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Research Article

RESEARCH ARTICLE

Dynamics of bacterial composition in the locust reproductive tract are affected by the density-dependent phase

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One sentence summary: Differences in the dynamics of the bacterial composition in the reproductive tract of locusts are derived from the host's density-dependent phase.

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ABSTRACT

The important role that locust gut bacteria play in their host biology is well accepted. Among other roles, gut bacteria are suggested to be involved in the locust swarming phenomenon. In addition, in many insect orders, the reproductive system is reported to serve as a vector for trans-generation bacterial inoculation. Knowledge of the bacterial composition of the locust reproductive tract is, however, practically absent. Here we characterized the reproductive system bacterial composition of gregarious and solitary females. We investigated its temporal dynamics and how it interacts with the locust phase, by comparative sampling and 16S rRNA amplicon sequencing. We revealed that the bacterial composition of the locust female reproductive tract is mostly constructed of three core genera: *Micrococcus*, *Acinetobacter* and *Staphylococcus*. While solitary females maintained a consistent bacterial composition, in the gregarious phase this consortium demonstrated large temporal shifts, mostly manifested by *Brevibacterium* blooms. These data are in accord with our previous report on the dynamics of locust hindgut bacterial microbiota, further indicating that locust endosymbionts are affected by their host population density. These newly understood dynamics may have implications beyond their contribution to our knowledge of locust ecology, as aggregation and mass migration are prevalent phenomena across many migrating animals.

Keywords: locust; reproductive tract; insect bacteria interaction; *Schistocerca gregaria*; bacterial symbionts; locust bacteria

INTRODUCTION

Insect-bacteria association constitutes a common symbiotic interaction found in many insect orders and is known to frequently alter the life history of the host (Moran and Baumann 2000; Oliver *et al.* 2010; Bennett and Moran 2013; Salem *et al.* 2015). Some bacterial symbionts are involved in the degradation of complex polysaccharides (Ohkuma 2008; Morales-Jiménez *et al.* 2012) or nutrient synthesis for the benefit of the insect (Oliver *et al.* 2010; Opatovsky *et al.* 2018). Symbionts can also augment host resistance to heat, parasitism and pathogenic infections through numerous mechanisms; they can act as pathogens and challenge the host's immune response (Dillon *et al.* 2005; Kaltenpoth 2009; Oliver *et al.* 2010) and even alter the insect's fertility (Hunter, Perlman and Kelly 2003; He *et al.* 2019).

Extensive literature is available on insect obligatory symbionts (i.e. those that are essential for the host fitness), as well as on reproduction-manipulating bacteria such as *Wolbachia* (e.g.

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Bourtzis and Miller 2006; Gill and Latorre 2019; Landmann 2019). However, very few studies have addressed the role of extracellular facultative bacteria in the insect reproductive tract (Raina *et al.* 2007; Rizzi *et al.* 2013; Marchini *et al.* 2014; Otti 2015; Segata *et al.* 2016; Bellinvia 2019). Findings from these studies, and data collected from vertebrates, indicate the importance of facultative extracellular bacteria in the reproductive tract. Copulation exposes both male and female to their partner's gonad-associated bacteria, and dysbiosis in either one of the sexes may infect the other and severely hamper both their joint and individual fitness (Ottie 2015; Bellinvia 2019). For example, post-copulation insect females generally store the sperm within the spermatheca (a sperm-storing organ) for different periods of time (Pascini and Martins 2016). Undesirable bacteria derived from either males or females may consume the sperm or sperm-associated compounds within the spermatheca (e.g. proteins, carbohydrates, lipids) (Da´vila 2018), thus destroying the sperm cells before fertilization can take place.

The desert locust *Schistocerca gregaria* (Forskål) (order: Orthoptera) offers a very well-established experimental system for the study of microbial residents and the role these play in their host biology and population dynamics (Dillon and Charnley 1995; Dillon, Vennard and Charnley 2000; Dillon and Charnley 2002; Dillon, Vennard and Charnley 2002; Dillon *et al.* 2008, 2010; Shi *et al.* 2014; Lavy *et al.* 2019). This is a swarm-forming species, known since ancient times as a pest with devastating damage potential to crops and vegetation. It is also known for demonstrating density-dependent phenotypes (Pener and Simpson 2009; Cullen *et al.* 2017; Ayali 2019): under certain conditions, favoring a rise in the locust density, individuals will express the notorious gregarious swarming phenotype; whereas under low density they will express the solitary phenotype. The two phases differ in appearance, behavior and physiology (Pener and Simpson 2009; Cullen *et al.* 2017; Ayali 2019), as well as in the bacterial dynamics among conspecifics (Lavy *et al.* 2019).

Previous studies have suggested that bacteria residing within the locust gut play a role in augmenting host immunity (Dillon and Charnley 1995; Dillon *et al.* 2005) and also in the gregarization phenomenon, through the emission of volatiles (Dillon, Vennard and Charnley 2000, 2002). Some of these bacteria seem to be highly conserved within the population and across generations (Dillon and Charnley 2002; Lavy *et al.* 2019), suggesting vertical symbiont transmission (and a tentative mechanism involved in density-dependent phase vertical transmission). However, although locust gut bacteria have been comprehensively studied (e.g. Dillon and Charnley 1986; Dillon, Vennard and Charnley 2000, 2002; Shi *et al.* 2014; Lavy *et al.* 2019), the microbiome of the reproductive tract has been largely ignored.

Here we characterized the bacterial microbiota of the female locust reproductive tract; explored the effect of the male on the post-copulatory spermatheca community composition; and uncovered the bacterial dynamics of the spermatheca in both gregarious and solitary individuals across time. We focused primarily on the spermatheca, both because this is the most probable region of the system to be affected by copulation, and because its content must be transmitted to the eggs shortly before they are laid (Pascini and Martins 2016).

METHODS

Insect rearing

Locusts were reared in a temperature-controlled room under the same conditions described in Lavy *et al.* (2019). Gregarious locusts were reared for many consecutive generations under heavy crowding of 300–500 individuals in 140 L wooden cages. Solitary-phase locusts were attained by collecting and separating hatchlings of gregarious-laid pods within 3 h of hatching and rearing the nymphs in isolation until adulthood (Geva *et al.* 2010; Berman, Ayali and Gefen 2013). Insects were fed daily with fresh wheat seedlings and dry oats. Locusts of both phases were reared in different rooms, under similar conditions except for density.

Mated and virgin females

A set of gregarious females was collected within 12 h of adult emergence and introduced into a cage (30 x 20 x 20 cm), where they were maintained until sexual maturation. In order to account for between-phase variation in timing of sexual maturity (reviewed in Pener and Simpson 2009), reproductive status in both phases was determined by the first signs of futile egg pod dispatch.

