

Immune priming against bacteria in spiders and scorpions?

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Empirical evidence of immune priming in arthropods keeps growing, both at the within- and trans-generational level. The evidence comes mostly from work on insects and it remains unclear for some other arthropods whether exposure to a non-lethal dose of a pathogen provides protection during a second exposure with a lethal dose. A poorly investigated group are arachnids, with regard to the benefits of immune priming measured as improved survival. Here, we investigated immune priming in two arachnids: the wolf spider *Lycosa cerrofloresiana* and the scorpion *Centruroides granosus*. We injected a third of the individuals with lipopolysaccharides of *Escherichia coli* (LPS, an immune elicitor), another third were injected with the control solution (PBS) and the other third were kept naive. Four days after the first inoculations, we challenged half of the individuals of each group with an injection of a high dose of *E. coli* and the other half was treated with the control solution. For scorpions, individuals that were initially injected with PBS or LPS did not differ in their survival rates against the bacterial challenge. Individuals injected with LPS showed higher survival than that of naive individuals as evidence of immune priming. Individuals injected with PBS tended to show higher survival rates than naive individuals, but the difference was not significant — perhaps suggesting a general immune upregulation caused by the wounding done by the needle. For spiders, we did not observe evidence of priming, the bacterial challenge reduced the survival of naive, PBS and LPS individuals at similar rates. Moreover; for scorpions, we performed antibacterial assays of hemolymph samples from the three priming treatments (LPS, PBS and naive) and found that the three treatments reduced bacterial growth but without differences among treatments. As non-model organisms, with some unique differences in their immunological mechanisms as compared to the most studied arthropods (insects), arachnids provide an unexplored field to elucidate the evolution of immune systems.

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12 Abstract

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14 trans-generational level. The evidence comes mostly from work on insects and it remains unclear
15 for some other arthropods whether exposure to a non-lethal dose of a pathogen provides
16 protection during a second exposure with a lethal dose. A poorly investigated group are
17 arachnids, with regard to the benefits of immune priming measured as improved survival. Here,
18 we investigated immune priming in two arachnids: the wolf spider *Lycosa cerrofloresiana* and
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30 individuals at similar rates. Moreover; for scorpions, we performed antibacterial assays of
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32 three treatments reduced bacterial growth but without differences among treatments. As non-
33 model organisms, with some unique differences in their immunological mechanisms as
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35 elucidate the evolution of immune systems.

36

37 **Introduction**

38 The invertebrate immune system was traditionally believed to contain no memory and
39 specificity. This is due to the lack of immune machinery that is needed in order to develop the
40 desired immune response in vertebrates (Rowley & Powell, 2007). However, recent literature has
41 reported that invertebrates exposed to a low dose of a pathogen can obtain protection against a
42 subsequent lethal dose of the same pathogen, a phenomenon termed as immune priming (Little &
43 Kraaijeveld, 2004). This improved immune response can be observed within a few days after the
44 priming, in later stages of the individual ('within-generation immune priming', Milutinović &

45 Kurtz, 2016) or even transferred to the offspring ('trans-generational immune priming', Tetreau
46 et al., 2019).

47 Evidence of immune priming comes mostly from insects (Milutinović et al., 2016; Cooper &
48 Eleftherianos, 2017). However, a number of studies found no evidence of immune priming in
49 insects against fungi (Reber & Chapuisat, 2012; Gálvez & Chapuisat, 2014), bacteria (González-
50 Tokman et al., 2010; Patnogić et al., 2018) and bacterial immune elicitors (ter Braak et al.,
51 2013; Wu et al., 2015b). In some cases the detection depended on the pathogen used (Pham et
52 al., 2007; Vargas et al., 2016; Ferro et al., 2019). Overall, the detection of immune priming in
53 insects seems to depend on multiple factors such as host – pathogen combination, host lifespan,
54 priming method, pathogen dose, virulence, among others (Contreras-Garduño, 2016; Milutinović
55 et al., 2016; Cooper & Eleftherianos, 2017; Tetreau et al., 2019).

