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

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

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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.

Keywords	Immune priming; Haemocytes; Invertebrates; Crustaceans; Armadillidium vulgare; Salmonella.
Taxonomy	Crustacean Disease, Salmonella, Animal Immunology, Cell-Mediated Immunity

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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 confered by immune priming in A. vulgare against S. enterica by determining its setting up and duration. We also analysed haemocyte variations in order to linked them with a potential protection against the bacterial infection. For these purposes, we conducted two distinct experiments with several independant experimental replicates: The first consisted to primed (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). But 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. Datas of these experiments are available in the file "Prigot_DataSurvivals" in the open access repository Mendeley Data. Differents animals were use for the two experiments. You can also find enclosed a .txt file (Prigot_DataLegends) giving all legends of the datasets colomn 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 7th 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

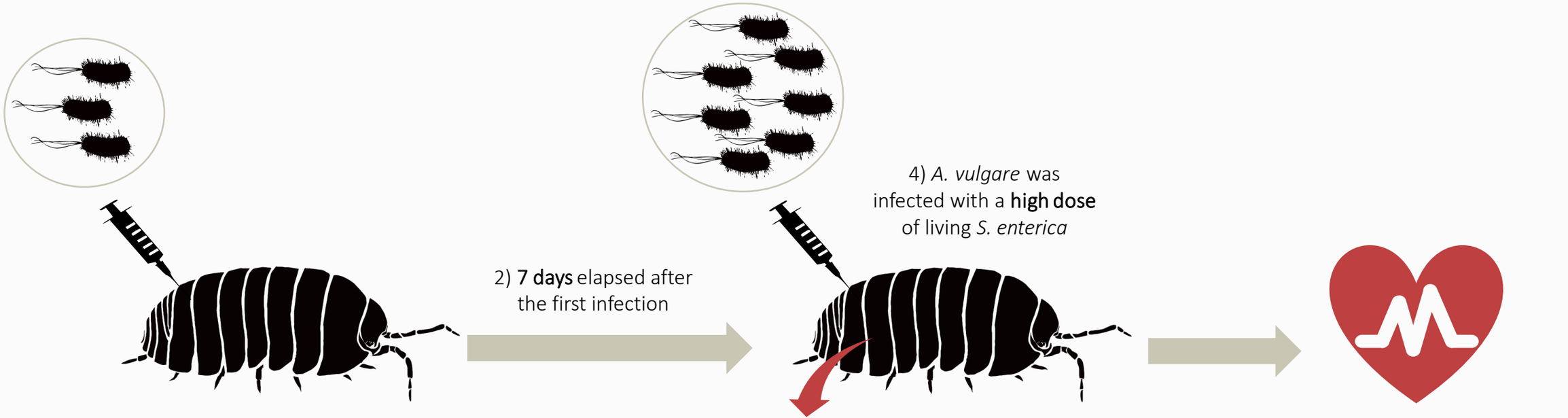
Immune priming with *Salmonella enterica* improved survival of *Armadillidium vulgare*

Survival of *A. vulgare* to an LD_{50} 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) A. vulgare immune sytem was primed with a **low dose** of living *S. enterica*

2) **7 days** elapsed after the first infection

3) Haemocytes of *A. vulgare* present a **better viability** but a **lower concentration**

4) *A. vulgare* was infected with a **high dose** of living *S. enterica*

5) Primed *A. vulgare* displayed **better survival rate** compared to non primed animals

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

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1. Introduction

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

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

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

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

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

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

2. Materials and methods

2.1 Biological model

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

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

2.2 Experiments

2.2.1 Experimental design

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

2.2.2 Priming procedure

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

2.2.3 Haemolymph analysis

2.2.3.1 Haemocyte parameters

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

2.2.3.2 Evaluation of S. enterica *persistency*

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

2.2.4. Survival monitoring

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

2.3 Statistical analysis

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

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

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

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

3. Results

3.1 Haemolymph analysis

3.1.1 Variation in haemocyte parameters

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

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

3.1.2 Presence of living S. enterica *in haemolymph*

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

3.2 Survival rate in response to a LD_{50} injection of S. enterica

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

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

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

4. Discussion

4.1 Main results overview

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

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

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

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

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

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

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

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

We demonstrated increased survival when *A. vulgare* had encountered *S. enterica* 7 d before receiving a LD_{50} of the same pathogen, with boosted survival rates – approximately 50% higher than non-primed individuals. This protection tended to be effective until 15 d post-priming procedure (30% increase in survival), which is consistent with previous studies suggesting immune priming in terrestrial and aquatic crustaceans may last for 7 to 30 d (Roth 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_{50} 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_{50} 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)***

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

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

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

4.5 Concluding remarks

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

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Declaration of interest

None

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Table I

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

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

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.

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

S. enterica-primed / LB-primed	-0.158	0.057	**0.016****
LB-primed / Non-primed	-0.269	0.057	**< 0.001*****

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

Table III

Pairwise comparisons (Tukey adjustment) of survival rate for the priming treatments (Non-primed: non-injected; LB-primed: injected with LB; *S. enterica*-primed: injected with 10^3 *S. enterica* during the priming procedure) under the time points (time elapsed between priming procedure and LD_{50} injection.

