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## Survival capacity of the common woodlouse *Armadillidium vulgare* is improved with a second infection of *Salmonella enterica*

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### ► To cite this version:

Cybèle Prigot-Maurice, Alexandra Cerqueira de Araujo, Sylvine Durand, Tiffany Laverré, Romain Pigeault, et al.. Survival capacity of the common woodlouse *Armadillidium vulgare* is improved with a second infection of *Salmonella enterica*. *Journal of Invertebrate Pathology*, Elsevier, 2019, 168, pp.107278. 10.1016/j.jip.2019.107278 . hal-02994662

**HAL Id: hal-02994662**

**<https://hal.archives-ouvertes.fr/hal-02994662>**

Submitted on 18 Nov 2020

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## Manuscript Details

<b>Manuscript number</b>	JIP_2019_204_R2
<b>Title</b>	Survival capacity of the common woodlouse <i>Armadillidium vulgare</i> is improved with a second infection of <i>Salmonella enterica</i>
<b>Article type</b>	Full Length Article

### Abstract

Immune priming has been widely observed in invertebrates. However, this phenomenon remains incompletely characterized concerning the time course of the protective effect and immune responses. Here, we investigated the existence of a protective effect in the terrestrial isopod *Armadillidium vulgare* against a second infection with the bacterium *Salmonella enterica*. We primed animals with a low dose of living bacteria or with the bacterial culture medium. One day, 7 or 15 days later, we measured haemocytes concentration and viability and we injected a LD50 of living *S. enterica* to monitor the survival rates of infected individuals. We show that *A. vulgare* is better protected (i.e. survival improvement) upon secondary infection when it has encountered *S. enterica* 7 days before. This protection, which tends to persist 15 days after the first infection, is however not observed when the priming is performed only 1 day before the LD50 injection. Besides providing a new invertebrate example, we highlight this protection is dynamic and may be partly due to a high haemocyte production.

<b>Keywords</b>	Immune priming; Haemocytes; Invertebrates; Crustaceans; <i>Armadillidium vulgare</i> ; <i>Salmonella</i> .
<b>Taxonomy</b>	Crustacean Disease, <i>Salmonella</i> , Animal Immunology, Cell-Mediated Immunity
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<b>Suggested reviewers</b>	Delphine Destoumieux-Garzon, Martin Zimmer

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Data for: Survival capacity of the common woodlouse *Armadillidium vulgare* is improved with a second infection of *Salmonella enterica*

Our study aimed to investigate the protective effect conferred by immune priming in *A. vulgare* against *S. enterica* by determining its setting up and duration. We also analysed haemocyte variations in order to link them with a potential protection against the bacterial infection. For these purposes, we conducted two distinct experiments with several independent experimental replicates: The first consisted of priming (i.e. "vaccinated") individuals either with a low dose of living *S. enterica* bacteria, with LB broth or without injection (control). 1 day, 7 days or 15 days later, we sampled haemolymph of individuals and examined haemocyte concentrations and viabilities. Data of these experiments are available in the file "Prigot\_DataHaemocytes" in the open access repository Mendeley Data. The second experiment was based on the same priming treatment than in the first (with low dose of living bacteria, LB broth or nothing). 1 day, 7 days or 15 days later, we injected a LD50 of bacteria in all individuals and monitored their survival rates for 7 days. Data of these experiments are available in the file "Prigot\_DataSurvivals" in the open access repository Mendeley Data. Different animals were used for the two experiments. You can also find enclosed a .txt file (Prigot\_DataLegends) giving all legends of the datasets column heads. Datasets are ready to be used in R program for statistical analysis, see enclosed the R-script file. For any problems in the dataset comprehension, do not hesitate to contact us.

Thursday 22nd August 2019

Dear editorial board of *Journal of Invertebrate Pathology*,

Enclosed you will find an electronic version of our manuscript: “The common woodlouse *Armadillidium vulgare* responds to a second infection of *Salmonella enterica* by improving its survival capacity” for submission as a research article in *Journal of Invertebrate Pathology*.

In this manuscript, we contribute to the characterization of immune priming in arthropods, a phenomenon defined as an enhanced protection from past infection with a pathogen.

We have for the first time brought to light the existence of this protection in the terrestrial crustacean *Armadillidium vulgare*, by demonstrating (i) survival improvement in response to a second infection with *Salmonella enterica* and (ii) differences in haemocyte-related parameters depending on the priming treatment. The originality of our study also lies in new elements about the delay required to observe a protective effect and the duration for which the host remains protected against living bacteria. By the way, the protection against *Salmonella* in *A. vulgare* follows a temporal dynamic over the time between the two infections. We used living bacteria, whereas experiments on crustacean immune priming generally used inactivated pathogens to prime animals. To date, only one published study was carried on a terrestrial crustacean while these animals display appropriated features for the evolution of immune priming.

We certify the findings have not been published elsewhere and are only being submitted to *Journal of Invertebrate Pathology*. All authors have read and approved the material being submitted.

We truly hope the readers of *Journal of Invertebrate Pathology* will find the paper quite interesting and mostly helpful for researchers working on invertebrate immune priming.

Yours faithfully

Thursday 7<sup>th</sup> of November 2019

Dear editorial board of *Journal of Invertebrate Pathology*,

Enclosed you will find our revised manuscript: “Survival capacity of the common woodlouse *Armadillidium vulgare* is improved with a second infection of *Salmonella enterica*” for a second revision as a research article in *Journal of Invertebrate Pathology*.

We would like to express our thanks to the reviewer and editors for the positive feedback, constructive comments and the thorough checking of the text. We took into account all the suggested modifications, which had substantially improved the manuscript quality.

We are very grateful to know our work was approved for the *Journal of Invertebrate Pathology*.

Thank you again for your consideration of our work.

Yours faithfully

## Highlights

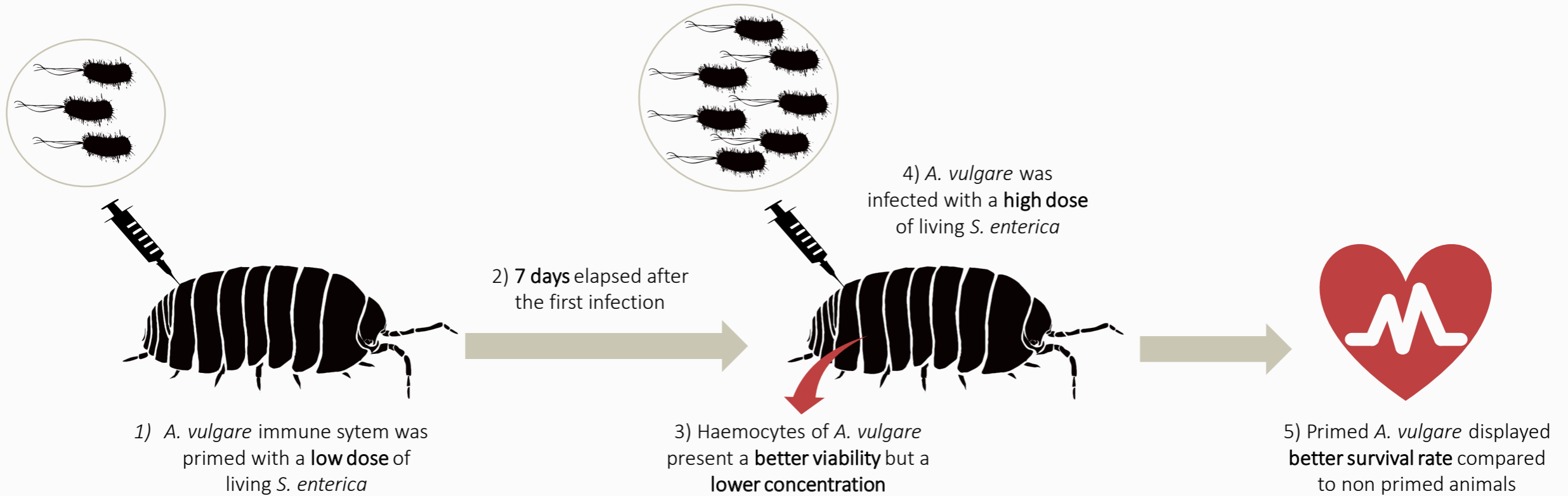
Immune priming with *Salmonella enterica* improved survival of *Armadillidium vulgare*

Survival of *A. vulgare* to an LD<sub>50</sub> dosage was higher when primed 7 days earlier

The protection isn't observed when 1 or 15 days separate the two infections

Primed animals display higher haemocyte viabilities but lower concentrations

# Survival improvement in *Armadillidium vulgare* against *Salmonella enterica*



1 **Survival capacity of the common woodlouse *Armadillidium vulgare* is**  
2 **improved with a second infection of *Salmonella enterica***

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## 23 **1. Introduction**

24 Because invertebrates rely solely on the innate immune system and are not known to  
25 possess a vertebrate-like adaptive immunity (Kurtz, 2005; Milutinovic and Kurtz, 2016), they  
26 have long been considered unable to establish an immune memory (Hoffmann et al., 1999). In  
27 the past 20 years, several studies have shaken this paradigm by providing evidence that the  
28 invertebrate immune system could be stimulated during a first infection to pathogens, leading  
29 to better protection upon a second exposure. Studying this phenomenon, called “immune  
30 priming” (Milutinovic and Kurtz, 2016; Gourbal et al., 2018; Pradeu and Pasquier, 2018), is all  
31 the more relevant in that natural populations display variations in their immune priming (Khan  
32 et al., 2016). This variability may reflect adaptive responses to various environmental  
33 conditions independently from the evolution of resistance/tolerance strategies commonly  
34 assumed (Khan et al., 2017; Ayres & Schneider, 2008).

