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Haemoplasmas in wild rodents: routes of transmission and infection dynamics

Running title: Haemoplasma dynamics in wild rodents

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Abstract

The way that some parasites and pathogens persist in the hostile environment of their host for long periods remains to be resolved. Here longitudinal field surveys were combined with laboratory experiments to investigate the routes of transmission and infection dynamics of such a pathogen—a wild rodent haemotropic bacterium, specifically a *Mycoplasma haemomuris*-like bacterium. Flea-borne transmission, direct rodent-to-rodent transmission, and vertical transmission from fleas or rodents to their offspring were experimentally quantified, and indications were found that the main route of bacterial transmission is direct, although its rate of successful transmission is low (~20%). The bacterium's temporal dynamics was then compared in the field to that observed under a controlled infection experiment in field-infected and lab-infected rodents, and indications were found, under all conditions, that the bacterium reached its peak infection level after 25–45 days and then decreased to low bacterial loads, which persist for the rodent's lifetime. These findings suggest that the bacterium relies on persistency with low bacterial loads for long-term coexistence with its rodent host, having both conceptual and applied implications.

Keywords: Haemotropic mycoplasmas, haemoplasmas, host-parasite interactions, infection dynamics, persistent infection, wild rodent, transmission mechanisms

Introduction

A host's behavioral, physiological, and immunological responses evolve to cope with the selection pressure imposed by parasites/pathogens (Schmid-Hempel 2011a,b). As a result, most parasites/pathogens living in the host (i.e. endoparasites) do not persist for the host's lifetime. Correspondingly, the way that other parasites and pathogens persist in the hostile environment of their host for long periods remains to be resolved.

Blood constitutes an extreme example of a hostile environment for the parasites/pathogens living therein, as it is unstable, with a high red blood cell turnover rate and a great selective pressure imposed by diverse immune cells and mediators. This hostile nature led scientists to consider blood as a sterile environment that possesses living organisms only during disease (Hall & Lyman 2006; Brooks *et al.* 2007; Motoshima *et al.* 2012). However, recent evidence points to more permanent blood bacterial residents such as *Bartonella* and haemoplasma (haemotropic *Mycoplasma*) species, which may use red blood cells as their primary microhabitat (Gavish *et al.* 2014; Gutiérrez *et al.* 2014; 2015; Cohen *et al.* 2015a; b). Revealing the mechanisms of these bacteria's transmission and persistence will shed light on the evolutionary strategies underlying host-parasite/pathogen coexistence and will have an applied aspect considering that some of these bacteria are pathogenic to wild animals and humans (Breitschwerdt & Kordick 2000; Liang *et al.* 2002; Messick 2004; Eisen & Eisen 2011; Hoelzle *et al.* 2014; Atif 2015; Ogden *et al.* 2015).

Haemoplasmas provide convenient models in which to explore transmission routes and infection dynamics under hostile conditions. They are small pathogens belonging to the class Mollicutes that reside on red blood cells (Willi *et al.* 2007b; Hicks *et al.* 2014). This group is remarkably diverse in terms of impact on the mammalian host and prevalence. Their pathogenicity can range, depending on the haemoplasma and mammalian host species, from

acutely life-threatening haemolytic anemia to chronic infection with no apparent clinical manifestation (Henry 1979; Hoelzle *et al.* 2003; Tasker *et al.* 2009b; Strait *et al.* 2012). Haemoplasmas are also common in wild animals, infecting a range of mammalian hosts at various prevalence levels, ranging from 3–97% (Willi *et al.* 2007c; Boes *et al.* 2012; Iso *et al.* 2013; Santos *et al.* 2013; Sashida *et al.* 2013; Bajer *et al.* 2014; Sashida *et al.* 2014; Sharifiyazdi *et al.* 2014; Mascarelli *et al.* 2015; Millan *et al.* 2015; Volokhov *et al.* 2017). However, to date, experimental studies and long-term surveys have all been conducted with only a few target haemoplasma species of veterinary importance (e.g. *Mycoplasma parvum*, *M. suis*, '*Ca*. M. haemominutum', 'Ca. M. turicensis', *M. haemofelis*, *M. haematoparvum*, *M. haemocanis* and *M. wenyonii*) infecting pet and domestic animals (Willi *et al.* 2007b; Hoelzle 2008; Wengi *et al.* 2008; Tasker *et al.* 2009b; 2010; do Nascimento *et al.* 2014; Sasaoka *et al.* 2015). Thus, the transmission and persistence mechanisms of naturally occurring haemoplasmas in wild animals not subjected to antibiotics or vaccinations that do not cause disease in their mammalian host remain to be explored.

Here longitudinal field surveys and laboratory experiments were combined to investigate the routes of haemoplasma transmission and its infection dynamics in the blood of a wild rodent, *Gerbillus andersoni*. In the Negev Desert sand dunes (Israel), the haemoplasmas found in all the blood samples of this rodent species belong to a single cluster, which is closely related to (90–95% similarity in the 16S gene), but distinguishable from, *M. haemomuris* (Kedem *et al.* 2014). Below, these will thus be termed *M. haemomuris*-like bacteria (MHLB). Regarding transmission routes, since lice have rarely been detected on wild *G. andersoni* rodents (two lice specimens were detected from thousands of rodents sampled over the past 17 years), whereas *Synosternus cleopatrae* fleas are common on these wild rodents (Hawlena *et al.* 2006a) and MHLB were detected in 33% of them (Cohen *et al.*

2015a), it seems most likely that MHLB are flea-borne. However, the high natural MHLB prevalence (60 ± 3% SE) in rodents' blood samples and the evidence for positive associations between MHLB and the fleas' reproductive success (Messika *et al.* 2017) led to the prediction that in concert with flea-borne transmission, other transmission routes may operate. Such transmission routes may include transovarial transmission from the parent to the offspring flea (Azad *et al.* 1992; Morick *et al.* 2011), transmission via blood regurgitations of the flea (Morick *et al.* 2013b; Rollend *et al.* 2013), and transmission by flea feces (Woods *et al.* 2005). Transmission may also operate through direct rodent-to-rodent contact (see indirect evidence for '*Ca*. M. haemominutum' and '*Ca*. M. *et* turicensis'; Willi *al.* 2007a, Dean *et al.* 2008, Lappin *et al.* 2008, Museux *et al.* 2009), through rodent feces (Woods *et al.* 2005; Willi *et al.* 2007a), and through vertical transmission from female to offspring rodents (Harvey & Gaskin 1977; Almy *et al.* 2006; Fujihara *et al.* 2011; Sasaoka *et al.* 2015). Regarding infection dynamics, our longitudinal field data suggest that MHLB have high persistency in *G. andersoni*; 90% of the MHLB-positive individuals were infected four months after the first sampling (Cohen *et al.* 2015a). However, due to the observational nature of that study, the possibility that these rodents had cleared the infection and were reinfected towards the second sampling period could not be rejected.

