



Eidelman, A., Cohen, C., Navarro-Castilla, Á., Filler, S., Gutiérrez, R., Bar-Shira, E., Shachar, N., Garrido, M., Halle, S., Romach, Y., Barja, I., Tasker, S., Harrus, S., Friedman, A., & Hawlena, H. (2019). The dynamics between limited-term and lifelong coinfecting bacterial parasites in wild rodent hosts. *Journal of Experimental Biology*, 222. <https://doi.org/10.1242/jeb.203562>

Peer reviewed version

Link to published version (if available):
[10.1242/jeb.203562](https://doi.org/10.1242/jeb.203562)

[Link to publication record in Explore Bristol Research](#)
PDF-document

This is the author accepted manuscript (AAM). The final published version (version of record) is available online via Company of Biologists at [10.1242/jeb.203562](https://doi.org/10.1242/jeb.203562) . Please refer to any applicable terms of use of the publisher.

University of Bristol - Explore Bristol Research

General rights

This document is made available in accordance with publisher policies. Please cite only the published version using the reference above. Full terms of use are available:
<http://www.bristol.ac.uk/red/research-policy/pure/user-guides/ebr-terms/>



Tasker, S. (Accepted/In press). The dynamics between limited-term and lifelong coinfecting bacterial parasites in wild rodent hosts. *Journal of Experimental Biology*.

Peer reviewed version

[Link to publication record in Explore Bristol Research](#)
PDF-document

University of Bristol - Explore Bristol Research

General rights

This document is made available in accordance with publisher policies. Please cite only the published version using the reference above. Full terms of use are available:
<http://www.bristol.ac.uk/pure/about/ebr-terms>

1
2 **The dynamics between limited-term and lifelong coinfecting bacterial parasites in wild**
3 **rodent hosts**
4
5

6 Anat Eidelman^{1*}, Carmit Cohen^{1,2*}, Álvaro Navarro-Castilla³, Serina Filler⁴, Ricardo
7 Gutiérrez⁵, Enav Bar-Shira⁶, Naama Shachar¹, Mario Garrido¹, Snir Halle¹, Yoav Romach⁷,
8 Isabel Barja^{3,8}, Séverine Tasker⁴, Shimon Harrus⁵, Aharon Friedman⁶, and Hadas Hawlena^{1c}
9

10 ¹Mitrani Department of Desert Ecology, Jacob Blaustein Institutes for Desert Research, Ben-Gurion University
11 of the Negev, Midreshet Ben-Gurion, Israel; ²Infection Prevention & Control Unit, Sheba Medical Center, Tel
12 Hashomer, Israel; ³Department of Biology, Faculty of Sciences, University Autonomous of Madrid,
13 Spain; ⁴School of Veterinary Sciences, University of Bristol, Langford, UK; ⁵Koret School of Veterinary
14 Medicine, The Hebrew University of Jerusalem, Rehovot, Israel; ⁶Section of Immunology, Department of Animal
15 Sciences, Faculty of Agricultural, Nutritional and Environmental Sciences, The Hebrew University of
16 Jerusalem, Rehovot, Israel; ⁷The Institute of Physics, The Hebrew University of Jerusalem, Jerusalem, Israel;
17 ⁸Center for Research on Biodiversity and Global Change (CIBC-UAM), University Autonomous of Madrid,
18 Spain.
19

20
21 *Equal contribution

22 ^cCorresponding author: hadashaw@bgu.ac.il
23

24 A running title

25 Multifaceted coinfection
26

27 Keywords:

28 Coinfection, concomitant infections, mixed infections, multiple parasites, within-host
29 competition, within-host facilitation.
30

31 A summary statement

32 Coinfection with both limited-term and lifelong bacteria induces physiological and behavioral
33 changes in a rodent host that may make these bacteria's interaction dynamic over time.
34
35
36
37
38
39

40 **Abstract**

41 Interactions between coinfecting parasites¹ may take various forms, either direct or indirect,
42 facilitative or competitive, and may be mediated by either bottom-up or top-down
43 mechanisms. While each form of interaction lead to different evolutionary and ecological
44 outcomes, it is challenging to tease them apart throughout the infection period. To establish
45 the first step towards a mechanistic understanding of the interactions between coinfecting
46 limited-term bacterial parasites and lifelong bacterial parasites, we studied the coinfection of
47 *Bartonella* sp. (limited-term) and *Mycoplasma* sp. (lifelong), which commonly co-occur in
48 wild rodents. We infected *Bartonella* and *Mycoplasma*-free rodents with each species, and
49 simultaneously with both, and quantified the infection dynamics and host responses.
50 *Bartonella* benefited from the interaction; its infection load decreased more slowly in
51 coinfecting rodents than in rodents infected with *Bartonella* alone. There were no indications
52 for bottom-up effects, but coinfecting rodents experienced various changes, depending on the
53 infection period, in their body mass, stress levels, and activity pattern, which may further
54 affect bacterial replication and transmission. Interestingly, the infection dynamics and
55 changes in the average coinfecting rodent traits were more similar to the chronic effects of
56 *Mycoplasma* infection, whereas coinfection uniquely impaired the host's physiological and
57 behavioral stability. These results suggest that parasites with distinct life history strategies
58 may interact, and their interaction may be asymmetric, non-additive, multifaceted, and
59 dynamic through time. Since multiple, sometimes contrasting, forms of interactions are
60 simultaneously at play and their relative importance alternates throughout the course of
61 infection, the overall outcome may change under different ecological conditions.

62

63

64

65 **Introduction**

66 Due to the ubiquity of coinfecting parasites¹ and their potential impact on disease outcomes,
67 biodiversity, and host-parasite coevolution, there is a growing interest in revealing the
68 mechanisms underlying the interactions between them (McArdle et al., 2018; Pedersen and
69 Fenton, 2007; Rynkiewicz et al., 2015; Seppala and Jokela, 2016; Telfer et al., 2010).
70 Parasitic organisms may chemically or mechanically affect the growth, reproduction, or
71 transmission of their coinfecting counterparts (e.g., Grube et al., 2011; Hawlena et al., 2010).
72 Coinfecting parasites may also interact indirectly through bottom-up mechanisms such as
73 exploitative competition or by producing substances that aid in resource exploitation (Poulin,
74 2001; Ramiro et al., 2016; Wale et al., 2017; West and Buckling, 2003) or through top-down
75 mechanisms. The latter mechanisms may be mediated by the host behavioral or
76 immunological responses (Reviewed in Cox, 2001; Dianne et al., 2010; Mabbott, 2018;
77 Supali et al., 2010) or through the damage caused to the host (e.g., Gleichsner et al., 2018;
78 Mendez-Lozano et al., 2003; Yin et al., 2017).

79 Teasing apart the different above interaction mechanisms is important since they can
80 lead to different evolutionary outcomes (Mideo, 2009). For example, exploitation
81 competition is predicted to lead to selection, either for divergence in resource use between
82 competitors or increased virulence. In contrast, interference competition is expected to select
83 for lower virulence, and top-down mechanisms are predicted to select for adaptations such as
84 immunomodulation to host responses (reviewed by Mideo, 2009). Different interaction forms
85 can also lead to different ecological and epidemiological outcomes. For instance, knowledge
86 on the underlying mechanisms can explain patterns of parasite diversity (Bashey, 2015; Zele
87 et al., 2018), as well as spatial and temporal variability in parasite-parasite, and host-parasite
88 interactions in natural communities (Jolles et al., 2008; Pedersen and Fenton, 2007;
89 Penczykowski et al., 2016). However, teasing apart interaction mechanisms among
90 coinfecting parasites is not trivial and demands simultaneous exploration of multiple,
91 reciprocal host and parasite responses throughout the infection period (Mideo, 2009; Zele et
92 al., 2018). This challenge might explain the scarcity of mechanistic studies on interactions
93 between unrelated coinfecting parasitic organisms despite the pervasiveness of their co-
94 occurrence (Karvonen et al., 2019). Such studies, above all, require knowledge of and
95 practice with various organisms, differing in their life histories and methods of host

¹ "Parasite" is used throughout the article in its broad definition as an organism that lives in or on an organism of another species (its host) and benefits by consuming parts of the host resources or components. It damages the host but is rarely lethal in the short term. This term includes herein viruses, bacteria, protozoa, helminths, ectoparasites, and other blood-sucking organisms.

96 exploitations. Moreover, since often facilitation and competition are regarded as different
97 ecological paths (e.g., Mideo, 2009; Zele et al., 2018), the potential involvement of both
98 components in the interaction has been overlooked (but see; Cattadori et al., 2014; Kamiya et
99 al., 2018).