Mature females were then assigned to two experimental groups. Virgin-unmated females were sacrificed, placed individually in 70% ethanol, and kept at −20°C until further use. For the 'mated-females' group, females were placed in a cage (11 x 12 x 14.5 cm) for 12 h with a mature male of their own phase and continuously video-monitored (Sony HDR-PJ820E) to establish mating status. If a copulation of at least 15 min occurred during this period, females were considered as mated. Mated females were then sacrificed, placed individually in 70% ethanol and kept at −20°C.

Tissue sampling

In order to examine the bacterial composition of the female reproductive system we collected various samples in four independent sampling rounds, as described in Fig. 1, using the subsequent protocol.

Following wing and leg removal, each individual (a total of 30 solitary and 57 gregarious females) was submerged for 2 min in a 1% NaOCl solution for surface sterilization and washed twice with filtered, double-distilled water. Then animals were dissected aseptically in a filtered saline solution (0.9% NaCl); 3% NaOCl and flame were used for sterilization of the workstation and dissection tools, respectively. Excised tissue samples were kept individually in 70% ethanol at −20°C until further use.

Locust gonads are in close proximity to the digestive system, so in order to ensure that our findings were not largely affected by gut bacteria (3 in Fig. 1), we sampled the female digestive tract in addition to the spermatheca in one of the sampling rounds.

DNA extraction and sequencing

Ethanol was removed and bacterial genomic DNA was extracted using the 'Powersoil' DNA isolation Kit (Mo Bio Laboratories, Carlsbad, CA) according to the manufacturer's instructions, using 60 µl for final DNA elution. To determine bacterial composition, polymerase chain reaction (PCR) of variable regions V3 and V4 of the prokaryotic 16S rRNA gene was performed using the extracted DNA as a template and a universal primer containing 5′end common sequences (CS1–341F 5′- ACACTGACGACATGGTTCTACANNNNCCTACGGGAGGC AGCAG and CS2–806R 5′-TACGGTAGCAGAGACTTGG TCTGGAC-TACHVGGGTW TCTAAT). PCR conditions: initial 94°C step for 2 min followed by 30 PCR cycles of denaturation at 94°C/ 30 s, annealing at 50°C/30 s and extension at 72°C/30 s, ending with

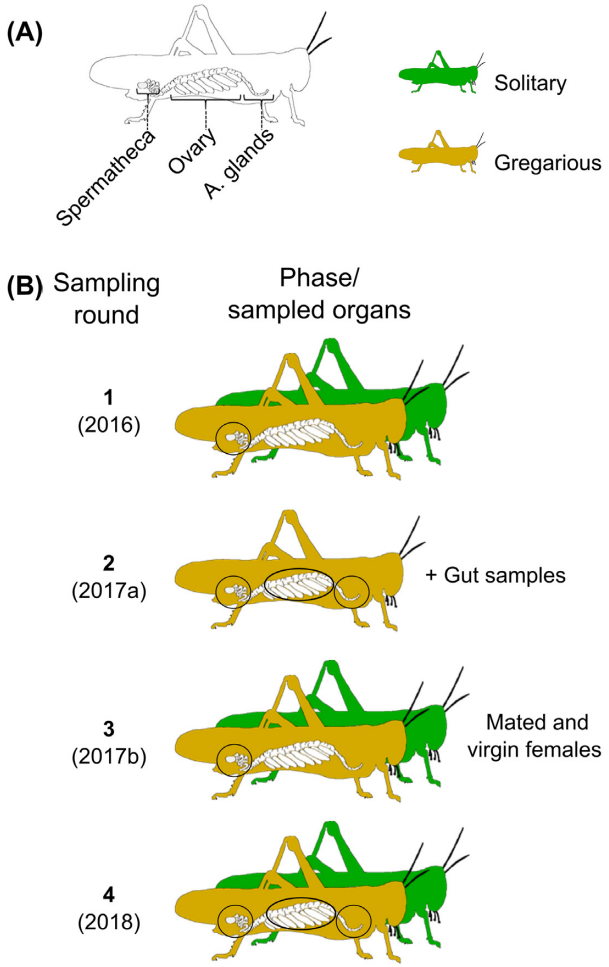

Figure 1. Sampling strategy of the study. **(A)** The locust-female reproductive organs tested in the study; yellow and green colors represent gregarious and solitary locusts, respectively, in (B). **(B)** The locust reproductive tract sampling strategy. Each row represents one sampling round, and black circles mark the sampled tissues in that round.

4 min at 72°C. The reactions were performed using the PCR master mix Go Taq® Green Master Mix (Promega Corporation, Madison, WI). PCR products were validated by electrophoresis and visualization of the amplified products on agarose gel. Deep sequencing of the amplified amplicons was conducted on an Illumina MiSeq platform at the Chicago Sequencing Center of the University of Illinois.

Testing whether soil can serve as an infection vector

We conducted a dedicated experiment to test a potential role of the soil as a vector of the spread of bacteria among females. Nine newly molted, virgin, mature, gregarious *S. gregaria* females were introduced individually to a cage together with one mature male and one 5th instar male (to maintain gregarious conditions). A 50 ml centrifuge tube (Corning, NY) filled with autoclaved sand saturated with *Brevibacterium salitolerance*-containing saline was supplied for oviposition (a fresh tube was replaced daily). Once oviposition was observed, each female was transferred to a new clean cage under identical ambient conditions and was provided with a 50 ml centrifuge tube containing sand saturated with bacteria-free saline until 2nd oviposition. The females were then washed and dissected aseptically according to the protocol described above, and their reproductive tracts and hindgut were excised and kept at −20°C until further use. The 2nd laid egg pods were incubated at 37°C and the hatchlings were also sampled.

DNA was extracted by crushing the samples individually (n = 9) in 50 μl (spermatheca and accessory glands) or 100 μl (ovary, hindgut and hatchling) of proteinase K buffer solution 2 mg/ml, (QIAGEN, Hilden, Germany), followed by incubation for 45 min at 35°C and then for 10 min at 95°C (Lavy *et al.* 2015). A diagnostic PCR was then performed with *Brevibacterium*-specific primers to determine the presence of the bacterium in the different samples: Forward- 5′-CGGTACCTGCAGAAGAAGT-3′, Reverse-5′-GTCAGTHACAGCCCA GAGT-3′ (Gelsomino *et al.* 2004). PCR conditions: initial 95°C step for 3 min followed by 35 PCR cycles of denaturation at 95°C/15 s, annealing at 60°C/30 s and extension at 72°C/10 s, ending with 2 min at 72°C. The reactions were performed using the PCR master mix KAPA2G FastTM (KAPA Biosystems, Wilmington, MA). The above protocol was thoroughly examined using controls of cultured *B. salitolerance*, DNA extraction of *B. salitolerance*, a mixture of the two and a mixture of extracted DNA with the examined organs (taken from additional locusts that were not part of that experiment) to exclude locust-derived inhibitors.