35 Immune priming has been observed in more than 40 invertebrate species including  
36 arthropod, mollusk, cnidarian, annelid and nematode species (Milutinovic and Kurtz, 2016).  
37 Among them, the ability to mount immune priming relies on several mechanisms and may occur  
38 against some pathogens but not others. It can be expressed against a broad repertoire of  
39 pathogens or be very specific to a single species of pathogen (Contreras-Guarduño et al., 2016;  
40 Milutinovic and Kurtz, 2016; Dhinaut et al., 2017), and may result in higher survival capacity  
41 when the primed individual faces a second infection (Milutinovic and Kurtz, 2016; Mellilo et  
42 al., 2018). This protective effect is often explained by immune changes involving either (i)  
43 humoral response (e.g. production of anti-microbial peptides or AMPs, reactive oxygen species  
44 (ROS) and phenoloxydase activity), (ii) cellular response (e.g. haemocyte production,  
45 phagocytosis, encapsulation) or/and (iii) immune-related gene expression, depending on the  
46 species (Wu et al., 2015; 2016; Pinaud et al., 2016; Kutzer et al., 2018; Fugmann, 2018, Mellilo  
47 et al., 2018). Although the invertebrate immune priming phenomenon has been widely  
48 described, the delay required to provide a protective effect remains partly characterized (Little  
49 and Kraaijeveld, 2004). Few studies have addressed the time course of the immune response  
50 following the first infection by a pathogen (Rosengaus et al., 1999; Milutinovic and Kurtz,  
51 2016; Browne et al., 2015).

52 It has been suggested that mounting an immune priming response may be costly for  
53 organisms (Moret and Schmid-Hempel, 2000; Jacot et al., 2005; Contreras-Garduño et al.,  
54 2014). Consequently, immune priming should preferably evolve in organisms for which  
55 probability of re-infection with the same pathogen(s) is high (Schmid-Hempel, 2005; Best et

56 al., 2012) and is, thus, expected in species with long lifespan, low dispersion rate and group-  
57 living behaviors (Little and Kraaijeveld, 2004; Pigeault et al., 2016; Moret et al., 2019).  
58 Terrestrial isopods fulfill all the conditions assumed necessary for the evolution of immune  
59 priming. Most species can live up to 3 years and some have an estimated life expectancy of 5  
60 years (Warburg et al., 1984). These organisms live in moist soil, an environment suitable for  
61 micro-organisms (Ranjard and Richaume, 2001) where  $10^9$  bacteria per gram of soil can be  
62 found (Torsvik et al., 1990). Isopods constitute the suborder of crustaceans best adapted to a  
63 terrestrial lifestyle with the evolution of various morphological, physiological and behavioral  
64 traits (Hornung, 2011). For example, they aggregate to fight efficiently against desiccation  
65 (Broly et al., 2013, 2014) and display a low dispersion rate (Beck and Price, 1981). They are  
66 mostly promiscuous (Zimmer, 2002; Durand et al., 2017), which increases the risk of pathogen  
67 transmission within populations. Considering these features, Roth and Kurtz (2009) supported  
68 the plausibility of immune priming in the terrestrial isopod *Porcellio scaber*. In this species,  
69 individuals that were previously primed with heat-killed *Bacillus thuringiensis* had higher  
70 proportions of phagocytosing haemocytes during the second encounter with the same bacteria  
71 compared to non-primed individuals. This immune response lasts at least 14 days after the first  
72 infection. No other studies have investigated immune priming in terrestrial isopods and it is not  
73 known whether the first immune stimulation provides a protection to the animal in terms of  
74 survival against a subsequent infection.

75 In this work, we explored the hypothesis that a protective effect exists in *Armadillidium*  
76 *vulgare*. This terrestrial isopod is highly gregarious, lives more than 3 years and has a well-  
77 described immune system (Braquart-Varnier et al., 2008, 2015; Chevalier et al., 2011). The aim  
78 of our study was to examine whether a first infection provides protection in terms of improved  
79 survival against a second infection with the same pathogen, and to determine the time period  
80 between two infections needed to observe this protection. To further our understanding of this  
81 phenomenon, we also measured haemocyte concentration and viability immediately before the  
82 second inoculation. In crustaceans, these immune cells defend organisms against pathogens via  
83 phagocytosis, nodulation and encapsulation but also support humoral immune responses  
84 such as coagulation, phenoloxidase activities or antimicrobial peptide storage (Söderhäll and  
85 Cerenius, 1992; Lee and Söderhäll, 2002; Chevalier et al., 2011). Haemocytes are equally  
86 suspected to enhance their functions after a first infection, allowing a better immune response  
87 against subsequent pathogen infection (Gourbal et al., 2018).

88 To meet these aims, we first inoculated *A. vulgare* females with a non-lethal low dose  
89 of living *Salmonella enterica* bacteria (priming treatment), which have been shown to be  
90 pathogenic for this species (Braquart-Varnier et al., 2015). After 1 day, 7 days or 15 days post-  
91 infection, we investigated the cellular response by measuring the haemocyte concentration and  
92 viability. Then, the protective effect of the first injection of bacteria was explored by monitoring  
93 the survival rates of *A. vulgare* following a LD<sub>50</sub> injection of *S. enterica* occurring at the same  
94 time points (1, 7 or 15 days) after the first injection. Should a protective effect be triggered, we  
95 expected to observe higher survival rate in individuals previously primed with *S. enterica*  
96 compared to those not primed with *S. enterica*. We also expect to observe higher haemocyte  
97 concentrations and viabilities in response to the first infection as already observed in other  
98 biological models (Pope et al., 2001; Wu et al., 2014; Zang et al., 2014). These parameters may  
99 differ at each time point post-infection, which would provide insights about the temporal  
100 dynamics of the protective effect.

101

## 102 **2. Materials and methods**

### 103 ***2.1 Biological model***

104 The *Armadillidium vulgare* laboratory line used descends from cross breeding of  
105 individuals sampled in 1982 at Helsingör (Denmark). Individuals were maintained in plastic  
106 boxes (10 x 30 cm, Ets Caubere) containing moistened potting mix and were supplied *ad libitum*  
107 with linden leaves and carrot slices. To carry out our experiments, we used 439 1-year-old ( $\pm$  2  
108 months) virgin females for survival monitoring and 153 additional females for the haemocyte  
109 analysis.

110 The pathogen used for infection was *Salmonella enterica* serovar *Typhimurium* J18 (Verdon  
111 et al., 2016), a Gram-negative bacterium which is found in soil and water (Murray, 1991;  
112 Andino and Hanning, 2015). Cultures of *S. enterica* were produced as described in Braquart-  
113 Varnier et al. (2015) (see S1 in supplementary materials for detailed method).

114

### 115 ***2.2 Experiments***

#### 116 ***2.2.1 Experimental design***

117 We performed two experiments on individuals that received a first injection (i.e. priming  
118 procedure, see part 2.2.2). The first experiment was conducted to measure haemocyte  
119 concentration and viability after 1, 7 or 15 d post priming procedure (see part 2.2.3.1) but also  
120 the persistence of living *S. enterica* in haemolymph (see part 2.2.3.2). We selected these time  
121 points based on results of previous studies on crustacean immune priming (Roth & Kurtz, 2009;  
122 Chang et al., 2018). The second experiment was performed to monitor individual survival rate  
123 after receiving a LD<sub>50</sub> of bacteria injected 1, 7 or 15 d after the priming procedure (see part  
124 2.2.4 and Fig. 1). Different individuals were used for each experiment and time point. Each  
125 experiment was replicated several times (three independent experimental replicates for  
126 haemolymph analysis and seven for survival monitoring). Within each experimental replicate,  
127 we performed several series of the priming procedure (see S2 in supplementary materials).  
128 Datasets are available on the open access repository Mendeley Data (Prigot-Maurice et al.,  
129 2019).

### 130 2.2.2 Priming procedure

131 Each individual was washed (3% NaClO then water) prior conducting three priming  
132 treatments: (1) non-injected (“non-primed” group) as a control, (2) injected with Luria-Bertani  
133 (LB) medium (“LB-primed” group), and (3) injected with a non-lethal dose of  $10^3 \pm 1.10^3$  living  
134 *S. enterica* in 100 nL of LB medium (“*S. enterica*-primed” group). The sample sizes are  
135 summarized in Table I. All injections were performed under sterile conditions using a  
136 Drummond TM Nanoject (3-000-205A), in two 50 nL consecutive injections. The *A. vulgare*  
137 were injected dorsally in their posterior abdominal segment. Each individual was then isolated  
138 in a plastic box (5 cm x 8 cm Ø, Ets Caubere) containing a moist paper, under a natural  
139 photoperiod at 20°C and without food, to avoid any external contamination and variations in  
140 food intake. After each bacterial injection, the injected dose was controlled by spreading 100  
141 µL of the solution diluted to 1 bacteria/µL onto LB agar plates. After an overnight culture at 37  
142 °C, the number of *S. enterica* colonies was counted. All animals remained in their individual  
143 box for either 1, 7 or 15 d until haemolymph analysis (first experiment, see part 2.2.3) or LD<sub>50</sub>  
144 injection for survival monitoring (second experiment, see part 2.2.4).

