**Tetranychus urticae** mites do not mount an induced immune response against bacteria

Gonçalo Santos-Matos1,2,†, Nicky Wybouw3,4,†, Nelson E. Martins1,4,‡, Flore Zélé2, Maria Riga5, Alexandre B. Leitão1,4,*, John Vontas6,7, Miodrag Grbić8,9, Thomas Van Leeuwen3,4, Sara Magalhaes1,10,∥ and Elio Sucena1,10,∥

1Instituto Gulbenkian de Ciência, Rua da Quinta Grande, 6 2780-156 Oeiras, Portugal
2E3c: Centre for Ecology, Evolution and Environmental Changes, Faculdade de Ciências, Universidade de Lisboa, Campo Grande, 1749-016 Lisbon, Portugal
3Laboratory for Agrozoology, Department of Crop Protection, University of Ghent, Coupure links 653, 9000 Ghent, Belgium
4Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, Science Park 904, 1098 XH Amsterdam, The Netherlands
5Faculty of Applied Biotechnology and Biology, Department of Biology, University of Crete, Vasilika Vouton, PO Box 2208, 71409 Heraklion, Greece
6Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology Hellas, 100 N. Plastira Street, 70013 Heraklion, Crete, Greece
7Laboratory of Pesticide Science, Department of Crop Science, Agricultural University of Athens, 75 Iera Odos Street, 11855 Athens, Greece
8Department of Biology, University of Western Ontario, London, Canada N6A 5B7
9Instituto de Ciencias de la Vid y del Vino Consejo Superior de Investigaciones Científicas, Universidad de la Rioja, 26006 Logroño, Spain
10Departamento de Biología Animal, Faculdade de Ciências, Universidade de Lisboa, Campo Grande, 1749-016 Lisboa, Portugal

The genome of the spider mite *Tetranychus urticae*, a herbivore, is missing important elements of the canonical *Drosophila* immune pathways necessary to fight bacterial infections. However, it is not known whether spider mites can mount an immune response and survive bacterial infection. In other chelicerates, bacterial infection elicits a response mediated by immune effectors leading to the survival of infected organisms. In *T. urticae*, infection by either *Escherichia coli* or *Bacillus megaterium* did not elicit a response mediated by immune effectors leading to the survival of infected organisms. In *T. urticae*, infection by either *Escherichia coli* or *Bacillus megaterium* did not elicit a response as assessed through genome-wide transcriptomic analysis. In line with this, spider mites died within days even upon injection with low doses of bacteria that are non-pathogenic to *Drosophila*. Moreover, bacterial populations grew exponentially inside the infected spider mites. By contrast, *Sancassania berlesei*, a litter-dwelling mite, controlled bacterial proliferation and resisted infections with both Gram-negative and Gram-positive bacteria lethal to *T. urticae*. This differential mortality between mite species was absent when mites were infected with heat-killed bacteria. Also, we found that spider mites harbour in their gut 1000-fold less bacteria than *S. berlesei*. We show that *T. urticae* has lost the capacity to mount an induced immune response against bacteria, in contrast to other mites and chelicerates but similarly to the phloem feeding aphid *Acyrthosiphon pisum*. Hence, our results reinforce the putative evolutionary link between ecological conditions regarding exposure to bacteria and the architecture of the immune response.

1. Introduction

To deal with infection, arthropods rely on several defensive mechanisms that include behavioural avoidance, physical and chemical barriers, and the
immune response [1,2]. For example, virtually all arthropods studied thus far mount some combination of cellular and humoral responses against bacteria that rely on coagulation, production of reactive oxygen species (ROS), melanization, phagocytosis and the synthesis of antimicrobial peptides (AMPs) and/or enzymes [3,4].

In the insect model system *Drosophila*, the humoral response has been dissected genetically in great detail. It relies strongly on the induction of two signalling pathways, Toll and Imd, through the recognition of Lys-type or diaminopimelic acid (DAP) type peptidoglycans, present in Gram-positive and Gram-negative bacteria, respectively, and culminating in the production of AMPs [3,5].