Preparation of *Brevibacterium salitolerance*-containing saline

Brevibacterium was isolated from *S. gregaria* spermatheca on lysogeny broth (LB) agar. A fragment of its 16S rRNA gene was amplified and sequenced using the universal primers 341F and 806R (the sequence is detailed above). That bacterium was taxonomically classified as *B. salitolerance* using the EZbiocloud database (top hit type-strain: TRM 415, similarity: 99.47%).

Bacteria were incubated overnight in liquid LB medium at 37°C to reach a stationary stage; the culture was then centrifuged (4000 rpm for 4 min) and the supernatant was discarded. The cell pellet was re-suspended in autoclaved 0.9% NaCl saline and centrifuged again under the same protocol. The supernatant was discarded and the cells were suspended again in fresh autoclaved 0.9% NaCl solution and diluted 100-fold in sterile saline to reach a final concentration of $\sim 10^7$ cells/ml.

Data analyses

Demultiplexed raw sequences were quality-filtered (bases with a PHRED score of <20 were removed) and merged using PEAR (Zhang *et al.* 2014). Sequences of less than 380 bp (after merging and trimming) were discarded. Data were then analyzed using the Quantitative Insights Into Microbial Ecology (QIIME) package (Caporaso *et al.* 2010). Vsearch (Rognes *et al.* 2016) was used for chimera detection and elimination; OTU picking (0.99 similarity) and taxonomy assignment were performed using the Silva database (version 128). OTUs assigned as Chloroplast or *Escherichia coli* were excluded from the downstream analysis as they potentially originated in the food and our inability to completely avoid Master Mix-derived *E. coli* fragments. To ensure data evenness, before analysis the data were rarefied to 980 seqs/sample. All statistical analyses were conducted using 'R' v.3.4.1. (R Core Team 2013). Bray-Curtis-based Analysis of similarities-'ANOSIM', principal coordinate analysis (PCoA) and canonical analysis of principal coordinates were carried out using the 'vegan 2.4–3' package (functions used: 'anosim', 'vegdist' and 'capscale', respectively; Oksanen *et al.* 2008). For the

Table 1. Genus level, Bray-Curtis-based Analysis of Similarities (ANOSIM) of the spermatheca bacterial composition of mated and virgin females sampled at round 3.

Groups compared	ANOSIM P	ANOSIM R
Gregarious (n = 24) vs. solitary (n = 13)	$P = 0.378$	$R = 0.015$
Mated (n = 20) vs. virgins (n = 17)	$P = 0.08$	$R = 0.043$

Table 2. Genus level, Bray-Curtis-based Analysis of Similarities (ANOSIM) of the bacterial composition of the different reproduction organs within sampling rounds 2 and 4 (depicted in Fig. 2).

Sampling round and phase	ANOSIM P	ANOSIM R
Gregarious round 2	$P < 0.001$	$R = 0.2$
Gregarious round 4	$P < 0.001$	$R = 0.45$
Solitary round 4	$P = 0.47$	$R = 0.0003$

PCoA analysis we also used the 'stats 3.6.1' package (functions: 'hclust' and 'cmdscale'; R Core Team, 2013).

RESULTS

Overall, we successfully established the bacterial composition of 202 tissue samples of the reproductive and digestive tracts (158 and 44 samples, respectively) of gregarious and solitary *S. gregaria* females via 16S rRNA gene amplicon sequencing (SRA archive accession number: PRJNA598984).

Copulation does not affect spermathecal bacteria

No significant differences were found between mated and virgin females of both phases. Pooling the data together to compare between mated and virgin females (of both phases together) also did not indicate a significant difference between the treatments (Table 1). It did, however, border the accepted statistical significance level ($P = 0.05$) (Table 1) and therefore the potential effect of the males during mating cannot be completely ruled out.

Phase effect on bacteria of the reproductive organs

Comparative investigation of the bacterial composition of the gonads and other reproductive organs (i.e. ovary, accessory glands and spermatheca) of gregarious and solitary females (data: sampling round 4 in Fig. 1) revealed different spatial bacterial distribution for solitary and gregarious forms (Fig. 2, Table 2). The reproductive tissues of the solitary locusts were relatively similar in their bacterial composition (emphasized by the dense clustering of the solitary reproductive samples in Fig. 2a) and maintained a dominant fraction of *Micrococcus*, *Staphylococcus*, *Acinetobacter* and *Ralstonia* throughout the reproductive system (Fig. 2b, Table 2). By contrast, in gregarious locusts of the same sampling round, the different reproductive organs significantly differed in bacterial content (manifested as different clusters for the different organs of the gregarious samples in Fig. 2a and Table 2), demonstrating an interesting trend of rostral-to-caudal reduction in core bacterial diversity from the accessory glands toward the spermatheca (Fig. 2b). The latter was completely dominated by *Brevibacterium salitolerance* (Actinobacteria). In order to confirm these results, we added as a control to our analyses additional female tissues that included gregarious female gut and reproductive tissues (data: round 2 in Fig. 1), as well as additional gregarious and solitary spermatheca samples (data: round 1 in Fig. 1). This indeed confirmed that the bacterial composition of the reproductive organs is different from that of the gut (between-groups genus level, Bray-Curtis-based ANOSIM: $P < 0.001$, $R = 0.9$; Fig. 3) and therefore ascertained that these are true bacteria of the locust reproductive organs. In addition, it enabled us to explore the bacterial community composition of the spermatheca of both phases across the four different sampling rounds (Fig. 1) and to compare gregarious bacterial content of the accessory glands and ovaries at two different time points (data: rounds 2 and 4 in Fig. 1).

These analyses revealed that while the gregarious samples of round 4 demonstrated the distinct pattern of decrease in bacterial biodiversity from the accessory glands towards the spermatheca, the gregarious samples of round 2 reached higher bacterial biodiversity throughout the reproductive system and shared the core bacterial genera of *Micrococcus* and *Acinetobacter,* both among themselves and with the round 4 solitary samples (Fig. 2).

This inconsistency in the gregarious bacterial biodiversity is even more apparent when exploring all four sampling rounds. While solitary spermatheca samples maintained a high diversity index throughout the sampling rounds, gregarious spermatheca were characterized by low biodiversity in rounds 1 and 4 when the samples were dominated by *B. salitolerance*, and significantly higher index values, similar to those of the solitary locusts, during sampling rounds 2 and 3 (Fig. 4).