SURVIVAL RATE (frequency of living animals)

Days		estimate	std error	*p-value*
1				
	S. enterica-primed / Non-primed	0.412	0.324	0.411
	S. enterica-primed / LB-primed	-1.103	0.450	**0.037***
	LB-primed / Non-primed	1.515	0.435	**0.001****
7				
	S. enterica-primed / Non-primed	1.208	0.296	**< 0.001*****
	S. enterica-primed / LB-primed	1.030	0.316	**0.003****
	LB-primed / Non-primed	0.178	0.274	0.792
15				
	S. enterica-primed / Non-primed	0.858	0.413	0.095
	S. enterica-primed / LB-primed	0.488	0.430	0.492
	LB-primed / Non-primed	0.369	0.332	0.506

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

References

Adamo, S.A., 2017. Stress responses sculpt the insect immune system, optimizing defense in an ever-changing world. Dev. Comp. Immunol. 66, 24–32. https://doi.org/10.1016/j.dci.2016.06.005

Agresti, A., 2002. Categorical data analysis. 2nd Edition, Hoboken, NJ: Wiley. doi:10.1002/0471249688

Akoda, K., Van Den Bossche P., Marcotty T., Kubi, C., Coosemans, M., Dedeken R., 2009. Nutritional stress affects the tsetse fly's immune gene expression. Med. Vet. Entomol. 23, 195–201. https://doi.org/10.1111/j.1365-2915.2009.00799.x

Andino, A., Hanning, I., 2015. *Salmonella enterica*: Survival, colonization, and virulence differences among serovars, 2015. Scientific. World Journal. https://doi.org/10.1155/2015/520179

Ayres, J.S., Schneider, D.S., 2009. The role of anorexia in resistance and tolerance to infections in Drosophila. PLoS Biol. 7. https://doi.org/10.1371/journal.pbio.1000150

Bates, D., Mächler, M., Bolker, B., Walker, S., 2014. Fitting Linear Mixed-Effects Models using lme4. Journal of Statistical Software, [S.l.], v. 67, Issue 1, p. 1 - 48, doi:http://dx.doi.org/10.18637/jss.v067.i01.

Beck, M.L., Price, J.O., 1981. Genetic variation in the terrestrial isopod, *Armadillidium vulgare*. J. Hered. 72, 15–18. https://doi.org/10.1093/oxfordjournals.jhered.a109417

Best, A., Tidbury, H., White, A., Boots, M., 2012. The evolutionary dynamics of within-generation immune priming in invertebrate hosts. J. R. Soc. Interface 10, 20120887–20120887. https://doi.org/10.1098/rsif.2012.0887

Braquart-Varnier, C., Lachat, M., Herbinière, J., Johnson, M., Caubet, Y., Bouchon, D., Sicard, M., 2008. Wolbachia mediate variation of host immunocompetence. PLoS One 3, e3286. https://doi.org/10.1371/journal.pone.0003286

Braquart-Varnier, C., Raimond, M., Mappa, G., Chevalier, F.D., Clec'h, W. Le, Sicard, M., 2015. The hematopoietic organ: A cornerstone for *Wolbachia* propagation between and within hosts. Front. Microbiol. 6, 1–8. https://doi.org/10.3389/fmicb.2015.01424

Brehélin, M., Roch, P., 2008. Specificity, learning and memory in the innate immune response. Invertebrate Survival Journal, 5 (2), 103-109. https://prodinra.inra.fr/record/42302

Bretz, F., Hothorn, T., Westfall, P.H., 2011. Multiple comparisons using R. CRC Press.

Broly, P., Deneubourg, J.L., Devigne, C., 2013. Benefits of aggregation in woodlice: A factor in the terrestrialization process? Insectes Soc. 60, 419–435. https://doi.org/10.1007/s00040-013-0313-7

505 Broly, P., Devigne, L., Deneubourg, J.L., Devigne, C., 2014. Effects of group size on
506 aggregation against desiccation in woodlice (Isopoda: Oniscidea). Physiol. Entomol. 39,
507 165–171. https://doi.org/10.1111/phen.12060

508 Browne, N., Surlis, C., & Kavanagh, K., 2014. Thermal and physical stresses induce a short-
509 term immune priming effect in *Galleria mellonella* larvae. Journal of Insect Physiology,
510 63(1), 21–26. https://doi.org/10.1016/j.jinsphys.2014.02.006

511 Cerenius, L., Söderhäll, K., 2011. Coagulation in Invertebrates. J. Innate Immun. 3, 3–8.
512 https://doi.org/10.1159/000322066

513 Chang, Y.H., Kumar, R., Ng, T.H., Wang, H.C., 2018. What vaccination studies tell us about
514 immunological memory within the innate immune system of cultured shrimp and crayfish,
515 Developmental and Comparative Immunology. Elsevier Ltd.
516 https://doi.org/10.1016/j.dci.2017.03.003

517 Chevalier, F., Herbinière-Gaboreau, J., Bertaux, J., Raimond, M., Morel, F., Bouchon, D.,
518 Grève, P., Braquart-Varnier, C., 2011. The immune cellular effectors of terrestrial isopod
519 *Armadillidium vulgare*: Meeting with their invaders, *Wolbachia*. PLoS One 6.
520 https://doi.org/10.1371/journal.pone.0018531

521 Contreras-Garduño, J., Rodríguez, M.C., Rodríguez, M.H., Alvarado-Delgado, A., Lanz-
522 Mendoza, H., 2014. Cost of immune priming within generations: Trade-off between
523 infection and reproduction. Microbes Infect. 16, 261–267.
524 https://doi.org/10.1016/j.micinf.2013.11.010

525 Contreras-Garduño, J., Rodríguez, M.C., Hernández-Martínez, S., Martínez-Barnetche, J.,
526 Alvarado-Delgado, A., Izquierdo, J., Herrera-Ortiz, A., Moreno-García, M., Velazquez-
527 Meza, M.E., Valverde, V., Argotte-Ramos, R., Rodríguez, M.H., Lanz-Mendoza, H.,
528 2015. *Plasmodium berghei* induced priming in *Anopheles albimanus* independently of
529 bacterial co-infection. Dev. Comp. Immunol. 52, 172–181.
530 https://doi.org/10.1016/j.dci.2015.05.004