To test which of the above transmission routes are exploited by MHLB and to experimentally quantify any long-term persistency in *G. andersoni*, a series of transmission experiments were conducted, and the MHLB temporal dynamics in the field was compared to that observed under a controlled infection experiment in field-infected and lab-infected rodents.

Materials and methods

General study approach and methods

In order to reveal the transmission routes of MHLB, four laboratory experiments were designed. Experiment 1 tested for flea-borne transmission (Fig. 1a), experiment 2 tested for transovarial and non-transovarial vertical transmission from fleas to offspring (Fig. 1b), experiment 3 tested for direct rodent-to-rodent transmission (Fig. 1c), and experiment 4 tested for vertical transmission from parent rodent to offspring (Fig. 1d). To quantify the infection dynamics of MHLB in the laboratory and to confirm that this dynamics fits the field patterns, in experiment 5, the infection dynamics of the MHLB-positive rodents that were either captured-recaptured in the field, captured in the field and brought to the laboratory, or inoculated under laboratory conditions was followed through time.

All rodents used for the above five experiments were non-reproductive adult *G. andersoni*. The rodents that were brought from the field were polymerase chain reaction (PCR) tested for MHLB (see below), and the PCR confirmed them to be *Bartonella*-negative (see below); then they were kept isolated and repeatedly cleaned from ectoparasites for at least two weeks (see details in experiment 1 and in Supplementary Information, SI 1). The other rodents used in the five experiments were offspring from our laboratory colony that were confirmed to be MHLB- and *Bartonella*-negative and were free of ectoparasites; thus, none of the rodents received any drug treatment.

In the laboratory, rodents were maintained on sand bedding, in an animal room with an air temperature of $25 \pm 1\ ^{0}C$ and a photoperiod of 12D: 12L, and were provided daily with millet seeds *ad libitum* and alfalfa as a water source according to Hawlena *et al.* (2007). Excluding the rodents used for experiment 3 (which were kept in pairs; see below), all rodents were kept individually in disinfected $20 \times 30\ cm^2$ plastic cages with a 1-cm layer of autoclaved sand as substrate.

To infect MHLB-negative rodents for experiments 1, 3, and 5, rodents were subcutaneously inoculated with 150–300 µl blood from MHLB-positive *G. andersoni*, preserved in 20% DMSO (Sigma-Aldrich, Buchs, Switzerland) at –80 °C. MHLB-positive blood contained MHLB loads ranging from 2×10^3–4×10^5 copies per inoculum. The bacteria could not be directly inoculated since haemoplasma species are currently uncultivable (Tasker *et al.* 2003).

To assess the probability of being infected by MHLB and the MHLB loads in the rodents' blood collected during experiments 1, 3, 4, and 5 (designated as infection parameters), following Cohen *et al*. (2015a), 100–200 µl of blood was collected from the retro-orbital sinus of each individual by capillaries immersed in 0.15% EDTA, and was stored in EDTA blood collection tubes at –20 °C until further molecular analyses. To assess MHLB infection parameters in fleas collected during experiments 1 and 2, fleas were collected from the rodent body and their DNA was extracted. DNA was extracted from blood samples (experiments 1, 3, 4, and 5) and flea-regurgitated blood (experiment 2) using a MoBio Bacteremia DNA Isolation Kit, following the manufacturer's instructions (Cohen *et al.* 2015a). DNA from fleas (experiments 1 and 2) and buccal swabs (experiment 3) was extracted using a DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA, USA) (Hawlena *et al.* 2013). DNA from feces was extracted using the QIAamp DNA Stool Mini Kit (QIAGEN, Valencia, CA, USA). In each extraction session, a negative control was included, in which all of the reagents were added to phosphate-buffered saline (PBS) instead of to the blood, fleas, rodent feces, buccal swabs, or regurgitated blood.

The PCR was performed to test for the presence of MHLB by amplification of the 16S gene using the HM16S-1(F) and HM16S-2(R) primers, following Kedem *et al.* (2014). Sanger sequencing was performed on 20% of randomly chosen PCR-positive samples, and it

confirmed that the tested bands of 762 base pair lengths indeed showed 95% similarity to *M. haemomuris*. Quantification of the MHLB copy numbers in positive samples was performed by a real-time quantitative PCR (qPCR) (Applied Biosystems 7300, Waltham, Massachusetts, USA), using Qiagen HotStarTaq Master Mix (Precision FAST Blue 2x qPCR Master Mix with ROX) with 10 µM of the 16S rRNA gene (F)GGAGCGGTGGAATGTGTAG and 10 µM of the 16S rRNA gene (R) GGGGTATCTAATCCCATTTGC, 10 µM of probe (TYAAGAACACCAGAGGCGAAGGCG), 25 µM of $MgCl_2$ and 5 µL of DNA in a total volume of 10 µL, following the reaction conditions described in Tasker *et al*. (2010). To estimate the absolute copy number and validate the repeatability, efficiency, and sensitivity of the reactions, in each run, we added a 10-fold serial dilution (i.e., standard curve ranged from 10^1–10^7 copies per reaction) of previously sequenced plasmids containing the 16S rRNA gene from blood samples that were positive for MHLB. More details are provided in the Supplementary Information (SI 2).