Interestingly, *B. salitolerance* dominance in some of the spermatheca samples (Fig. 4) was not reflected in other reproductive organs (Fig. 2). A constrained canonical analysis of the reproduction organs reinforced this finding (Fig. 5). Hence, in the solitary and gregarious female samples of round 2 no specific bacterium stands out as the main cause for the noted variability. By contrast, in the gregarious females of round 4, *B. salitolerance* was a main driver of the explained variability (Fig. 5).

The sand is not a vector of bacterium spread

In our investigation of the sand as a possible vector for bacterial spread within the gregarious population, we observed that *B. salitolerance* failed to infect the examined tissues of females that laid their eggs in the *B. salitolerance*-contaminated sand. Traces of *Brevibacterium* were also absent from the offspring of these females.

DISCUSSION

Understanding the dynamics of reproductive system-associated bacteria in the desert locust can provide further understanding of locust biology; it may also contribute to potential means for locust control, and on a larger scale to provide important information on insects-associated bacteria.

It is well established that symbiotic bacteria play an important role in many aspects of insect biology (Moran and Baumann 2000; Oliver *et al.* 2010; Bennett and Moran 2013; Salem *et al.* 2015; Douglas 2015). Here, for the first time we describe the temporal dynamics of extracellular bacteria in an insect reproductive system. Using the desert locust as our model, we also investigated possible interactions between the reproductive system bacteria and locust density-dependent phase polyphenism across time. High similarity between laboratory-reared and field-collected locusts (Lavy *et al.* 2019) indicates that our findings are not laboratory artifacts.

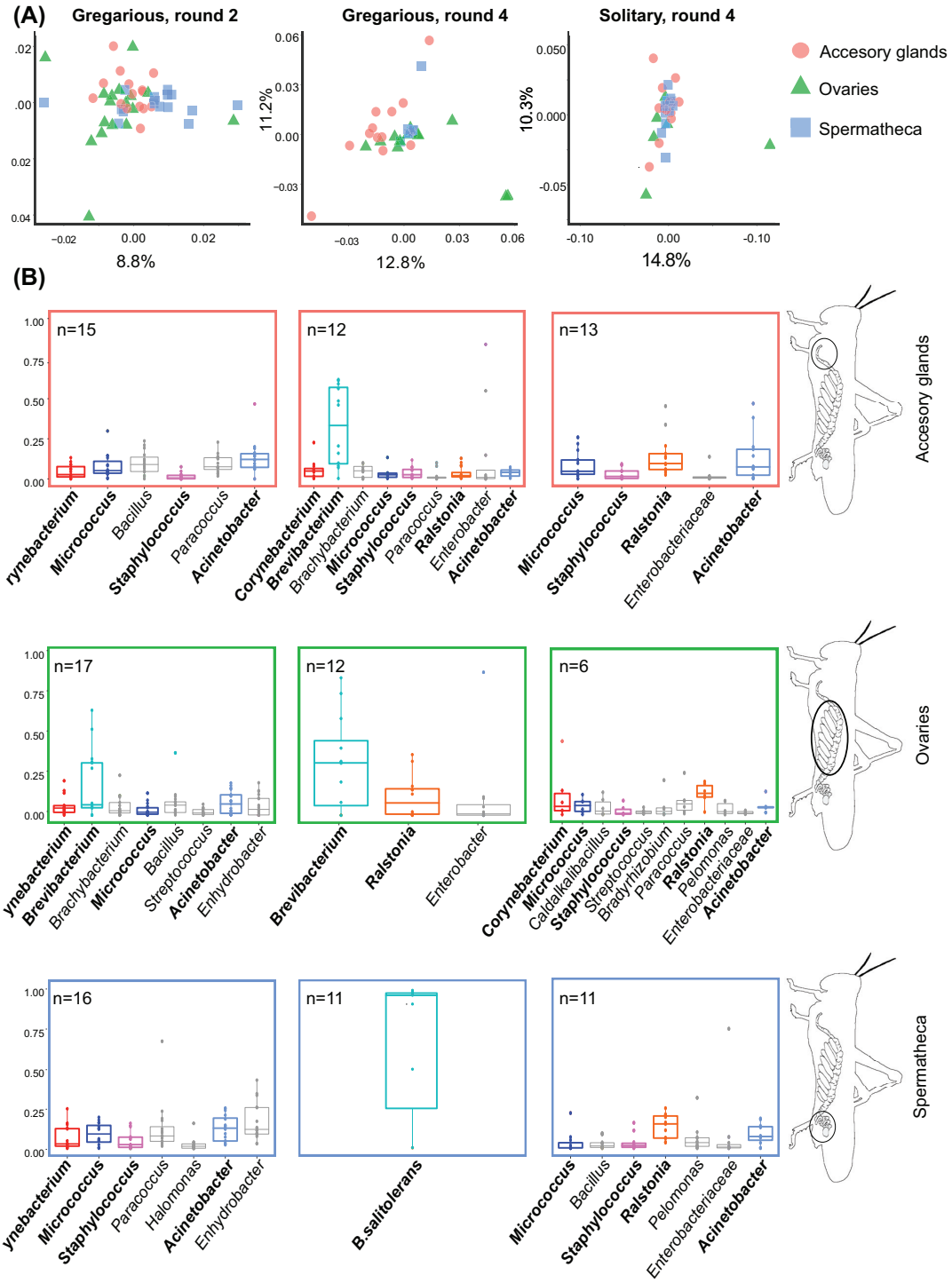

Figure 2. **(A)** Genus level, Bray-Curtis distances-based principal coordinate analysis (PCoA) of female reproductive organ microbiota composition, presented per-phase and per-year of sampling. **(B)** Relative abundance of the core bacterial genera in female gonads, in the data presented in (A). Only genera that were present in at least 80% of samples within a group are presented. For example in the gregarious females' ovaries of round 4, 80% harbored *Brevibacterium*, *Ralstonia* and *Eterobacter*. Bacteria noted in the main text are colored and highlighted. The same sampling rounds designation are used in both (A) and (B). ∗Only sampling rounds including a full set of the female reproductive tract (i.e. accessory glands, ovaries and spermatheca) are presented.