145

### 146 2.2.3 Haemolymph analysis

#### 147 2.2.3.1 Haemocyte parameters

148 Haemocyte concentration and viability were generally measured on the same individual  
149 (see sample size in Table I). The haemolymph sampling procedure as well as the measure of  
150 the number of immune cells per  $\mu\text{L}$  of haemolymph (i.e. concentration) and the percentage of  
151 living cells (i.e. viability) were performed as described by Sicard et al. (2010). Briefly, each  
152 individual was washed a second time (3% NaClO) then water). The integument was pierced  
153 dorsally on the sixth tergite with a sterile needle, and the resulting drop of haemolymph was  
154 collected with a pipette. Three  $\mu\text{L}$  of haemolymph were diluted in 15  $\mu\text{L}$  of MAS (27 mM  
155 sodium citrate: 336 mM NaCl, 115 mM glucose, 9 mM EDTA, pH 7; Herbinière et al. 2005)  
156 and 6  $\mu\text{L}$  of 0.4 % Trypan blue. Then, 10  $\mu\text{L}$  of each sample were deposited in a counting  
157 chamber and analyzed in an automated cell counter (Countess TM Version B, Invitrogen).

#### 158 2.2.3.2 Evaluation of *S. enterica* persistency

159 To test whether living *S. enterica* persist in the haemolymph of individuals primed with the  
160 bacteria at the three time points tested (1, 7, 15 d), 5 additional  $\mu\text{L}$  of haemolymph collected  
161 from individuals used to measure haemocyte parameters were added to 95  $\mu\text{L}$  of LB medium.  
162 The solutions were plated onto LB agar plates and incubated overnight at 37°C before counting  
163 the number of LB agar plates presenting at least one colony of *S. enterica* for each time point.  
164 *S. enterica* was identified by using MALDI Biotyper® Compass (v4.1 from Bruker Daltonik  
165 GmbH), according to the Bruker manufacturer protocol. This technique is commonly use in  
166 clinical microbiology for strain identification because it is more precise than the morphological  
167 approach (Scott et al., 2016; Kostrzewa, 2018).

168

#### 169 2.2.4. Survival monitoring

170 All individuals (non-primed, LB-primed and *S. enterica*-primed) intended for survival  
171 monitoring were injected with a LD<sub>50</sub> (dosage to kill 50% of animals in 7 days) of  $3.10^5 \pm 1.10^5$   
172 living *S. enterica* in 100 nL (Braquart-Varnier et al., 2015; see sample size in Table I), either 1,  
173 7 or 15 d after the priming procedure. Individuals were returned to their boxes and survival was  
174 monitored every 8 h for 7 d. Humidity was controlled and adjusted daily. The injected dosage  
175 was controlled using the same method as the priming procedure.

176

### 177 2.3 Statistical analysis

178 All statistical analyses were performed with R, version 3.4.1 (R Core Team, 2017).

179 For the haemocyte analysis, we constructed two generalized linear mixed-effect models:  
180 one with concentration and the other with viability as response variables, both built using the  
181 *lme4* package (Fox and Weisberg, 2011) and tested with Wald  $\chi^2$  using the *car* package (Bates  
182 et al., 2015). Haemocyte concentration (number of haemocytes per  $\mu\text{L}$ ) was modeled with a  
183 Poisson error distribution and haemocyte viability (percentage of living cells) was modeled  
184 with a Binomial error distribution. For these two models, the priming treatment (non-primed,  
185 LB-primed and *S. enterica*-primed) and the time point (1, 7, 15 d) were entered as categorical  
186 fixed effects with the interaction between these two terms. In addition, to correct the non-  
187 independence of samples within the same series in a given replicate, we entered two categorical  
188 random factors: the experimental replicates and the series nested within each experimental  
189 replicate (Harrison et al., 2018). For the haemocyte concentration, we added an Observation  
190 Level Random Effect (OLRE). This variable corresponds to a unique level of a random factor  
191 for each data point, in order to cope with over dispersion (Harrison, 2014).

192 For the survival data (frequency of living animals), a global mixed-effects Cox  
193 proportional hazard regression model was built using *coxme* packages (Therneau et al., 2003).  
194 We included the priming treatment, the time point and their interaction as fixed effects and the  
195 experimental replicate as well as the series nested within each replicate as random factors. We  
196 obtained the Hazard Ratios (HR) from this model as an estimate of the ratio between the  
197 instantaneous risks of death between control (non-primed) and the priming treatments (LB-  
198 primed, *S. enterica*-primed).

199 In all statistical models, our focal variable of interest was always the priming treatment.  
200 The fixed effect “time point” was treated as a covariate and the inclusion of the interaction term  
201 between “time point” and “priming treatment” allowed us to compare the three priming  
202 treatments at each time point, independently of the two other time points. These comparisons  
203 were made by analyzing all relevant pairs of means with Tukey adjustment (*lsmeans* package;  
204 Lenth, 2018). Using this approach, the interaction term and the main time point term were  
205 retained in the models even if not significant (Agresti, 2002). However, we did not use these  
206 models to statistically test for the effect of time point on haemocyte parameters and animal  
207 survival because of the correlation between the fixed effect “time point” and the random effect  
208 “experimental replicate”, preventing the correct attribution of variance to each effect (see S2 in  
209 supplementary materials for more details).

## 210 3. Results

### 211 3.1 Haemolymph analysis

#### 212 3.1.1 Variation in haemocyte parameters

213 Priming treatment had an effect on haemocyte concentration ( $\chi^2 = 16.42$ ,  $df = 2$ ,  $p <$   
214  $0.001$ ), but there was no significant effect of time post-priming ( $\chi^2 = 1.19$ ,  $df = 2$ ,  $p = 0.550$ )  
215 nor of the interaction between priming treatment and time post-priming ( $\chi^2 = 4.20$ ,  $df = 4$ ,  $p =$   
216  $0.379$ ). At 1 and 7 d post-priming, the non-primed and LB-primed individuals had more  
217 haemocytes than *S. enterica*-primed ones (1 d:  $p = 0.004$  and  $p = 0.023$ , respectively; 7 d:  $p =$   
218  $0.043$  and  $p = 0.023$ , respectively; Table II, Fig. 2). In contrast, no difference in haemocyte  
219 concentration between these priming treatments was observed at 15 d post priming (non-primed  
220 vs *S. enterica*-primed:  $p = 0.799$ ; LB-primed vs *S. enterica*-primed:  $p = 0.966$ , Table II, Fig. 2).  
221 For all time points, no significant difference in haemocyte concentration was observed between  
222 non-primed and LB-primed individuals ( $p > 0.05$  for all comparisons, Table II, Fig. 2).

223 Priming treatment had an effect on haemocyte viability (percentage of living cells) ( $\chi^2 =$   
224  $139.49$ ,  $df = 2$ ,  $p < 0.001$ ), but there was no significant effect of the time post-priming ( $\chi^2 = 1.50$ ,  
225  $df = 2$ ,  $p = 0.470$ ) nor of the interaction between priming treatment and time, even if a trend  
226 was observed ( $\chi^2 = 8.57$ ,  $df = 4$ ,  $p = 0.072$ ). Whatever the time period after the priming  
227 procedure, haemocyte viability was higher for *S. enterica*-primed individuals than non-primed  
228 individuals ( $p < 0.001$  for all comparisons, Table II, Fig. 3) and LB-primed individuals (1 d:  $p$   
229  $= 0.003$ ; 7 d:  $p < 0.001$ ; 15 d:  $p = 0.016$ , Table II, Fig. 3). Haemocyte viability was not  
230 significantly different between non-primed and LB-primed individuals at 1 d after priming ( $p$   
231  $= 0.20$ , Table II, Fig. 3), whereas non-primed individuals had a lower haemocyte viability  
232 compared to LB-primed one's at 7 d ( $p = 0.008$ , Table II, Fig. 3) and 15 d after the priming  
233 procedure ( $p < 0.001$ , Table II, Fig. 3).

234

#### 235 3.1.2 Presence of living *S. enterica* in haemolymph

236 One day after the priming injections, 91% (20 out of 22 plates) of the LB agar plates  
237 spread with haemolymph from *S. enterica*-primed individuals contained *S. enterica* colonies.  
238 At 7 d, 100% displayed colonies (10 plates) whereas only 59% (10 out of 17 plates) of the plates  
239 presented colonies 15 d after priming. This shows that *S. enterica* was still present in the  
240 haemolymph of some individuals when we injected the  $LD_{50}$  ( $3.10^5 \pm 1.10^5$  living *S. enterica*).  
241 No *S. enterica* was detected in the haemolymph from non-primed and LB-primed individuals.

### 242 **3.2 Survival rate in response to a LD<sub>50</sub> injection of *S. enterica***

243 Priming treatment had an effect on the survival rate ( $\chi^2 = 18.88$ ,  $df = 2$ ,  $p < 0.001$ , Fig.  
244 4) as well as the interaction between priming treatment and time post-priming ( $\chi^2 = 15.12$ ,  $df =$   
245 4,  $p = 0.004$ ). Therefore, the effect of the priming treatment depends on the time post priming.  
246 No effect of the time point factor alone was observed on the survival rate ( $\chi^2 = 2.58$ ,  $df = 2$ ,  $p =$   
247 0.274).

248 When the LD<sub>50</sub> injection occurred 1 d after the priming procedure, *S. enterica*-primed  
249 individuals did not survive significantly better than non-primed individuals ( $p = 0.411$ , Table  
250 III, Fig. 4A). LB-primed individuals displayed a significantly higher survival rate than non-  
251 primed ones ( $p = 0.001$ , Table III, Fig. 4A) and *S. enterica*-primed individuals ( $p = 0.037$ , Table  
252 III, Fig. 4A). The risks of death of LB-primed and *S. enterica*-primed individuals were 78%  
253 (HR = 0.22; 95% CI = [0.094 - 0.52]) and 33% (HR = 0.67; 95% CI = [0.35 - 1.26]) lower than  
254 that of non-primed controls.