The trapping and handling protocol was approved by the Committee for the Ethical Care and Use of Animals in Experiments of Ben-Gurion University of the Negev (# IL-59-09-2015) and by the Israel Nature and Parks Authority (# 41428).

Experiment 1: Evaluation of transmission by fleas

Eighteen rodent donors were randomly assigned to either experimental (sample size N = 14) or control (N = 4) groups. The 14 experimental donors were composed of four MHLB-positive individuals that were brought from the field to the laboratory and 10 MHLB-negative individuals (six field-captured and four individuals from our breeding colony) that were inoculated by MHLB in the laboratory and became MHLB-positive 10–15 days post-

inoculation. The four control donors, who were born in the laboratory and were MHLB- and *Bartonella*-negative, were inoculated with PBS (Fig. 1a).

Each rodent was infested by 100 MHLB-negative *S. cleopatrae* fleas. *S. cleopatrae* is the most dominant ectoparasite infesting *G. andersoni* in the Negev Desert sand dunes and has the highest prevalence of MHLB among all *G. andersoni* ectoparasites (Cohen *et al.* 2015a). These fleas were all *Bartonella*-negative and part of our breeding core, which is maintained as previously described (Krasnov *et al.* 2001a; b; Supplementary Information, SI 3). During infestation, the sandy substrate was covered with a wire mesh to prevent the rodents from killing the fleas. This allowed the fleas to feed and reproduce on the rodent, regurgitate blood, and lay their eggs in the sand with only minimal disturbances from the rodents (Morick *et al.* 2013a). Sterilized absorbent paper was added to minimize flea death by rodent urination.

Beginning from the first flea infestation, fleas were collected every 72 h from the donor rodents and were placed on nine MHLB-negative laboratory-born rodents, designated as "recipient" rodents. In each infestation event, all recipients were infested by 50 fleas per rodent; the experimental recipients were infested by fleas removed from experimental donors, and the control recipients were infested by fleas removed from control donors (Fig. 1a). Then, 40–60 newly emerged MHLB-negative fleas were placed on the donor rodents.

To estimate the MHLB infection parameters of the transmitted fleas (see general methods), during every infestation event, all the dead fleas were collected, in addition to five live fleas, from the body of each donor and recipient rodent and were stored in 70% ethanol at –20 °C until further molecular analyses. It was then tested whether the MHLB organisms remained viable within the flea or were just remnants in the blood meal and would thereby be lost after complete digestion. This was done by randomly selecting one flea per rodent in each

flea collection event and allowing it to fully digest its blood meal for 10 additional days at 25 °C and 85% relative humidity before placing it into ethanol at –20 °C until further molecular analyses for MHLB detection.

This design resulted in MHLB screening of DNA extracts from a total of 69 dead and 88 live fleas fed on experimental donor rodents, 40 dead and 38 live fleas fed on experimental recipients, and 16 live fleas that were fed on donors and were allowed to digest their blood meal for 10 days. We then compared the infection parameters of these fleas and 30 fleas fed on control donors and recipients (sample size = 19 and 11, respectively).

The experiment lasted 65 days to allow sufficient time for fleas to defecate on recipient rodents and for MHLB multiplication and transmission, as well as to allow an overlap between flea generations, which might be essential for flea-borne transmission.

Experiment 2: Evaluation of vertical transmission in fleas

In order to examine transovarial MHLB transmission, female fleas from MHLB-positive rodents in the field were randomly collected and allowed to lay eggs for 48 h of incubation at 95% ± 3 relative humidity and 23 ± 2 °C (one flea per rodent; Fig. 1b), following Messika *et al.* (2017). The eggs, developed larva, and cocoons were further incubated, and their emerging offspring were collected. The female parents and their offspring were then subjected to DNA extraction and PCR to screen for MHLB. Simultaneously, 32 females from our laboratory colony (see Supplementary Information, SI 3) were allowed to lay eggs under the same conditions, and their newly emerged fleas served as controls. This design resulted in 42 offspring of 16 MHLB-positive female fleas and 65 control offspring of 48 MHLB-negative female fleas (16 field-collected females who had 33 offspring plus 32 females from our laboratory colony who had 32 offspring) (Fig. 1b).

To test for non-transovarial vertical transmission via MHLB-positive regurgitated blood, nine pools of fleas (28 ± 17 fleas per pool) collected from the female flea-positive donors in experiment 1 were allowed to regurgitate blood into a glass flask for an hour. Then, the voids were subjected to molecular analyses, following Morick *et al*. (2013b). In parallel, the regurgitated blood of one pool of fleas collected from an MHLB-negative donor served as a control (Fig. 1b).

Experiment 3: Evaluation of direct transmission between rodents

Haemoplasma species may be directly transmitted between individuals from the same mammalian host species via aggression either between sexes during reproduction or between males during male-male competition (within-sex) (Willi *et al.* 2007a; Dean *et al.* 2008; Museux *et al.* 2009). Between-sex transmission was evaluated by pairing (one pair per cage) 20 field-captured couples of MHLB-positive (designated as "donors") and MHLB-negative (designated as "recipients") individuals (in half of them, the females in the pair were donors) for two weeks to allow reproductive activity. Three MHLB-negative couples served as controls (Fig. 1c). In the within-sex transmission experiment, direct transmission was evaluated by placing each of five MHLB-positive males (designated as "donors") in a cage with one of four different MHLB-negative males (designated as "recipients") for three hours during a period of peak foraging activity (19:30–22:30), resulting in 20 unique pairs (Fig.1c). Individuals used for the within-sex transmission experiment were part of our breeding colony. The infection status of all the individuals that participated in experiment 3 was assessed for six weeks.