100 Rodents coinfecting with *Bartonella* and hemotropic *Mycoplasma* bacteria
101 (hemoplasmas) in the Negev Desert sand dunes, Israel, constitute a convenient system in
102 which to tease apart the mechanisms of interactions between unrelated coinfecting parasitic
103 organisms throughout the infection period, including facilitative and competitive
104 components. On one hand, these are widespread and prevalent coinfecting genera among wild
105 and domestic mammals. Both employ the host red blood cells (RBCs) as targeted cells and
106 multiply within the vascular system, providing various opportunities to interact and coevolve.
107 On the other hand, the two bacterial genera are phylogenetically distant and have different
108 ecological niches [*Mycoplasma* spp. (hemoplasmas) parasitize the RBC outer membrane
109 while *Bartonella* spp. penetrate the RBCs], transmission routes, and persistency levels
110 (Barker and Tasker, 2013; Cohen et al., 2018; Gutiérrez et al., 2015; Harms and Dehio,
111 2012). Importantly, the species from these two genera that coinfect Gerbillina rodents
112 demonstrate two ends of the invasion-persistency continuum. '*Candidatus* *Bartonella*
113 *krasnovii*' bacteria (Gutiérrez et al., 2018) are easily transmitted between rodents through
114 fleas, leading to limited-term bacteremia (i.e., up to six months; Morick et al., 2011; 2013),
115 whereas *Mycoplasma haemomuris*-like infections are characterized by low transmission rates
116 (mainly via host-to-host contact) and lifelong infections (Cohen et al., 2018). Thus, the
117 exploration of within-rodent *Bartonella-Mycoplasma* interactions is expected to provide
118 insights into the mechanisms underlying coinfection between parasitic organisms with
119 different life history strategies that share the same resources. Finally, co-occurrence analyses
120 indicate positive *Bartonella-Mycoplasma* associations in blood sampled from wild *Gerbillus*
121 *andersoni* captured in the southeast Negev (Kedem et al., 2014) and negative associations in
122 blood sampled from *G. andersoni* captured in the northwest Negev (Cohen et al., 2015a).
123 Thus, it is likely that *Bartonella-Mycoplasma* interactions include both positive and negative
124 components, but their balance changes under different ecological conditions.

125 To establish the first step towards a mechanistic understanding of *Bartonella*-
126 *Mycoplasma* interactions, we compared the infection dynamics and the changes in the rodent
127 host's physiological and behavioral variables in response to infection with '*Ca. B. krasnovii*'
128 (designated herein as *Bartonella*) and *Mycoplasma haemomuris*-like bacteria (designated
129 herein as *Mycoplasma*) alone and to simultaneous coinfection with these two bacterial species

130 under laboratory conditions. The infection dynamics was used to deduce, from the parasite
131 perspective, the outcome of the interaction under laboratory conditions. In particular, lower
132 loads of bacteria in coinfection compared to a single infection would support competition,
133 while higher loads would support facilitation. The quantification of changes in rodent
134 variables was used to highlight candidate interaction mechanisms, where RBC loss in
135 coinfecting rodents (measured by packed blood cell volume) would suggest exploitative
136 competition, higher antibodies would suggest immune-mediated competition, and the
137 opposite effects would suggest the parallel facilitative interactions. Since the main
138 transmission route of *M. haemomuris*-like bacteria is assumed to be through host-to-host
139 aggressiveness (Cohen et al., 2018), we also predicted that to increase transmission,
140 *Mycoplasma*, like other parasitic organisms (Hughes and Libersat, 2019; Moore, 2002),
141 would induce greater rodent movement (i.e., a higher activity duration, frequency, or both).
142 Following this logic, from the *Mycoplasma* perspective, increased activity of coinfecting
143 rodents would support facilitation, whereas reduced rodent activity would support
144 competition. Finally, to highlight candidate interaction mechanisms mediated through host
145 damage, we compared the rodent body mass gain, body temperature, physiological stress
146 levels, activity pattern, and the daily variability in these variables, between singly infected
147 and coinfecting rodents. However, since it is not yet clear whether the damage to the host
148 measured by these variables benefits or harms each bacterial species, we could not use these
149 measurements to locate our predictions on the competition-facilitation continuum.

150

151 **Materials and Methods**

152 *Study animals*

153 Rodents: All *Gerbillus andersoni* that were used in the study were born and raised in
154 the laboratory and were PCR-negative for *Mycoplasma* and *Bartonella* bacteria. The rodents
155 were at least seven months old, non-reproductive, with an average body mass of $38.79 \pm$
156 5.22 g (\pm standard error; SE) and 36.11 ± 4.01 g for males and females, respectively. Rodents
157 were maintained individually in 20×30 cm² plastic cages with a 1-cm layer of autoclaved
158 sand as substrate, in an animal facility with an air temperature of $25 \pm 1^{\circ}\text{C}$ and a photoperiod
159 of 12 h dark: 12 h light. They were provided daily with millet seeds *ad libitum* and 13.3 ± 1.4
160 g alfalfa as a water source according to Hawlena et al. (2007).

161 Bacteria: *Mycoplasma* and *Bartonella* are the most dominant genera in *G. andersoni*
162 blood (Cohen et al., 2015b; Gavish et al., 2014). Regardless of the geographical region,

163 rodent community composition, and rodent species, the *Mycoplasma* found in all blood
164 samples belong to a single cluster, which is closely related to, but distinguishable from, *M.*
165 *haemomuris*, and is therefore termed *M. haemomuris*-like (Kedem et al., 2014). In contrast,
166 diverse *Bartonella* species may infect and coinfect *G. andersoni* rodents, but commonly,
167 individuals are found infected with '*Ca. B. krasnovii*' (Gutiérrez et al., 2018). Thus, by
168 infecting *G. andersoni* rodents with *M. haemomuris*-like bacteria, '*Ca. B. krasnovii*', or both,
169 we emulated the most common infection and coinfection scenarios in nature.

170

171 ***Experimental design***

172 Forty *G. andersoni* rodents (1:1 male to female ratio) were subjected randomly to four
173 groups, each composed of ten individuals: (i) a control group inoculated with 500 µl of
174 phosphate-buffered saline (PBS), (ii) rodents infected with 1.0×10^4 *M. haemomuris*-like
175 bacteria in 500 µl PBS, (iii) rodents infected with 2.1×10^8 '*Ca. B. krasnovii*' in 500 µl PBS,
176 and (iv) rodents coinfecting with 1.0×10^4 *M. haemomuris*-like bacteria and 2.1×10^8 '*Ca. B.*
177 *krasnovii*' in 500 µl PBS. To assess the infection dynamics of the two bacterial species in
178 rodents from the three infection groups, we bled them every 10 days following the
179 inoculation day until day 122 post-inoculation. In parallel, we quantified the host variables
180 over a period ranging from one to four days during each of the main infection stages, namely
181 (i) before the inoculation (days -15-0), (ii) an infection peak (days 11-24), (iii) a sharp
182 decrease in bacteremia (days 30-42), (iv) a slow decrease in bacteremia (with only a few
183 *Bartonella* bacteria; days 42-65), and (v) when *Mycoplasma* loads reached a plateau, as
184 *Mycoplasma* stabilized on a low bacterial load and *Bartonella* could not be detected (days
185 87-122) (Fig. 1 and Table 1). The sampling days were chosen to minimize the interference
186 among measurements. Accordingly, the behavioral measurements (see below) were always
187 recorded at least four days after bleeding and before fecal collection, and we omitted the
188 behavioral records during feeding and temperature measurements, including 30 min after
189 these activities. Similarly, fecal collection was conducted at least two days post-bleeding, and
190 the temperature measurements were recorded at least four days post-bleeding.

191

192 ***Bacterial inoculation and quantification***

193 *M. haemomuris*-like bacteria, similarly to other hemotropic *Mycoplasma* species, are
194 uncultivable organisms (Tasker et al., 2003); therefore, rodents were subcutaneously
195 inoculated with blood from *Mycoplasma*-positive *G. andersoni*, preserved in 20% DMSO

196 (Sigma-Aldrich, Buchs, Switzerland) and stored at -80°C . After thawing, the inoculum was
197 diluted with PBS to reach a concentration of 1.0×10^4 cells in 500 μl (confirmed by qPCR).
198 This concentration was chosen since it minimizes the blood volume needed for the
199 inoculation (58 ± 6.3 μl of required donor blood; mean and SE, respectively) to allow 100%
200 infection success (Cohen, Eidelman, and Hawlena, unpublished data). Regardless, the
201 transmission success, the infection load, and the timing of peak infection of *M. haemomuris*-
202 like bacteria are dose-independent (Cohen et al., 2018). To prepare a *Bartonella* inoculum, a
203 wild-type '*Ca. B. krasnovii*' was maintained in the laboratory, and after six passages on
204 chocolate agar plates, the colonies were diluted in PBS to reach a concentration of 2.1×10^8
205 colony-forming unit (CFU) in 500 μl (based on colony counting and confirmed by qPCR).
206 This concentration was chosen since it is the minimum number of bacteria required for 100%
207 success of infection with this species (Cohen, Eidelman, and Hawlena, unpublished data).

208 Despite the low volume of blood subcutaneously injected to rodents in the
209 *Mycoplasma* and coinfecting infection groups ($\sim 12\%$ of the inoculum and $\sim 2\%$ of their blood
210 volume), we intended to exclude the possibility that the blood itself generated the differences
211 between these groups and the control and *Bartonella* groups, which were injected with only
212 PBS. Accordingly, at the end of the experiment, we simultaneously subcutaneously injected
213 six *G. andersoni* rodents with an average of 50 ± 13 (\pm SE) μl of blood from bacteria-free
214 rodents in a total volume of 200 μl solution ($\sim 25\%$ of the inoculum and $\sim 2\%$ of their blood)
215 and six *G. andersoni* with 200 μl PBS. We then compared the physiological and behavioral
216 changes of these two rodent groups over 122 days.