531 Contreras-Garduño, J., Lanz-Mendoza, H., Franco, B., Nava, A., Pedraza-Reyes, M., Canales-
532 Lazcano, J., 2016. Insect immune priming: ecology and experimental evidences. Ecol.
533 Entomol. 41, 351–366. https://doi.org/10.1111/een.12300

534 Coustau, C., Kurtz, J., Moret, Y., 2016. A novel mechanism of immune memory unveiled at
535 the invertebrate-parasite interface. Trends Parasitol. 32, 353–355.
536 https://doi.org/10.1016/j.pt.2016.02.005

537 Dhinaut, J., Chogne, M., Moret, Y., 2018. Immune priming specificity within and across
538 generations reveals the range of pathogens affecting evolution of immunity in an insect. J.
539 Anim. Ecol. 87, 448–463. https://doi.org/10.1111/1365-2656.12661

540 Durand, S., Cohas, A., Braquart-Varnier, C., Beltran-Bech, S., 2017. Paternity success depends
541 on male genetic characteristics in the terrestrial isopod *Armadillidium vulgare*. Behav.
542 Ecol. Sociobiol. 71, 90. https://doi.org/10.1007/s00265-017-2317-1

543 Fox, J., Sanford, W., 2011. Cox Proportional-hazards regression for survival data in R. An R
544 Companion to Appl. Regression, Second Ed. 2008, 1–18.
545 https://doi.org/10.1016/j.carbon.2010.02.029

546 Fugmann, S. D., Gung, C., & Hospital, M., 2018. Invertebrate adaptive immunology, 1–8.
547 https://doi.org/10.1016/B978-0-12-809633-8.20729-2

548 Gourbal, B., Pinaud, S., Beckers, G.J.M., Van Der Meer, J.W.M., Conrath, U., Netea, M.G.,
549 2018. Innate immune memory: An evolutionary perspective. Immunol. Rev. 283, 21–40.
550 https://doi.org/10.1111/imr.12647

551 Greenwood, J.M., Milutinović, B., Peuß, R., Behrens, S., Esser, D., Rosenstiel, P., Schulenburg,
552 H., Kurtz, J., 2017. Oral immune priming with *Bacillus thuringiensis* induces a shift in the
553 gene expression of *Tribolium castaneum* larvae. BMC Genomics 18, 329.
554 https://doi.org/10.1186/s12864-017-3705-7

555 Herbiniere, J., 2006. Contribution à la mise en évidence des effecteurs impliqués dans
556 l'immunité innée d'*Armadillidium vulgare*, crustacé isopode terrestre infecté par une
557 bactérie du genre *Wolbachia*. Ecologie, Environnement. Université de Poitiers, 2005.
558 Français.

559 Hoffmann, J.A., Kafatos, F.C., Janeway, C.A., Ezekowitz, R.A., 1999. Phylogenetic
560 perspectives in innate immunity. Science 284, 1313–8. DOI:
561 10.1126/science.284.5418.1313

562 Hornung, E., 2011. Evolutionary adaptation of oniscidean isopods to terrestrial life: Structure,
563 physiology and behavior. Terr. Arthropod Rev. 4, 95–130.
564 https://doi.org/10.1163/187498311X576262

565 Jacot, A., Scheuber, H., Kurtz, J., Brinkhof, M.W.G., 2005. Juvenile immune system activation
566 induces a costly upregulation of adult immunity in field crickets *Gryllus campestris*. Proc.
567 R. Soc. B Biol. Sci. 272, 63–69. https://doi.org/10.1098/rspb.2004.2919

568 Khan, I., Prakash, A., Agashe, D., 2016. Divergent immune priming responses across flour
569 beetle life stages and populations 7847–7855. https://doi.org/10.1002/ece3.2532

570 Khan I, Prakash A, Agashe D. 2017 Experimental evolution of insect immune memory versus
571 pathogen resistance. Proc. R. Soc. B 284: 20171583.
572 http://dx.doi.org/10.1098/rspb.2017.1583

573 Karimi B., Chemidlin Prévost-Bouré N., Dequiedt S., Terrat S. & Ranjard L., 2018. —Atlas
574 français des bactéries du sol. Muséum national d'Histoire naturelle, Paris ; Biotope, Mèze,
575 192 p.

576 Kostrzewa, M., 2018. Application of the MALDI Biotyper to clinical microbiology: progress
577 and potential. Expert Rev. Proteomics 0. https://doi.org/10.1080/14789450.2018.1438193

578 Kurtz, J., 2005. Specific memory within innate immune systems. Trends Immunol. 26, 186–
579 192. https://doi.org/10.1016/j.it.2005.02.001

580 Kutzer, M. A. M., Kurtz, J., & Armitage, S. A. O., 2019. A multi-faceted approach testing the
581 effects of previous bacterial exposure on resistance and tolerance. *Journal of Animal*
582 *Ecology*, *88*(4), 566–578. https://doi.org/10.1111/1365-2656.12953

583 Lanz-Mendoza, H., Garduño, J.C., 2018. Insect innate immune memory, in: advances in
584 comparative immunology. Springer International Publishing, Cham, pp. 193–211.
585 https://doi.org/10.1007/978-3-319-76768-0_9

586 Lee, S.Y., Söderhäll, K., 2002. Early events in crustacean innate immunity. Fish Shellfish
587 Immunol. 12, 421–437. https://doi.org/10.1006/fsim.2002.0420

588 Little, T.J., Kraaijeveld, A.R., 2004. Ecological and evolutionary implications of
589 immunological priming in invertebrates. Trends Ecol. Evol. 19, 58–60.
590 https://doi.org/10.1016/j.tree.2003.11.011

591 Márkus, R., Kurucz, É., Rus, F., & Andó, I., 2005. Sterile wounding is a minimal and sufficient
592 trigger for a cellular immune response in Drosophila melanogaster 101, 108–111.
593 https://doi.org/10.1016/j.imlet.2005.03.021