Since direct haemoplasma transmission is expected to occur through biting (Willi *et al.* 2007a; Dean *et al.* 2008; Lappin *et al.* 2008; Museux *et al.* 2009), the presence of MHLB

on the buccal mucosa of the donor and recipient rodents was also examined. To do this, the infection parameters were assessed in 25 swabs collected from the buccal mucosa of 10 experimental donors (two swabs from female donors and three swabs from male donors from the between-sex experiment and four consecutive monthly swabs from each of the five donors in the within-sex experiment). As a control, the MHLB infection parameters in five swabs from recipients in the within-sex experiment and four swabs from control recipients from the between-sex experiment were assessed (Fig. 1c). The sampling of the buccal mucosa was done by gently swabbing the mucous membranes of the upper and lower jaws using a sterilized cotton swab immersed in PBS. To ensure that the source of these samples was the buccal mucosa, swabs contaminated with blood were excluded. The rest of the swabs were then stored in Eppendorf tubes containing 200 µl of PBS, at –20 °C until molecular analyses. Finally, to test for the possibility of direct MHLB transmission through rodent feces, 21 *G. andersoni* were inoculated with MHLB, and after 27 days, around peak infection, they were confirmed to be MHLB-positive, and their feces were collected and subjected to DNA extraction and MHLB PCR. Feces from two *G. andersoni*, who were inoculated with MHLB-negative blood, served as positive and negative controls by adding to them 50 µl of either MHLB-positive blood or PBS, respectively.

Experiment 4: Evaluation of vertical transmission in rodents

We assessed the infection status of 35 offspring belonging to eight litters of (i) an MHLB-positive pregnant female field-captured rodent (one litter), (ii) an MHLB-positive female and an MHLB-negative male (four litters of couples from experiment 3), (iii) an MHLB-negative female and an MHLB-positive male (one litter of a couple from experiment 3), and (iv) an

MHLB-negative female and male (two litters of control couples from experiment 3) for six weeks after birth (Fig. 1d).

Experiment 5: Comparison of infection dynamics under natural conditions and in field-infected and lab-infected rodents under laboratory conditions

The MHLB infection load of three groups of rodents was assessed over time. Group 1 comprised 13 female and six male MHLB-positive rodents, sampled in the field during the spring, the main reproductive season, and recaptured four months later in the autumn (Cohen *et al.* 2015a). Group 2 comprised two female and five male MHLB-positive rodents, captured during the summer in the same region and brought into the lab for further assessment of their infection dynamics. Group 3 comprised four female and six male MHLB-negative rodents inoculated by MHLB-positive preserved blood.

Statistical analysis

Generalized linear models (GLMs) were applied to our data. In experiment 1, the effects of rodent role (donor versus recipient), flea sex, flea condition (dead or live), sample origin (field or laboratory), and sample type (rodent blood or fleas) (independent variables) on the probability of being infected by MHLB (binomial distribution) and the MHLB load (Gamma distribution) (dependent variables) were explored. In experiment 3, a GLM with a binomial distribution was applied to quantify the probability of a recipient rodent becoming infected by MHLB (dependent variable) as a function of the route of direct transmission (between- or within-sex) while the donor identification was treated as a random variable. In experiment 5, a GLM with a Gamma distribution was applied to explore the effects of the rodent's sex and body mass, and infection load (independent variables) on the day of peak infection and of the

MHLB load at peak infection (dependent variables). Statistical analyses were performed in R (Version 3.3.3 R development core team; 2017).

Results

Experiment 1: Evaluation of transmission by fleas

The infection loads of the donor rodents were comparable to the loads observed in MHLB-positive rodents in the field (N = 10 and 19, respectively; $p = 0.4$; Fig. 2b). None of the fleas who were allowed to complete digestion or the fleas fed on control rodents were positive for MHLB. In contrast, some of the fleas fed on experimental donors and recipients became MHLB-positive (Figs. 1a and 2a). However, the values of both infection parameters were low, i.e., 0.14 ± 0.03 (N = 235, including dead fleas) and 76 ± 28 (N =32, including dead fleas), for means ± SE of the probability of being infected by MHLB and of MHLB load (copy numbers in 1 µl of DNA), respectively, and they were not significantly affected by the rodent role, flea sex, or flea condition ($p > 0.08$ for all tests; Figs. 1a and 2).

To test whether these infection values fell within the natural infection ranges, the infection values in the 126 live fleas sampled from donor and recipient experimental rodents were compared to those estimated in 151 live fleas removed from MHLB-positive field-captured rodents. The probability of live fleas fed on experimental rodents to become infected by MHLB was 0.13 ± 0.03 and was significantly lower than that of the live field-captured fleas (0.31 ± 0.04; $p < 0.001$; Fig 2a). However, the MHLB loads in the two groups of fleas were similar (N = 17 and 47, respectively; $p = 0.24$; Fig 2b). Despite the detection of MHLB in some of the experimental fleas, none of the recipient rodents became positive for MHLB (Fig. 1a).

Experiment 2: Evaluation of vertical transmission in fleas

None of the 107 flea offspring were positive for MHLB (Fig. 1b). In contrast, in two of the nine flea pools, MHLB-positive regurgitated blood was detected (Figs. 1a and 1b). However, the MHLB load in it was low (Fig. 2b).

Experiment 3: Evaluation of direct transmission between rodents

In all sex combinations of the donor-recipient experiments, some of the recipients became positive, and although the probability of the recipient becoming infected by MHLB was higher for the between-sex combination than for the within-sex combination, the differences were only marginally significant (N = 40 pairs; $p = 0.058$; Fig 1c). During the within-sex transmission experiment, the two recipient males that became infected were the only males that suffered from injuries, followed by the development of abscesses.

From the 20 buccal swabs that were sampled from the five male donors in the within-sex transmission experiment, two samples, which were sampled 20 days post-inoculation, were positive for MHLB and showed low loads (Fig. 2b). The other swabs that were sampled were MHLB-negative (Fig. 2a). Although the two positive controls of the feces were MHLB-positive, there were no indications for MHLB in the feces of the MHLB-positive rodents or of the negative controls; therefore, the possibility of transmission through rodent feces was not further investigated.

Experiment 4: Evaluation of vertical transmission in rodents

None of the 35 rodent offspring were positive for MHLB (Fig. 1d).

