

No evidence that *Mycoplasma* infection causes cognitive impairment during foraging in Allenby's gerbil (*Gerbillus andersoni allenbyi*)

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Supplementary methods

Inoculation procedures

Three laboratory-reared gerbils, Ga-318, Ga-335, and Ga-340, were inoculated by a chronically infected gerbil, Ga-255, which had survived a previous *Mycoplasma* experiment (Makin *et al.*, 2021). Blood was drawn from Ga-255 retro-orbitally using heparin sulfate capillary tubes. The inoculation procedures were adopted slightly adjusted as follows from Cohen *et al.* (2017). The withdrawn blood was supplemented with 0.15% of 0.5 M EDTA to prevent coagulation and then diluted with 20% DMSO in proportion to the volume of blood extracted. During the initial stages, gerbils Ga-318, Ga-335, and Ga-340 were infected with 150-300 µl of blood along with EDTA and DMSO via subcutaneous and peritoneal routes.

In the second stage, 225 µl of blood from Ga-340 was inoculated into Ga-557 and Ga-560. The volume of blood introduced was intentionally reduced to explore the efficacy of a smaller quantity in facilitating the transfer of *Mycoplasma*. This approach was also adopted with the aim of minimizing discomfort for the donor gerbils. Similarly, 180 µl of blood from Ga-388, mixed with EDTA and DMSO, was administered to Ga-548 and Ga-549. Then, 200 µl of blood with added EDTA and DMSO from Ga-335 was used to inoculate Ga-652 and Ga-653.

The process of hypotonic lysis, which is typically used to extract parasite DNA from RBCs, was avoided in order to prevent a reduction in DNA concentration. Both the pathogen and host DNA were extracted directly from gerbil blood (Crippen *et al.*, 2001; Samaï *et al.*, 2018). The *Mycoplasma* infection statuses of both the donors and recipients were confirmed by PCR before proceeding with the inoculations for vivarium experiments. Subsequently, it was determined that 150 µl of blood from a chronically infected gerbil could successfully inoculate at least one *Mycoplasma*-free gerbil, and 75 µl of blood from an acutely infected gerbil, combined with EDTA and DMSO, was sufficient to inoculate a minimum of one *Mycoplasma*-free gerbil.

Before the vivarium experiment, Ga-548 successfully inoculated three gerbils (Ga-610, Ga-624, and Ga-625), while Ga-557 inoculated two (Ga-615 and Ga-616). Additionally, a lab-reared *Gerbillus pyramidum* (Gp-139) was used to inoculate Ga-608, and Ga-549 was responsible for inoculating the remaining gerbils (Supplementary Table S2).

Table S1. List of donor and recipient gerbils from a single source (Ga-255).

Donor Gerbil	Recipient Gerbils	Inoculation Date	PCR confirmed
Ga-255	Ga-318, Ga-335, Ga-340	17-Aug-2020, 24-Aug-2020	Both the donor and recipients HM-16s primers

Ga-340	Ga-557, Ga-560	4-Jun-2022	Both the donor and recipients HM-16s 480 bp and 180 bp
Ga-388	Ga-548, Ga-549	10-Jun-2022	Both the donor and recipients HM-16s 480 bp and 180 bp
Ga-335	Ga-652, Ga-653	25-Nov-2022	Both the donor and recipients HM-16s 480 bp and 180 bp

Table S2. List of donor gerbils that inoculated gerbils for vivarium experiment and gerbils that received saline for the vivarium experiment.

Donor	Recipient Gerbils	Inoculation Date	PCR confirmed HM-16s 480 bp and 180 bp
Ga-548	Ga-610, Ga-624, Ga-625	08-Dec-2022	Ga-624 Ga-625 Ga-610
Ga-557	Ga-615, Ga-616	08-Dec-2022	Ga-616
Gp-139	Ga-608	08-Dec-2022	-
Ga-549	Ga-468, Ga-511, Ga- 539, Ga-545, Ga-550, Ga-552, Ga-609 Ga-627, Ga-635, Ga-641	08-Dec-2022	Ga 511 Ga 545 Ga 609
Saline	Ga-467, Ga-527, Ga-542 Ga-553. Ga-558, Ga-566, Ga-580, Ga-612, Ga-613, Ga-617, Ga-623, Ga-626 Ga-632, Ga-633, Ga-634	08-Dec-2022	Ga-542 Ga 612 Ga 626 Ga 633 Ga 634

PCR

PCR methods were adopted from Kedem *et al.* (2014), utilizing HM16-s forward and reverse primers to amplify clone 6 of the 16S ribosomal RNA gene, with the partial sequence accession number MT345323. The donor Ga-255 and the initial recipients Ga-318, Ga-335, Ga-340 were tested using HM-16s primers. The gerbils subjected to the vivarium experiment underwent testing in PCR utilizing two sets of HM-16S primers (180 bp and 489 bp). Detailed information on the primers designed, and other molecular methods will be provided in a separate publication (Sargunraj *et al.*, unpublished data).