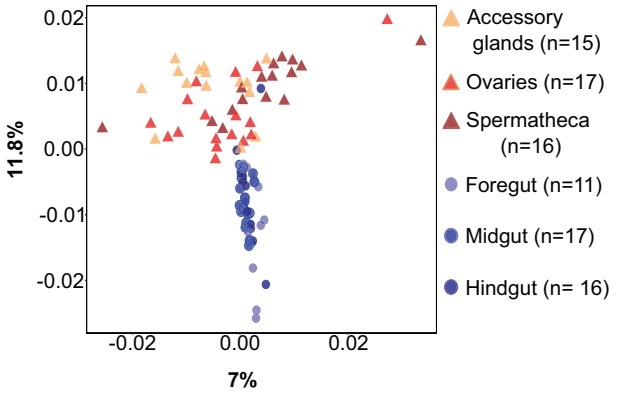

Figure 3. Genus level, Bray-Curtis distances-based principal coordinate analysis (PCoA) of female gregarious locust reproductive organs and gut bacterial composition. Digestive tract samples and reproductive tract samples (differentiated by shape and color) cluster separately. (Based on the data of Fig. 1, round 3).

The core bacterial composition of the female locust reproductive system consists of at least three bacterial genera that are frequently present (unless replaced by *Brevibacterium*): *Micrococcus*, *Staphylococcus* and *Acinetobacter*. These bacteria were found in both the gregarious and the solitary females across sampling rounds. *Ralstonia*, by contrast, was present only in some of the samples in round 4. It thus seems safe to assume that *Ralstonia* is not a constant inhabitant of the female reproductive tract. *Micrococcus*, *Staphylococcus* and *Acinetobacter* have all been found previously in the copulatory organ of the female bedbug (Otti *et al.* 2017; Bellinvia *et al.* 2019). *Staphylococcus* was also found in the immature eggs extracted from the body of *Anoplophora chinensis* (beetles) (Rizzi *et al.* 2013).

Overall, temporal dynamics of the spermatheca microbiome is reminiscent of that observed in gut bacterial shifts, as described by Lavy *et al.* (2019), albeit different genera are involved. While in the gregarious locust hindgut there is a core population of *Enterobacter* with transient *Weissella* blooms, in the gregarious locust spermatheca we can see blooms of *Brevibacterium*. In both cases, the dominance of these bacteria (i.e. *Brevbacterium/Weissella*) eventually ceases and the original bacterial consortium is restored. These conspicuous shifts in bacterial composition occurred only in the gregarious population while the solitary individuals retained a consistent core bacterial composition. Hence, our findings suggest not only that the locust reproductive system maintains a bacterial composition that is unique and different from that of the gut but also that this composition reemerges through different generations of both gregarious and solitary individuals, suggesting a symbiont transgenerational transmission route. To some extent, these temporal fluctuations are analogous to human vaginal microbiomes that are usually *Lactobacillus*-dominated, but under some circumstances can shift towards *Gardnerella vaginalis* dominancy (Saunders *et al.* 2006; Jang *et al.* 2017; Castro *et al.* 2018).

The nature of this symbiotic interaction within the reproductive system remains unclear. However, bacterial colonization resistance is a main factor in the immunity of the locust gut (Dillon *et al.* 2005). Therefore, it is highly plausible that the same is true for the female reproductive system, whose integrity has obvious implications for fitness. This hypothesis is reinforced when taking into account the high prevalence of Actinobacteria (i.e. *Micrococcus*, *Corynebacterium* and *Brevibacterium*), which are known for the production of antimicrobial substances and have been shown to take an active part in the immunity of several insects (Kaltenpoth 2009; Zucchi, Prado and Cônsoli 2012; Engl *et al.* 2018). Previous studies concerning the human vaginal microbiome provide further reinforcement of this hypothesis as it has been shown that the health of the human vagina is closely linked to the dominancy of *Lactobacillus* species, which are able to secrete substances that were shown to inhibit the pathogenic bacterium *G. vaginalis* from causing vaginal infections (Saunders *et al.* 2006; Jang *et al.* 2017; Castro *et al.* 2018).

It was previously suggested that gregarious individuals maintain a higher bacterial diversity because of their higher potential exposure to pathogens (Dillon *et al.* 2005). Our previous work, as well as the current study, demonstrate that both gregarious and solitary locusts retain a similar biodiversity baseline, with transient decreases in the gregarious biodiversity indices (Lavy *et al.* 2019; Fig. 1). However, the need to regulate this bacterial consortium may incur a considerable energetic cost (Lochmiller and Deerenberg 2000; Wilson *et al.* 2002; Douglas 2018), which is expected to be higher for the gregarious individuals that experience higher potential exposure to pathogens as a result of their crowding tendency (Wilson *et al.* 2002; Groulx and Forrest 2018). Ample evidence has been gathered concerning the higher lipid content of gregarious compared with solitary locusts, and in regard to their elevated response to the release of adipokinetic hormone (AKH) (Ayali and Pener 1992, 1995 reviewed by Pener, Ayali and Golenser 1997; Cullen *et al.* 2017). In addition, Goldsworthy *et al.* (2002) demonstrated how hemolymph AKH activates the prophenoloxidase cascade in gregarious *Locusta migratoria*. It is possible that these mechanisms, which have been attributed mainly to the ability to perform rapid long-distance flights in the gregarious phase, may also serve to compensate for the higher energetic cost of activating the immune system vigorously and accurately to regulate a constant bacterial composition within a swarm.

In an early study, *Brevibacterium* was found in whole-body grasshopper homogenates and attributed to gut microbiota (Bucher and Stephens 1959). However, its presence in the gut has not been confirmed by any of the subsequent studies that used isolated-gut examination (Dillon, Vennard and Charnley 2000; Dillon and Charnley 2002; Dillon *et al.* 2005, 2008, 2010; Shi *et al.* 2014; Lavy *et al.* 2019). Therefore, it is possible that the original reference to *Brevibacterium* as a gut bacterium was a result of the experimental protocol used at the time.

The spatial distribution pattern of *Brevibacterium* across reproductive organs reveals an increase in its dominance from the anterior accessory glands towards the posterior spermatheca (gregarious round 4 in Fig. 2). This finding may seem to somewhat contradict the hypothesis of bacterial colonization resistance by the reproduction system. Nevertheless, the core bacterial genera of *Micrococcus*, *Staphylococcus* and *Acinetobacter* remained constant in the accessory glands, which could serve as a refuge, conserving these bacteria in the reproductive tracts. It does not seem likely that *Brevibacterium* completely eliminates the other bacteria since we observed their constant presence across sampling years and organs (Figs 1 and 3).