Experiment 5: Comparison of infection dynamics

Regardless of whether the rodents were naturally or laboratory-infected with MHLB and whether or not they were brought into the laboratory, all individuals were persistently infected (Fig. 3). MHLB were detected throughout each rodent's life, reaching 800 days of continuous infection in field-captured rodents that were maintained in the laboratory (Fig. 3b). In the field, the MHLB load was significantly higher in the spring than in the autumn ($N = 19$; $p < 0.001$; Fig. 3a). In the laboratory, all rodents infected with MHLB-positive blood became infected (Figs. 2a and 3c). MHLB were first detected in the blood after 10–15 days of infection, and peaked by 25–45 days post-inoculation. MHLB loads then gradually reduced in all individuals until they stabilized at only a few tens of copy numbers per 1 μl of blood (Fig. 3c). MHLB loads at peak bacteraemia and the timing of the peak were not significantly associated with the MHLB load in the inoculum ($N = 10$, $p = 0.42$ and $p = 0.11$, respectively).

The infection loads and dynamics under controlled infection in the laboratory (Fig. 3c) reflected well the infection loads under natural infection in the field (Figs. 3a-b). MHLB loads in the spring were similar to the loads observed at peak infection under controlled infection (25–45 days post-infection), and MHLB loads in the autumn were similar to the loads observed during the stabilized period (65–400 days post-infection) under controlled infection (Fig. 1d).

Discussion

Knowledge of parasite/pathogen transmission routes and their infection dynamics is crucial to our understanding of how parasites/pathogens persist within host populations in nature. Here, the transmission routes and infection dynamics of MHLB in wild rodents were experimentally examined. Both the laboratory manipulations and the infection patterns reported in the field support a major role of direct rodent-to-rodent transmission rates and long persistency. Below

the results are discussed in light of the study goals and of their conceptual and applied implications.

Transmission routes

Transmission is a fundamental process in disease ecology, determining the evolved virulence levels of the parasite/pathogen, the host response, and host-parasite/pathogen dynamics (Sorrell *et al.* 2009; Ebert 2013; Antonovics *et al.* 2017). However, due to the challenges associated with sampling the mammalian hosts, the arthropod vectors, and their parasites/pathogens in nature, for many parasites and pathogens, the exact routes of transmission are still unknown. Throughout four experiments in which both field-infected and laboratory-infected rodents were used, evidence suggested that the MHLB are mainly transmitted by rodent-to-rodent contact.

In experiment 3, on average, 20% of the MHLB-negative recipient rodents became infected by MHLB after being in contact with their donor counterparts for at least three hours. Moreover, between-sex transmission was established from donors that were infected by only tens to hundreds of copy numbers per 1 μl of blood, during their stabilized infection period. Considering that gerbils are solitary, the rates of encounters in nature may be lower and may fluctuate seasonally. Seasonality is also supported by the observed infection dynamics in the field wherein MHLB loads during the spring, the main reproductive season, were similar to peak infection in the laboratory but were significantly lower during autumn (Figs. 3a & d).

The exact mechanism of rodent-to-rodent transmission needs to be experimentally confirmed. In the meantime, our study suggests that transmission does not occur through rodent feces. Furthermore, three pieces of evidence suggest that the transmission is likely to occur by passage of the donor rodent's infected saliva into the recipient's open wounds. First,

the detection of MHLB in the buccal swabs of donor rodents during peak infection demonstrated the presence of MHLB on rodent buccal mucous membranes. Second, the two male recipients that became infected suffered from open wounds. Finally, between- and within-sex aggressiveness in *G. andersoni* is common under laboratory conditions (Khokhlova and Cohen, unpublished data) and was documented during foraging in the field (Ovadia *et al.* 2005). Taken together, the above evidence suggests that rodent aggressiveness may enhance MHLB transmission, thereby offering an explanation for the skewed distribution of MHLB toward *G. andersoni* (Kedem *et al.* 2014)—the most aggressive species among the three co-occurring rodent species (i.e. *G. andersoni*, G. *pyramidum*, and *G. gerbillus*) in the Negev Desert sand dunes (Ovadia *et al.*, 2005; Halle, personal communication).

Our study also suggests that other possible transmission routes for haemoplasmas, namely flea-borne and flea or rodent vertical transmission (e.g. Taroura *et al.* 2005; Fujihara *et al.* 2011; Hornok *et al.* 2015), are less likely in our system. No evidence for transovarial vertical transmission between fleas or between rodents was found. Moreover, although about 16% of the fleas became positive for MHLB after feeding on MHLB-positive rodents and allowing for flea defecation, none of the recipient rodents became infected (Fig. 1a). It is unlikely that our failure to simulate flea-to-rodent transmission was the result of the low probability of fleas to be infected by MHLB in experiment 1 (Fig. 2a). This is because the mean number of infected fleas per rodent in experiment 1 was 16 (100 fleas on an average adult rodent multiplied by 0.16, the probability that a flea will become infected in experiment 1), which exceeded six, the mean number of infected fleas per *G. andersoni* host in the Negev sand dunes (21 fleas on an average adult rodent and in its burrow multiplied by 0.3, the probability that a flea will become infected in the field; Hawlena *et al.* 2006), whereas MHLB loads per infected flea were comparable under both conditions (Fig. 2b). An alternative

explanation for our failure to simulate flea-to-rodent transmission is that flea-borne transmission does not occur in this system. It is likely that the MHLB we detected in the fleas and their regurgitated blood during the laboratory and field sampling were dead bacterial copies detected in the fleas' blood meal, and thus were not transmissible to the recipient rodents. Four pieces of evidence support this "dead bacterial copies" explanation, namely: (i) the absence of a correlation between the probability of a rodent and its fleas to be infected by MHLB in nature (Messika-Madmon 2015), (ii) the absence of a correlation between flea burden and the probability of a flea being infected by MHLB (Kedem *et al.* 2014), (iii) the similarity between the MHLB loads of dead and live fleas and between those of fleas fed on donor and those fed on recipient rodents in experiment 1, and (iv) the lack of MHLB in fleas that were allowed to complete blood meal digestion during experiment 1. Even if we are wrong, and fleas can transmit MHLB by their gut or their mouth parts without replication (Vobis *et al.* 2003; Shaw *et al.* 2004; Schorderet-Weber *et al.* 2017), our results indicate that flea-borne transmission is likely not their main transmission route.