Genomic analyses of other holometabolous insects have revealed that most genes of the Toll and Imd pathways are conserved, namely in mosquitoes, the honeybee and the beetle *Tribolium castaneum* [6–8]. However, in the pea aphid *Acyrthosiphon pisum*, a hemimetabolous insect, the Imd pathway is incomplete and several genes coding for receptors and common AMPs could not be identified. Moreover, this aphid species does not mount an immune response to bacterial infection [9,10]. Yet, this is not a general feature of hemipterans, because the Toll and Imd pathways along with several receptors and AMPs were annotated in the genome of the brown plant hopper, *Nilaparvata lugens*, and several Toll pathway genes were shown to be upregulated upon bacterial infection in this species [11]. This pattern is also verified in another closely related hemipteran, *Rhodnius prolixus*, in which activity of the Imd pathway was experimentally confirmed [12]. Furthermore, Imd has been found in the genomes of other hemipterans such as the large milkweed bug (*Oncopeltus fasciatus*) and of the water strider *Gerris buenoi* (M van der Zee 2015, personal communication). Taken together, these observations suggest that, to a great extent, the immune response in most insects is directly comparable to that of the dipteran *Drosophila*.

In chelicerates, however, the Imd pathway seems to be incomplete in all species thus far analysed [13,14]. Notwithstanding, in *Carcinoscorpius rotundicauda*, an orthologue of the *Drosophila* NF-κB-like transcription factors, Relish, has been described and implicated in the immune response against *Pseudomonas aeruginosa* infection [15,16]. In fact, in several studied chelicerates, a response is elicited through the canonical production of antimicrobial compounds [17,18].

The two-spotted spider mite *Tetranychus urticae* feeds on the cell contents of a multitude of plant species. Its genome annotation failed to identify several canonical immunity genes, amongst which an important part of the Imd pathway and effectors such as haemolectins (von Willebrand factor-like proteins) or defensins [13,14,19] (electronic supplementary material, table S1). Two general hypotheses may explain this observation: (i) the spider mite mounts an immune response based on a different genetic basis, as do other chelicerates or (ii) as in aphids, *T. urticae* does not possess an inducible anti-bacterial immune response.

To distinguish between these hypotheses, we present experimental data describing the response of mites to bacterial systemic infection, including host survival, bacterial proliferation in the host and transcriptional responses. Additionally, we tested the generality of our results by repeating this characterization on the litter-dwelling grain mite *Sancassania berlesei* [20].

2. Results

(a) *Tetranychus urticae* is susceptible to infection with *Escherichia coli* and *Bacillus megaterium*

We tested survival of *T. urticae* following infection with *Escherichia coli*, a Gram-negative bacterium, or the Gram-positive *Bacillus megaterium*. Injecting spider mites with *E. coli* at three different concentrations—ODs 0.1, 1 or 10—significantly affected survival (Cox model, bacterial concentration effect, $\chi^2 = 19.213, p = 0.0002$; figure 1a). A pairwise comparison of the hazard ratios between spider mites injected with *E. coli* or with Luria broth (LB) confirmed that spider mite survival was severely affected (OD 0.1: $z = 9.828, p < 0.0001$; OD 1: $z = 11.124, p < 0.0001$ and OD 10: $z = 14.267, p < 0.0001$; figure 1c).

Injecting spider mites with *B. megaterium* at three different concentrations (ODs 0.1, 1 or 10) also significantly affected survival (Cox model, bacterial concentration effect, $\chi^2 = 19.471; p < 0.0006$; figure 1b). Hazard ratios revealed no significant change in survival between mites injected with LB or with *B. megaterium* at OD 0.1 ($z = 1.769; p = 0.0769$), but survival of *T. urticae* decreased significantly relatively to the LB control at OD 1 and OD 10 (*B. megaterium* OD 1: $z = 8.792,$
The high mortality rate in *T. urticae* caused by injection with bacteria known to be non-pathogenic to *Drosophila melanogaster* [21] raised the possibility that our bacterial strains had an unexpected level of pathogenicity. To test this, we infected *D. melanogaster* adult females with the same bacteria and at the same concentrations applied to *T. urticae*. As previously reported [22,23], within the same time frame and bacteria inoculum range as our experiment with *T. urticae*, the survival of *D. melanogaster* was not reduced upon injection with either bacterium (Cox model, bacterial concentration effect, χ² = 3.501, p = 0.7439; electronic supplementary material, figure S1).