217 To assess the bacterial loads in the rodent blood over the 122 experimental days, in
218 every bleeding event, 100–200 μl of blood was collected from the retro-orbital sinus of each
219 individual by sterile capillaries immersed in 0.15% EDTA and stored in EDTA tubes at
220 -20°C until further molecular analyses. The bleeding was conducted under local anesthesia
221 (Localin, Fischer Pharmaceutical Labs, Tel Aviv, Israel). DNA was extracted from blood
222 samples using a MoBio Bacteremia DNA Isolation Kit, following the manufacturer's
223 instructions (Cohen et al., 2015b). In each extraction session, a negative control was included,
224 in which all of the reagents were added to PBS instead of the blood.

225 Quantification of the bacterial copy numbers (designated as bacterial loads) was
226 performed by a real-time quantitative PCR (qPCR) (CFX Connect™ System, Hercules,
227 California, USA), using 2 x qPCRbio Fast Qpcr Probe Blue Mix, Hi-ROX (PCR
228 Biosystems).

229 For *Mycoplasma*, the following were added to the master mix: 200 nM of the 16S
230 rRNA gene forward primer (MhmI 458F) CGCCGAATACTGCTCGTC and 200 nM of the
231 16S rRNA gene reverse primer (MhmI 590R) TCAAGCCTAAGCGTCAATAGC ,100
232 nM of probe (Mhm 634P) FAM/AACACCAGA/Zen/GGCGAAGGCGAAA/3IABkFQ, 4
233 μ M of MgCl₂ and 5 μ L of DNA in a total volume of 25 μ L. PCR conditions were 2 min at
234 95°C followed by 40 cycles of 15 s at 95°C and 30 s at 60°C.

235 For *Bartonella*, the following were added to the master mix; 300 nM of the *gltA* gene
236 forward primer GGATTTGGTCACCGAGTCTATAAA, 300 nM of *gltA* gene reverse primer
237 AAGAAGCGGATCGTCTTGAATAT, 200 nM of probe
238 CCACGTGCAAAAATCATGCAAAAACCTGTCA, and 2 μ l of DNA in a total volume of
239 20 μ L (PrimerDesign Ltd., Chandlers Ford, UK). PCR conditions were 3 min at 95°C
240 followed by 35 cycles of 10 s at 95°C and 30 s at 60°C.

241 We ran 2–3 replications per sample, and in each run, we included a positive control (a
242 sample with a known bacterial concentration) and a negative control (ultrapure water).

243 To estimate the absolute copy numbers and validate the repeatability, efficiency, and
244 sensitivity of the reactions, in each run, we added a 10-fold serial dilution (i.e., standard curve
245 ranged from 10²–10⁷ copies per reaction) of previously sequenced plasmids containing either
246 the 16S rRNA gene of *M. haemomuris*-like bacteria or the *gltA* gene of '*Ca. B. krasnovii*'. To
247 avoid overestimation of absolute numbers by plasmid standards (Kim et al., 2014), the
248 standard curve was calibrated by Digital Droplet PCR (ddPCR), which separates each sample
249 into a large number of partitions and then runs the PCR reaction in each partition
250 individually, allowing a direct count of the nucleic acid molecules. To assess the specificity
251 of the *Bartonella* qPCR, we ran, in addition, 20 DNA samples extracted from blood collected
252 from the *Mycoplasma*-infected rodents at an infection peak (days 11 and 21) as well as 18
253 samples of control individuals, using the *gltA* primers. Similarly, to assess the specificity of
254 the *Mycoplasma* qPCR, we ran 40 DNA samples extracted from blood collected from the
255 *Bartonella*-infected rodents at an infection peak (days 11 and 21) and at a period of sharp
256 decrease in bacteremia (days 30 and 42), as well as 40 samples of control individuals, using
257 the 16S rRNA gene primers.

258

259 ***Physiological and behavioral measurements***

260 Packed RBC volume (PCV): After collecting the blood through a 0.15% EDTA-
261 coated capillary into EDTA tubes (see bacterial inoculation and quantification), we left the

262 remaining blood in the capillary, sealed it on one side, and centrifuged it at 13.3 RPM for 12
263 min. Then, we measured the packed cell volume.

264 *Bartonella* specific IgG: We used anti-*Bartonella* IgG as a measure of the specific
265 immune response. We prepared heat-killed '*Ca. B. krasnovii*' antigens by heating bacterial
266 cells in PBS at 56°C for 1 h. We then determined the *Bartonella*-specific IgG antibody levels
267 in rodent sera sampled by ELISA, following Bar-Shira et al. (2003). Briefly, immunoplates
268 (Nunc, ThermoFisher Scientific, Waltham, MA USA) were coated with *Bartonella* antigens
269 at a concentration of 5×10^8 cells/ml in a carbonate-bicarbonate coating buffer with pH 9.6.
270 Coated plates were placed in a humidified chamber at 4°C overnight. Plates were blocked for
271 2 h at 37°C, using 0.5 % skim milk (BD, Difco, Sparks, MD, USA) in PBS. Then, serum
272 samples diluted 1:1000 in a blocking solution were added, and plates were incubated at 37°C
273 for 1 h. *Bartonella*-specific antibodies were detected using HRP-conjugated rabbit anti-gerbil
274 IgG (GeneTex, Irvine, CA, USA), where TMB (Kirkegaard and Perry Laboratories,
275 Gaithersburg, MD, USA) was used as a substrate. Color development was terminated by
276 TMB stop solution (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA). Optical
277 absorbance was determined at 450 nm, using an EL × 808™ Absorbance Microplate Reader
278 (Bio Tek, Winooski, VT, USA). To enable the comparison of samples among runs, all
279 readings were normalized to *Bartonella*-positive and *Bartonella*-negative serum samples,
280 which were run in all the ELISA plates. This allowed us to ensure that the *Bartonella*-specific
281 reading obtained for each of the tested samples reflected the differences in the immune
282 response raised against *Bartonella* and were not dependent on the experimental conditions.
283 Unfortunately, due to the uncultivable nature of *Mycoplasma*, we could not run parallel
284 *Mycoplasma*-specific ELISA assays. However, the similar dynamics of these bacteria in
285 single and coinfecting rodents (see Results section below) suggest that immunity to
286 *Mycoplasma* does not play a key role in mediating the interactions between the two bacterial
287 species.

288 **Body mass:** Rodents were weighed in the mornings of the bleeding days before
289 bleeding.

290 **Surface body temperature at rest:** One week before the first measurement of the pre-
291 inoculation period (day -14), we subcutaneously implanted calibrated temperature
292 transponders (Bio Medic Data Systems, IPTT-300) in all rodents. Daily measurements were
293 recorded in the morning, 90 min after first light, when rodents were resting, by holding a

294 transponder reader above the host cage without opening the cage or disturbing the host rest.
295 These measurements are designated hereafter as body temperatures.

296 Physiological stress levels: We quantified the fecal corticosterone metabolites
297 (FCMs). The fecal glucocorticoid levels are indicators for stress levels in vertebrates,
298 including rodents (Navarro-Castilla et al., 2017; Sanchez-Gonzalez et al., 2018; Touma et al.,
299 2004). For the fecal collection, one hour after dark, each rodent was placed in a clean plastic
300 cage with a bowl of seeds and a wire mesh floor above a paper, allowing the feces to fall to
301 and be collected from the paper. After 12 h, we collected the fresh feces, saved them in -20°C
302 until further analysis, and returned the rodents to their original cage. Corticosterone
303 quantification was done by enzyme immunoassay (EIA), following Navarro-Castilla et al.
304 (2018). Briefly, fecal samples were primarily dried in a heater (3 h, 90°C). Then, 0.05 g of the
305 dry feces was mixed with 500 μl of 100% methanol and 500 μl of phosphate buffer, and
306 shaken for 16 h in a multivortex. Later, samples were centrifuged (2500 g, 15 min), and fecal
307 extracts were stored at -20°C until analysis. For the quantification of FCM levels, we used a
308 commercial corticosterone EIA (DEMEDITEC Diagnostics GmbH, D-24145 Kiel, Germany)
309 previously used in rodent species (Abelson et al., 2016; Navarro-Castilla et al., 2017). This
310 EIA was specifically validated for *G. andersoni* through an ACTH challenge test, and the
311 laboratory EIA performance was verified by parallelism, accuracy, and precision tests.

312 Activity pattern: We placed a motion detector (model Swan Quad; Crow Group,
313 Airport City, Israel) 40 cm above each cage, which continuously counted the rodent
314 movements (every 20 s, the activity was either coded as “1” or “0”, for movement or no
315 movement, respectively) into data loggers. We then extracted the data and calculated the
316 mean average time of a movement bout, designated as activity duration, and the movement
317 frequency (i.e., number of movement bouts), designated as activity frequency in the light
318 (diurnal) and dark (nocturnal) hours.

319 The handling protocol was approved by the Committee for the Ethical Care and Use
320 of Animals in Experiments of Ben-Gurion University of the Negev (# IL-59-09-2015).

321

322 ***Statistical analysis***

323 To compare between the bacterial load (dependent variable) in singly infected and coinfecting
324 rodents (independent variable), we performed, for each infection period and bacterial species,
325 a generalized linear model (GLM). For each of the rodent’s immunological, physiological,
326 and behavioral measurements, we calculated first the means and SEs of the days included in
327 each infection period per individual rodent. The means and SEs were then used to evaluate

328 the changes in the trait values and trait variability, respectively, by calculating the difference
329 between the values measured in each infection period and the values of the pre-inoculation
330 period (designated as the change in the measured variable). We then tested with GLMs
331 whether sex, treatment, and the interaction between them (independent variables) could
332 significantly explain the variability in these observed changes (dependent variables). Since
333 we were mainly interested in the comparisons between the single-infection groups and the
334 control group and between the coinfection group and the three other treatment groups and not
335 between the two single-infection groups, we also ran specific planned comparisons of least
336 square means between the groups of interest. In both the bacterial and rodent analyses, we
337 first tested a full factorial design, and when the sex \times treatment interaction was not
338 significant, we repeated the analysis, excluding the effect of sex.