Infection dynamics

Considering the above evidence for only low transmission rates between rodents and the hostile conditions found in the mammalian host's blood, the question of how haemoplasmas survive in nature is intriguing. The results of experiment 5 provide us with some clues to this question. First, it seems that very low loads (~1×10^4 MHLB copies in 150–300 µl whole blood subcutaneous inoculum) of MHLB are sufficient for successful infection and that transmission success, infection load, and the timing of peak infection are all inoculum dose-independent. Thus, even if transmission opportunities are rare, they are likely to be successful. Second, the laboratory experiment suggests that once infection is established, it is lifelong,

maintaining high numbers of infectious rodents in the population. Third, the short infection peak is followed by a low and stable load of asymptomatic MHLB infection in which only a few tens of copy numbers per 1 µl of blood persist. This low and persistent MHLB infection dynamics is similar to several other haemoplasmas of domestic animals (Groebel *et al.* 2009; Museux *et al.* 2009; Tasker *et al.* 2009b; Hoelzle *et al.* 2014), as well as to other parasites/pathogens (*Bartonella* spp., *Mycobacterium tuberculosis*, *Plasmodium* spp., *Brucella* spp., *Helicobacter pylori*, *Burkholderia pseudomallei*, *Coxiella burnetii* and *Salmonella enterica* serovar Typhi; Rhen *et al.* 2003; Merrell & Falkow 2004; Monack *et al.* 2004; Chomel *et al.* 2009; Cory 2015; Okamura 2016). Such an infection dynamics may be the result of the parasites/pathogens' strategy to evade the immune response, compensating for their low loads with their low level of damage to their mammalian host, which reduces their mortality probability (the transmission-virulence tradeoff; Anderson & May 1982; Ewald 1995; Sorrell *et al.* 2009). However, it is also possible that this infection dynamics is the result of the mammalian host's immune system's ability to control the parasites/pathogens below harmful levels or even to protect the mammalian host against subsequent infection (Miller *et al.* 2005; Haine 2008).

Interestingly, in contrast to most of the other parasites/pathogens, which are characterized by low and persistent infection dynamics, some species of haemoplasmas including MHLB (i) are persistent in all individuals and (ii) are horizontally transmitted; additionally, there is (iii) no indication that they may convert to disease-causing states (as for *Ca*. M. haemominutum, Willi *et al.* 2007c; Tasker *et al.* 2009b; Barker *et al.* 2012). Future experiments should thus reveal the mechanisms of persistent haemoplasma infection. In particular, it is important to understand whether (i) the low stable infection levels result from the bacterium's or the mammalian host's actions, (ii) the bacterium hides in other tissues in

the rodent's body (Tasker *et al.* 2009a; Wolf-Jackel *et al.* 2012; Novacco *et al.* 2013), or (iii) the bacterium replicates during the stabilized infection period or remains in a dormant state (Monack & Hultgren 2013; Potgieter *et al.* 2015; Mandell & Beverley 2017; Vadivelu *et al.* 2017) .

Ecological and applied implications

Knowledge of MHLB transmission routes and persistence sheds light on the way that parasites/pathogens perceive their mammalian host as an environment. Together with previous evidence, it appears that MHLB spend most of their time in a stable population size (the current study), exhibit narrow rodent ranges (Kedem *et al.* 2014), have low rates of dispersal (= transmission rate between rodents; the current study), and cause only minimal damage to the rodent (Cohen *et al.* 2015a). It is likely that the described pattern reflects a bacterial strategy to persist in a population of solitary mammalian hosts (Weiss 2002) and to survive in hostile environments such as a mammalian host's blood. Under more favorable conditions for the parasite/pathogen (e.g., a less protected environment or an environment that enables high mammalian host-to-host transmission), it may be more adaptive for it to actively destroy the mammalian host's biomass (Esch *et al.* 1977; Andrews & Rouse 1982). The knowledge gained in this study can also be applied to control MHLB in natural gerbil communities. In particular, it suggests that (i) the most critical season for MHLB transmission coincides with the breeding season, that (ii) all MHLB-positive *G. andersoni* rodents should be considered as infectious individuals, and that (iii) ectoparasites do not provide a serious risk factor for bacterial transmission.

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Data accessibility

Raw data can be accessed via the public archive “Figshare.com”. Accession address is *http://dx.doi.org/10.6084/m9.figshare.6741518*

Author contribution

CC participated in the design of the study, carried out the field sampling, experiments and molecular analysis, performed the statistical analysis, and wrote the manuscript. MS participated in the design of the study and carried out experiments and molecular analyses. MG participated in the laboratory experiments and assisted in the statistical analyses. IM contributed to the laboratory experiments. ME participated in the design and analysis of the molecular assays. IK participated in experimental design and flea rearing. ST participated in the experimental design and consultation regarding the haemoplasma group. HH conceived of the study and participated in its design, the design of the molecular assays, and the statistical analysis and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