(b) The transcriptomic profile of *Tetranychus urticae* is unaltered upon infection

Next, we analysed genome-wide gene-expression patterns to assess the effect of bacterial infection on spider mites using a qPCR-validated microarray set-up [24]. Differential transcript levels were determined in mites injected with *E. coli* or with *B. megaterium* relative to mites injected with LB. Expression levels were measured 3, 6 and 12 h post-injection. Only a limited number of genes showed significant differences in expression between mites injected with or without bacteria and displayed an inconsistent response to bacteria across the three time points (electronic supplementary material, table S2). Moreover, even though the 34 orthologues of immunity-related *Drosophila* genes identified in the *T. urticae* genome had multiple probes on the array, none of these showed significant differential expression upon bacterial infection (electronic supplementary material, table S2).

By contrast, a more pronounced transcriptional response was observed between injected and non-injected mites. In these comparisons, we observed altered transcription of a total of 259 genes (out of 17 798 genes with probes on the array). More specifically, transcriptomic comparisons of the *E. coli* and *B. megaterium* injections, with their respective LB controls, uncovered a total of 177 and 211 differentially expressed genes, respectively, at any of the three time points (electronic supplementary material, table S2). Only three genes were significantly differentially expressed in a consistent manner across every time point of every injection treatment (LB buffer, *E. coli* and *B. megaterium*). These are *tetur03g07900*, *tetur05g04720* and *tetur19g00860*, none of which shows any significant homology to known immunity genes (sequence data accessible at: http://bioinformatics.psb.ugent.be/orcae/overview/Tetur and http://www.uniprot.org/proteomes/ under UP000015104). No orthologues of *Drosophila* genes classified as immunity-related were present in any of these gene sets [19]. Of the 259 differentially expressed genes that showed significant differential expression in any comparison, 118 were given a Gene Ontology (GO) term by Blast2GO analysis [25], Fisher’s exact test showed that 16 and 12 GO terms were significantly over- and under-represented in the differentially expressed gene set, respectively (electronic supplementary material, table S3). No terms related to a physiological response to wounding were observed.

As indicated by the hierarchical clustering analysis based on time-series alignments of the relative transcription levels across the different time points (figure 2) and by corresponding gene-expression heatplots (electronic supplementary material, figure S2), the transcriptional response to injection did not appear to be time-dependent within the first 12 h. Indeed, no strong linear differential expression across the three time points was observed in any of the three injection treatments. Moreover, the lack of consistent differential expression across all time points of the different injection treatments indicates that the injection procedure itself did not elicit an immune response.

The transcriptional responses observed in individual comparisons do show that our pipeline is capable of identifying differential expression. Therefore, we interpret the lack of significantly distinct transcript levels in the direct comparisons of bacterial-injected versus LB-injected mites as caused by the virtual absence of immune response induction and not by a technical artefact.

(c) *Sancassania berlesei* and *Tetranychus urticae* respond differently to systemic bacterial infection

To test whether the lack of an induced immune response in *T. urticae* is a general feature of the Acari, we mirrored the infections performed on the spider mite in the grain mite, *S. berlesei*.

Overall, infection with bacteria decreased significantly the survival of *S. berlesei* (Cox model, bacterial concentration effect, χ² = 17.461, p = 0.0077; figure 3a,b). A reduction in survival was observed upon bacterial injection with either bacterial species at all tested concentrations (*E. coli* OD 0.1: z = 3.513, p < 0.0022; *E. coli* OD 1: z = 3.446, p = 0.0022; *E. coli* OD 10: z = 3.599, p = 0.0022; *B. megaterium* OD 0.1: z = 2.899, p = 0.0057; *B. megaterium* OD 1: z = 3.495, p = 0.0022; *B. megaterium* OD 10: z = 3.414, p = 0.0022; figure 3c).
time point and species (ANOVA, $F_{1,20} = 25.131, p < 0.0001$). The latter indicates that bacterial populations have distinct growth dynamics in T. urticae and in D. melanogaster. Indeed, in T. urticae, the number of CFUs increased across time points (figure 4a), whereas for D. melanogaster the number of CFUs started to decrease 2–3 days after injection, reaching in the last time point a value similar to the bacterial number initially injected ($t_{25} = -0.201, p = 0.8427$). By contrast, no significant differences were found between the dynamics of bacteria infecting S. berlesei and D. melanogaster (ANOVA, $F_{4,20} = 0.51, p = 0.482$; figure 4b), between time points (ANOVA, $F_{4,20} = 2.287, p = 0.0957$) and in the interaction between time points and species (ANOVA, $F_{4,20} = 0.069, p = 0.9906$).