255 When the LD<sub>50</sub> injection was performed 7 d after the priming procedure, survival rate  
256 was higher for *S. enterica*-primed individuals than LB-primed and non-primed ones ( $p = 0.003$   
257 and  $p = 0.0001$  respectively, Table III, Fig. 4B). No significant difference between LB-primed  
258 and non-primed individuals was observed ( $p = 0.792$ , Table III, Fig. 4B). The risk of death of  
259 *S. enterica*-primed individuals was 52% lower than that of non-primed controls (HR = 0.48;  
260 95% CI = [0.27 - 0.84]), whereas that of LB-primed ones was 25% lower than that of non-  
261 primed individuals (HR = 0.75; 95% CI = [0.44 - 1.27]). At 15 d after priming procedure, no  
262 significant differences were observed between the three priming treatments ( $p > 0.05$  for all  
263 comparisons, Table III, Fig. 4C). Nevertheless, *S. enterica*-primed individuals tended to have a  
264 higher survival rate than non-primed individuals ( $p = 0.095$ , Table III, Fig. 4C), with a risk of  
265 death 62% lower than that of non-primed (HR = 0.38; 95% CI = [0.17 - 0.83]). The  
266 instantaneous risk of death of LB-primed individuals was 34% lower than that of non-primed  
267 controls (HR = 0.66; 95% CI = [0.34 - 1.26]).

268

## 269 **4. Discussion**

### 270 **4.1 Main results overview**

271 Immune priming in invertebrates is defined as a phenomenon by which a first activation  
272 of the immune system (by killed/living pathogens or inactivated pathogen units) is associated



273 with enhanced protection of the host against subsequent exposure to pathogens (Kurtz, 2005;  
274 Gourbal et al., 2018). In this study, we tested for enhanced protection in *Armadillidium vulgare*  
275 following a subsequent inoculation of the Gram-negative bacterium *Salmonella enterica*.

276 Our results demonstrated the existence of a protective effect in this species because *A.*  
277 *vulgare* survival after a sublethal infection with *S. enterica* was higher when its immune system  
278 was activated during inoculation with the same bacteria. This protection is dynamic: immune  
279 primed individuals showed a higher survival rate than control individuals when the second  
280 infection occurred 7 d after a first infection with the same bacterial strain. There was a trend  
281 towards the persistence of this protection 15 d after the first infection (p-value = 0.09, Table III,  
282 Fig.2), but this protection was not effective when the LD<sub>50</sub> injection was performed only 1 d  
283 after priming. We observed fluctuations in haemocyte concentration and viability, which also  
284 highlight a related dynamic immune response differing at each time following the infection.

285

#### 286 ***4.2. When the first bacterial infection is too recent to trigger an immune protection, a*** 287 ***simple stimulation without bacteria is enough***

288 Firstly, we observed that a first activation of the immune system with Luria Bertani  
289 broth resulted in higher survival of an injection of bacteria at the LD<sub>50</sub>. In fact, LB-primed  
290 individuals survival rate was about twice that of non-primed ones when the LD<sub>50</sub> injection  
291 occurred 1 d after the LB-injection. Higher survival is likely the result of a protection  
292 phenomenon termed as “immune enhancement” by Contreras-Guarduño et al. (2016).

293 Because LB-primed individuals were not primed with the pathogen, we hypothesized  
294 that an immune response was triggered by the piercing in the integument during the priming  
295 procedure (Cerenius and Söderhäll, 2011). When the lethal dosage of *S. enterica* arrived in the  
296 haemolymph 1 d after the LB broth injection, the bacteria could face an already “ready-to-fight”  
297 immune system, leading to better protection (by resistance and/or tolerance) and to a higher  
298 survival rate. As haemocyte concentration is known to rapidly decrease following an injury in  
299 crustaceans (Söderhäll et al., 2003, 2016), we might expect to observe fewer haemocytes in LB-  
300 primed individuals compared to non-primed ones at 1 d post-priming. But LB-primed  
301 individuals had haemocyte concentration and viability similar to non-primed individuals. This  
302 suggests a highly effective immune system having already renewed haemocytes after the injury  
303 of the cuticle (Söderhäll et al., 2003, 2016).

304 Furthermore, we observed an intermediate survival rate in *S. enterica*-primed  
305 individuals at 1 d. As the immune system of *S. enterica*-primed individuals should be activated  
306 as in LB-primed, the absence of immune protection compared to non-primed individuals could  
307 reflect either a saturation or a depletion event of *A. vulgare* immune system facing a high  
308 number of bacteria. Braquart-Varnier et al. (2015) showed that 24 hours post-injection with  $10^4$   
309 living *S. enterica*, the bacterial load in the haemolymph was higher than the number of bacteria  
310 injected. The immune system of *S. enterica*-primed individuals might not have enough time,  
311 energy or reserve of immune effectors to cope with a new infection of *S. enterica* occurring too  
312 soon after the first infection. This hypothesis is supported by the lower haemocyte concentration  
313 observed in *S. enterica*-primed individuals, suggesting the immune cellular response was still  
314 fighting bacteria persisting in the haemolymph since the priming procedure. The loss of  
315 circulating haemocytes following an infection was previously observed in *A. vulgare* exposed  
316 to heat-killed *Escherichia coli* (Herbinière, 2005) and in other crustaceans (Persson et al., 1987;  
317 Muñoz et al., 2002; Söderhäll et al., 2003). Following this loss of immune cells, new  
318 haemocytes are found to be released rapidly in the haemolymph to compensate for the loss  
319 (Söderhäll et al., 2003; Chevalier et al., 2011). We hypothesize the high viability of haemocytes  
320 in *S. enterica*-primed individuals reflects an important hematopoietic activity, but new  
321 haemocytes might be directly used in protection against bacteria without totally compensating  
322 previous cell loss, both at 1 and 7 d after the priming procedure. However, haemocyte  
323 concentration of *S. enterica*-primed individuals became similar to those of other groups at 15 d  
324 post-priming. This suggests the compensation was effective between 7 and 15 d following the  
325 first infection, either by even greater haemocyte production (Pipe and Coles, 1995; Zhang et  
326 al., 2014; Söderhäll, 2016) or a smaller loss of new haemocytes.

#### 327 ***4.3 Evidence of a protective effect against the same pathogen: a minimal delay*** 328 ***required between successive infections***

329 We demonstrated increased survival when *A. vulgare* had encountered *S. enterica* 7 d  
330 before receiving a LD<sub>50</sub> of the same pathogen, with boosted survival rates – approximately 50%  
331 higher than non-primed individuals. This protection tended to be effective until 15 d post-  
332 priming procedure (30% increase in survival), which is consistent with previous studies  
333 suggesting immune priming in terrestrial and aquatic crustaceans may last for 7 to 30 d (Roth  
334 et al., 2009; Chang et al., 2018).

335           Because the improved survival for LB-primed individuals observed at 1 d disappeared  
336 for individuals treated at 7 d and 15 d, we suppose the immune enhancement against *S. enterica*  
337 induced by the piercing disappeared between 1 day and 7 d post-priming procedure. By contrast,  
338 the first infection with *S. enterica* protected individuals from the LD<sub>50</sub> infection, supporting that  
339 the protective effect is established between 1 and 7 d following the first infection. With regards  
340 to the duration of the protective effect, we cannot determine whether the trend of better  
341 protection observed 15 d after the priming procedure is related to a decrease in the protective  
342 effect over time or an analytical limit. This absence of significant differences between *S.*  
343 *enterica*-primed and non-primed individuals at 15 d could also be influenced by the animal  
344 physiological states. Individuals displayed different levels of nutritional stress at 1, 7 or 15 d  
345 post-priming, which may affect the resistance/tolerance ability of animals facing a second  
346 infection (Siva-Jothy and Thompson, 2002; Akoda et al., 2009; Ayres & Schneider 2009;  
347 Ponton et al., 2013; Adamo, 2017).

348           Interestingly, we showed that living *S. enterica* bacteria from the first injection were  
349 still present in haemolymph of *S. enterica*-primed individuals until 15 d post-priming  
350 procedure. While we would expect to observe a higher mortality in such individuals because  
351 they should be weak due to the constant fight against bacteria from the first infection, *A. vulgare*  
352 still presented higher survival rates when receiving a LD<sub>50</sub> of bacteria 7 (and potentially 15) d  
353 later. We thus highlight that the enhanced protection against a second infection with *S. enterica*  
354 in *A. vulgare* is possible despite the lack of clearance of the same pathogenic bacteria in the  
355 haemolymph. This species is thus able to better survive inoculation with a large quantity of  
356 living pathogen, provided the successive infections are separated enough in time. Since the  
357 natural habitat of *A. vulgare* is highly contaminated by several species of bacteria (Ranjard and  
358 Richaume, 2001; Karimi et al., 2018), it would not be surprising that during the immune system  
359 evolution, *A. vulgare* developed strong resistance and/or tolerance to repeated pathogenic  
360 infections. Most of the studies focusing on immune priming used inactivated pathogens or  
361 purified molecules to trigger this phenomenon (Milutinovic and Kurtz, 2016; Chang et al.,  
362 2018), but the use of living pathogens seems more appropriate to describe the immunological  
363 capacities occurring under natural conditions.