Figures

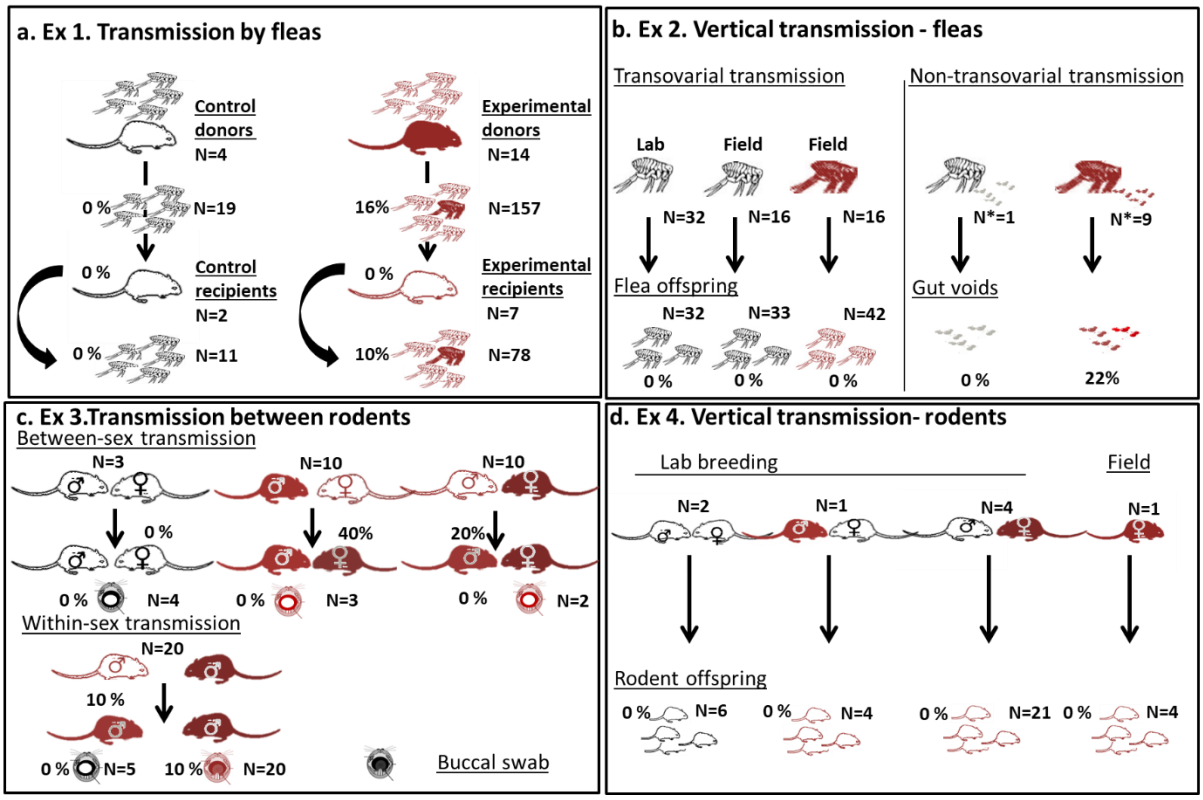

Fig. 1. Schematic description of the experimental design and results of the four transmission experiments wherein the red and black figures represent experimental and control animals, respectively, and the filled and empty figures represent MHLB-positive and MHLB-negative animals, respectively. (a) In experiment 1, flea-borne transmission was quantified by feeding and breeding MHLB-negative fleas on MHLB-positive rodents and transmitting these fleas to MHLB-negative recipient rodents. Although 16% of the fleas became infected, all the recipients remained MHLB-negative. (b) In experiment 2, transovarial and non-transovarial transmission in fleas was quantified by quantifying the MHLB status of offspring from field-collected MHLB-positive female fleas and regurgitated blood from pools of fleas fed on MHLB-positive rodents in the lab, respectively. MHLB were detected in only two of the regurgitated blood pools. (c) In experiment 3, direct transmission was quantified between MHLB-positive (designated "donors") and MHLB-negative (designated "recipients") rodents and MHLB copy numbers in buccal swabs taken from donor and recipient rodents. Between-sex transmission was examined by raising male and female pairs of MHLB-positive and MHLB-negative rodents for 14 days in the same cage, whereas transmission between male rodents (within-sex) was examined by introducing MHLB-positive and MHLB-negative males to the same cage for three hours. Direct transmission was detected between the two sexes (20–40%) and between rodent males (10%), and 8% of buccal swabs from donors were found to be positive for MHLB. (d) In experiment 4, vertical transmission was quantified in one MHLB-positive female rodent who was brought into the laboratory pregnant from the field, and in pairs, including either a female or a male MHLB-positive parent, but no supporting evidence for MHLB transmission was found. “N” = sample size; “N*” = the number of flea pools.