This experiment provides a further line of evidence that S. berlesei is capable of fighting bacterial proliferation, contrary to T. urticae.

(e) *Tetranychus urticae* has an impoverished gut microbiota

We proceeded to quantify the microbiota in both mite species motivated by three important facts: (i) the ecological similarities between *T. urticae* and the aphid, (ii) the genomic evidence for the absence of the *Imd* gene and a general degeneration of the *Imd* pathway in the spider mite [13,14,19] and (iii) the central role of this pathway in gut homeostasis regarding regulation of the microbiota [28,29].

Quantified bacteria in individuals that were (i) surface-sterilized in bleach and alcohol, which should not have bacteria in their external surface; (ii) fed on rifampicin, which should not have bacteria in the gut or (iii) both, which should have neither and (iv) mites taken directly from their natural substrate, which should present both internally and externally associated bacteria. We homogenized individual adult females of *T. urticae* and *S. berlesei* from each treatment and plated them on LB agar plates (figure 4c) or extracted DNA to perform semi-quantitative PCR for the 16S gene (electronic supplementary material, figure S4). We found that the two mite species harbour a significantly different number of bacteria capable of growing on LB agar plates (ANOVA, mite species effect, $F_{1,72} = 169.855, p < 0.0001$) and that the treatments applied significantly decreased the number of bacteria (ANOVA, treatment effect, $F_{3,72} = 93.543, p < 0.0001$; figure 4c). We found a significant species by treatment interaction (ANOVA, $F_{3,72} = 13.029, p < 0.0001$), and the treatment with external sterilization and antibiotic treatment brings bacteria numbers to non-significantly different levels in both species ($t_{25} = 1.924, p = 0.058$). All other comparisons between treatments across species are highly significant ($t_{25} > 5.492, p < 0.0001$). It is particularly striking that, once sterilized and only harbouring the bacteria inside the gut, single crushed *T. urticae* individuals only generate around 10 CFUs, a difference of three orders of magnitude relative to their *S. berlesei* counterparts (figure 4c).

It is expected that an undetermined number of bacterial species will not be detected with the specific culture conditions used in this test. However, it is unlikely that, between the two mite species, the distribution of bacterial species, which can and cannot be grown in LB, will be significantly different to change the qualitative conclusion we reach.

A semi-quantitative PCR provided independent confirmation that *S. berlesei* has a microbiota in the order of one thousand times higher than that of *T. urticae*. Whereas amplification products are clearly visible using gDNA from sterilized *S. berlesei* after 25 cycles, only after 35 cycles are bands detectable from sterilized *T. urticae* (electronic supplementary information, figure S5).
supplementary material, figure S4). For example, contrasting bands from the pool of 100 non-sterilized _T. urticae_ females (TP) at 35 cycles to the pool of 50 non-sterilized _S. berlesei_ females (SP) at 25 cycles show comparable amplification products in the two bacterial types ($2^{30} = 1024$). Despite the poor quantitative power of this technique, its qualitative interpretation provides a rough estimate of the difference in bacteria present in either species, namely in their digestive tracts. Importantly, this difference is hardly attributable to any of the four most commonly described endosymbionts of spider mites [30], which are absent from our tested populations (electronic supplementary material, figure S5).

Together, these results concur in that most bacteria found in these species are inside the mite gut and that between _T. urticae_ and _S. berlesei_ their numbers differ by roughly three orders of magnitude.

3. Discussion

(a) Spider mites are susceptible to bacterial infections

Using bacteria that are non-pathogenic to _D. melanogaster_, we have shown that spider mites infected over a 100-fold concentration range with both Gram-positive and Gram-negative bacteria display high mortality when compared with controls (both mock-infected and infected with heat-killed bacteria). In addition, no qualitatively different transcriptional change is induced consistently by the presence of bacteria. This is in sharp contrast with _Drosophila_ which displays a strong upregulation of _Imd_ and/or _Toll_ pathways upon bacterial infection [3,31,32]. Although individual transcriptomic comparisons between injected and non-injected mites reveal differential expression, no consistent response to injection was observed across all time points and treatments. Therefore, wounding itself does not seem to induce an immune response. This is supported by the absence of enriched GO terms related to wound response in lumped individual transcriptomic responses (electronic supplementary material, table S3). Finally, in _T. urticae_, bacterial proliferation is maintained steadily across 4 days post-injection in consonance with its mortality profile. This strongly indicates that no resistance or tolerance mechanisms are operating in the spider mite and that uncontrolled bacterial proliferation caused the observed mortality rates.