594 Melillo, D., Marino, R., Italiani, P., Boraschi, D., 2018. Innate immune memory in invertebrate
595 metazoans: a critical appraisal. Front. Immunol. 9.
596 https://doi.org/10.3389/fimmu.2018.01915

597 Mikonranta, L., Mappes, J., Kaukoniitty, M., Freitak, D., 2014. Insect immunity: Oral exposure
598 to a bacterial pathogen elicits free radical response and protects from a recurring infection.
599 Front. Zool. 11, 1–7. https://doi.org/10.1186/1742-9994-11-23

600 Milutinović, B., Kurtz, J., 2016. Immune memory in invertebrates. Semin. Immunol. 28, 328–
601 342. https://doi.org/10.1016/j.smim.2016.05.004

602 Moret, Y., Schmid-Hempel, P., 2000. Survival for immunity: The price of immune system
603 activation for bumblebee workers. Science (80-.). 290, 1166–1168.
604 https://doi.org/10.1126/science.290.5494.1166

605 Moret, Y., Coustau, C., Braquart-Varnier, C., Gourbal, B., 2019. Immune priming and trans-
606 generational protection from parasites, in: encyclopedia of animal behavior. Elsevier, pp.
607 764–774. https://doi.org/10.1016/B978-0-12-809633-8.90726-X

608 Muñoz, M., Vandenbulcke, F., Saulnier, D., Bachère, E., 2002. Expression and distribution of
609 penaeidin antimicrobial peptides are regulated by haemocyte reactions in microbial
610 challenged shrimp. Eur. J. Biochem. 269, 2678–2689. https://doi.org/10.1046/j.1432-
611 1033.2002.02934.x

612 Murray, C.J., 1991. Salmonellae in the environment. Rev. Sci. Tech. Off. Int. des Epizoot. 10,
613 765–785. https://doi.org/10.5772/2470

614 Persson, M., Cerenius, L., Söderhäll, K., 1987. The influence of haemocyte number on the
615 resistance of the freshwater crayfish, *Pacifastacus leniusculus Dana*, to the parasitic
616 fungus *Aphanomyces astaci*. J. Fish Dis. 10, 471–477. https://doi.org/10.1111/j.1365-
617 2761.1987.tb01098.x

Pigeault, R., Garnier, R., Rivero, A., Gandon, S., 2016. Evolution of transgenerational immunity in invertebrates. Proc. R. Soc. B Biol. Sci. 283. https://doi.org/10.1098/rspb.2016.1136

Pinaud, S., Portela, J., Duval, D., Nowacki, F.C., Olive, M.A., Allienne, J.F., Galinier, R., Dheilly, N.M., Kieffer-Jaquinod, S., Mitta, G., Théron, A., Gourbal, B., 2016. A shift from cellular to humoral responses contributes to innate immune memory in the vector snail *Biomphalaria glabrata*. PLoS Pathog. 12, 1–18. https://doi.org/10.1371/journal.ppat.1005361

Pipe, R.K., Coles, J.A., 1995. Environmental contaminants influencing immune function in marine bivalve molluscs. Fish Shellfish Immunol. 5, 581–595. https://doi.org/10.1016/S1050-4648(95)80043-3

Ponton, F., Wilson, K., Holmes, A.J., Cotter, S.C., Raubenheimer, D., Simpson, S.J., 2013. Integrating nutrition and immunology: A new frontier. J. Insect Physiol. 59, 130–137. https://doi.org/10.1016/j.jinsphys.2012.10.011

Pope, E.C., Powell, A., Roberts, E.C., Shields, R.J., Wardle, R., Rowley, A.F., 2011. Enhanced cellular immunity in shrimp (*Litopenaeus vannamei*) after "vaccination." PLoS One 6, 1–7. https://doi.org/10.1371/journal.pone.0020960

Pradeu, T., Du Pasquier, L., 2018. Immunological memory: What's in a name? Immunol. Rev. 283, 7–20. https://doi.org/10.1111/imr.12652

[dataset] Prigot-Maurice C., Cerqueira de Araujo A., Durand S., Laverré T., Pigeault R., Verdon J., Bulet P., Beltran-Bech S., and Braquart-Varnier C., 2019. Survival and haemocyte data for *Armadillidium vulgare* responses against *Salmonella enterica* subsequent infections. Mendeley Data, v1.

R Development Core Team, 2017. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org.

Ranjard, L., Richaume, A., 2001. Quantitative and qualitative microscale distribution of bacteria in soil. Res. Microbiol. 152, 707–16. https://doi.org/10.1016/S0923-2508(01)01251-7

Rechavi, O., Minevich, G., Hobert, O., 2011. Transgenerational inheritance of an acquired small RNA-based antiviral response in *C. elegans*. Cell 147, 1248–1256. https://doi.org/10.1016/j.cell.2011.10.042

Rodrigues, J., Brayner, F.A., Alves, L.C., Dixit, R., Barillas-mury, C., 2010. Hemocyte differentiation mediates innate immune memory in *Anopheles gambiae* mosquitoes. NIH Public Access. Science (80-.). 329, 1353–1355. https://doi.org/10.1126/science.1190689

Rosengaus,R.B., Traniello,J.F.A., J.J., T. Chen, Brown, R.D. Karp., 1999. Immunity in a social insect. Naturwissenschaften 86, 588–591, http://dx.doi.org/10. 1007/s001140050679.