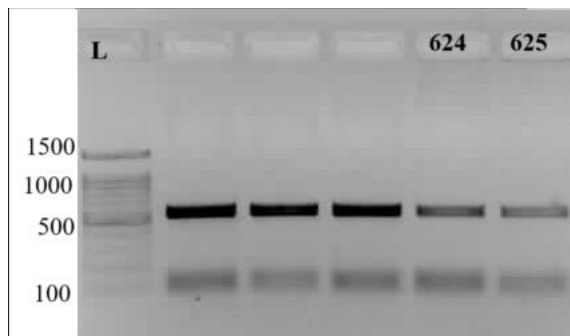


Figure S1. Ga-624 and 625 show bands eight days post inoculation using HM-16s (489 bp) primers. In gerbils Ga-624 and Ga-625 were tested using HM-16s (489 bp) primers. L corresponds to SuperLadder2100 (Cat# 9597580SL2100), which signifies a 100 bp DNA ladder encompassing 11 unique fragments: 1.5k, 1k, 900, 800, 700, 600, 500, 400, 300, 200, and 100 base pairs. It's noteworthy that the bands, particularly the topmost 1.5k and the 500 bp band (pertaining to the amplicon generated by HM-16s-489 bp primers in our case), have been intensified to facilitate easy and accurate identification. The wells which are not labelled are gerbils that are not relevant for the study.

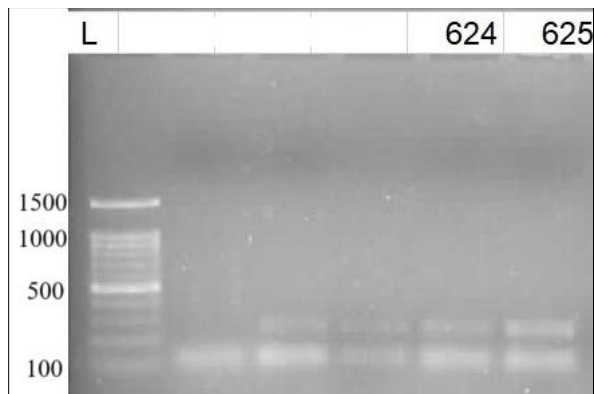


Figure S2. Ga- 624 and 625 show bands eight days post inoculation using HM-16s (180 bp).

PCR post vivarium:

After the initial round of foraging experiments in vivarium, further testing was conducted for both the gerbils inoculated with saline and the *Mycoplasma*-positive gerbils from January 28th to 31st, 2023. This testing occurred approximately 50 days after the initial inoculation on December 8th, 2022. To prevent any cross-contamination, DNA extraction and PCR for the gerbils inoculated with saline and *Mycoplasma* were performed on separate days.

For the *Mycoplasma*-inoculated gerbils, testing was done using two pairs of primers - HM-16s primers of 489 bp and 180 bp. In contrast, the saline-inoculated gerbils were tested solely with the HM-16s 180 bp primers. Because, the HM-16s 180 bp primers are more sensitive due to the shorter target amplicon length, making them sufficient for testing saline-inoculated gerbils. Both 489 bp and 180 bp primers were used for *Mycoplasma*-inoculated gerbils to compare primers' efficiency. The concept is that even the most minuscule gene fragment MT345323 remains imperceptible in saline-inoculated gerbils, necessitating testing with HM-16s 180 bp primers. Conversely, both the larger fragment targeted by HM-16s-489 bp primers and the smaller fragment targeted by HM-16s-180 bp primers of MT345323 should be amplified in *Mycoplasma*-positive gerbils. The nested PCR utilizing HMA-16s-180 bp primers successfully amplified both these amplicons from the initial PCR.

Initially, both the *Mycoplasma*-positive and negative gerbils did not display any visible bands in the results (data not shown). However, a second PCR was conducted, following the similar approach described as Nested PCR reactions (Green and Sambrook, 2019), utilizing 2 µl of the template derived from the initial PCR product.

In the case of the saline-inoculated gerbils, the second PCR involved using the HM-16s 180 bp primers with the same primer type from the first PCR template. Meanwhile, the *Mycoplasma*-inoculated gerbils employed the template from the initial PCR product (using HM-16s 180 bp) in the second PCR reaction, with both HM-16s 180 bp and HM-16s 489 bp primers.