Unfortunately, though it is clear that the observed shifts in the locust bacterial composition between sampling rounds are a phenomenon of the gregarious phase, we could not determine the specific mechanism underlying these changes. The sand used as an oviposition substrate, though being saturated with *B. salitolerance* did not serve as an inoculation vector, and

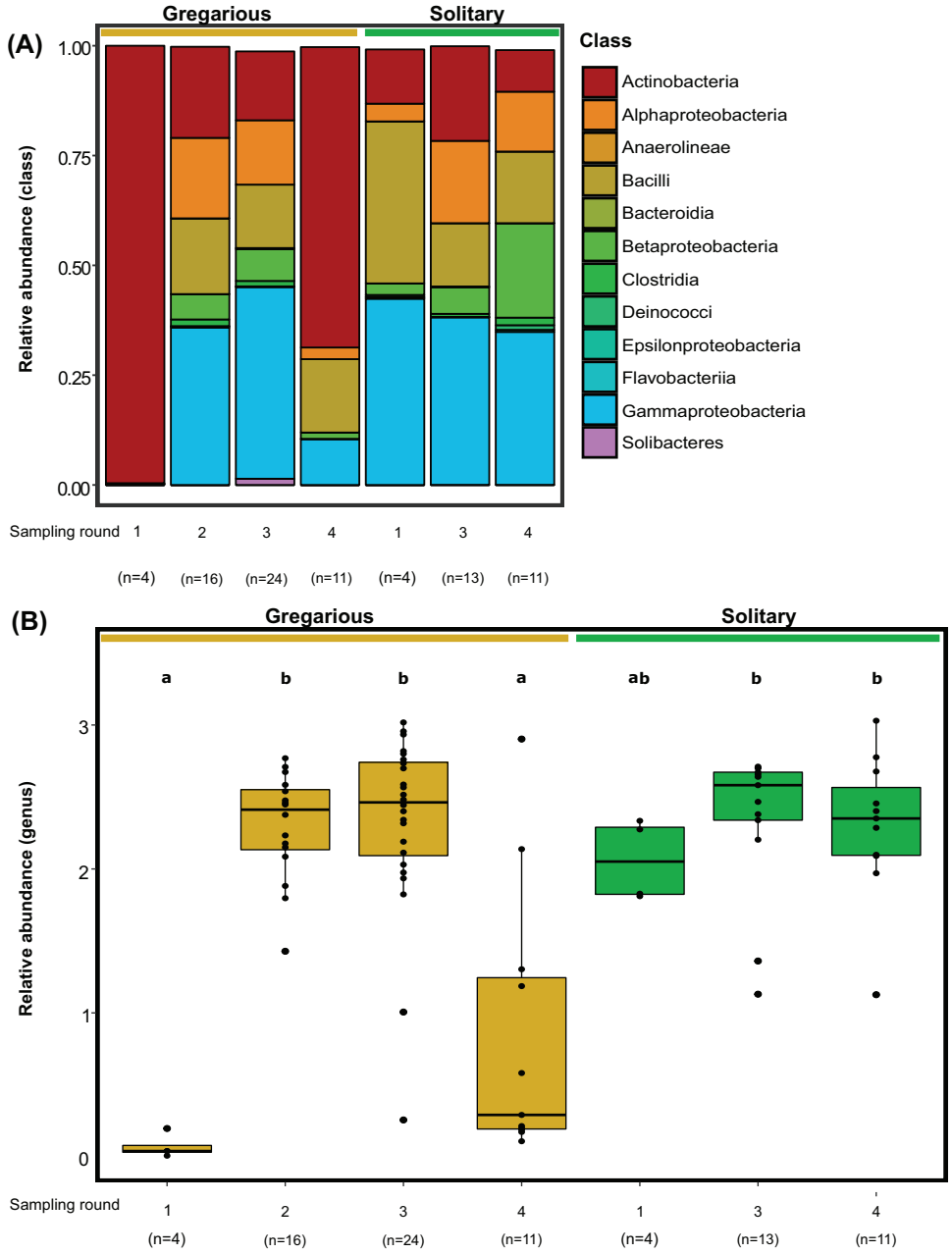

Figure 4. **(A)** Mean relative abundance of the bacterial classes in the spermathecae of gregarious and solitary females from different sampling years. **(B)** Genus-level Shannon diversity index of the bacterial composition in the spermatheca of gregarious and solitary locusts in different years. Differences between the groups were analyzed by Kruskal-Wallis test and Dunn's test as post hoc.

the female mating status (virgin or mated) did not significantly affect the spermathecal bacterial composition. One possible explanation for this mystery is that the males are the vector responsible for spreading this bacterium among females. There is a possibility that since the 'mated/virgin' experiment was conducted between *Brevibacterium* blooms (Table 1, Figs 1, 3 and 4), the potential effect of mating on the spermatheca bacterial composition was not revealed. This hypothesis is reinforced by the partial separation that was observed in the 'mated/virgin' comparison ($P = 0.08$; Table 2), suggesting that males might have some impact on spermathecal bacterial composition.

In conclusion, this study reveals the unique bacterial composition of the locust reproductive system for the first time. As density-dependent polyphenism has developed in many insect groups, including hemipterans, lepidopterans, coleopterans and phasmids (Reeson *et al.* 1998; Wilson and Reeson 1998; Barnes and Siva-Jothy 2000; Lo, Simpson and Sword 2018), the importance of this study lies beyond that of locust ecology and reveals a novel aspect of this phenomenon. Here, we have established that insect populations may retain a constant reproductive system microbiota across generations and that this microbiota is affected by population density. The costs and benefits of a specific bacterial consortium under