364

#### 365           **4.4 Going inside the possible underlying mechanism(s)**

366 Three main mechanisms are currently proposed to explain the protection conferred by  
367 immune priming (i) the “immune memory”, a recalled biphasic immune response implying a  
368 specific pathogen recognition, (ii) the “immune shift” consisting in a shift from one immune  
369 effector to another between successive infections and (iii) the “sustained immune boost”, an  
370 up-regulated and long lasting immune response lasting from the first to the second infection  
371 events (Contreras-Garduño et al., 2015; Coustau et al., 2016; Pinaud et al., 2016; Greenwood  
372 et al., 2017; Lanz-Medosa and Garduño, 2018; Gourbal et al., 2018; Melillo et al., 2018; Moret  
373 et al., 2019). In this study, as *S. enterica* persists in the haemolymph until 15 d after the first  
374 sublethal infection, the more plausible mechanism explaining such protection could be a  
375 sustained immune boost in *A. vulgare* individuals primed with the bacteria. As an example, the  
376 butterflies *Parasemia plantaginis* and *Galleria mellonella* display a sustained immune response  
377 to some pathogens, conferring a protective effect and with highly maintained ROS levels and  
378 haemocyte density following a first exposure (Mikonranta et al., 2014; Wu et al., 2014).  
379 Sustained immune response in our study was illustrated by the high haemocyte viability at any  
380 time for *S. enterica*-primed individuals. However, this high viability of haemocytes could also  
381 reveal the production and/or differentiation of a competent cell subpopulation (Sokolova et al.,  
382 2004; Snyman and Odendaal, 2009). As stated by Gourbal et al. (2018), “insects are able to  
383 select and activate a competent subpopulation of cells with enhanced capacities of lysis or  
384 phagocytosis”. For example, Rodrigues et al. (2010) demonstrated that the immune priming  
385 protection in mosquitoes against *Plasmodium* is not linked to a variation in haemocyte  
386 concentration but to an increased proportion of circulating granulocytes. Similarly, the survival  
387 improvement of *S. enterica*-primed individuals could be due to the selection and/or production  
388 of a more efficient subset of haemocytes, resulting in a protective effect against the second  
389 infection of *S. enterica*. We noticed that LB-primed individuals also displayed higher  
390 haemocyte viability than non-primed ones at 7 and 15 d post-priming, suggesting LB broth  
391 injection could also induce a cellular immune response (e.g Markus et al., 2005).

392 The protective effect of immune priming in invertebrates is not based on a universal  
393 system but mobilizes a diversity of immune effectors (Brehélin and Roch, 2008; Pradeu and  
394 Pasquier, 2018, Melillo et al., 2018). We thus assume that haemocyte concentration and  
395 viability in *A. vulgare* cannot fully explain the increase in survival. The next step to further  
396 investigate the immune priming mechanism would be to analyze the dynamics of other immune  
397 parameters, for example, haemocyte types, ROS and AMP production. We also suggest  
398 studying the immune response over a longer period of time and testing the specificity of this

399 response against different pathogenic species. In addition, inactivated pathogens could be used  
400 for the priming injection in order to determine the end of the first immune response if there is  
401 one (Milutinovic and Kurtz, 2016).

#### 402 ***4.5 Concluding remarks***

403 Ours results contribute to the understanding of immune priming, by providing additional  
404 details about the protective effect (in terms of survival) and the haemocyte responses in another  
405 example of a long-lived non-model arthropod. Since cell proliferation is not often monitored  
406 during the priming response (Contreras-Guarduño et al. 2016), this work also constitutes a  
407 baseline for further studies on cellular responses and the temporal dynamics of immune priming  
408 in invertebrates.

#### 409 **Acknowledgements**

410 We thank Maryline Raimond and Clémentine Bernier for their help in the laboratory and  
411 Charlotte Depeux for discussions on the subject.

#### 412 **Declaration of interest**

413 None

#### 414 **Funding**

415 This work was supported by the grant Vivipary and Immune Priming (PEPS EXOMOD  
416 CNRS); the State-Region Planning Contracts (CPER); the European Regional Development  
417 Fund (FEDER); the French Centre National de la Recherche Scientifique; the French Ministère  
418 de l'Enseignement Supérieur et de la Recherche and the University of Poitiers.

419 **Table I**

420 Sample size details of *A. vulgare* females used for haemocyte parameters and survival  
 421 monitoring, for each priming treatment (Non-primed: non-injected; LB-primed: injected with  
 422 LB; *S. enterica*-primed: injected with  $10^3$  *S. enterica* during the priming procedure) and each  
 423 time point after priming procedure. For the haemocyte concentration and viability,  
 424 measurements were mostly made on same individuals. As a result, sample sizes were the same  
 425 for the haemocyte concentration and viability, except for sample sizes with exponent letter. <sup>a</sup>:  
 426 First number is haemocyte concentration sample size; second (in parentheses) is haemocyte  
 427 viability sample size.

<b>HAEMOCYTE PARAMETERS</b>				
Time points (days)	Non-primed	LB-primed	<i>S. enterica</i> -primed	Total animals per time point
1	15 <sup>a</sup> (17)	18 <sup>b</sup>	19 <sup>a</sup> (14)	52 (49)
7	17	16	15	48
15	19	14	12 <sup>a</sup> (14)	45 (47)
<b>SURVIVAL MONITORING</b>				
Time points (days)	Non-primed	LB-primed	<i>S. enterica</i> -primed	Total animals per time point
1	35	35	36	106
7	59	58	57	174
15	55	54	50	159

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433 **Table II**

434 Pairwise comparisons (Tukey adjustment) of haemocyte parameters for the priming treatments  
 435 (Non-primed: non-injected; LB-primed: injected with LB; *S. enterica*-primed: injected with  $10^3$   
 436 *S. enterica* during priming procedure) at time points between priming and haemolymph  
 437 sampling, 1, 7 and 15 d.

<b>HAEMOCYTE CONCENTRATION (number of cells per <math>\mu</math>L of haemolymph)</b>			
<b>Days</b>	estimate	std error	<i>p</i> -value
<b>1</b>			
<i>S. enterica</i> -primed / Non-primed	0.600	0.188	<b>0.004**</b>
<i>S. enterica</i> -primed / LB-primed	0.471	0.179	<b>0.023*</b>
LB-primed / Non-primed	0.128	0.191	0.778
<b>7</b>			
<i>S. enterica</i> -primed / Non-primed	0.470	0.193	<b>0.043*</b>
<i>S. enterica</i> -primed / LB-primed	0.515	0.196	<b>0.023*</b>
LB-primed / Non-primed	-0.045	0.190	0.968
<b>15</b>			
<i>S. enterica</i> -primed / Non-primed	0.128	0.201	0.799
<i>S. enterica</i> -primed / LB-primed	0.053	0.215	0.966
LB-primed / Non-primed	0.075	0.192	0.919
<b>HAEMOCYTE VIABILITY (frequency of living cells)</b>			
<b>Days</b>	estimate	std error	<i>p</i> -value
<b>1</b>			
<i>S. enterica</i> -primed / Non-primed	-0.269	0.056	<b>&lt; 0.001***</b>
<i>S. enterica</i> -primed / LB-primed	-0.177	0.054	<b>0.003**</b>
LB-primed / Non-primed	-0.091	0.054	0.207
<b>7</b>			
<i>S. enterica</i> -primed / Non-primed	-0.434	0.055	<b>&lt; 0.001***</b>
<i>S. enterica</i> -primed / LB-primed	-0.267	0.054	<b>&lt; 0.001***</b>
LB-primed / Non-primed	-0.167	0.056	<b>0.008**</b>
<b>15</b>			
<i>S. enterica</i> -primed / Non-primed	-0.427	0.055	<b>&lt; 0.001***</b>

438	<i>S. enterica</i> -primed / LB-primed	-0.158	0.057	<b>0.016**</b>
439	LB-primed / Non-primed	-0.269	0.057	<b>&lt;0.001***</b>

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440 Values where  $p \leq 0.05$  are given in bold. Stars indicate statistical differences between priming  
441 treatments with \* =  $0.05 < p < 0.06$ , \*\* =  $p \leq 0.05$ ,  $p \leq 0.01$ , \*\*\* =  $p \leq 0.001$ .

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444 **Table III**

445 Pairwise comparisons (Tukey adjustment) of survival rate for the priming treatments (Non-  
 446 primed: non-injected; LB-primed: injected with LB; *S. enterica*-primed: injected with  $10^3$  *S.*  
 447 *enterica* during the priming procedure) under the time points (time elapsed between priming  
 448 procedure and LD<sub>50</sub> injection.

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452 **SURVIVAL RATE (frequency of living animals)**

453	Days	estimate	std error	<i>p-value</i>
454	<b>1</b>			
454	<i>S. enterica</i> -primed / Non-primed	0.412	0.324	0.411
455	<i>S. enterica</i> -primed / LB-primed	-1.103	0.450	<b>0.037*</b>
456	LB-primed / Non-primed	1.515	0.435	<b>0.001**</b>
457	<b>7</b>			
457	<i>S. enterica</i> -primed / Non-primed	1.208	0.296	<b>&lt; 0.001***</b>
458	<i>S. enterica</i> -primed / LB-primed	1.030	0.316	<b>0.003**</b>
459	LB-primed / Non-primed	0.178	0.274	0.792
460	<b>15</b>			
461	<i>S. enterica</i> -primed / Non-primed	0.858	0.413	0.095
462	<i>S. enterica</i> -primed / LB-primed	0.488	0.430	0.492
462	LB-primed / Non-primed	0.369	0.332	0.506

463 Values where  $p \leq 0.05$  are given in bold. Stars indicate statistical differences between priming  
 464 treatments with \*\* =  $p \leq 0.01$  and \*\*\* =  $p \leq 0.001$ .