56 Even though it is thought that the immune system of arthropods is well conserved across species,
57 based on an innate immune system, consisting of cellular and humoral responses (Rowley &
58 Powell, 2007), recent studies showed there exists some variation across taxa and the insect
59 immune system that which does not necessarily characterize other arthropods. For instance,
60 Bechsgaard et al. (2016) discovered that some genes involved in pathways for pathogen
61 recognition (e.g. bacteria) have been lost in arachnids and the humoral immune effector proteins
62 (antimicrobial peptides, AMPs) are apparently not induced as it is the case for insects, but they
63 are constitutively produced, a trend also observed by previous studies (Lorenzini et al., 2003;
64 Fukuzawa et al., 2008; Baumann et al., 2010; González-Tokman et al., 2014). In other arachnids,
65 the evidence seems to suggest a complete absence of an induced immune response (Santos-
66 Matos et al., 2017). Another example of dissimilarities between insects and arachnids is the
67 evidence indicating that phagocytosis plays a role in the immune priming of insects (Pham et al.,

68 2007; Wu et al., 2015a). However, in spiders, phagocytosis seems to play a minor role in defense
69 when compared to AMPs and coagulation (Fukuzawa et al., 2008). Overall, whether these
70 differences in arachnids' immune systems influence their capacity to mount an immune priming
71 response is unclear.

72 Immunological studies and evidence of immune priming in arachnids come mainly from work
73 with ticks, given their medical importance, with evidence of upregulation (Nakajima et al., 2001;
74 Matsuo et al., 2004) and improved survival after exposure to an immune elicitor, controlled by
75 molecular pathways that are apparently unique to ticks (Shaw et al., 2017). Moreover, blood-
76 feeding can strongly upregulate defensin genes in the midgut, which normally occurs in the fat
77 body after bacterial infection in insects [review in Taylor, 2006]. Ticks as hematophagous are an
78 atypical group of arachnids in terms of the use of immune defenses; for instance, ticks can use
79 fragments of the host blood for their own defense against bacteria in the midgut level (Nakajima
80 et al., 2003, 2005), together with their own antibacterial peptides (Nakajima et al., 2005) or with
81 the influence of commensal and symbiont bacteria (Chávez et al., 2017). In contrast, knowledge
82 about the immune system of other arachnids remains mostly unknown.

83 In fact, no experimental study has investigated immune priming in terms of increased survival in
84 non-hematophagous arachnids like spiders or scorpions (Milutinović & Kurtz, 2016; Milutinović
85 et al., 2016). By studying the immune response of other arachnids, analogies and differences
86 with other taxa can be established in order to understand the evolution of the immune systems in
87 invertebrates. Here, we performed the first test of immune priming in spiders and scorpions in
88 terms of improved survival. We investigated whether the wolf spider *Lycosa cerrofloresiana*
89 (Lycosidae) and the scorpion *Centruroides granosus* (Buthidae) can mount an immune priming
90 response when injected with lipopolysaccharides (LPS) of *Escherichia coli* and subsequently

91 challenged with a lethal dose of the same bacteria. If antimicrobial peptides are constitutively
92 produced, then their immune system may always be prepared for an immune challenge and
93 exposure to a priming agent may not be required. Alternatively, priming would both trigger the
94 release of constitutive components and induce recruitment of production of higher levels of
95 antimicrobials components.