Our data are consistent with the absence of an induced immune response but does not address the putative role of other constitutive defences involving expression of effectors, such as lysozymes, AMPs and ROS, or cellular immunity and phagocytosis [33]. Be that as it may, we show that these other candidate mechanisms in _T. urticae_ are clearly insufficient in face of bacterial infections that are innocuous to _Drosophila_ and other chelicerates such as ticks [34] or the wet grain mite _S. berlesei_, which occupies a very different ecological niche, namely bird litter and other substrates prone to bacterial proliferation and infection [20].

Bacterial infection affected the survival of _S. berlesei_ but to a much lesser extent than that observed for _T. urticae_. Moreover, unlike _T. urticae_, _S. berlesei_ was capable of controlling and reducing the bacterial load, and also mimicking the characterized immune response in _D. melanogaster_ [35,36]. The observation that bacterial load in _S. berlesei_ increases initially and decreases over time suggests that this mite species mounts an immune response against bacterial infection. The nature of this response in _S. berlesei_, induced and/or constitutive, remains to be determined. An induced response is supported in other chelicerates such as horseshoe crabs and spiders, which upregulate AMPs upon bacterial challenge [17,37], possibly without resorting to _Imd_ or _Toll_ pathways, as these are (at least partially) degenerated in genome-sequenced species [13,14]. In addition, antibacterial response may rely on higher basal levels of immune effectors such as circulating haemocyanin, C-reactive proteins and α2-macroglobulin deployed upon infection [38].

(b) Life history correlates with immune degeneration in spider mites

Genomic and physiological studies of the pea aphid _A. pisum_ uncovered a very similar pattern to our results on _T. urticae_ [9,10,39,40]. Similarly to _T. urticae_, the genome of _A. pisum_ misses important genes of the _Imd_ pathway and several other _Drosophila_ immune genes [9]. Infected aphids, in which lysozyme activity could be detected, did not upregulate AMPs, suggesting that aphids do not deploy an induced...
immune response upon bacterial challenge [9,10]. Instead, pea aphids seem to rely on another layer of defence, that is provided by their endosymbionts, namely Regiella insecticola, Rickettsia and Spiroplasma, to fight some fungal infections [41,42] and Hamiltonella defensa against parasitoid attack [43]. Possibly, endosymbionts commonly detected in T. urticae populations might also confer protection to attacks by fungi and other natural enemies [30] but, to our knowledge, there has been no report of endosymbiont protection against bacterial infections in arthropods (reviewed in [44]). In addition, infection with E. coli also induces significant mortality in the pea aphid [39]. In any case, by using bacteria-challenged spider mites devoid of four common endosymbionts, we aimed to specifically test the response on the host genomic and physiological levels [30].

As shown for the pea aphid [9,11,12], the degenerated immune genetic repertoire and immune response in T. urticae is also a secondary loss. One hypothesis for the genomic and physiological patterns observed in pea aphid immunity is that the virtually aseptic phloem diet of aphids would relax selection for the maintenance of costly immune response mechanisms [45]. This hypothesis may be extended to T. urticae as this species also feeds on a seemingly bacterial-free resource, the cytoplasmic content of leaf cells and phloem. This ecological scenario is opposite to that of the house fly, where the genomic expansion of immune-related genes may underlie its adaptation to septic environments [46]. Moreover, the shared degeneration of the Imd pathway in the spider mite and aphid reinforces this notion because of the central role played by this pathway in mediating the epithelial response to bacterial contacts in the gut and in the modulation of its bacterial contents [31,47–49]. Recent work has shown that, in addition to aphids, other phloem-sap feeders, such as white flies and psyllids, carry a reduced gut microbiota in both laboratory and natural populations [50]. Unfortunately, no information is yet available regarding immune responses in these insects.