339 To exclude the possibility that the blood injection itself generated the differences
340 between the *Mycoplasma*-infected and coinfecting groups and the other two groups that were
341 injected with only PBS, for each significant difference found between those groups, we ran
342 parallel statistical comparisons between the clean blood- and PBS-inoculated rodents to
343 confirm that we did not get the same between-group differences. All analyses were performed
344 using STATISTICA 12 software (StatSoft Inc., USA).

345

346 **Results**

347 Apart from one coinfecting individual that became sick during the experiment and thereby
348 was excluded from all following analyses, the remaining 39 rodents were monitored
349 throughout the 122 experimental days. All individuals, singly and coinfecting, became
350 successfully bacteremic at day 11 post-inoculation (Fig. 1). The qPCR assays showed high
351 specificity. First, the DNA extracts of the control individuals showed only low values (mean
352 estimated loads and SE of 2 ± 2 and 3 ± 3 per 1 μ l of DNA for the *gltA* and 16S rRNA
353 primers, respectively). Second, the cross-reaction rate between *Bartonella* and *Mycoplasma*
354 was low, with $2 \pm 2\%$ cross-reaction for the *gltA* primers and $2 \pm 1\%$ cross-reaction for the
355 16S rRNA primers.

356 Individuals infected with *Bartonella* showed a typical limited-term infection pattern,
357 reaching a peak bacterial load by day 20 and a sharp bacteremic decline from day 21 onwards
358 to a complete elimination by day 87 post-inoculation in the blood of any individual (Fig. 1A).
359 All individuals remained *Bartonella*-negative during the remaining experimental days.
360 Individuals infected with *Mycoplasma* also showed a reduction in bacterial loads after day 21

361 of the infection, but their blood remained persistently infected throughout the 122
362 experimental days (Fig. 1B). The *Mycoplasma* dynamics in the blood of coinfecting rodents
363 also showed a chronic persistent pattern and was not significantly different from the
364 dynamics in the single bacterial species infected rodents (Fig. 1C, Table 1). In contrast, while
365 all coinfecting individuals, similarly to the *Bartonella* (only)-infected individuals, showed no
366 indications for the presence of *Bartonella* in their blood from day 87 onwards, the *Bartonella*
367 reduction rate was slower in the former, reaching significantly greater loads at the sharp
368 decrease in their bacteremia period (Table 1, Fig. 2A). The same qualitative results of
369 significant differences between the *Bartonella*-infected and coinfecting groups and non-
370 significant differences between the *Mycoplasma*-infected and the coinfecting group were
371 obtained after we subtracted from the observed bacterial loads the maximum false positive
372 values (as estimated through the additional cross-reaction and negative control evaluations;
373 data not shown).

374 A trend of a lower increase in *Bartonella*-specific antibodies (IgG) in peak infection
375 in coinfecting rodents compared to singly infected rodents was noticed, but this trend was not
376 significant ($p = 0.2$).

377 Due to technical problems, we could not analyze the stress hormones of seven
378 individuals or measure the body temperature of ten individuals, decreasing the sample size of
379 the stress hormone analysis to 32 (nine control, seven coinfecting, eight *Bartonella*-infected,
380 and eight *Mycoplasma*-infected rodents) and of the body temperature to 29 (seven control,
381 seven coinfecting, eight *Bartonella*-infected, and seven *Mycoplasma*-infected rodents). From
382 all the physiological and behavioral traits measured, only the changes in physiological stress
383 levels and in the variability in the diurnal activity frequency (DAF) were significant at the
384 whole GLM level (Table 1: significant treatment \times sex interaction for stress levels in plateau
385 and significant treatment for DAF in sharp decrease). In particular, in male rodents,
386 coinfecting individuals had significantly higher FCM levels than both *Bartonella*-infected and
387 control individuals. The effect of *Mycoplasma* on DAF is described below.

388 The planned comparisons between groups further revealed significant differences in
389 various infection periods (Table 1; Figs. 1–2). The *Bartonella*-infected rodents gained
390 significantly less body mass than the coinfecting individuals at the period of slow decrease in
391 bacteremia (Fig. 2C). The *Mycoplasma*-infected rodents exhibited a decrease in body
392 temperature during the “*Mycoplasma* plateau” period (Fig. 2D) and a higher increase in the
393 diurnal variability of activity duration during the peak infection period (Fig. 2I) compared to

394 the control individuals. *Mycoplasma*-infected rodents also had a stronger decrease in the
395 variability of diurnal activity frequency during all periods of infections compared to the
396 coinfecting and control individuals (Fig. 2J). In addition to the above differences between
397 coinfecting rodents and the other groups, they showed a decrease in the variability of
398 nocturnal activity duration during the peak infection period (Fig. 2H) and a higher increase in
399 physiological stress levels during the period of slow decrease in bacteremia (Fig. 2E)
400 compared to the control individuals. They also demonstrated a higher increase in the
401 variability of body temperature during the period of sharp decrease in bacteremia compared
402 to *Bartonella*-infected individuals (Fig. 2G), and a higher variability in body mass during the
403 period of slow decrease in bacteremia compared to *Mycoplasma*-infected rodents (Fig. 2F).
404 All the other planned comparisons were not statistically significant (Table 1).

405

406 **Discussion**

407 Much emphasis has previously been placed on the documentation of within-host interactions
408 and the exploration of their effects on epidemiological patterns, and the evolution of
409 virulence and host resistance (reviewed in Hawlena and Ben-Ami, 2015; Pedersen and
410 Fenton, 2007; Tollenaere et al., 2016). These effects are likely to be affected by the
411 mechanism underlying parasite interactions. Through a long-term experimental quantification
412 of the infection and host variable dynamics, we demonstrated that when limited-term and
413 lifelong infecting parasitic organisms meet in a host, the outcome of their interaction is
414 multifaceted. It is dependent on the balance among multiple, sometimes contrasting, subtle
415 top-down mechanisms, making the overall outcome highly dependent on the ecological
416 conditions. Below we discuss the results in light of the research challenges addressed in the
417 introduction and their potential implications for natural communities.

418

419 ***Teasing apart the mechanisms underlying the interaction between distant parasitic*** 420 ***organisms***

421 We showed indications that even distant coinfecting bacteria with different life history
422 strategies may interact. Coinfecting rodents showed a slower decline in *Bartonella* load than
423 rodents infected with *Bartonella* alone. This facilitative effect may be translated into higher
424 transmission rates for coinfecting rodents in nature. The packed RBC volume analysis
425 suggests that the interactions are not direct (Table 1). Although the differences between the
426 antibodies produced by coinfecting and by rodents infected with *Bartonella* alone were not
427 significant, due to the high within-group variability in this measurement, we cannot reject the

428 hypothesis that this facilitative effect is mediated by decreased specific immunity against
429 *Bartonella*. Our results also suggest that the interactions between the two bacteria may be
430 mediated by host damage, as the behavioral pattern of coinfecting rodents was significantly
431 different from rodents infected with each bacterial species alone, and they showed a higher
432 physiological stress response and body mass gain (Figs. 1–2).

433 These indications for *Bartonella-Mycoplasma* interactions, together with recent
434 evidence in other host-parasite systems (e.g., Ben-Ami et al., 2011; Ezenwa and Jolles, 2011;
435 Graham, 2008), suggest that interactions between unrelated coinfecting parasitic organisms
436 may be important determinants of host fitness, and of host-parasite population dynamics and
437 coevolution (Ezenwa and Jolles, 2011; Karvonen et al., 2019). They also suggest that
438 multiple top-down effects may operate simultaneously.

439 The effect of *Bartonella-Mycoplasma* coinfection on the host seems to be asymmetric.
440 Coinfection under laboratory conditions benefited mainly *Bartonella* and resulted in a host-
441 parasite dynamics that mostly resembles the long-term dynamics of infection with
442 *Mycoplasma* alone—that is, similar effects on average host traits to those caused by
443 *Mycoplasma* (Figs. 2D–E), a slower reduction in bacterial loads (Fig. 2A), and less damage to
444 the host (in terms of body mass gain; Fig. 2C) compared to infection with *Bartonella* alone.
445 These changes might have been the result of behavioral (e.g., change in activity pattern),
446 physiological (e.g., elevation of stress hormones), and molecular mechanisms (not tested in
447 this study) induced by *Mycoplasma* to provoke a tolerant response in the rodent host. The
448 chronic-like coinfection effect observed here is consistent with conceptual model predictions
449 for helminth-microparasite coinfection (Ezenwa and Jolles, 2011). The model suggests that
450 for acute or limited-term infections, which are expected to end with either the elimination of
451 the parasite or by host death, coinfection with a lifelong infecting parasite may alter host
452 recovery, mortality, or both. Accordingly, for those limited-term infections such as
453 *Bartonella* that cause only mild damage to the host, the main predicted effect of coinfection is
454 the deceleration of their clearance (Ezenwa and Jolles, 2011). Such chronic-like effects may
455 have a facilitative effect on the number of secondary infections that are produced in the
456 population of a limited-term infecting parasite (Ezenwa and Jolles, 2011).