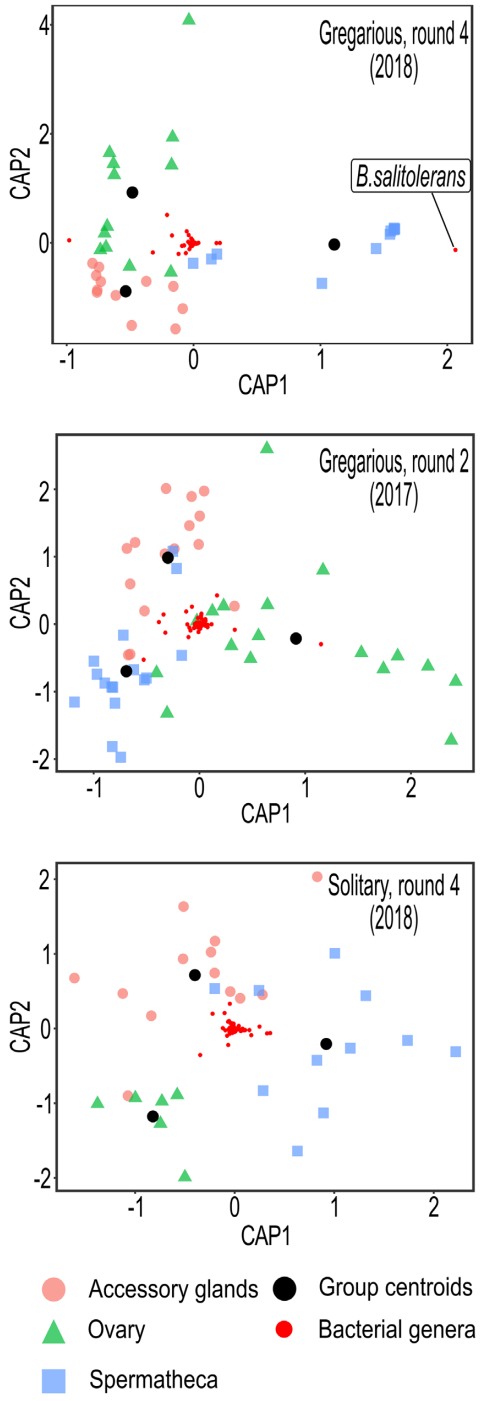

Figure 5. Genus level, Bray-Curtis distances-based, constrained canonical analysis of bacterial composition according to the female gonads (presented by phase and year of sampling). The axes display the variation in the explanatory variables (i.e. the gonad groups). The location of the bacterial genera relative to the group centroids represents the between-group variability explained by a specific bacterial taxon. For example, in the gregarious 2018 samples, we can see that *B. salitolerance* is a main factor of the explained variability in bacterial composition between the spermatheca samples and the other reproductive parts.

different ecological conditions remain to be determined. However, understanding the interaction of the bacterial composition with the host population density is an important step in this direction.

Conflict of interest. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

REFERENCES

Ayali A, Pener MP. Density-dependent phase polymorphism affects response to adipokinetic hormone in Locusta. *Comp Biochem Physiol – Part A Physiol* 1992;**101**:549–52.

Ayali A, Pener MP. The relations of adipokinetic response and body lipid content in locusts (*Locusta migratoria migratorioides*) with special reference to phase polymorphism. *J Insect Physiol* 1995;**41**:85–89.

Ayali A. The puzzle of locust density-dependent phase polyphenism. *Curr Opin Insect Sci* 2019;**35**:41–47.

Barnes AI, Siva-Jothy MT. Density-dependent prophylaxis in the mealworm beetle *Tenebrio molitor L.* (Coleoptera: Tenebrionidae): Cuticular melanization is an indicator of investment in immunity. *Proc R Soc B Biol Sci* 2000;**267**: 177–82.

Bellinvia S, Jhonston PR, Reinhardt K *et al.* Bacterial communities of the reproductive organs of virgin and mated common bedbugs, *Cimex lectularius*. *Ecol Entomol* 2019;**45**:142–154.

Bennett GM, Moran NA. Small, smaller, smallest: The origins and evolution of ancient dual symbioses in a phloem-feeding insect. *Genome Biol Evol* 2013;**5**:1675–88.

Berman TS, Ayali A, Gefen E. Neural control of gas exchange patterns in insects: locust density-dependent phases as a test case. *PLoS One* 2013;**8**:e59967.

Bourtzis K, Miller T. *Insect Simbiosis*. Volume **2**. CRC press; 2006.

Bucher GE, Stephens JM. Bacteria of grasshoppers of western Canada: II. The Pseudomonadaceae, Achromobacteraceae, Micrococcaceae, Brevibacteriaceae, Lactobacillaceae, and less important families. *J Insect Pathol* 1959;**1**: 374–90.

Caporaso JG, Kuczynski J, Stombaugh J *et al.* QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* 2010;**7**:335–6.

Castro J, Martins AP, Rodrigues ME *et al. Lactobacillus crispatus* represses vaginolysin expression by BV associated Gardnerella vaginalis and reduces cell cytotoxicity. *Anaerobe* 2018;**50**:60–63.

Cullen DA, Cease AJ, Latchininsky AV *et al.* From molecules to management: mechanisms and consequences of locust phase polyphenism. *Adv In Insect Phys* 2017;**53**:167–285.

Dillon RJ, Charnley AK. Chemical barriers to gut infection in the desert locust - in-vivo production of antimicrobial phenols associated with the bacterium Pantoea agglomerans. *J Invertebr Pathol* 1995;**66**:72–75.

Dillon RJ, Charnley AK. Invasion of the pathogenic fungus *Metarhizium anisopliae* through the guts of germ-free desert locusts, *Schistocerca gregaria*. *Mycopathologia* 1986;**96**: 59–66.

Dillon RJ, Charnley AK. Mutualism between the desert locust *Schistocerca gregaria* and its gut microbiota. *Res Microbiol* 2002;**153**:503–9.