The aseptic nature of the feeding source of spider mites is supported by the rough comparative characterization of the gut bacteria present in the two mite species studied here, which differs by several orders of magnitude. This ecological feature, by eliminating a constant necessity for balancing bacterial interactions (commensal or pathogenic), may relax the pressure to evolve or maintain a transcriptionally induced and regulated response. Further studies looking into immunity in other arthropods with obligate endosymbionts and/or with comparable dietary regimes will provide clearer answers about the forces driving convergent degeneration of this type of immune response.

4. Material and methods

(For most sections below, more detail is provided in electronic supplementary materials and methods.)

(a) Arthropod and bacterial strains

(i) Tetranychus urticae

All spider mites used in this work are of the London strain, a reference line originally collected in Ontario, Canada. The line used for the Spider Mite Genome Sequencing project [19] was derived from this population. Spider mites were reared under laboratory conditions (25°C, 60% humidity and 16 L : 8 D photoperiod).

(ii) Sancassania berlesei

Grain mites (a kind gift from J. Radwan) were maintained in large numbers in Petri dishes (6 cm diameter) with fly food under laboratory conditions (25°C, 50% humidity and 12 L : 12 D photoperiod).

(iii) Drosophila melanogaster

The w1118 laboratory stock kept under standard laboratory conditions was used in the survival assays and the dynamics of bacterial infection.

(iv) Bacteria

Escherichia coli (Gram –) and B. megaterium (Gram +) stocks were kept at ~80°C and bacteria were plated onto Petri dishes with LB. Per experiment, one colony was picked from selective medium cultures, transferred to liquid LB and grown overnight at 37°C for E. coli and at 30°C for B. megaterium.

(b) Survival assays

Tetranychus urticae, S. berlesei and D. melanogaster survival was monitored after injection with E. coli or B. megaterium for up to 96 h at 24-h intervals. Individuals were injected with LB with or without bacteria. For the former treatment, we used three different concentrations of bacteria, OD 0.1, OD 1 and OD 10 measured with a BioRad SmartSpec 3000. OD 10 corresponds to 5 × 107 cell ml⁻¹; OD 1 and OD 0.10 were obtained by diluting bacteria at OD 10 in LB at a 1 : 10 ratio and 1 : 100 ratio, respectively.

(c) Tetranychus urticae transcriptome analysis

Female adult spider mites were injected with E. coli or B. megaterium at OD 1 concentration with LB as a negative control, or were left unmanipulated. Three, 6 and 12 h post-infection, four biological replicates of every injection treatment were collected. Two biological replicates were collected from non-treated, non-injected mites. Every RNA sample was extracted from a pool of 300 mites and labelled with Cy5 or with Cy3. Significant differential expression was identified by an empirical Bayes approach employing cut-offs for the Benjamini–Hochberg FDR adjusted p-values and log2-converted fold change at 0.05 and 1, respectively [51]. The proxy, NbClust and dtw packages in R were used in the distance matrix construction and clustering of the transcriptomic responses. Distance measures were generated through alignments of the relative transcription levels (injected versus non-injected) using dynamic time warp algorithms. This technique allows for the comparison of the transcriptomic responses over time [52,53].

(d) Infection with heat-killed bacteria

Tetranychus urticae and S. berlesei survival was measured after infection (injection or pricking, respectively) with live or heat-killed E. coli or B. megaterium at OD 10. Three replicates of 30, 1- to 3-day-old, adult females were used per treatment: LB, live E. coli, heat-killed E. coli, live B. megaterium and heat-killed B. megaterium. Survival was monitored every 24 h over 4 days.

(e) Dynamics of bacterial growth

We infected 150 females of T. urticae, S. berlesei and D. melanogaster with 5–100 CFUs of E. coli per individual. The dynamics of the bacterial population was followed every 24 h from 0 to 96 h. At each time point, three replicates of four individuals were homogenized in 50 μl of LB and serially diluted. Homogenates (4 μl) were plated in triplicate on LB plates supplemented with 100 mg ml⁻¹ kanamycin and incubated overnight. The next day, the number of CFUs was counted. For S. berlesei and D. melanogaster, only the homogenates of the individuals alive were plated.
However, for *T. urticae*, individuals collected and plated at 72 and 96 h were dead because no survivors could be recovered at those time-points. The standard error was obtained by dividing the standard deviation of the three biological replicates datapoints (values in log10 CFU), divided by the square root of three, the number of samples for each time point/species.