655 Roth, O., Sadd, B.M., Schmid-Hempel, P., Kurtz, J., 2009. Strain-specific priming of resistance
656 in the red flour beetle, *Tribolium castaneum*. Proc. R. Soc. B Biol. Sci. 276, 145–151.
657 https://doi.org/10.1098/rspb.2008.1157

658 Schmid-Hempel, P., 2005. Evolutionary ecology of insect immune defenses. Annu. Rev.
659 Entomol. 50, 529–551. https://doi.org/10.1146/annurev.ento.50.071803.130420

660 Scott, J.S., Sterling, S.A., To, H., Seals, S.R., Alan, E., Scott, J.S., Sterling, S.A., To, H., Seals,
661 S.R., Alan, E., 2016. Diagnostic performance of matrix-assisted laser desorption ionisation
662 time-of-flight mass spectrometry in blood bacterial infections : a systematic review and
663 meta-analysis 4235. https://doi.org/10.3109/23744235.2016.1165350

664 Sicard, M., Chevalier, F., De Vlechouver, M., Bouchon, D., Grève, P., Braquart-Varnier, C.,
665 2010. Variations of immune parameters in terrestrial isopods: A matter of gender, aging
666 and Wolbachia. Naturwissenschaften 97, 819–826. https://doi.org/10.1007/s00114-
667 0100699-2

668 Siva-Jothy, M.T., Thompson, J.J.W., 2002. Short-term nutrient deprivation affects immune
669 function. Physiol. Entomol. 27, 206–212. https://doi.org/10.1046/j.1365-
670 3032.2002.00286.x

671 Snyman, R.G., Odendaal, J.P., 2009. Effect of cadmium on haemocyte viability of the
672 woodlouse *Porcellio laevis* (Isopoda, Crustacea). Bull. Environ. Contam. Toxicol. 83,
673 525–529. https://doi.org/10.1007/s00128-009-9814-5

674 Söderhäll, I., 2016. Crustacean hematopoiesis. Dev. Comp. Immunol. 58, 129–141.
675 https://doi.org/10.1016/j.dci.2015.12.009

676 Söderhäll, I., Bangyeekhun, E., Mayo, S., Söderhäll, K., 2003. Haemocyte production and
677 maturation in an invertebrate animal; proliferation and gene expression in hematopoietic
678 stem cells of *Pacifastacus leniusculus*. Dev. Comp. Immunol. 27, 661–672.
679 https://doi.org/10.1016/S0145-305X(03)00039-9

680 Söderhäll, K., Cerenius, L., 1992. Crustacean immunity. Annu. Rev. Fish Dis. 2, 3–23.
681 https://doi.org/10.1016/0959-8030(92)90053-Z

682 Sokolova, I.M., Evans, S., Hughes, F.M., 2004. Cadmium-induced apoptosis in oyster
683 haemocytes involves disturbance of cellular energy balance but no mitochondrial
684 permeability transition. J. Exp. Biol. 207, 3369–3380. https://doi.org/10.1242/jeb.01152

685 Therneau, T.M., Grambsch, P.M., Pankratz, V.S., 2003. Penalized survival models and frailty.
686 J. Comput. Graph. Stat. https://doi.org/10.2307/1391074

687 Therneau, T., 2012. Mixed effects cox models. Ref. Man. 1–14.
688 https://doi.org/10.1111/oik.01149

689 Torsvik, V., Goks0yr, J., Daae, F.L., 1990. High diversity in DNA of soil bacteria, Applied and
690 Environmental Microbiology. 56, 782-787.

691 Warburg, M.R., Llnsenmair, K.E., Bercovitz, K., 1984. The effect of climate on the distribution
692 and abundance of isopods. Symp. zool. Soc. L. 53, 339–367.
693 https://doi.org/10.1108/eb003826

694 Verdon, J., Coutos-Thevenot, P., Rodier, M.H., Landon, C., Depayras, S., Noel, C., La Camera,
695 S., Moumen, B., Greve, P., Bouchon, D., Berjeaud, J.M., Braquart-Varnier, C., 2016.
696 Armadillidin H, a glycine-rich peptide from the terrestrial crustacean *Armadillidium*
697 *vulgare*, displays an unexpected wide antimicrobial spectrum with membranolytic activity.
698 Front. Microbiol. 7. https://doi.org/10.3389/fmicb.2016.01484

699 Wu, G., Zhao, Z., Liu, C., Qiu, L., 2014. Priming *Galleria mellonella*; (Lepidoptera: Pyralidae)
700 Larvae with heat-killed bacterial cells induced an enhanced immune protection against
701 *Photorhabdus luminescens*; TT01 and the role of innate immunity in the process. J. Econ.
702 Entomol. 107, 559–569. https://doi.org/10.1603/EC13455

703 Wu, G., Li, M., Liu, Y., Ding, Y., & Yi, Y., 2015. The specificity of immune priming in
704 silkworm, *Bombyx mori*, is mediated by the phagocytic ability of granular cells. Journal of
705 Insect Physiology, 81, 60–68. https://doi.org/10.1016/j.jinsphys.2015.07.004

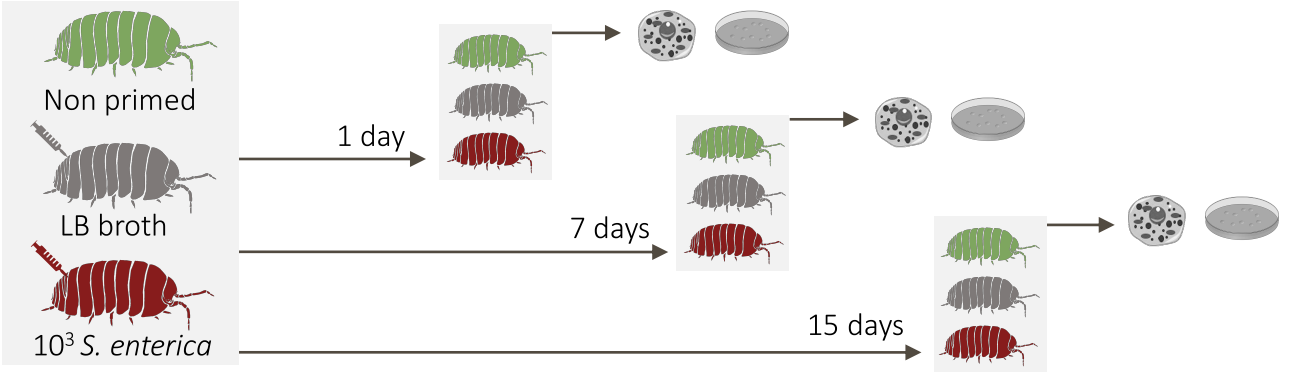
706 Wu, G., Xu, L., & Yi, Y. (2016). *Galleria mellonella* larvae are capable of sensing the extent
707 of priming agent and mounting proportionatal cellular and humoral immune responses.
708 Immunology Letters, 174, 45–52. https://doi.org/10.1016/j.imlet.2016.04.013