457 Our results also suggest that the effect of *Bartonella-Mycoplasma* coinfection is not
458 additive. In particular, the results indicate that coinfection destabilized host physiological and
459 behavioral responses—an effect that was not demonstrated in any of the infections with each
460 bacterial species alone (Figs. 2F, G, J). Future studies on coinfection dynamics and
461 mechanisms between other limited-term and lifelong infecting parasites are required to

462 determine whether the dominant effect of the lifelong parasites and the induction of non-
463 additive instability is typical for such interactions.

464 Taken together, our results, along with the experimental results on coinfection with
465 parasites with different transmission strategies, target host tissues, virulence levels, and
466 different host specificity levels, suggest that interactions between phylogenetically distant
467 parasitic organisms are mostly underlined by asymmetrical top-down mechanisms (Ben-Ami
468 et al., 2011; Duncan et al., 2015; Fellous and Koella, 2009; Vojvodic et al., 2012; Yin et al.,
469 2017; but see Graham, 2008 for a bottom-up example). This is in comparison to closely
470 related parasites, which more likely to interact through interference and bottom-up
471 mechanisms (Hawlena et al., 2012; Ramiro et al., 2016; Wale et al., 2017). However, this
472 hypothesis should be systematically tested.

473

474 ***Considering simultaneously positive and negative aspects of within-host interactions***
475 ***throughout the infection period***

476 The fact that most of the observed differences between groups were revealed only by the
477 planned comparison analyses suggests that the impacts of the single infections and
478 coinfections are subtle relative to the variability in the measured traits and may be obscured
479 by the irrelevant comparisons. These subtle effects, along with the high natural infection and
480 coinfection rates (Cohen et al., 2015a; Kedem et al., 2014), support a long coevolution
481 between these parasitic bacteria, as well as between each of the bacterial species and the
482 rodent hosts.

483 It is under this long coevolution scenario that a simultaneous exploration of multiple host
484 and parasite reciprocal responses throughout the infection period is most important for
485 understanding coinfection interactions in natural communities. First, in contrast to our
486 expectations and to evidence from other coinfection studies (e.g., Bell et al., 2006; Graham,
487 2008), the effect of coinfection on host physiological and behavioral variables persisted even
488 at or after *Bartonella* clearance (Figs. 1 and 2C, E, F, J). This prolonged effect suggests that
489 in nature, the signature of previous coinfections should be considered. Second, our data
490 suggest that multiple forms of interactions are at play, with the relative importance of each
491 one alternating throughout the course of infection (Fig. 1). Temporal changes in the
492 interaction mechanisms were also found in a malaria-rodent system, where competition
493 between strains occurred around the peak of acute infection possibly through resource
494 exploitation and an immune-mediated competitive suppression, which occurred only towards
495 the end of the acute phase of the infection (Raberg et al., 2006). Such temporal dynamics in

496 the relative importance of alternative mechanisms may explain why interactions between
497 microparasites in rodents can be facilitative when infections are new, but competitive when
498 chronic (Telfer et al., 2010). More generally, this temporal dynamics may provide the key for
499 understanding the role that the sequence and timing of coinfection play in determining
500 coinfection outcomes (Karvonen et al., 2019).

501 Whether each of the observed effects of coinfection on host variables results in
502 facilitation or competition between the parasites would ultimately require measuring the
503 transmission of each parasite to new hosts, to ascertain whether the net direction of the
504 interaction is positive or negative (Ezenwa and Jolles, 2011). The increased instability in the
505 coinfecting rodent behavior and physiology is consistent with the expectations for coinfection
506 effects, as multiple infections may disrupt the balance of energy input, use, and output within
507 a host (Rynkiewicz et al., 2015) and is likely to negatively affect both parasites (Cao and
508 Goodrich-Blair, 2017). However, their increased body mass gain, for example, may have
509 either positive or negative effects on the parasites, depending on the link between body mass,
510 immune response, and resource availability (Hawlena et al., 2005). Regardless of the effect of
511 each change in coinfecting rodent traits on the parasites, the fact that some of them are
512 conflicting (e.g., a higher body mass gain and higher stress levels) suggests that facilitative
513 and competitive components act simultaneously. Similarly, a simultaneous infection of
514 rabbits with two helminth species increased the density, but decreased the fecundity, of one
515 parasite, while having the opposite effect on the other (Cattadori et al., 2014).

516 In such natural communities, the outcome of the interaction may depend on the specific
517 ecological conditions, determining the balance between the multiple positive and negative
518 components (Ezenwa and Jolles, 2011; Fellous and Koella, 2010). For example, the improved
519 body condition of *G. andersoni* rodents (Brand and Abramsky, 1987) and lower interspecific
520 competition (Cohen et al., 2015a; Kedem et al., 2014) that they face in the northwest Negev,
521 compared to their conspecifics in the southeast Negev, may explain why we found, in the
522 former region, indications for competition (Cohen et al., 2015a) and, in the latter, indications
523 for facilitation (Kedem et al., 2014). In particular, it is possible that when rodents are under
524 improved conditions, the stress and instability induced by coinfection are more influential,
525 leading to parasite competition, but when their conditions are poor, the reduced immune
526 response and increased body mass gain associated with coinfection are more pronounced,
527 leading to facilitation. A similar pattern was found in African buffalo, coinfecting with a
528 gastrointestinal nematode and the bacterium, *Mycobacterium bovis*, where facilitative
529 interactions, mediated by trade-off in immune response, were detected only during the dry

530 season when resources were more scarce (Jolles et al., 2008). Such an interplay between
531 positive and negative effects may underlie the spatial and temporal variability in within-host
532 interactions in nature (Callaway, 2007; Jolles et al., 2008; Pedersen and Fenton, 2007;
533 Penczykowski et al., 2016).

534

535 **Conclusions**

536 The major contribution of this study is the manipulation and exploration of a natural
537 assembly of phylogenetically distant parasitic organisms to illustrate the dynamic nature of
538 coinfection. To this end, the results encourage a holistic investigation of the within-host
539 ecology of coinfection, including the simultaneous quantification of multiple parasite and
540 host responses and the consideration of the host's trait variability along with the host's mean
541 traits throughout the infection period. Additional studies on the coinfection dynamics of
542 parasites with different life history strategies are encouraged for a better understanding of its
543 unique behavior compared to the dynamics of conspecific parasites. Regarding *Bartonella*-
544 *Mycoplasma* coinfection, future studies are encouraged to make comparisons between (i) the
545 transmission rate of singly infecting and coinfecting parasites to new hosts at different times
546 throughout the infection period, (ii) simultaneous and sequential coinfection dynamics
547 (Karvonen et al., 2018), and (iii) parasite interactions under different ecological conditions
548 (e.g., in various host species or hosts with different physiological states).

549

550 **Acknowledgments**

551 We thank N. Kronfeld-Schor, M. Einav, Y. Shani, O. Altstein, N. Azran, and A. Tsairi for
552 valuable help during this study, and B. Rosental, M. Segoli, and Y. Shafran for helpful
553 discussions on early versions of the manuscript. The analyses of physiological stress were
554 partially supported by the Etho-Physiology Laboratory (Head of Laboratory, Dra. Isabel
555 Barja), Universidad Autónoma de Madrid. This is publication number X of the Mitrani
556 Department of Desert Ecology.

557

558 **Competing interests**

559 No competing interests declared

560

561 **Funding**

562 This work was supported by the Israel Science Foundation [1391/15 to Hadas Hawlena and
563 688/17 to Shimon Harrus]. C.C. was sponsored by the short-term postdoctoral fellowship of
564 Ben-Gurion University of the Negev (BGU), and M.G. was sponsored by The Kreitman
565 School of Advanced Graduate Studies (BGU) and the Blaustein Center for Scientific
566 Cooperation (Jacob Blaustein Institutes for Desert Research, Ben-Gurion University of the
567 Negev). S.F. holds a PhD studentship at Bristol Veterinary School that is sponsored primarily
568 by the Langford Trust, but also Zoetis Animal Health.