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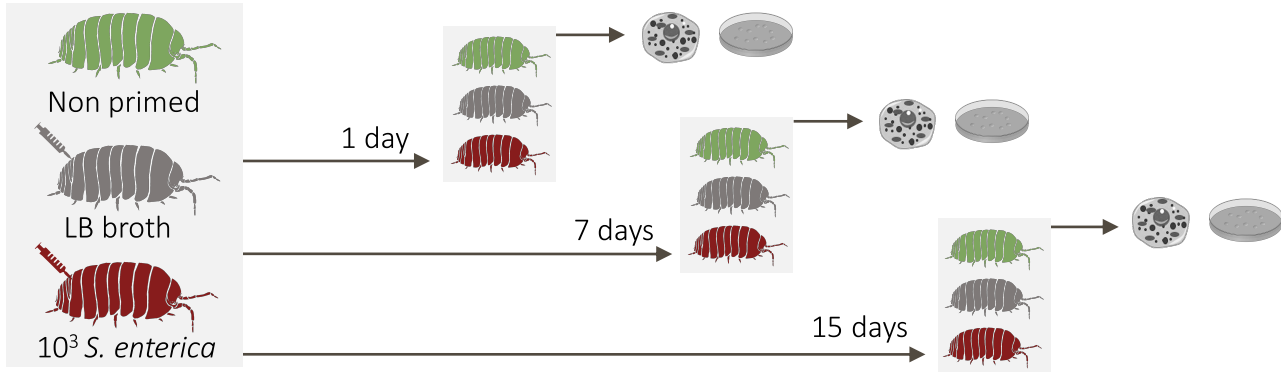
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# 1<sup>st</sup> EXPERIMENT : HAEMOLYMPH ANALYSIS

Priming procedure

Sampling (haemocyte concentration, viability  - bacterial presence )



# 2<sup>nd</sup> EXPERIMENT : SURVIVAL MONITORING

Priming procedure

LD<sub>50</sub> injection ( $3 \cdot 10^5 \pm 1 \cdot 10^5 S. enterica$  ) and survival monitoring ()