709 Zhang, T., Qiu, L., Sun, Z., Wang, L., Zhou, Z., Liu, R., Yue, F., Sun, R., Song, L., 2014. The
710 specifically enhanced cellular immune responses in Pacific oyster (*Crassostrea gigas*)
711 against secondary challenge with *Vibrio splendidus*. Dev. Comp. Immunol. 45, 141–150.
712 https://doi.org/10.1016/j.dci.2014.02.015

713 Zimmer, M., 2002. Nutrition in terrestrial isopods (Isopoda: Oniscidea): an evolutionary-
714 ecological approach. Biol. Rev. Camb. Philos. Soc. 77, S1464793102005912.
715 https://doi.org/10.1017/S1464793102005912

1st EXPERIMENT : HAEMOLYMPH ANALYSIS

Priming procedure **Sampling** (haemocyte concentration, viability - bacterial presence)



2nd EXPERIMENT : SURVIVAL MONITORING

Priming procedure **LD_{50} injection** ($3.10^5 \pm 1.10^5$ *S. enterica*) **and survival monitoring** ( )

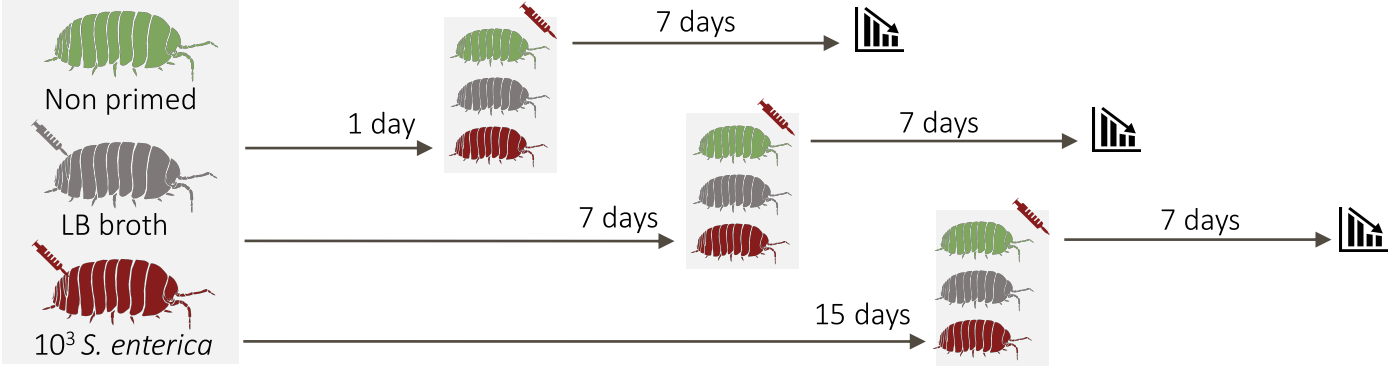


Fig. 2

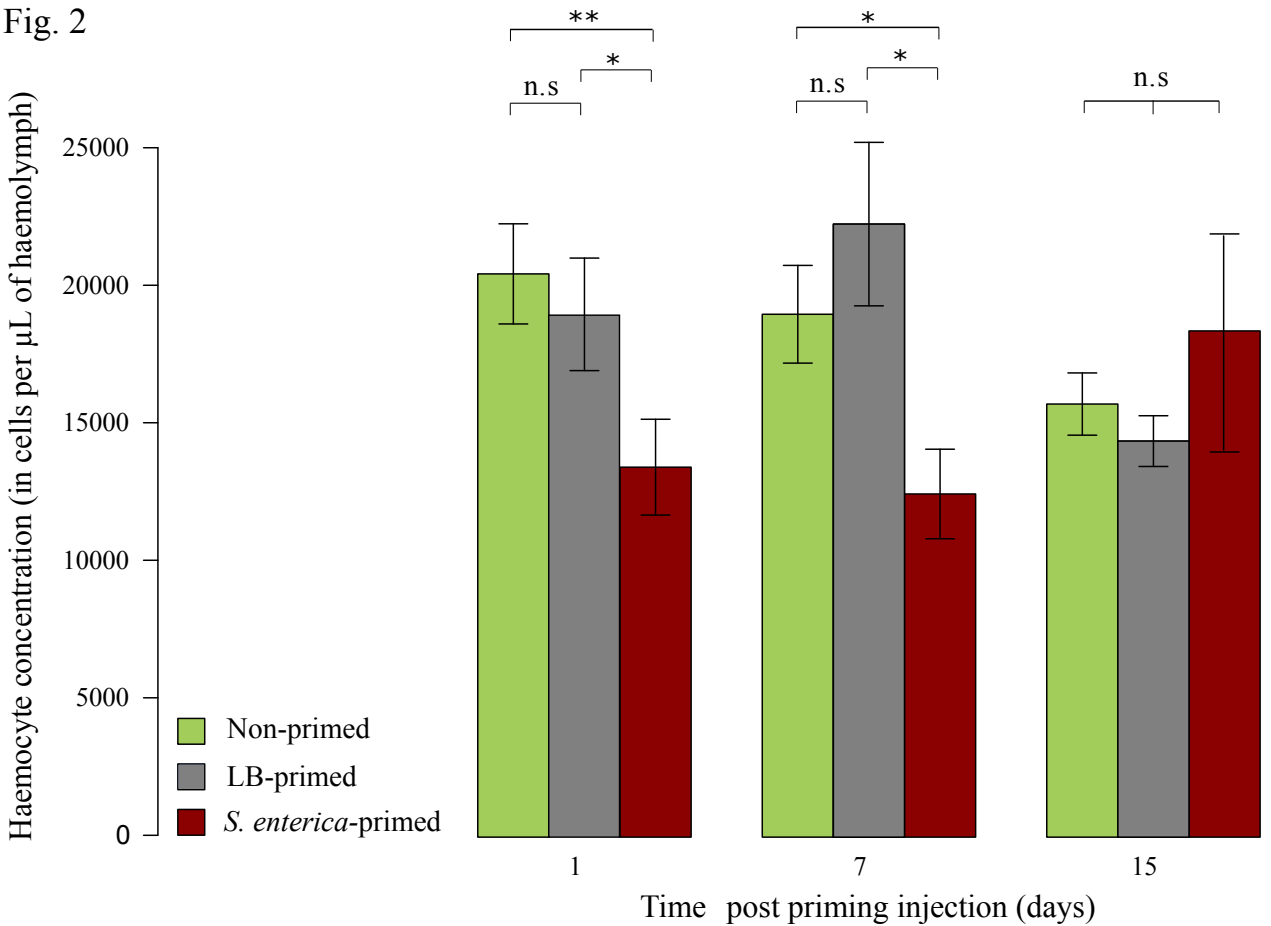

**
*
n.s
*
n.s
n.s
25000
20000
15000
10000
5000
0
Haemocyte concentration (in cells per µL of haemolymph)
Non-primed
LB-primed
S. enterica-primed
1
7
15
Time post priming injection (days)

Fig. 3

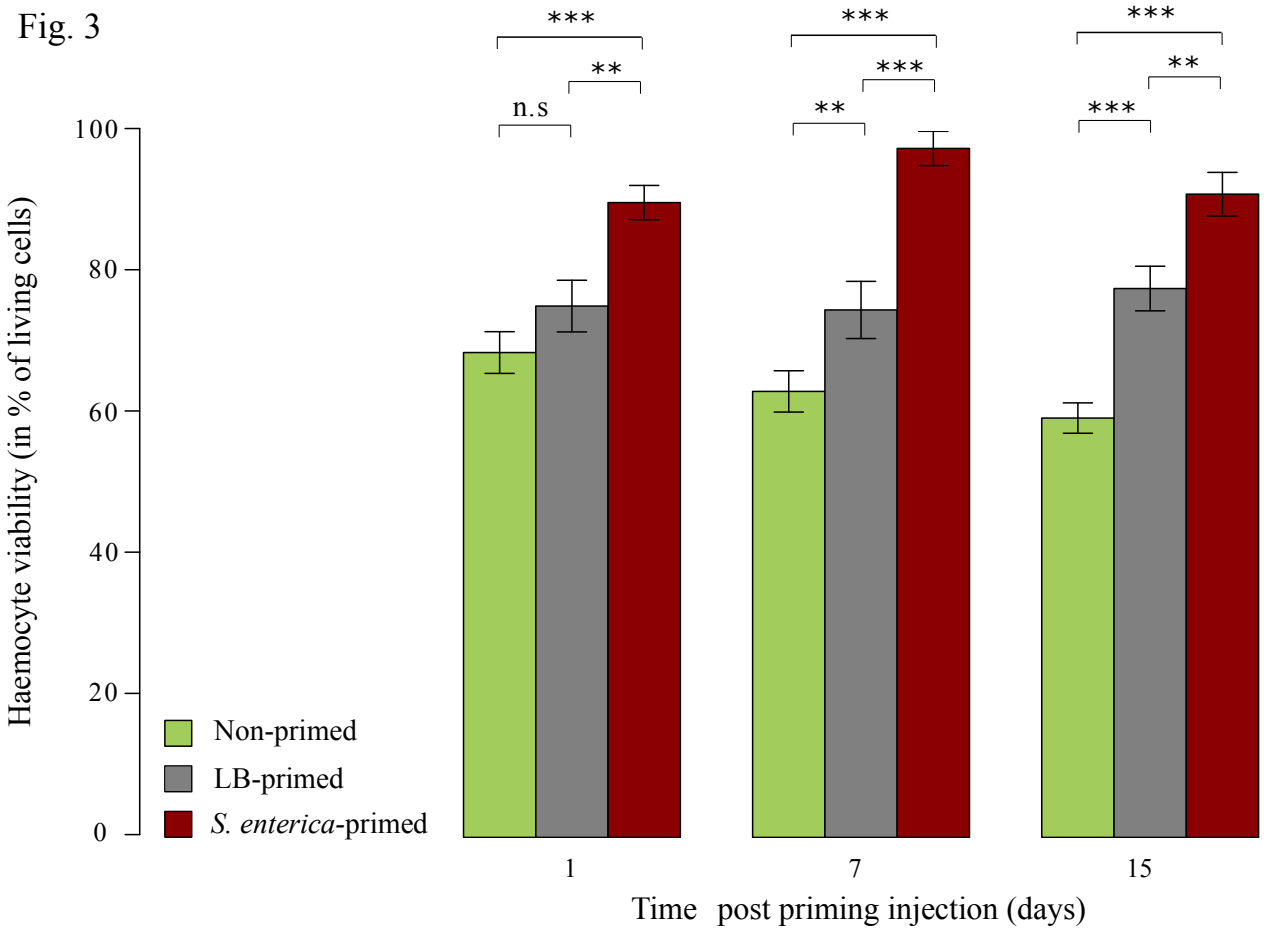

***
**
n.s
***
***
**
***
**
100
80
60
40
20
0
Haemocyte viability (in % of living cells)
Non-primed
LB-primed
S. enterica-primed
1
7
15
Time post priming injection (days)