96

97 **Materials & Methods**

98 *Study species*

99 This study was carried out with two nocturnal terrestrial predators, the wolf spider *Lycosa*
100 *cerrofloresiana* Petrunkevitch, 1925 and the scorpion *Centruroides granosus* Thorell, 1876
101 (Buthidae). *Lycosa cerrofloresiana* is found from El Salvador to Panama (World Spider Catalog,
102 2019), while *C. granosus* is endemic to Panama (de Armas, Teruel & Kovařík, 2011). For both
103 species, all the existing literature is on aspects of taxonomy and distribution (e.g. de Armas,
104 Teruel & Kovařík, 2011; World Spider Catalog, 2019 and references therein). Still, *Centruroides*
105 *granosus* prey on a variety of arthropods, including insects and other arachnids (Miranda et al.,
106 2015). Literature on the diet of the wolf spider is missing but we have noticed spiders eating
107 crickets and cockroaches in the field.

108 Spiders were collected from a baseball field in the town of Gamboa (09°07'05.1596", -
109 079°42'03.5266") and scorpions were collected from a dirt road in the town of Polanco
110 (08°45'44.3196", -079°48'22.8618"). All individuals were fed with the cricket *Acheta*
111 *domesticus*, one week before the experiments. The study did not involve unethical handling of
112 animals and did not require permits for experimentation by the Bioethics Office from the
113 University of Panama. We collected all specimens under the collection permit SE/AH-2-18

114 issued by the ‘Ministerio de Ambiente’, the government entity in charge of the management of
115 natural resources.

116 *Immune priming*

117 A strain of *Escherichia coli* was used for the experiments, which was obtained through isolation
118 with selective media by the Department of Microbiology of the Biology School at the University
119 of Panama. Tests of virulence of this strain produced high mortality in both spiders and scorpions
120 (Supplementary material). Previous studies have used *E. coli* via injection or pricking as an
121 immune elicitor in other arthropods (Eleftherianos et al., 2006; Roth & Kurtz, 2009; Erler, Popp
122 & Lattorff, 2011; Santos-Matos et al., 2017) and arachnids (Sonenshine et al., 2003; Santos-
123 Matos et al., 2017).

124 We used chilling anesthesia for all injections, which consisted of placing scorpions and spiders at
125 4 °C for 20 minutes. In order to stimulate priming, we injected spiders with 138 nL of LPS in
126 PBS (0.5 mg / mL; Sigma: L8274; hereafter LPS) by using a Nanoliter 2010 injector (WPI,
127 Florida, USA). For scorpions, we picked 100 µL of the LPS solution with a micropipette to fill
128 insulin syringes used for the injections. Control groups consisted of individuals injected only
129 with PBS and another group of untreated individuals (naive) to test whether the mechanical
130 damage caused by the injections was enough to prime the immune system. For spiders, the
131 injection procedure during the priming caused around 1% mortality and there was no mortality in
132 scorpions.

133 For the bacterial challenge, bacteria were cultured overnight on lysogeny broth (LB) at 27°C. We
134 centrifuged 14 ml of the culture ($LD_{50} 1 \times 10^7$ cells / mL) at 4000 rpm for 5 minutes, the pellet
135 was washed with PBS and resuspended in 14 ml of PBS. Four days after the initial injections,
136 half of the individuals in each treatment were injected with the bacterial solution (138 nL for

137 spiders and 100 μ L for scorpions; Naive – Challenged, PBS – Challenged, LPS – Challenged,
138 see Figure 1 for details on sample sizes). As controls, the other half of the individuals of each
139 treatment were injected only with PBS (138 nL for spiders and 100 μ L for scorpions; Naive –
140 Control, PBS – Control, LPS – Control, see Figure 1 for details on sample sizes). We performed
141 the experiments twice, on separate dates and monitored the survival of spiders and scorpions for
142 15 days after the final challenge.

143 *Antibacterial activity*

144 For these measurements, we were only able to collect sufficient hemolymph samples from
145 individual scorpions. To test whether the priming with LPS upregulated the production of
146 antimicrobial components found in the hemolymph, we measured antibacterial activity following
147 a protocol modified from Wu et al (Wu et al., 2014). Three days after the priming phase, we
148 collected 10 μ L of hemolymph from each treatment (Naive: n = 9; PBS: n = 6 and LPS: n = 9) by
149 pricking chilled animals and placed it immediately in ice and later stored at - 20 °C. The
150 antibacterial test consisted of mixing 10 μ L of cell-free hemolymph (centrifuged at 4000 rpm for
151 5 minutes) with 10 μ L of *E. coli* culture (1×10^7 cells / mL) in 180 μ L of LB and incubated
152 during 14 hours at 27°C in 1.5 mL Eppendorf tubes.