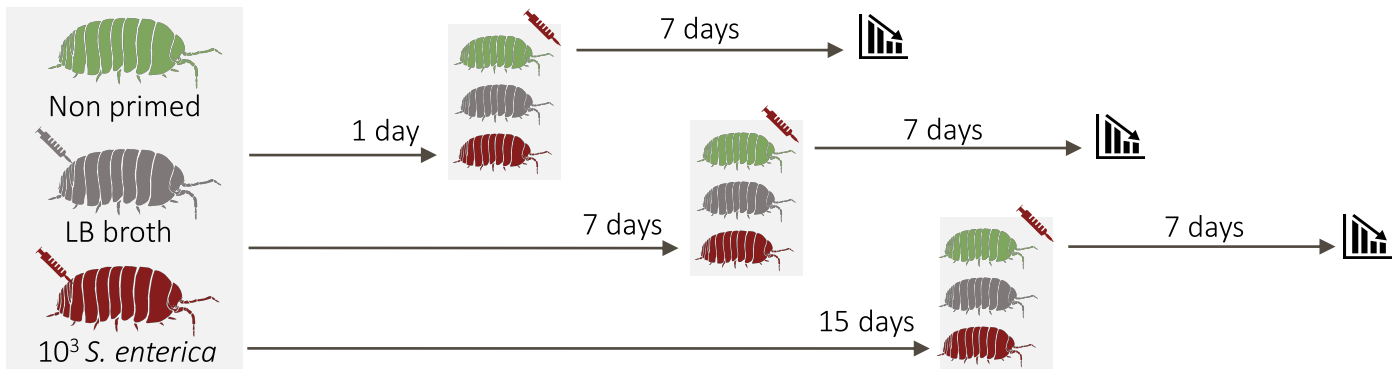


Fig. 2

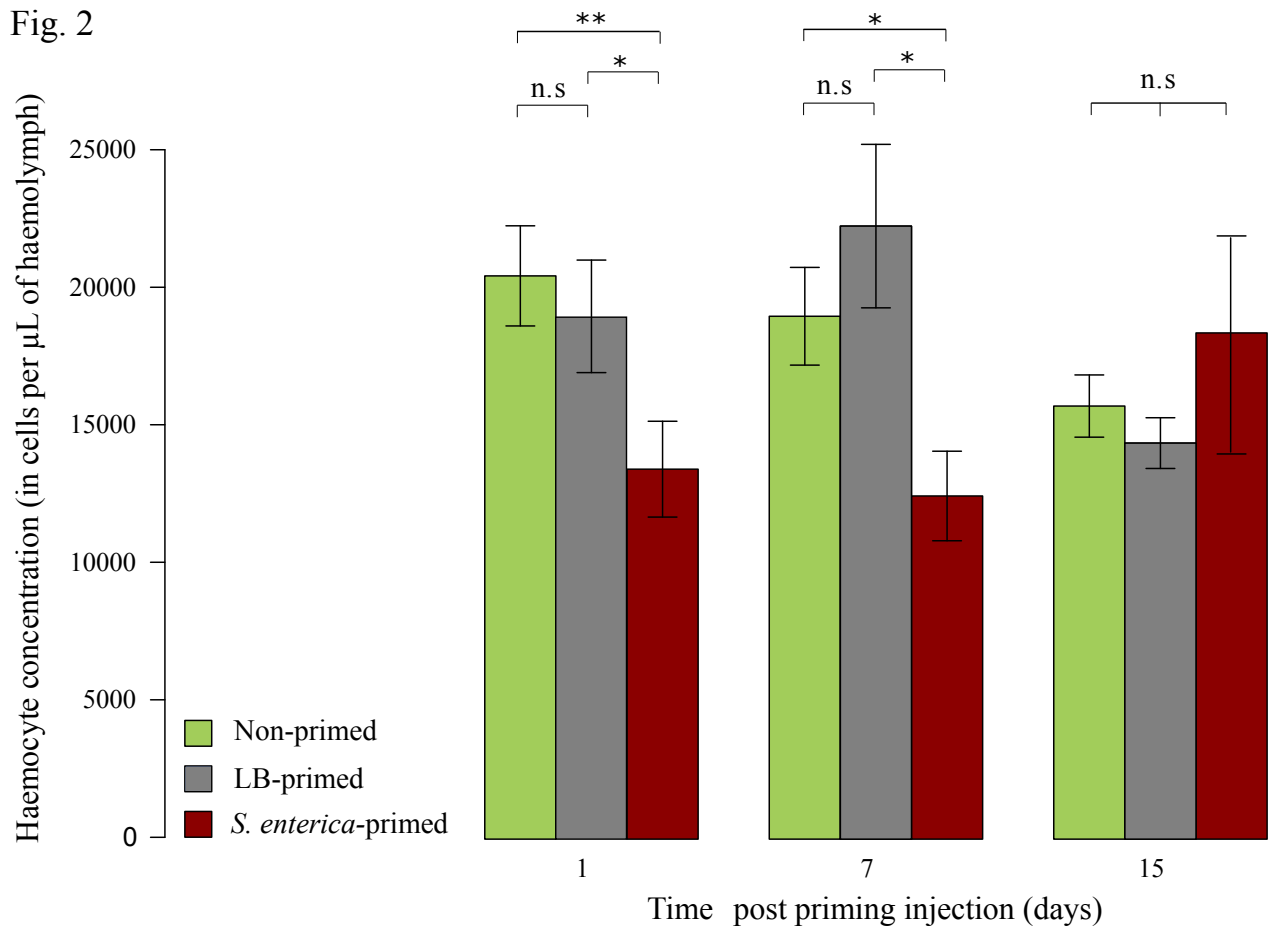


Fig. 3

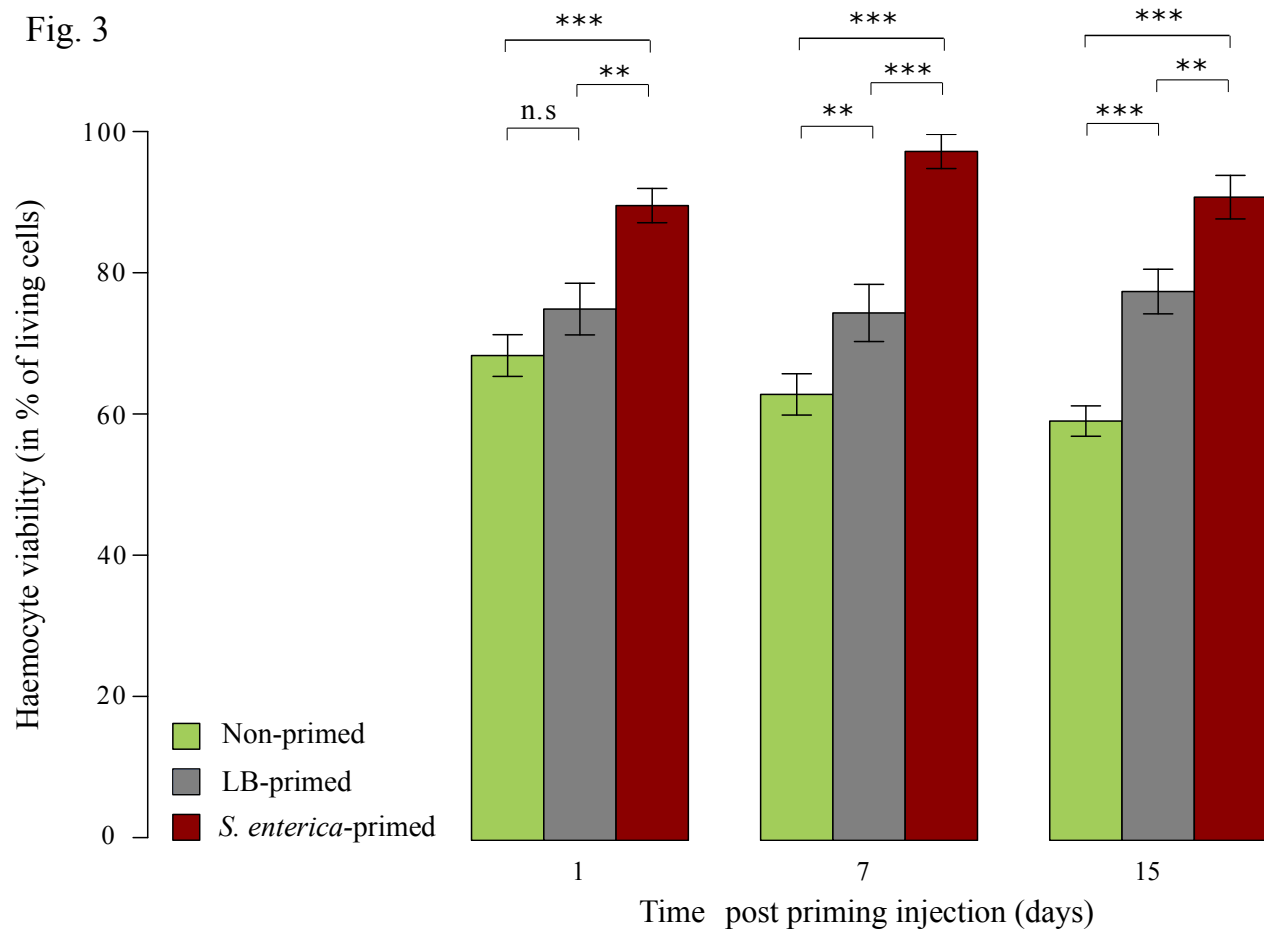
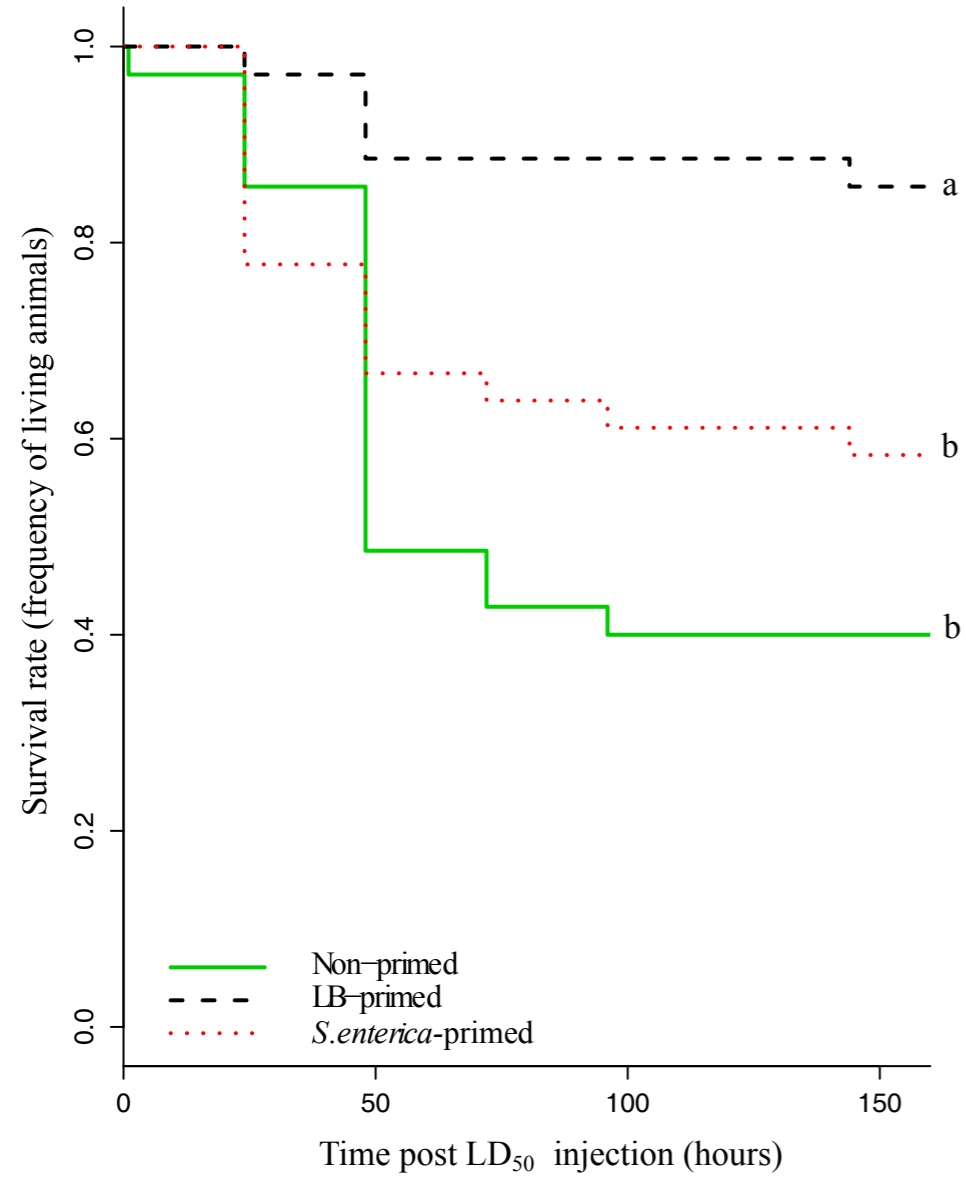
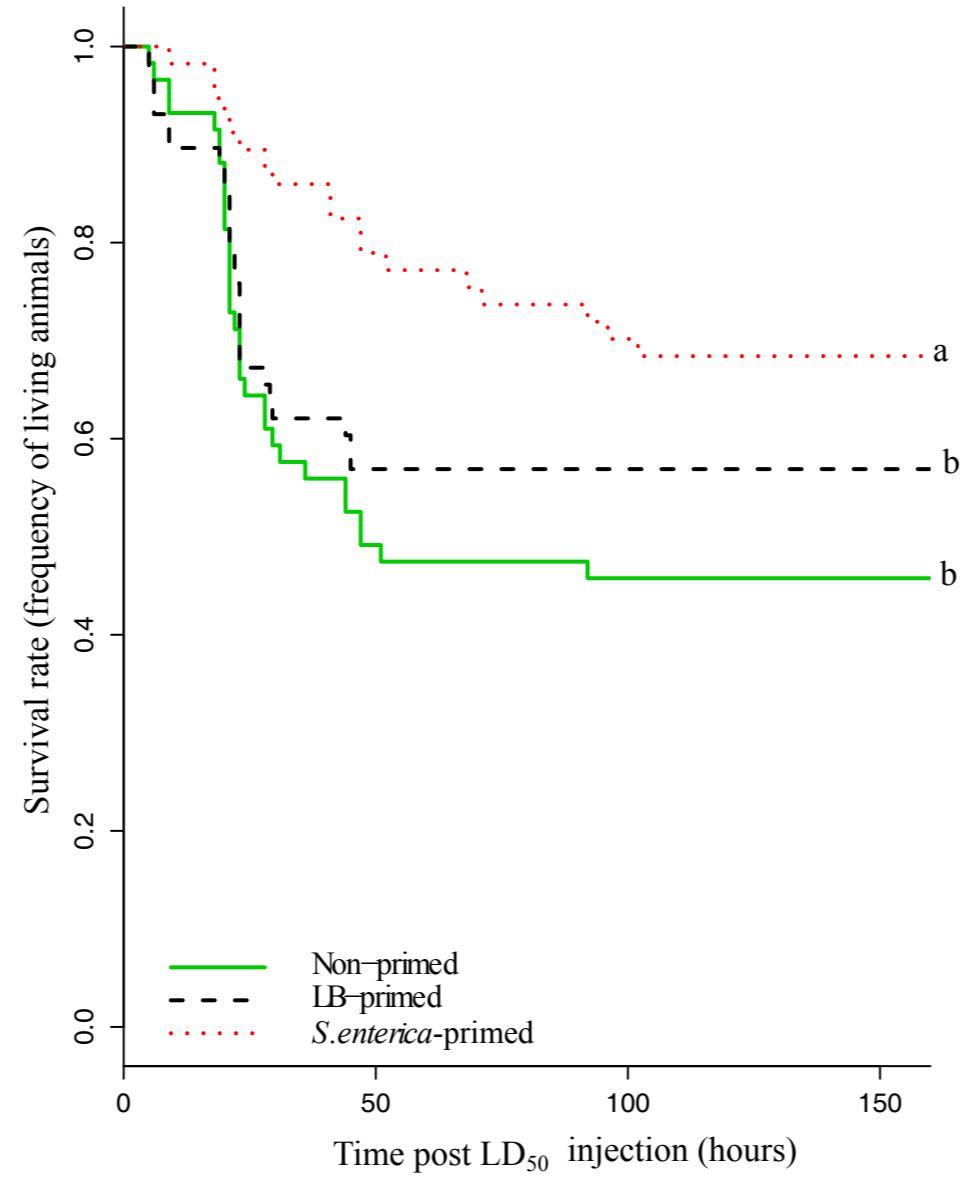