(f) Estimating the microbiota associated with each mite species

(i) Sterilization and rifampicin treatments

To measure the approximate number of bacteria present either outside or inside each mite species, adult *T. urticae* and *S. berlesei* females were exposed to one of four different treatments: no sterilization, sterilization, feeding on rifampicin and feeding on rifampicin plus sterilization. Single individuals were homogenized in 50 µL of LB, plated (4 µL) in LB agar and incubated overnight at 30°C. The next day, the number of CFUs was counted and used as a proxy to estimate the microbiota associated with each mite (five per treatment and per species).

(ii) Semi-quantitative PCR of bacterial 16S DNA was extracted from individual mites after sterilization (GenElute™ Mammalian Genomic DNA Miniprep Kit, Sigma–Aldrich Co., St Louis, USA). PCRs were performed using standardized concentration of DNA templates (around 4.5 ng µL⁻¹) and using universal primers for the bacterial 16S gene defined by Lane [54], 27f: GAG AGT TTG ATC CTG GCT CAG and 1495r: CTA CGG CCT TCT TGT TAC GA. PCR amplification conditions were as follows: 15 min at 95°C for 1 min followed by a final step of 10 min at 72°C.

(g) Statistical analysis

Analyses were carried out using the R statistical package (v. 3.1.2). To compare survival between uninfected and infected individuals, we used Cox proportional hazards mixed-effect models (coxme) defined by Lane [54], 27f: GAG AGT TTG ATC CTG GCT CAG and 1495r: CTA CGG CCT TCT TGT TAC GA. PCR amplification conditions were as follows: 15 min at 95°C, followed by 15/20/25/30 three-step cycles of 94°C for 30 s, 58°C for 1 min 30 s, 72°C for 1 min followed by a final step of 10 min at 72°C.


Authors’ contributions. G.S.M., N.W., N.E.M., T.V.L., S.M. and E.S. designed the research; G.S.M., N.W., S.M. and E.S. wrote the paper; G.S.M., N.W., N.E.M., F.Z., A.B.L. and M.R. performed research; G.S.M., N.W. and N.E.M. analysed data; M.G., J.V., T.V.L., S.M. and E.S. contributed reagents and resources.

Competing interests. We declare we have no competing interests.

Funding. N.W. was supported by a Marie Sklodowska-Curie Action (MSCA) Individual fellowship (685795-DOGMITE) of Horizon 2020. T.V.L. acknowledges funding from the Fund for Scientific Research Flanders (FWO) (grant nos. G09312N and G05818N), the European Commission (EC contract 618105) via FACCE ERA-NET Plus and FACCE-IP (Genomite, project ID 157 via NWO). This work was supported by Instituto Gulbenkian de Ciência/Fundaçao Calouste Gulbenkian to E.S. and by Fundação para a Ciência e a Tecnologia (FCT, Portugal) grant nos. ANR/BIA-EVF/0013/2012 to S.M. and Isabelle Olivieri and FCT-TUBITAK/0001/2014 to S.M. and Ibrahim Cakmuk. M.G. acknowledges funding from the Government of Canada through Genome Canada and the Ontario Genomics Institute (OGH046), Ontario Research Fund – Global Leadership in Genomics and Life Sciences GL2-01-035, NSERC Strategic Grant STPGP322206-05 and JGI Community Sequencing Program grant no. 777506.

Acknowledgements. We are indebted to several researchers for providing the biological material used here: Jacek Radwan (Jagiellonian University, Poland) for Saccassaria berlesei, Adriano Henriques (ITQB, Portugal) for Bacillus megaterium and Olivier Duron (MV/EGEC, France) for the endosymbiont positive controls. We also thank the Magalhães, Sucena, Beldade and Mith Labs for discussion, Francisco Dionisio (cESC, FCUL, Portugal) for generously hosting some of the bacteria experiments, Jacques Denoyelle for help with the greenhouse, Inês Santos for keeping mite colonies and Ioannis Livadaras (Laboratoire Évolution et Diversité Biologique, Toulouse, France) for suggesting the experiment in figure 4a,b and for comments following the critical reading of the manuscript.

References


34. Aung KM et al. 2012 HSRRB, a class B scavenger receptor, is key to the granulocyte-mediated microbial phagocytosis in ticks. PLoS ONE 7, e33504. (doi:10.1371/journal.pone.0033504)