153 Antibacterial activity was quantified as inhibition of bacterial growth in the samples by
154 measuring optical density at 630 nm on a 96-well microplate reader. To evaluate whether the
155 hemolymph samples inhibited the bacterial growth, we used a positive control in which we
156 placed 10 μ L of *E. coli* culture in 190 μ L of LB (three replicates).

157 *Statistical Analysis*

158 All analyses were performed in R (R Development Core Team, 2019). The Kaplan-Meier
159 survival analysis was carried out to test for differences in survival rates between treatments as

160 implemented in the package ‘survival’. Moreover, we tested for differences between sexes in
161 both species as a fixed factor. We used the Gehan-Breslow-Wilcoxon test to compare survival
162 rates across treatments at early time points and the log-rank test to compare treatments at the end
163 of the experiments (package survMisc). For the antibacterial activity, we performed a one-
164 sample Wilcoxon test for each treatment to assess whether the priming treatment reduced
165 bacterial growth as compared to the mean bacterial growth in the absence of hemolymph (OD_{630}
166 = 0.763). To compare treatments, we carried out a Kruskal-Wallis test.

167 **Results**

168 *Immune priming*

169 For scorpions, overall, sex has no effect on survival (log-rank: $z = -0.04$, $p = 0.97$). The bacterial
170 challenge significantly reduced the survival in each treatment (Naive - Bacteria vs Naive – PBS,
171 PBS - Bacteria vs PBS – PBS, LPS - Bacteria vs LPS – PBS, Fig. 1A, Table 1). We found
172 evidence of immune priming because scorpions initially injected with LPS showed higher levels
173 of survival against the bacterial challenge than that of naive scorpions (LPS - Bacteria vs Naive -
174 Bacteria, Fig. 1A, Table 1). Although the results suggests that the priming could be elicited by
175 the wounding caused by the injection, this trend was not significant overall (PBS – Bacteria vs
176 Naive – Bacteria, Table 1) and neither during the early stages of the infection (Gehan-Breslow-
177 Wilcoxon test in Table 1).

178 The survival between scorpions injected initially with PBS or LPS against the bacterial challenge
179 was not significantly different (PBS – Bacteria vs LPS - Bacteria, Fig. 1A, Table 1). The survival
180 of controls of the three treatments were not significantly different (Naive - PBS vs PBS – PBS,
181 Naive – PBS vs LPS – PBS, PBS – PBS vs LPS - PBS, Fig. 1A, Table 1).

182 For spiders, the influence of sex on survival was investigated in the first trial and was not
183 significant ($z = -1.89$, $p = 0.06$). The bacterial challenge significantly reduced the survival of all
184 the priming treatments (Naive - Bacteria vs Naive - PBS, PBS - Bacteria vs PBS - PBS, LPS -
185 Bacteria vs LPS - PBS, Fig. 1B, Table 1). The three priming treatments did not vary in the
186 survival against the bacterial challenge (Naive - Bacteria vs PBS - Bacteria, Naive - Bacteria vs
187 LPS - Bacteria, PBS - Bacteria vs LPS - Bacteria, Fig. 1B, Table 1). The controls of the three
188 priming treatments were not significantly different (Naive - PBS vs PBS - PBS, Naive - PBS vs
189 LPS - PBS, PBS - PBS vs LPS - PBS, Fig. 1B, Table 1).