569

570 **References**

- 571 **Abelson, K. S. P., Kalliokoski, O., Teilmann, A. C. and Hau, J.** (2016).
572 Applicability of commercially available ELISA kits for the quantification of faecal
573 immunoreactive corticosterone metabolites in mice. *In Vivo* **30**, 739-744.
- 574 **Bar-Shira, E., Sklan, D. and Friedman, A.** (2003). Establishment of immune
575 competence in the avian GALT during the immediate post-hatch period. *Developmental and*
576 *Comparative Immunology* **27**, 147-157.
- 577 **Barker, E. and Tasker, S.** (2013). Haemoplasmas: lessons learnt from cats. *New*
578 *Zealand Veterinary Journal* **61**, 184-192.
- 579 **Bashey, F.** (2015). Within-host competitive interactions as a mechanism for the
580 maintenance of parasite diversity. *Philosophical Transactions of the Royal Society B* **370**.
- 581 **Bell, A. S., De Roode, J. C., Sim, D. and Read, A. F.** (2006). Within-host
582 competition in genetically diverse malaria infections: Parasite virulence and competitive
583 success. *Evolution* **60**, 1358-1371.
- 584 **Ben-Ami, F., Rigaud, T. and Ebert, D.** (2011). The expression of virulence during
585 double infections by different parasites with conflicting host exploitation and transmission
586 strategies. *Journal of Evolutionary Biology* **24**, 1307-1316.
- 587 **Brand, S. and Abramsky, Z.** (1987). Body masses of gerbilline rodents in sandy
588 habitats of Israel. *Journal of Arid Environments* **12**, 247-253.
- 589 **Callaway, R.** (2007). Positive Interactions and Interdependence in Plant
590 Communities: Springer.
- 591 **Cao, M. Y. and Goodrich-Blair, H.** (2017). Ready or not: microbial adaptive
592 responses in dynamic symbiosis environments. *Journal of Bacteriology* **199**.
- 593 **Cattadori, I. M., Wagner, B. R., Wodzinski, L. A., Pathak, A. K., Poole, A. and**
594 **Boag, B.** (2014). Infections do not predict shedding in co-infections with two helminths from
595 a natural system. *Ecology* **95**, 1684-1692.
- 596 **Cohen, C., Einav, M. and Hawlena, H.** (2015a). Path analyses of cross-sectional and
597 longitudinal data suggest that variability in natural communities of blood-associated parasites
598 is derived from host characteristics and not interspecific interactions. *Parasites & Vectors* **8**,
599 429.
- 600 **Cohen, C., Shemesh, M., Garrido, M., Messika, I., Einav, M., Khokhlova, I.,**
601 **Tasker, S. and Hawlena, H.** (2018). Haemoplasmas in wild rodents: Routes of transmission
602 and infection dynamics. *Molecular Ecology* **27**, 3714-3726.
- 603 **Cohen, C., Toh, E., Munro, D., Dong, Q. and Hawlena, H.** (2015b). Similarities
604 and seasonal variations in bacterial communities from the blood of rodents and from their flea
605 vectors. *Isme Journal* **9**, 1662-1676.
- 606 **Cox, F. E. G.** (2001). Concomitant infections, parasites and immune responses.
607 *Parasitology* **122**, S23-S38.

608 **Dianne, L., Rigaud, T., Leger, E., Motreuil, S., Bauer, A. and Perrot-Minnot, M.**
609 **J.** (2010). Intraspecific conflict over host manipulation between different larval stages of an
610 acanthocephalan parasite. *Journal of Evolutionary Biology* **23**, 2648-2655.

611 **Duncan, A. B., Agnew, P., Noel, V. and Michalakis, Y.** (2015). The consequences
612 of co-infections for parasite transmission in the mosquito *Aedes aegypti*. *Journal of Animal*
613 *Ecology* **84**, 498-508.

614 **Ezenwa, V. O. and Jolles, A. E.** (2011). From host immunity to pathogen invasion:
615 the effects of helminth coinfection on the dynamics of microparasites. *Integrative and*
616 *Comparative Biology* **51**, 540-551.

617 **Fellous, S. and Koella, J. C.** (2009). Infectious dose affects the outcome of the
618 within-host competition between parasites. *American Naturalist* **173**, E177-E184.

619 **Fellous, S. and Koella, J. C.** (2010). Cost of co-infection controlled by infectious
620 dose combinations and food availability. *Oecologia* **162**, 935-940.

621 **Gavish, Y., Kedem, H., Messika, I., Cohen, C., Toh, E., Munro, D., Dong, Q.,**
622 **Fuqua, C., Clay, K. and Hawlena, H.** (2014). Association of host and microbial species
623 diversity across spatial scales in desert rodent communities. *Plos One* **9**, e109677.

624 **Gleichsner, A. M., Reinhart, K. and Minchella, D. J.** (2018). The influence of
625 related and unrelated co-infections on parasite dynamics and virulence. *Oecologia* **186**, 555-
626 564.

627 **Graham, A. L.** (2008). Ecological rules governing helminth-microparasite
628 coinfection. *Proceedings of the National Academy of Sciences* **105**, 566-570.

629 **Grube, M., Furnkranz, M., Zitzenbacher, S., Huss, H. and Berg, G.** (2011).
630 Emerging multi-pathogen disease caused by *Didymella bryoniae* and pathogenic bacteria on
631 Styrian oil pumpkin. *European Journal of Plant Pathology* **131**, 539-548.

632 **Gutiérrez, R., Cohen, C., Flatau, R., Marcos-Hadad, E., Garrido, M., Halle, S.,**
633 **Nachum-Biala, Y., Covo, S., Hawlena, H. and Harrus, S.** (2018). Untangling the knots:
634 Co-infection and diversity of *Bartonella* from wild gerbils and their associated fleas.
635 *Molecular Ecology* **27**, 4787-4807.

636 **Gutiérrez, R., Krasnov, B., Morick, D., Gottlieb, Y., Khokhlova, I. S. and**
637 **Harrus, S.** (2015). *Bartonella* infection in rodents and their flea ectoparasites: an overview.
638 *Vector-Borne and Zoonotic Diseases* **15**, 27-39.

639 **Harms, A. and Dehio, C.** (2012). Intruders below the radar: Molecular pathogenesis
640 of *Bartonella* spp. *Clinical Microbiology Reviews* **25**, 42-78.

641 **Hawlena, H., Abramsky, Z. and Krasnov, B. R.** (2005). Age-biased parasitism and
642 density-dependent distribution of fleas (Siphonaptera) on a desert rodent. *Oecologia* **146**,
643 200-208.

644 **Hawlena, H., Bashary, D., Abramsky, Z. and Krasnov, B. R.** (2007). Benefits,
645 costs and constraints of anti-parasitic grooming in adult and juvenile rodents. *Ethology* **113**,
646 394-402.

647 **Hawlena, H., Bashey, F. and Lively, C. M.** (2012). Bacteriocin-mediated
648 interactions within and between coexisting species. *Ecology and Evolution* **2**, 2516-2521.

649 **Hawlena, H., Bashey, F., Mendes-Soares, H. and Lively, C. M.** (2010). Spiteful
650 interactions in a natural population of the bacterium *Xenorhabdus bovienii*. *American*
651 *Naturalist* **175**, 374-381.

652 **Hawlena, H. and Ben-Ami, F. A.** (2015). A community perspective on the evolution
653 of virulence. In *Parasite Diversity and Diversification: Evolutionary Ecology Meets*
654 *Phylogenetics*, vol. 22 eds. S. Morand B. Krasnov and T. Littlewood). Cambridge Cambridge
655 University Press.

656 **Hughes, D. P. and Libersat, F.** (2019). Parasite manipulation of host behavior.
657 *Current Biology* **29**, R45-R47.

658 **Jolles, A. E., Ezenwa, V. O., Etienne, R. S., Turner, W. C. and Olf, H.** (2008).
659 Interactions between macroparasites and microparasites drive infection patterns in free-
660 ranging African buffalo. *Ecology* **89**, 2239-2250.

661 **Kamiya, T., Mideo, N. and Alizon, S.** (2018). Coevolution of virulence and
662 immunosuppression in multiple infections. *Journal of Evolutionary Biology* **31**, 995-1005.

663 **Karvonen, A., Jokela, J. and Laine, A. L.** (2019). Importance of sequence and
664 timing in parasite coinfections. *Trends in Parasitology* **35**, 109-118.

665 **Kedem, H., Cohen, C., Messika, I., Einav, M., Pilosof, S. and Hawlena, H.** (2014).
666 Multiple effects of host species diversity on co-existing host-specific and host-opportunistic
667 microbes. *Ecology* **95**, 1173-1183.

668 **Kim, T. G., Jeong, S. Y. and Cho, K. S.** (2014). Comparison of droplet digital PCR
669 and quantitative real-time PCR for examining population dynamics of bacteria in soil.
670 *Applied Microbiology and Biotechnology* **98**, 6105-6113.

671 **Mabbott, N. A.** (2018). The influence of parasite infections on host immunity to co-
672 infection with other pathogens. *Frontiers in Immunology* **9**.

673 **McArdle, A. J., Turkova, A. and Cunnington, A. J.** (2018). When do co-infections
674 matter? *Current Opinion in Infectious Diseases* **31**, 209-215.

675 **Mendez-Lozano, J., Torres-Pacheco, I., Fauquet, C. M. and Rivera-Bustamante,**
676 **R. F.** (2003). Interactions between geminiviruses in a naturally occurring mixture: *Pepper*
677 *huasteco* virus and *Pepper golden mosaic virus*. *Phytopathology* **93**, 270-277.

678 **Mideo, N.** (2009). Parasite adaptations to within-host competition. *Trends in*
679 *Parasitology* **25**, 261-268.

680 **Moore, J.** (2002). *Parasites and the Behavior of Animals*. New York: Oxford
681 University Press.

682 **Morick, D., Krasnov, B. R., Khokhlova, I. S., Gottlieb, Y. and Harrus, S.** (2011).
683 Investigation of *Bartonella* acquisition and transmission in *Xenopsylla ramesis* fleas
684 (Siphonaptera: Pulicidae). *Molecular Ecology* **20**, 2864-2870.

685 **Morick, D., Krasnov, B. R., Khokhlova, I. S., Gottlieb, Y. and Harrus, S.** (2013).
686 Transmission dynamics of *Bartonella* sp Strain OE 1-1 in Sundevall's Jirds (*Meriones*
687 *crassus*). *Applied and Environmental Microbiology* **79**, 1258-1264.

688 **Navarro-Castilla, A., Barja, I. and Diaz, M.** (2018). Foraging, feeding, and
689 physiological stress responses of wild wood mice to increased illumination and common
690 genet cues. *Current Zoology* **64**, 409-417.