Fig.4

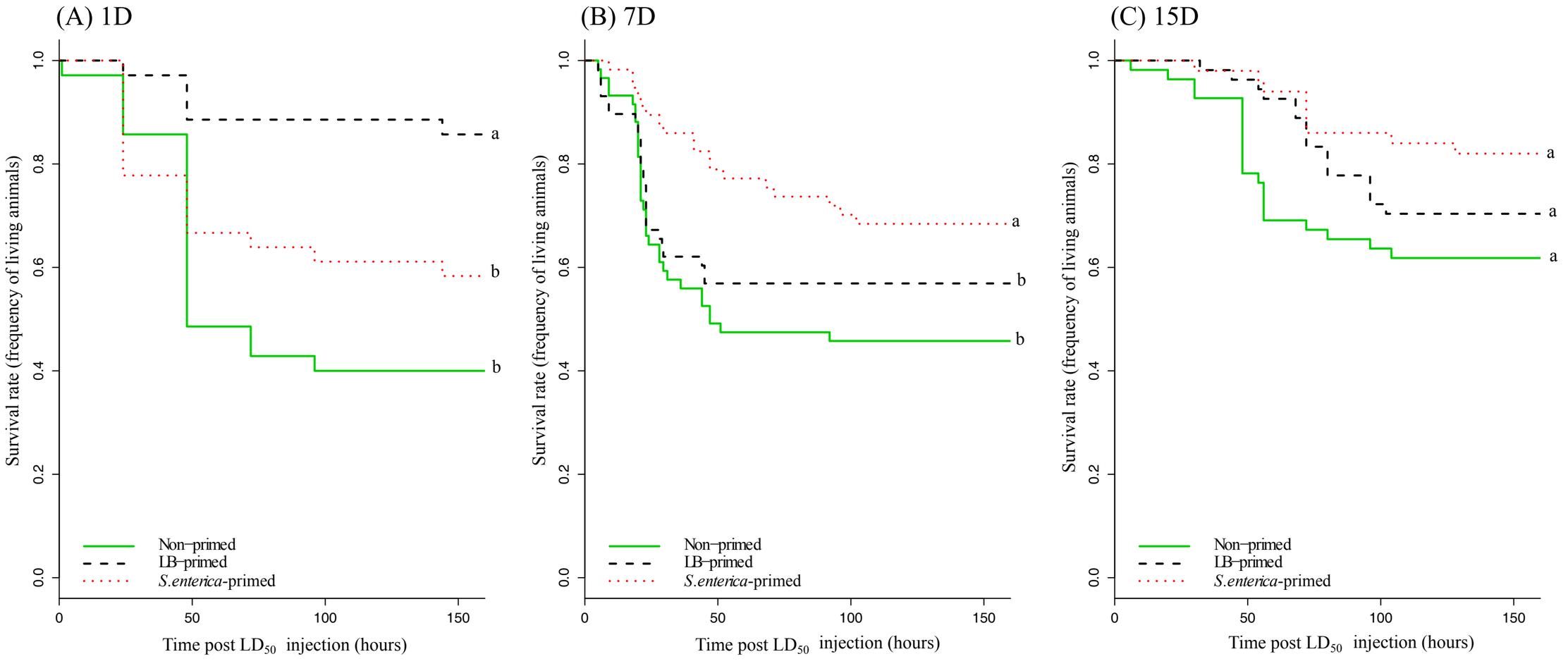

(A) 1D
(B) 7D
(C) 15D
Survival rate (frequency of living animals)
0.0
0.2
0.4
0.6
0.8
1.0
0
50
100
150
Time post LD_{50} injection (hours)
Non−primed
LB−primed
S.enterica-primed
a
b
b
a
b
b
a
a
a

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_{50} 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 10^3 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 10^3 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 10^3 living *S. enterica* during the priming procedure. Different letters beside curves indicate significant differences between the survival curves based on Tukey's range test ($p \leq 0.05$).

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

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

SUPPLEMENTARY MATERIALS

S1: detailed description of the bacterial culture methods

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

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

Supplementary materials references

1. Velge P, Wiedemann A, Rosselin M, Abed N, Boumart Z, Chaussé AM, Grépinet O, Namdari F, Roche SM, Rossignol A, Virlogeux-Payant I, 2012. Multiplicity of *Salmonella* entry mechanisms, a new paradigm for *Salmonella* pathogenesis. Microbiology open. 1, 243-258. doi: 10.1002/mbo3.28

2. Verdon J, Coutos-Thevenot P, Rodier MH, Landon C, Depayras S, Noel C, La Camera S, Moumen B, Greve P, Bouchon D, Berjeaud JM, Braquart-Varnier C, 2016. Armadillidin H, a glycine-rich peptide from the terrestrial crustacean *Armadillidium vulgare*, displays an unexpected wide antimicrobial spectrum with membranolytic activity. Front. Microbiol. 7, 1484. doi: 10.3389/fmicb.2016.01484

3. Braquart-Varnier C, Altinli M, Pigeault R, Chevalier FD, Grève P, Bouchon D, Sicard M., 2015. The mutualistic side of *Wolbachia*-isopod interactions: *Wolbachia* mediated protection against pathogenic intracellular bacteria. Front. Microbiol. 6, 1388. doi: 10.3389/fmicb.2015.01388

S2: Experimental design

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