190 *Antibacterial activity*

191 Hemolymph of naive scorpions inhibited *E. coli* growth when compared to the average growth of
192 the bacteria without hemolymph (Wilcoxon: $V = 0$, $p = 0.002$, $n = 9$, Fig. 2) as well as the
193 hemolymph of scorpions injected with PBS (Wilcoxon: $V = 2$, $p = 0.05$, $n = 6$, Fig. 2) and the
194 hemolymph of scorpions injected with LPS (Wilcoxon: $V = 6$, $p = 0.03$, $n = 9$, Fig. 2). Overall,
195 there were no differences between priming treatments in their capacity to inhibit bacterial growth
196 (Kruskal-Wallis: $X^2 = 0.27$, $d.f. = 2$, $p = 0.87$, Fig. 2).

197 **Discussion**

198 Scorpions as organisms with relatively long lifespans (Lourenço, 2000) are more likely to be
199 exposed to a pathogen multiple times during their lifetime; therefore, they are good candidates to
200 show immune priming (Best et al., 2013). Indeed, we found evidence of immune priming in
201 terms of improved survival for individuals that were treated with LPS as compared to naive
202 individuals. It is unclear whether wounding by itself is sufficient to elicit priming since control
203 individuals (injected with PBS) showed similar survival against the bacteria to individuals
204 injected with LPS or kept naive. Thus, further work should evaluate whether wounding may be

205 sufficient to trigger priming in arachnids as seen in other arthropods (Korner & Schmid-Hempel,
206 2004; Roth et al., 2010; Nam et al., 2012). Perhaps danger-associated molecular patterns
207 (DAMPs) associated to wound healing could trigger immune priming (Krautz, Arefin &
208 Theopold, 2014) or they may allow the entrance of pathogens that trigger the priming.
209 The presence of LPS in the hemolymph should have triggered the production of AMPs
210 (Rodríguez De La Vega et al., 2004) or other antimicrobial effectors; however, our antibacterial
211 activity assay with scorpions' hemolymph suggests that there was no upregulation of AMPs in
212 primed individuals, in line with previous work in scorpions comparing control and challenged
213 individuals (Cocianich et al., 1993; Ehret-Sabatier et al., 1996). However, the freezing and
214 thawing of the samples may have influenced the antibacterial effect, as it was not a part of the
215 original protocol or perhaps the detection of an effect requires larger sample sizes. Another
216 concern is that the immunological history of the individuals used for experimentation was
217 unknown (e.g. priming occurring before the experiments) and whether this influences the
218 immune priming response. Future studies should try to establish potential model species that
219 could be reared in the laboratory for immunological studies.

220 The improved resistance by priming may result from other factors or in interaction with AMPs in
221 the hemolymph, which might not perform well in the medium used for our assay. Rodríguez De
222 La Vega et al. (2004) found in *Centruroides limpidus* the existence of inducible AMPs and
223 proposed a cooperative antibacterial activity with constitutive hemolymph components. Still, the
224 differences between the survival experiment and the antibacterial activity illustrate how disease
225 resistance and immunity assays may not correlate or are pathogen dependent (review in Adamo,
226 2004); consequently, providing different resolutions to the experimental detection of immune
227 priming in arthropods. Furthermore, assays developed for insects may not be appropriate for

228 arachnids as pointed out by other studies (Gilbert, Karp & Uetz, 2016). Future studies should
229 investigate the efficacy of different methods to measure immune components in arachnids.
230 In spiders, Gilbert, Karp & Uetz (2016) provided some indirect evidence of immune priming,
231 finding in the wolf spider *Schizocosa ocreata* that juveniles fed with another gram-negative
232 pathogenic bacteria showed higher encapsulation response against a nylon monofilament implant
233 in the adult stage. In contrast, we did not find benefits in terms of increased survival for wolf
234 spiders that were ‘primed’ and challenged in the adult stage, suggesting that the age in which
235 priming occurs should be examined. Future studies on arachnids should be aimed at identifying
236 mechanisms, including multiple host – pathogen or host – elicitor (e.g. dead pathogen, other
237 molecules) combinations to evaluate specificity, duration, the effect of symbionts or other
238 potential influential factors. For example, the mode of infection: Keiser et al. (2016) showed that
239 a bacterial cocktail increased mortality of a social spider via cuticular topical application while
240 on the contrary spiders fed with crickets injected with the same bacterial cocktail showed longer
241 lifespans than spiders fed with control crickets.