691 **Navarro-Castilla, A., Diaz, M. and Barja, I.** (2017). Does ungulate disturbance
692 mediate behavioural and physiological stress responses in Algerian mice (*Mus spretus*)? A
693 wild enclosure experiment. *Hystrix-Italian Journal of Mammalogy* **28**.

694 **Pedersen, A. B. and Fenton, A.** (2007). Emphasizing the ecology in parasite
695 community ecology. *Trends in Ecology & Evolution* **22**, 133-139.

696 **Penczykowski, R. M., Laine, A. L. and Koskella, B.** (2016). Understanding the
697 ecology and evolution of host-parasite interactions across scales. *Evolutionary Applications*
698 **9**, 37-52.

699 **Poulin, R.** (2001). Interactions between species and the structure of helminth
700 communities. *Parasitology* **122**, S3-S11.

701 **Raberg, L., de Roode, J. C., Bell, A. S., Stamou, P., Gray, D. and Read, A. F.**
702 (2006). The role of immune-mediated apparent competition in genetically diverse malaria
703 infections. *American Naturalist* **168**, 41-53.

704 **Ramiro, R. S., Pollitt, L. C., Mideo, N. and Reece, S. E.** (2016). Facilitation
705 through altered resource availability in a mixed-species rodent malaria infection. *Ecology*
706 *Letters* **19**, 1041-1050.

707 **Rynkiewicz, E. C., Pedersen, A. B. and Fenton, A.** (2015). An ecosystem approach
708 to understanding and managing within-host parasite community dynamics. *Trends in*
709 *Parasitology* **31**, 212-221.

710 **Sanchez-Gonzalez, B., Planillo, A., Navarro-Castilla, A. and Barja, I.** (2018). The
711 concentration of fear: mice's behavioural and physiological stress responses to different
712 degrees of predation risk. *Science of Nature* **105**.

713 **Seppala, O. and Jokela, J.** (2016). Do coinfections maintain genetic variation in
714 parasites? *Trends in Parasitology* **32**, 930-938.

715 **Supali, T., Verweij, J. J., Wiria, A. E., Djuardi, Y., Hamid, F., Kaisar, M. M. M.,**
716 **Wammes, L. J., van Lieshout, L., Luty, A. J. F., Sartono, E. et al.** (2010). Polyparasitism
717 and its impact on the immune system. *International Journal for Parasitology* **40**, 1171-1176.

718 **Tasker, S., Binns, S. H., Day, M. J., Gruffydd-Jones, T. J., Harbour, D. A.,**
719 **Helps, C. R., Jensen, W. A., Olver, C. S. and Lappin, M. R.** (2003). Use of a PCR assay to
720 assess the prevalence and risk factors for *Mycoplasma haemofelis* and 'Candidatus
721 *Mycoplasma haemominutum*' in cats in the United Kingdom. *Veterinary Record* **152**, 193-+.

722 **Telfer, S., Lambin, X., Birtles, R., Beldomenico, P., Burthe, S., Paterson, S. and**
723 **Begon, M.** (2010). Species interactions in a parasite community drive infection risk in a
724 wildlife population. *Science* **330**, 243-246.

725 **Tollenaere, C., Susi, H. and Laine, A. L.** (2016). Evolutionary and Epidemiological
726 Implications of Multiple Infection in Plants. *Trends in Plant Science* **21**, 80-90.

727 **Touma, C., Palme, R. and Sachser, N.** (2004). Analyzing corticosterone metabolites
728 in fecal samples of mice: a noninvasive technique to monitor stress hormones. *Hormones and*
729 *Behavior* **45**, 10-22.

730 **Vojvodic, S., Boomsma, J. J., Eilenberg, J. and Jensen, A. B.** (2012). Virulence of
731 mixed fungal infections in honey bee brood. *Frontiers in Zoology* **9**.

732 **Wale, N., Sim, D. G. and Read, A. F.** (2017). A nutrient mediates intraspecific
733 competition between rodent malaria parasites in vivo. *Proceedings of the Royal Society B*
734 **284**.

735 **West, S. A. and Buckling, A.** (2003). Cooperation, virulence and siderophore
736 production in bacterial parasites. *Proceedings of the Royal Society of London Series B-*
737 *Biological Sciences* **270**, 37-44.

738 **Yin, C., Yang, W., Meng, J., Lv, Y., Wang, J. and Huang, B.** (2017). Co-infection
739 of *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia* in hospitalised pneumonia
740 patients has a synergic and significant impact on clinical outcomes. *European Journal of*
741 *Clinical Microbiology & Infectious Diseases* **36**, 2231-2235.

742 **Zelev, F., Magalhaes, S., Kefi, S. and Duncan, A. B.** (2018). Ecology and evolution
743 of facilitation among symbionts. *Nature Communications* **9**.

744

745

746 **Table 1. A summary of the statistical results.** The upper row for each dependent variable indicates the
 747 experimental days of the measurements, the row below indicates the ANOVA's F statistics for either the
 748 "treatment × sex" interaction (when it was significant, indicated by "ξ") or "treatment" effect (when there was
 749 no indication for a "treatment × sex" interaction). Contrast estimates were tested in all cases, but only the
 750 significant comparisons are mentioned in the bottom row of each cell.

	Dependent variable	Before	Peak	Sharp decrease	Slow decrease	Myc. plateau
Changes in trait mean	Log Bart. load F statistic	NA	11, 21 0.2	31, 42 *7	50, 61 NA (mostly zeros)	87, 100, 111 NA (all zeros)
	Log Myc. load F statistic	NA	11, 21 0.3	31, 42 0.013	50, 61 0.6	87, 100, 111 0.013
	<i>Bartonella</i> specific IgG F statistic	-14 NA	11, 21 2	42 0.6	42, 63 2	100 0.0086
	Packed RBC volume. F statistic	-14 NA	11, 21 0.9	42 0.3	42, 52 0.4	87, 111 0.4
	Body mass F statistic	0, -14 NA	11, 21 0.7	31, 42 1	42, 52 2 *Coin. > Bart.	100, 111 1
	Body temperature F statistic	-6, -5, -4, 0 NA	17-19, 21 0.3	25-38 0.5	49-52 2	101, 119, 122 2 *Myc < Co.
	FCM levels F statistic	-4 NA	24 0.4	NA	65 2 *Coin. > Co.	118 *ξ3 M: *Coin. >.Bart. *Coin. > Co.
	Log NAD F statistic	-11, -10, -9, -8 NA	15-16, 19-20 1	35-38 0.9	48-51 2	108-111 0.1
	DAD F statistic	-10, -9, -8, -7 NA	15-16, 19-20 2	35-38 1	48-51 0.7	108-111 0.3
	NAF F statistic	-11, -10, -9, -8 NA	15-16, 19-20 1	35-38 0.4	48-51 0.3	108-111 0.1
Changes in trait variability	DAF F statistic	-10, -9, -8, -7 NA	15-16, 19-20 0.3	35-38 0.5	48-51 0.8	108-111 0.1
	Body mass F statistic	0, -14 NA	11, 21 1	31, 42 1	42, 52 1 *Coin. > Myc.	100, 111 0.2
	Body temperature F statistic	-6, -5, -4, 0 NA	17-19, 21 0.5	25-38 2 *Coin.> Bart.	49-52 0.8	101, 119, 122 0.029
	Log NAD F statistic	-11, -10, -9, -8 NA	15-16, 19-20 2 *Coin. < Co.	35-38 0.8	48-51 2	108-111 0.4
	DAD F statistic	-10, -9, -8, -7 NA	15-16, 19-20 2 *Myc. > Co.	35-38 2 §*Myc. < Co.	48-51 0.085	108-111 0.4
	NAF F statistic	-11, -10, -9, -8 NA	15-16, 19-20 1	35-38 0.9	48-51 1	108-111 0.16
DAF F statistic	-10, -9, -8, -7 NA	15-16, 19-20 2 *Coin. > Myc.	35-38 *4 **Coin. > Myc. ***Co. > Myc.	48-51 2 *Coin. > Myc.	108-111 2 *Coin. > Myc. *Co. > Myc.	

751 Bart.: *Bartonella*; Myc: *Mycoplasma*; NAD: nocturnal activity duration; DAD: diurnal activity duration; NAF:
 752 nocturnal activity frequency; DAF: diurnal activity frequency; Co: control; Coin: coinfection; M: males.
 753 * $p < 0.05$; ** $p < 0.01$, *** $p < 0.005$. § The same trend was found between blood-injected and PBS-injected
 754 controls; thus, this effect was omitted.

755

756

757 **Figure legends**

758 **Fig 1. Bacterial dynamics and significant changes in rodent variables throughout the**
759 **experimental period.** Mean \pm SE of *Bartonella* (light grey) and *Mycoplasma* (dark grey)
760 load in 1 μ l of DNA extracted from the blood of either *Bartonella*-infected (A), *Mycoplasma*-
761 infected (B), or coinfecting (C) *Gerbillus andersoni* rodents. The changes in rodent variables
762 are indicated by increase \uparrow and decrease \downarrow arrows. Bart: *Bartonella* load in 1 μ l of DNA
763 extracted from rodent blood; IgG: *Bartonella*-specific antibody levels estimated by optical
764 density units at 450 nm; TEMP: surface body temperature ($^{\circ}$ C); BM: body mass gain (g);
765 FCM: fecal corticosterone metabolites (ng/g dry feces) used to assess stress levels; Var: trait
766 variability; NAD: nocturnal activity duration (s/12 h); DAD: diurnal activity duration (s/12
767 h); NAF: nocturnal activity frequency (number of movements per 12 h); DAF: diurnal
768 activity frequency (number of movements per 12 h); Co: control; Coin: coinfection; M:
769 males.