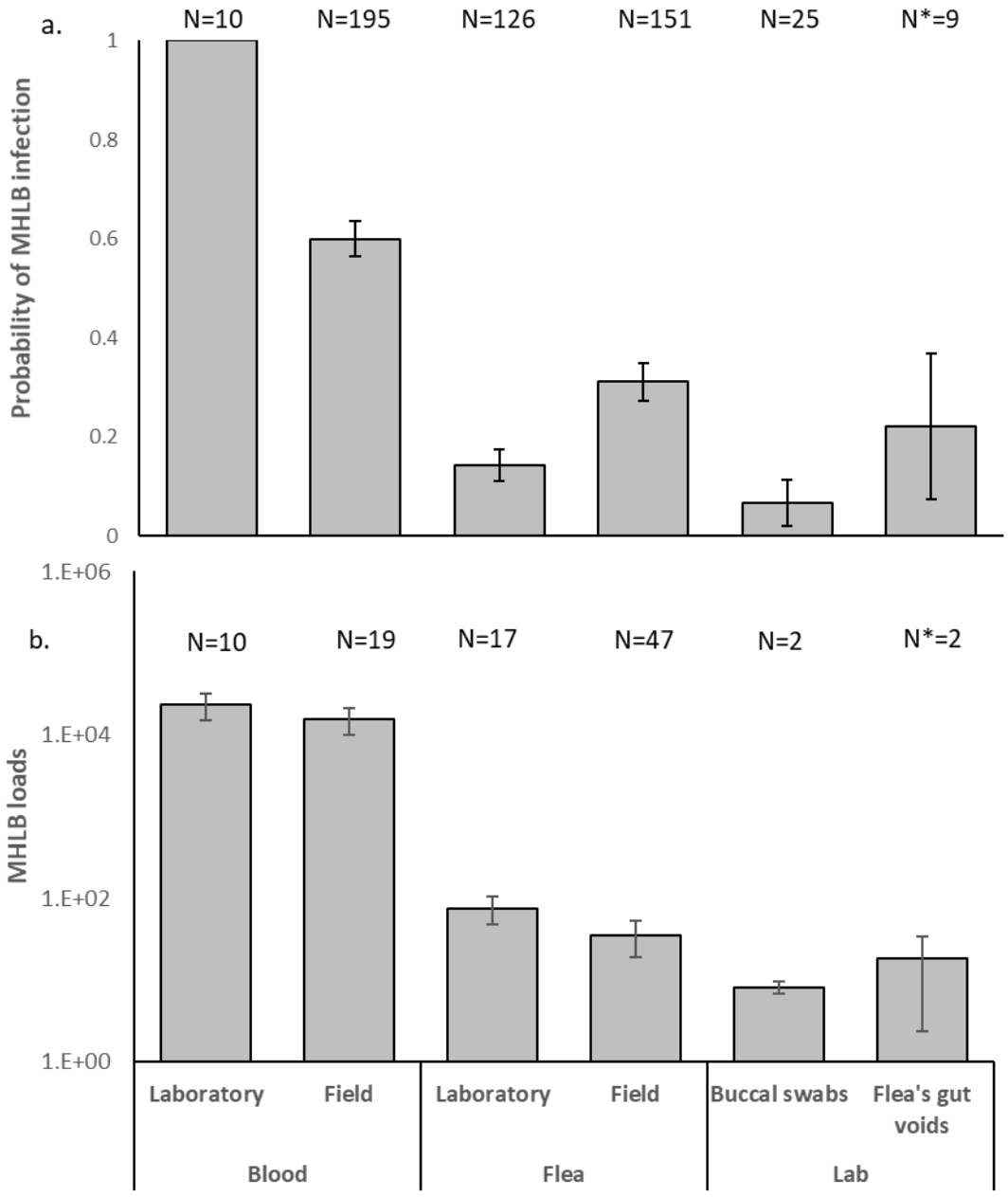

Fig. 2. Infection parameters that were quantified in DNA samples extracted from blood and live fleas collected from MHLB-positive rodents either captured in the field or infected in the laboratory or from buccal swabs and pools of flea-regurgitated blood collected in experiments 3 and 2, respectively; where (a) is the probability of a sample to be infected with MHLB and (b) is the MHLB load (copies per µl of MHLB-positive DNA samples) with respect to sample origin. All the control samples were negative for MHLB, and hence are not shown.
"N" = sample size; "N*" = the number of flea pools.

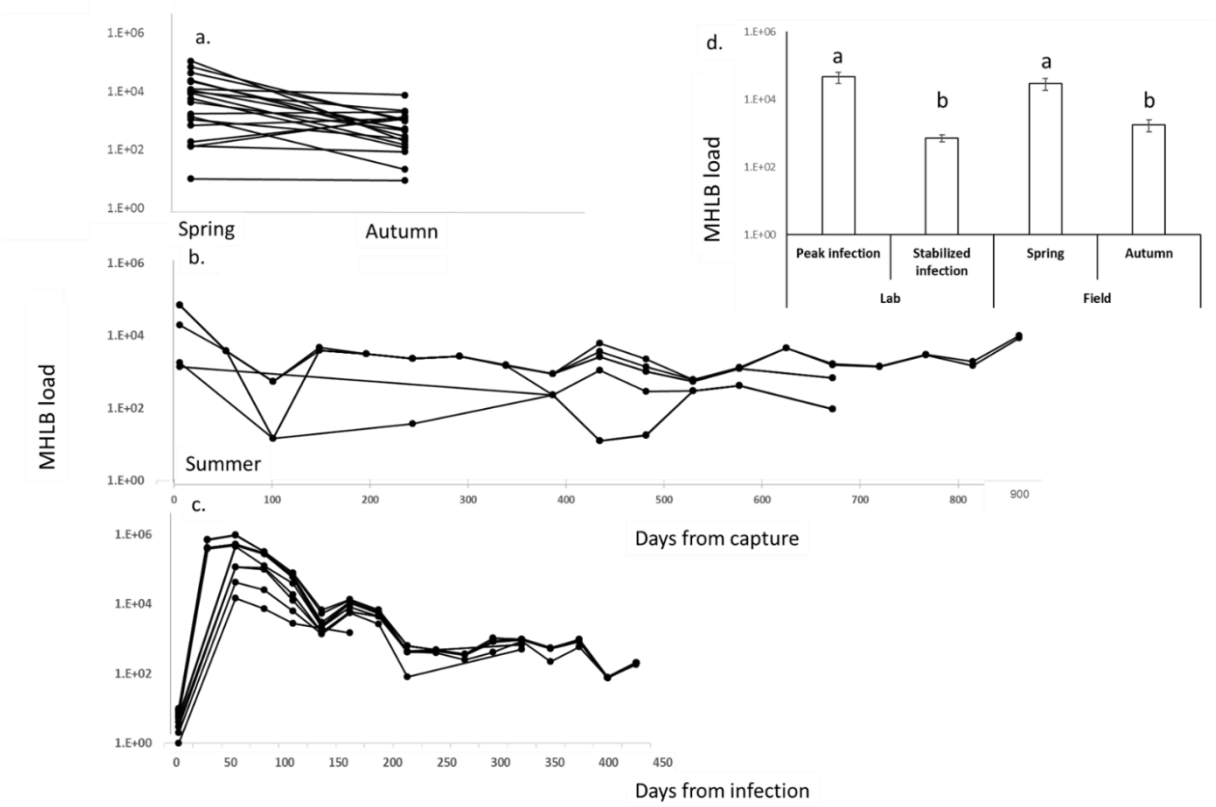

Fig. 3. Changes in MHLB load (copies per μl of DNA) over time in (a) 19 *G. andersoni* rodents that were sampled in the field during spring and then resampled during autumn (group 1), (b) seven *G. andersoni* rodents that were brought into the laboratory from the field during the summer, when they were positive for MHLB (group 2), and (c) 10 MHLB-negative *G. andersoni* that were infected in the laboratory (group 3). (d) To evaluate the relevance of the laboratory infection experiment, the means ± SE of MHLB loads that were quantified in the field during summer and autumn (a) were compared with those quantified during peak (days 25–45 post-infection) and stabilized (days 65–400 post-infection) infection experiments in the laboratory (c). Letters indicate significant post-hoc comparisons ($p < 0.05$).