242 Arachnids offer systems to study other means of defense against pathogens. For instance, the silk
243 of spiders can have antibacterial properties (Wright & Goodacre, 2012) and cuticular antifungals
244 have been found in subsocial spiders (González-Tokman et al., 2014). In addition, there is
245 extensive evidence revealing AMPs in the venom of spiders and scorpions that are active against
246 bacteria, fungi, viruses and parasites *in vitro*, which is being aimed at medical applications
247 (Santos, Reis & Pimenta, 2016; Wang & Wang, 2016). However, we are not aware of studies
248 that investigated the venom – immune system interaction in arachnids when coping with
249 pathogens. Our priming procedure and lethal injection did not allow the interaction between the
250 venom and the bacteria. One might expect that the deactivation of the bacteria by the venom

251 inoculated in the prey may generate a form of priming agent (e.g. dead bacteria) that would act
252 after ingestion.

253 Despite the inherent differences in the immune system of insects and spiders, immune priming
254 seems to be conserved as a general protection mechanism across arthropods taxa. As non-model
255 organisms, arachnids provide alternative systems to study the evolution of immune systems in
256 non-vertebrate animals and our study adds support to the hypothesis that all organisms should
257 have some sort of acquired immunity (Rimer, Cohen & Friedman, 2014).

258 **Conclusions**

259 The aim of the study was to test whether immune priming occurred in two arachnid species: a
260 scorpion and a wolf spider. Injection of bacterial components (LPS) seemed to trigger the
261 immune system of the scorpions as they showed improved survival against alive bacteria as
262 compared to individuals that remained untreated (naive). However, scorpions injected with LPS
263 showed similar survival rates as scorpions injected with only a saline solution (PBS), suggesting
264 that the damage caused by injection may be enough to trigger the upregulation of the immune
265 system. The lack of differences in antibacterial assays with scorpions' hemolymph from the
266 different treatments; together with the lack of evidence for immune priming in spiders, it
267 indicates that the experimental detection of this phenomenon may depend on multiple variables
268 (host – pathogen, priming method, host lifespan, virulence, among other) as proposed in the
269 literature.

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Figure 1

Kaplan-Meier survival curves of scorpions (A) and spiders (B), under different priming treatments.

After the priming period, half of the individuals of each treatment were injected with the control solution (Naive - PBS, PBS - PBS and LPS - PBS) or with the bacterial solution (Naive - Bacteria, PBS - Bacteria, LPS - Bacteria). Scorpions: Naive - PBS, n = 54; Naive - Bacteria, n = 52; PBS - PBS, n = 53; PBS - Bacteria, n = 55; LPS - PBS, n = 51; LPS - Bacteria, n = 57. Spiders: Naive - PBS, n = 55; Naive - Bacteria, n = 48; PBS - PBS, n = 50; PBS - Bacteria, n = 52; LPS - PBS, n = 47; LPS - Bacteria, n = 47. See text for statistical details.