770 * For male rodents only; ** Non-significant.

771

772 **Fig. 2. Comparisons between the four treatment groups throughout the four infection**
773 **periods.**

774 Mean \pm SE of *Bartonella* load (in 1 μ l of DNA; A) and of rodent variable changes between
775 the various infection periods (peak infection, sharp decrease in bacteremia, slow decrease in
776 bacteremia, and *Mycoplasma* plateau) and the pre-inoculation period (differences between
777 each measure at post-inoculation and pre-inoculation). Beside the changes in *Bartonella*-
778 specific antibodies [IgG (450 nm); B] that show an insignificant trend, all other shown host
779 variables include at least one significant between-group comparison. These are changes in
780 body mass (g; C), surface body temperature ($^{\circ}$ C; D), physiological stress levels [measured by
781 fecal corticosterone metabolites; FCM (ng/g dry feces); E], variability in body mass (g; F),
782 variability in surface body temperature ($^{\circ}$ C; G), variability in log nocturnal activity duration
783 (s/12 h; H), variability in diurnal activity duration (s/12 h; I), and variability in diurnal
784 activity frequency (number of movement bouts per 12 h; J).

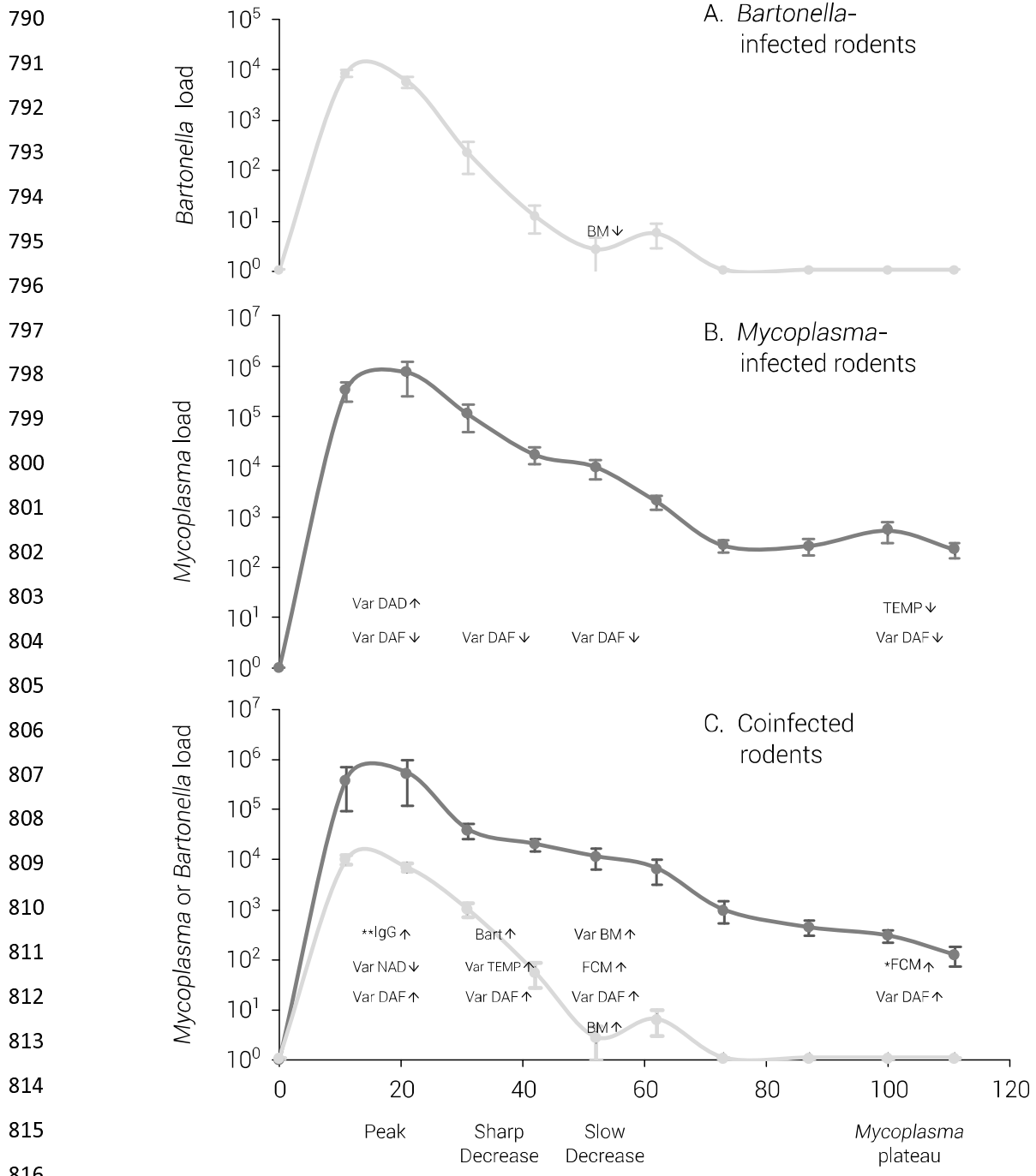
785 ^{*v}Significant between-group planned comparisons.

786

787

788

789



821 **Figure 1**

822
823

824
 825
 826
 827
 828
 829
 830
 831
 832
 833
 834
 835
 836
 837
 838
 839
 840
 841
 842
 843
 844
 845
 846
 847
 848
 849
 850
 851
 852
 853
 854

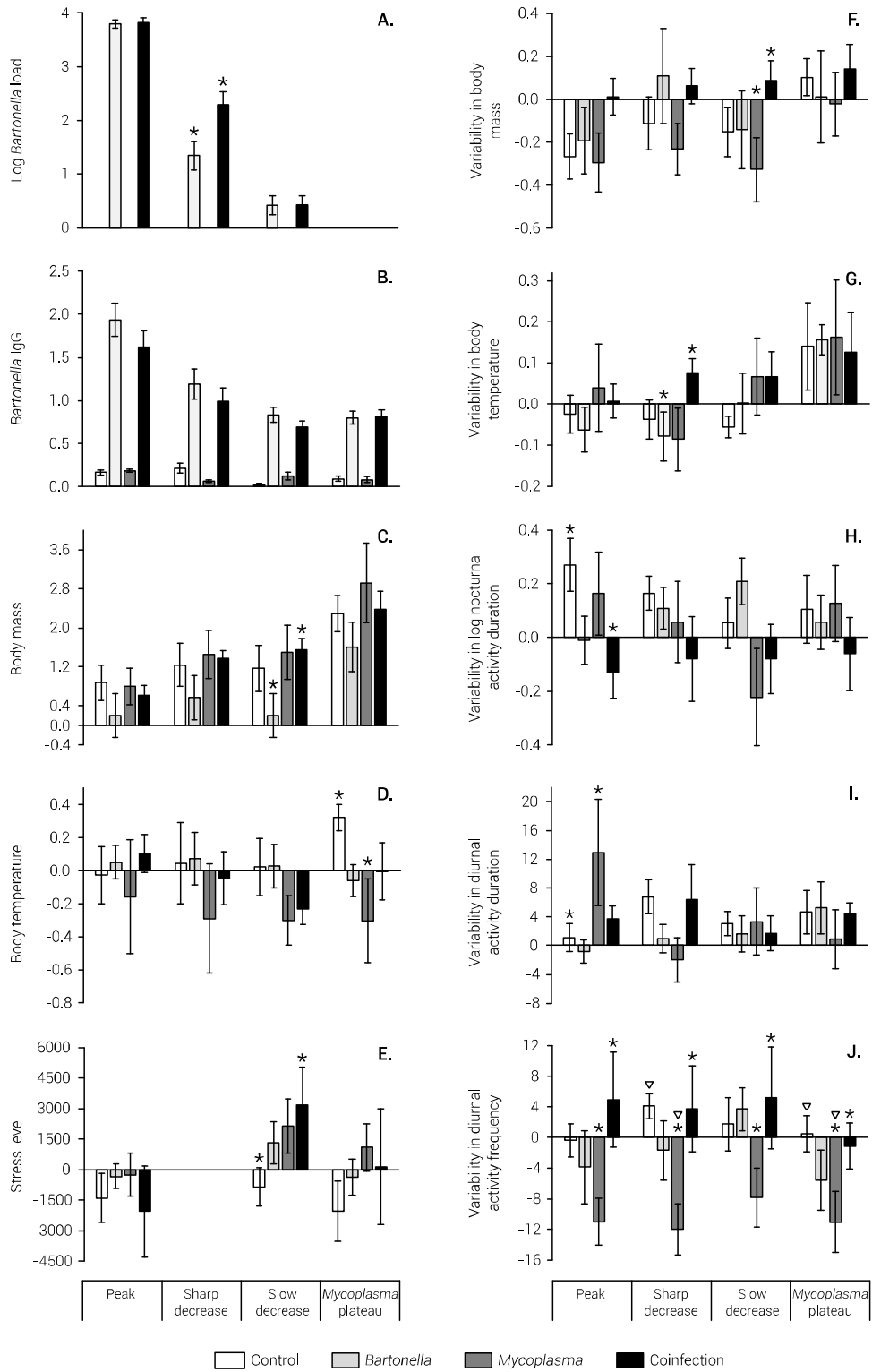


Figure 2