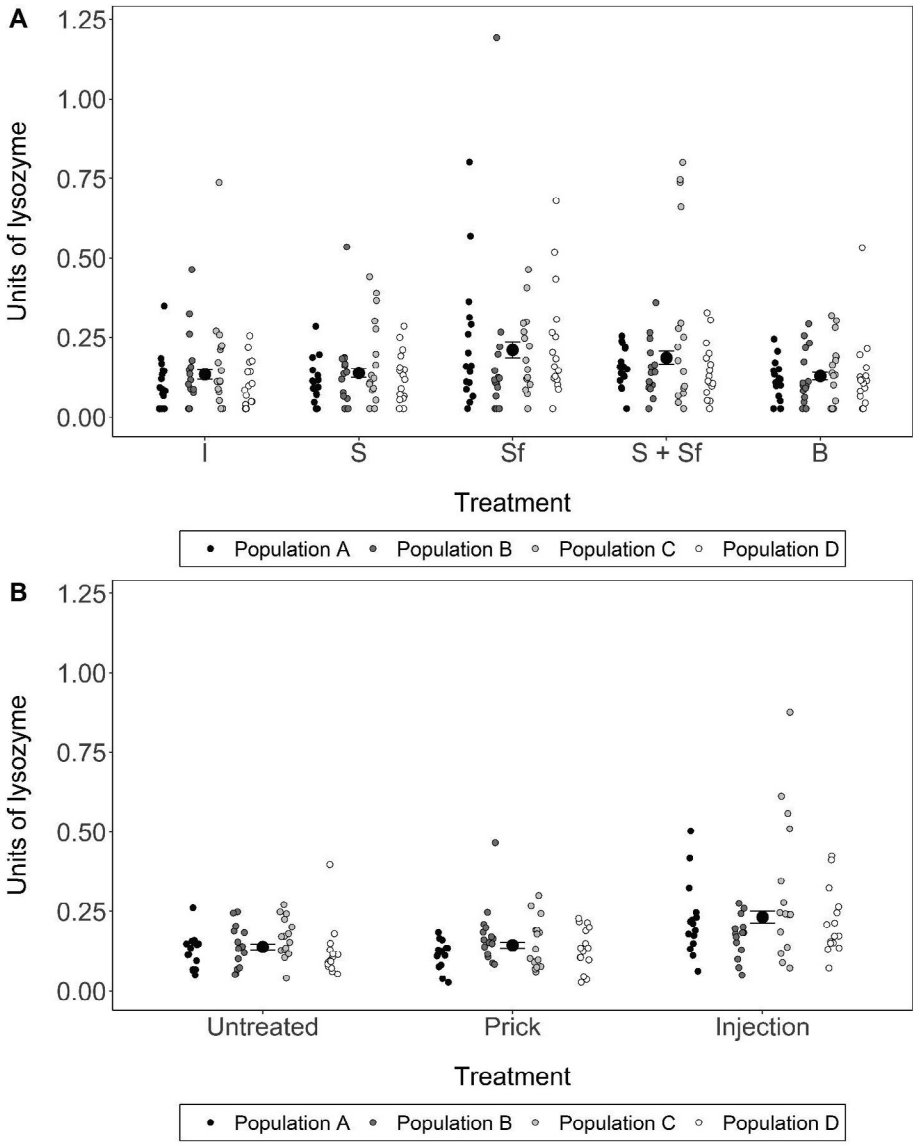

Fig. 3. Lysozyme-like activity in the female sperm-receiving organ in response to a) the injection of various mating-associated substances or b) wounding type. Given are mean and standard error of the mean for a) each treatment (I: Injection control, S: Sperm, Sf: Seminal fluid, S + Sf: Sperm + Seminal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

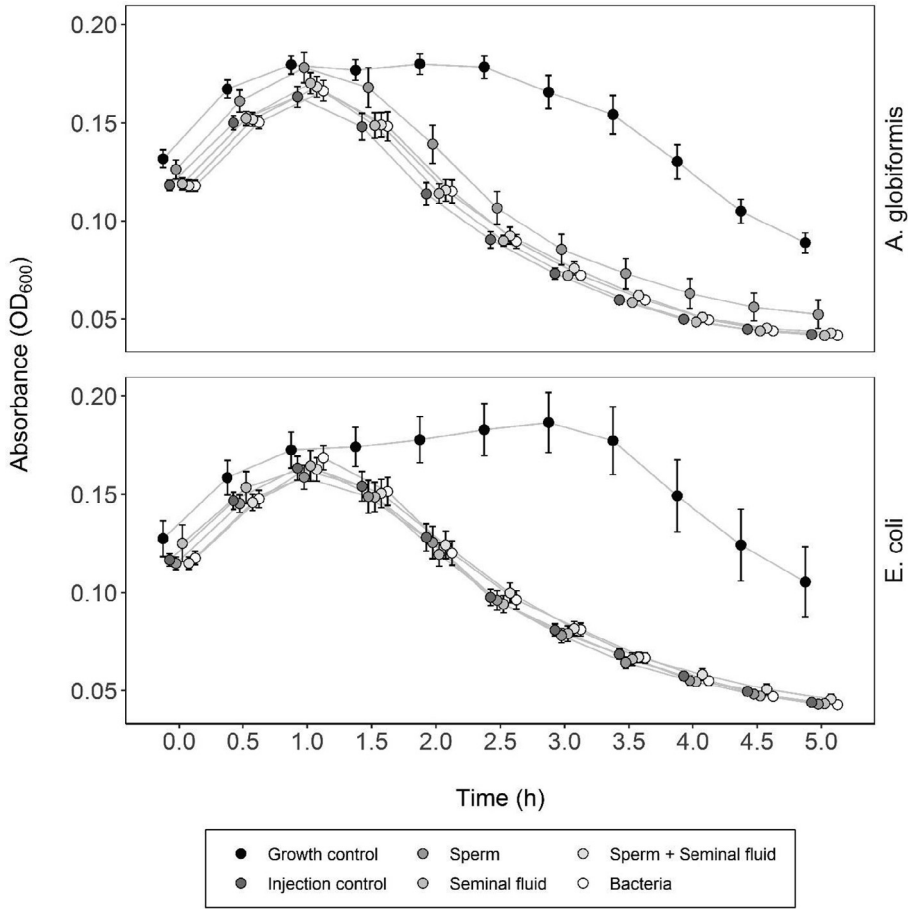

Fig. 4. Liquid growth inhibition of *Arthrobacter globiformis* and *Escherichia coli* by antimicrobial peptides produced in the female sperm-receiving organ in response to the injection of various mating-associated substances. Bacteria were incubated with the supernatant from centrifuged homogenised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