Fig.4

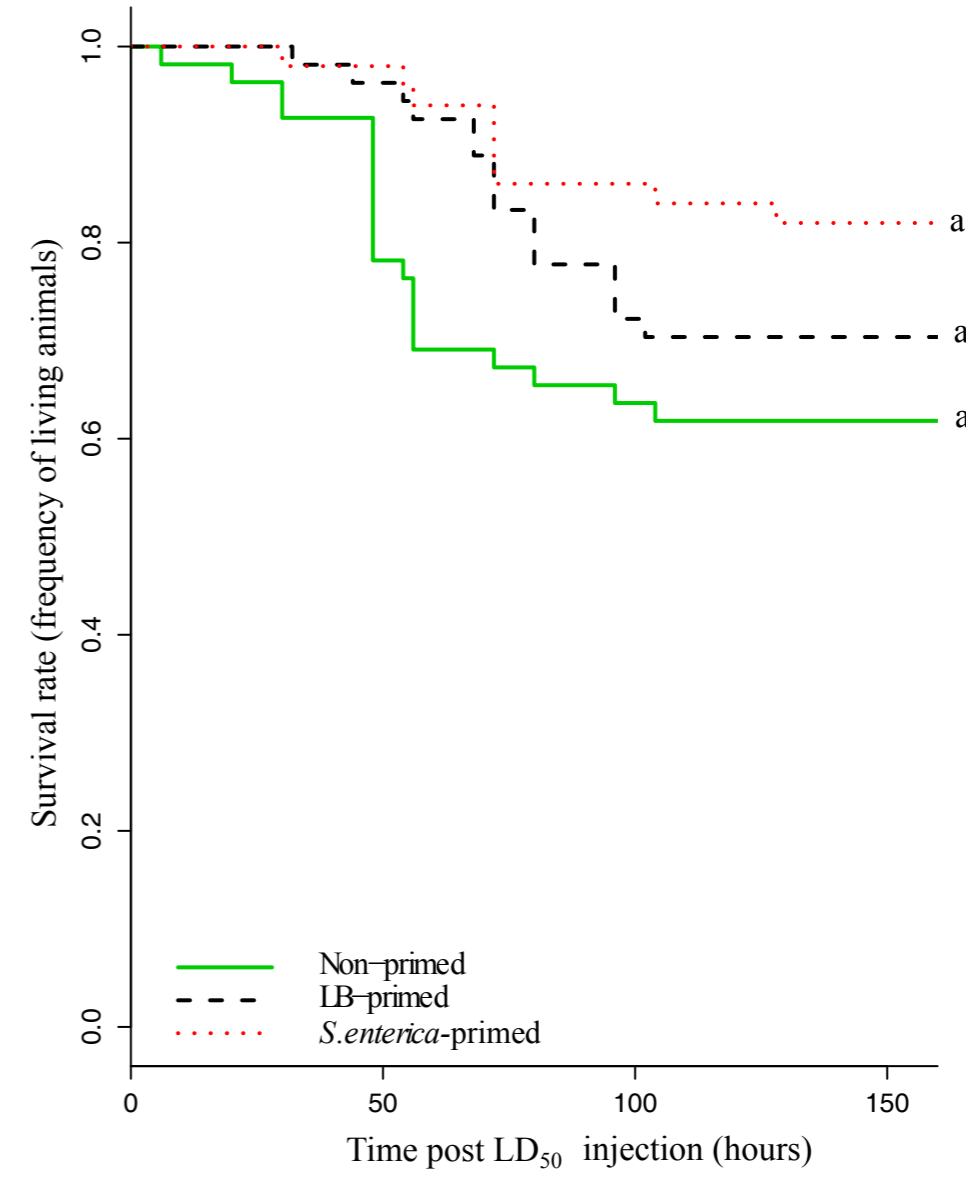
(A) 1D



(B) 7D



(C) 15D



## Figure captions

### Fig. 1

Experimental diagram. The first experiment consisted to prime animals either with a low dose of living *S. enterica*, LB broth medium or without injection (control). 1, 7 or 15 days later, we sampled haemolymph to account haemocytes (concentration, viability) and estimate the bacterial persistency. The second experiment was based on the same priming procedure, but 1, 7 or 15 days later, we injected a LD<sub>50</sub> of living *S. enterica* in all animals. Survival rates were then monitored for 7 days. These two types of experiments were divided into several independent experimental replicates including several independent series of priming injections.

### Fig. 2

Haemocyte concentration of *A. vulgare* females for each time point after the priming procedure (1 d, 7 d, 15 d). Non-primed: non injected; LB-primed: injected with LB; *S. enterica*-primed: injected with 10<sup>3</sup> living *S. enterica* during the priming procedure. Values correspond to the mean ± SE and stars indicate statistical differences between priming treatments (n.s:  $p > 0.05$ , \*:  $p \leq 0.05$ , \*\*:  $p \leq 0.01$ , \*\*\*:  $p \leq 0.001$ ).

### Fig. 3

Haemocyte viability of *A. vulgare* females for each time point after the priming procedure (1 d, 7 d, 15 d). Non-primed: non injected; LB-primed: injected with LB; *S. enterica*-primed: injected with 10<sup>3</sup> living *S. enterica* during the priming procedure. Values correspond to the mean ± SE and stars indicate statistical differences between priming treatments (n.s:  $p > 0.05$ , \*:  $p \leq 0.05$ , \*\*:  $p \leq 0.01$ , \*\*\*:  $p \leq 0.001$ ).

### Fig. 4

Survival rate (%) of *A. vulgare* females following the lethal injection of *S. enterica* occurring (A) 1 day, (B) 7 days or (C) 15 days after the priming procedure. Non-primed: non injected; LB-primed: injected with LB; *S. enterica*-primed: injected with 10<sup>3</sup> living *S. enterica* during the priming procedure. Different letters beside curves indicate significant differences between the survival curves based on Tukey's range test ( $p \leq 0.05$ ).

1 **Survival capacity of the common woodlouse *Armadillidium vulgare* is**  
2 **improved with a second infection of *Salmonella enterica***

3 Prigot-Maurice Cybèle, Cerqueira de Araujo Alexandra, Durand Sylvine, Laverré Tiffany,  
4 Pigeault Romain, Verdon Julien, Bulet Philippe, Beltran-Bech Sophie, Braquart-Varnier  
5 Christine

6  
7 **SUPPLEMENTARY MATERIALS**

8 **S1: detailed description of the bacterial culture methods**

9 *Salmonella enterica* is a non-spore-forming facultative aerobic Gram-negative strain  
10 [1]. *S. enterica* serovar *Typhimurium* J18 [2] cultures were performed either in Luria-Bertani  
11 (LB) broth composed of 25 g.L<sup>-1</sup> of LB base (Invitrogen 12795-027), or on LB agar plates (LB  
12 broth supplemented with 15 g.L<sup>-1</sup> of agar-agar, Fisher BioReagents™ BP1423-2). All *inocula*  
13 used for injections came from a same frozen glycerol stock streaked on LB agar plate incubated  
14 at 37 °C overnight. Before each injection experiment, the bacterial solution was prepared as  
15 described in Braquart-Varnier et al. (2015) [3]. Briefly, one colony of *S. enterica* was cultured  
16 overnight in 5 mL of LB broth at 37 °C, 180 rpm. The next day, 100 µL of this culture were  
17 added to 3 mL of fresh LB broth and incubated at 37 °C, 180 rpm to reach a 0.7 optical density  
18 (600 nm), which corresponds to a concentration around 3.10<sup>5</sup> bacteria/µL. After centrifugation  
19 of 1 mL of this solution (2 min, 4 °C, 13.000 g), the bacterial pellet was resuspended in 300 µL  
20 of fresh LB broth, to obtain a concentration of 10<sup>6</sup> bacteria/µL (the lethal dose being around 10<sup>5</sup>  
21 bacteria/100 nL). Then we performed a serial dilution, to obtain the concentration for the  
22 priming injection (10<sup>3</sup> bacteria/100 nL) and a sufficient low concentration of *S. enterica* (1  
23 bacteria/µL) to control the quantity of bacteria injected. To do so, we spread 100 µL of the

24 diluted solution onto a LB agar plate. After an overnight culture at 37°C, we counted the number  
25 of colonies, expected to be around 100.

## 26 **Supplementary materials references**

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## S2: Experimental design

49 Two experiments were design (haemolymph analysis and survival monitoring; see  
50 diagram below). Each experiment was replicated in several independent experimental replicates  
51 (Bloc 1, Bloc 2, Bloc 3, Bloc 4 etc.) themselves divided into independent experimental series  
52 (S1, S2, S3, S4 etc.) Each series correspond to a single priming procedure performed on the  
53 three treatments (non-primed, LB-primed and *S. enterica*-primed) for one time point (1 d, 7 d  
54 or 15 d, the time elapsed between priming procedure and sampling or second infection). For the  
55 haemolymph analysis, all time points are distributed equally in a same bloc. For the survival  
56 monitoring, each bloc corresponds to a single replicated time point, preventing to distinguish  
57 correctly the effect of “bloc” to the effect of “time point” in our statistical models. Because of  
58 the correlation between blocs and time points, we added and kept the “time point” factor as  
59 fixed effect, in order to adjust the attributed part of the variance and separate the comparisons  
60 of priming treatments per time point (1 d, 7 d or 15 d). We also corrected the non-independence  
61 of the samples within a same series by adding a random factor “series” nested in “blocs”.

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