Dillon RJ, Vennard CT, Buckling A *et al*. Diversity of locust gut bacteria protects against pathogen invasion. *Ecol Lett* 2005;**8**:1291–8.
Dillon RJ, Vennard CT, Charnley AK. A Note: Gut bacteria produce components of a locust cohesion pheromone. *J Appl Microbiol* 2002;**92**:759–63.
Dillon RJ, Vennard CT, Charnley AK. Exploitation of gut bacteria in the locust. *Nature* 2000;**403**:851–285.
Dillon RJ, Webster G, Weightman AJ *et al*. Composition of Acridid gut bacterial communities as revealed by 16S rRNA gene analysis. *J Invertebr Pathol* 2008;**97**:265–72.
Dillon RJ, Webster G, Weightman AJ *et al*. Diversity of gut microbiota increases with aging and starvation in the desert locust. *Int J Gen Mol Microbiol* 2010;**97**:69–77.
Douglas AE. Multiorganismal insects: diversity and function of resident microorganisms. *Annu Rev Entomol* 2015;**60**:17–34.
Douglas AE. ScienceDirect Omics and the metabolic function of insect – microbial symbioses. *Curr Opin Insect Sci* 2018,**29**:1–6.
Dávila F, Botteaux A, Bauman D *et al*. Antibacterial activity of male and female sperm-storage organs in ants. *J Exp Biol* 2018;**221**:jeb.175158.
Engl T, Kroiss J, Kai M *et al*. Evolutionary stability of antibiotic protection in a defensive symbiosis. *Proc Natl Acad Sci* 2018;**115**:E2020–9.
Gelsomino R, Vancanneyt M, Vandekerckhove TM *et al*. Development of a 16S rRNA primer for the detection of *Brevibacterium* spp. *Lett Appl Microbiol* 2004;**38**:532–5.
Geva N, Guershon M, Orlova M *et al*. Memoirs of a locust: Density-dependent behavioral change as a model for learning and memory. *Neurobiol Learn Mem* 2010;**93**:175–82.
Gil R, Latorre A. Unity makes strength: a review on mutualistic symbiosis in representative insect clades. *Life* 2019;**9**:21.
Goldsworthy G, Opoku-Ware K, Mullen L *et al*.. Adipokinetic hormone enhances laminarin and bacterial lipopolysaccharide-induced activation of the prophenoloxidase cascade in the African migratory locust, *Locusta migratoria*. *J Insect Physiol* 2002;**48**:601–8.
Groulx AF, Forrest JRK. Nesting aggregation as a predictor of brood parasitism in mason bees (*Osmia* spp.). *Ecol Entomol* 2018;**43**:182–91.
He Z, Zheng Y, Yu WJ *et al*. How do *Wolbachia* modify the Drosophila ovary? New evidences support the "titration-restitution" model for the mechanisms of *Wolbachia*-induced CI. *BMC Genomics* 2019;**20**:608.
Hunter MS, Perlman SJ, Kelly SE. A bacterial symbiont in the Bacteroidetes induces cytoplasmic incompatibility in the parasitoid wasp *Encarsia pergandiella*. *Proc R Soc B Biol Sci* 2003;**270**:2185–90.
Jang SE, Jeong JJ, Choi SY *et al*. *Lactobacillus* rhamnosus HN001 and lactobacillus acidophilus La-14 attenuate gardnerella vaginalis-infected bacterial vaginosis in mice. *Nutrients* 2017;**9**:531.
Kaltenpoth M. Actinobacteria as mutualists: general healthcare for insects? *Trends Microbiol* 2009;**17**:529–235.
Landmann F. The Wolbachia endosymbionts. *Microbiol Spectr* 2019;**7**:1–15.
Lavy O, Gophna U, Gefen E *et al*. The effect of density-dependent phase on the locust gut bacterial composition. *Front Microbiol* 2019;**10**:1–8.
Lavy O, Sher N, Malik A *et al*. Do bacterial symbionts govern aphid's dropping behavior? *Environ Entomol* 2015;**44**:588–92.
Lochmiller RL, Deerenberg C. Trade-offs in evolutionary immunology: Just what is the cost of immunity? *Oikos* 2000;**88**:87–98.
Lo N, Simpson SJ, Sword GA. Epigenetics and developmental plasticity in orthopteroid insects. *Curr Opin Insect Sci* 2018;**25**:25–34.
Marchini D, Ciolfi S, Gottardo M *et al*. Bacteria of the genus Erwinia found in the spermatheca of the laurel psyllid *Trioza alacris*. *Arch Microbiol* 2014;**196**:901–5.
Morales-Jiménez J, Zúñiga G, Ramírez-Saad HC *et al*. Gut-associated bacteria throughout the life cycle of the bark beetle *Dendroctonus rhizophagus* thomas and bright (Curculionidae: Scolytinae) and their cellulolytic activities. *Microb Ecol* 2012;**64**:268–78.
Moran NA, Baumann P. Bacterial endosymbionts in animals. *Curr Opin Microbiol* 2000;**3**:270–5.
Ohkuma M. Symbioses of flagellates and prokaryotes in the gut of lower termites. *Trends Microbiol* 2008;**16**:345–52.
Oksanen J, Kindt R, Legendre P *et al*. The vegan package. *Community Ecol Packag* 2008:1–190.
Oliver KM, Degnan PH, Burke GR *et al*. Facultative symbionts in aphids and the horizontal transfer of ecologically important traits. *Annu Rev Entomol* 2010;**55**:247–66.
Opatovsky I, Santos-Garcia D, Ruan Z *et al*. Modeling trophic dependencies and exchanges among insects' bacterial symbionts in a host-simulated environment. *BMC Genomics* 2018;**19**:1–14.
Otti O, Deines P, Hammerschmidt K *et al*. Regular wounding in a natural system: Bacteria associated with reproductive organs of bedbugs and their quorum sensing abilities. *Front Immunol* 2017;**8**:1–11.
Otti O. Genitalia-associated microbes in insects. *Insect Sci* 2015;**22**:325–39.
Pascini TV, Martins GF. The insect spermatheca: an overview. *Zoology* 2016;**121**:56–71.
Pener MP, Ayali A, Golenser E. Adipokinetic hormone and flight fuel related characteristics of density-dependent locust phase polymorphism: A review. *Comp Biochem Physiol - B Biochem Mol Biol* 1997;**117**:513–24.
Pener MP, Simpson SJ. Locust phase polyphenism: an update. *Adv In Insect Phys* 2009;**36**:1–272.
Raina A, Murphy C, Florane C *et al*. Structure of spermatheca, sperm dynamics, and associated bacteria in Formosan subterranean termite (Isoptera : Rhinotermitidae). *Ann Entomol Soc Am* 2007;**100**:418–24.
R Core Team. A Language and Environment for Statistical Computing. R foundation for statistical computing, Vienna. 2013.
Reeson AF, Wilson K, Gunn A *et al*. Baculovirus resistance in the noctuid *Spodoptera exempta* is phenotypically plastic and responds to population density. *Proc R Soc B Biol Sci* 1998;**265**:1787–91.
Rizzi A, Crotti E, Borruso L *et al*. Characterization of the bacterial community associated with larvae and adults of Anoplophora Chinensis collected in Italy by culture and culture-independent methods. *Biomed Res Int* 2013;**2013**:420287.
Rognes T, Flouri T, Nichols B *et al*.. VSEARCH: a versatile open source tool for metagenomics. *PeerJ* 2016;**4**:e2409v1.
Salem H, Florez L, Gerardo N *et al*. An out-of-body experience: the extracellular dimension for the transmission of mutualistic bacteria in insects. *Proc R Soc B Biol Sci* 2015;**282**:20142957.
Saunders S, Bocking A, Challis J *et al*. Effect of Lactobacillus challenge on Gardnerella vaginalis biofilms. *Colloids Surfaces B Biointerfaces* 2006;**55**:138–42.
Segata N, Baldini F, Pompon J *et al*. The reproductive tracts of two malaria vectors are populated by a core microbiome and by

gender- and swarm-enriched microbial biomarkers. *Sci Rep* 2016;**6**:24207.

Shi W, Guo Y, Xu C *et al*. Unveiling the mechanism by which microsporidian parasites prevent locust swarm behavior. *Proc Natl Acad Sci* 2014;**111**:1343–8.

Wilson K, Reeson AF. Density-dependent prophylaxis: Evidence from Lepidoptera-baculovirus interactions? *Ecol Entomol* 1998;**23**:100–1.

Wilson K, Thomas MB, Blanford S *et al*. Coping with crowds: density-dependent disease resistance in desert locusts. *Proc Natl Acad Sci U S A* 2002;**99**:5471–5.

Zhang J, Kobert K, Flouri T *et al*. PEAR: A fast and accurate Illumina Paired-End reAd mergeR. *Bioinformatics* 2014;**30**:614–20.

Zucchi TD, Prado SS, Cônsoli FL. The gastric caeca of pentatomids as a house for actinomycetes. *BMC Microbiol* 2012;**12**:101.