In the subsequent PCR round, which involved amplification of the products from the first PCR, no bands were observed for the saline-inoculated gerbils, while *Mycoplasma*-inoculated gerbils did show detectable bands. This phenomenon could be attributed to a relatively lower amount of *Mycoplasma* DNA initially, resulting in the absence of visible bands. However, upon utilizing the PCR product as a template in the second PCR, successful bands were clearly evident (refer to Figures S3 and S4).

Given that the *Mycoplasma*-negative samples and water control consistently yielded no bands during the two rounds of PCR, the presence of bands in the inoculated samples confirmed their infection (see Figures S3 and S4). Conversely, all gerbils that received saline injections tested negative for *Mycoplasma* based on the PCR results. Detailed insights into the molecular methods employed for *Mycoplasma* detection will be provided in separate publication (Sargunaraj *et al.*, unpublished data).

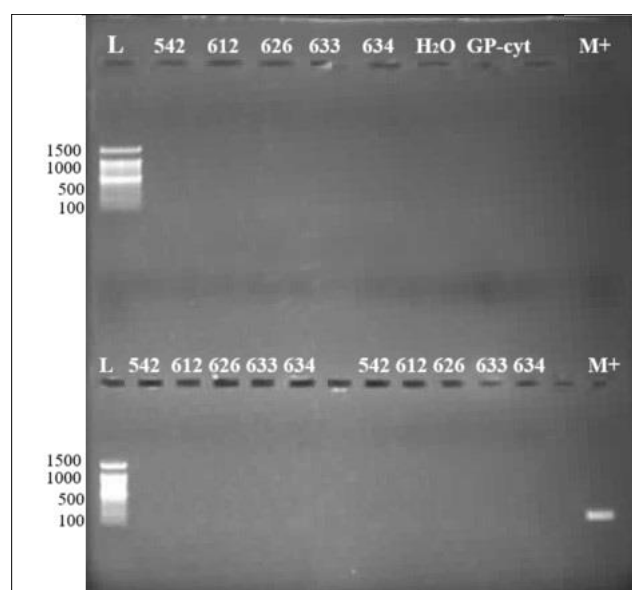


Figure S3. Nested PCR on DNA samples from saline inoculated gerbils using HM-16s 180 bp primers. Nested PCR on saline inoculated gerbils using HM-16s 180 bp primers. Both the PCR were done using HM-16s 180 bp primers. M+ refers to positive control, *Mycoplasma* synthetic DNA which showed a band once. GP-Cyt is a negative control in addition to water. The GP-Cyt refers to the synthetic DNA from accession number KT721357.1, which is the *Gerbillus pyramidum pyramidum* isolate cytochrome b (cytb) gene. M+ refers synthetic DNA of accession number MT345323.

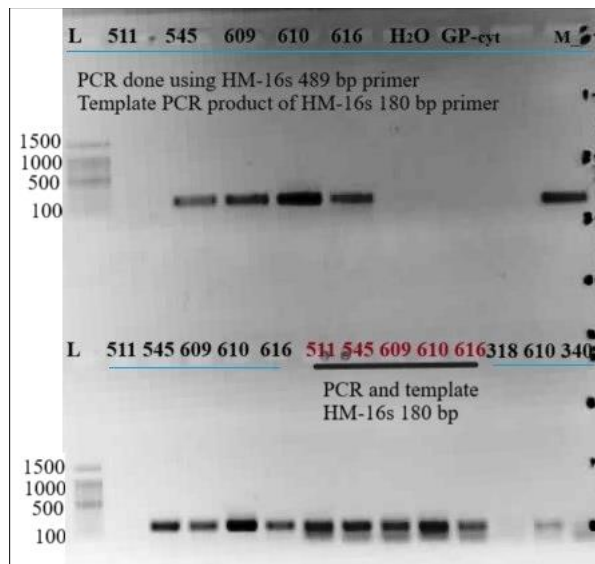


Figure S4. Nested PCR on *Mycoplasma* positive gerbils. The template is obtained from the PCR product amplified using HM-16s 180 bp primers. In the first row, this template is used in a PCR reaction with HM-16s 489 bp primers. For subsequent reactions, both HM-16s 489 bp primers (on the sides) and HM-16s 180 bp primers (in the middle) are utilized. The PCR bands indicated a presence of 180 bp bands in *Mycoplasma*-inoculated gerbils. Negative controls such as water and Gp-cytochrome did not exhibit any bands, while synthetic *Mycoplasma* DNA (M+) showed a distinct band.

References

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