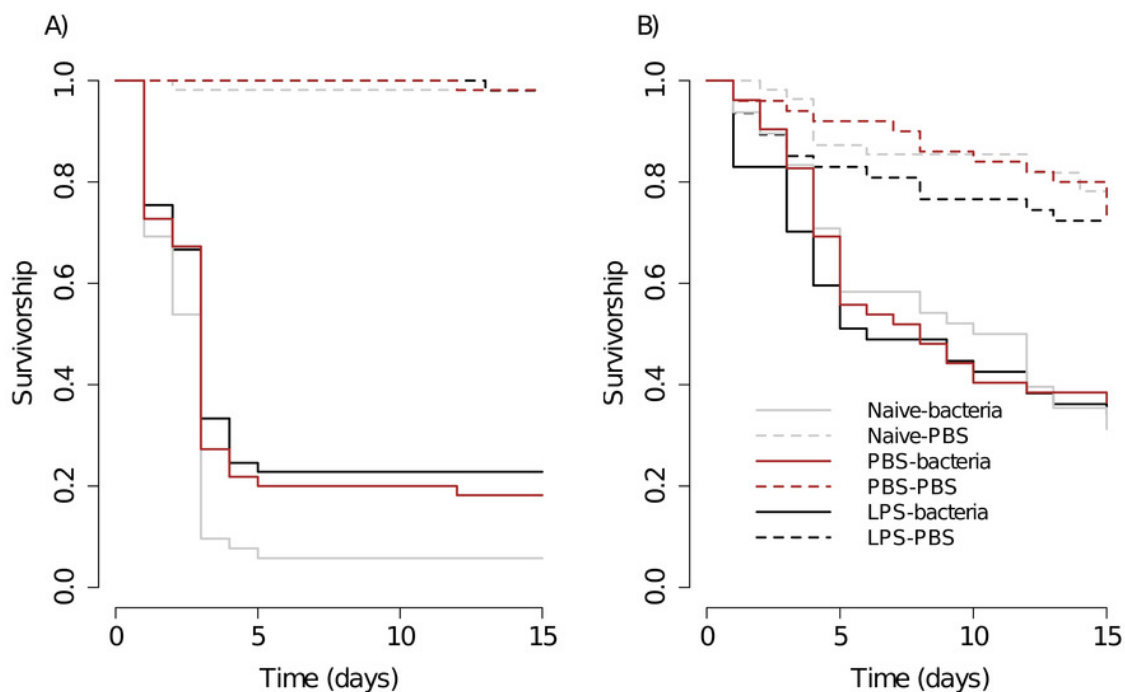


Figure 2

Growth in vitro of *Escherichia coli* when mixed with hemolymph samples of scorpions from the different priming treatments, measured as optical density (OD)

Stars indicate treatments that significantly reduced the bacterial growth as compared to the bacterial growth in the absence of any hemolymph (dashed line, $OD_{630} = 0.763$). Overall, treatments did not differ in their capacity to inhibit the bacterial growth. Naive: $n = 9$; PBS: $n = 6$ and LPS: $n = 9$.

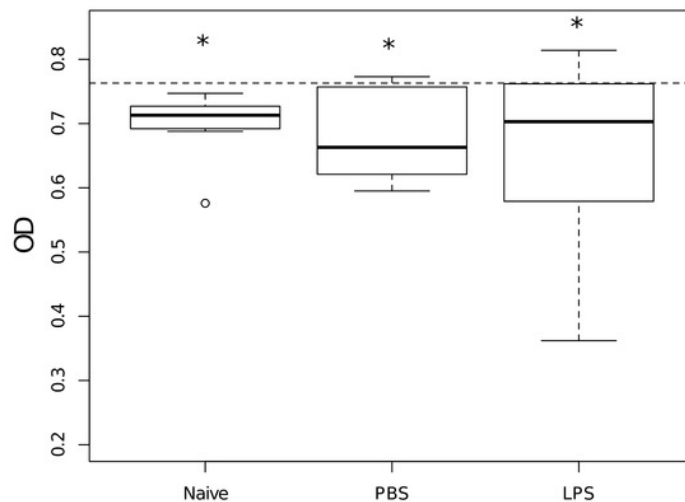


Table 1 (on next page)

Survival analysis pairwise comparisons of priming treatments exposed to a control solution (- PBS) or to a bacterial challenge (- Bacteria).

The Gehan-Breslow-Wilcoxon test compares survival rates at early time points and the Log-rank tests compares them at late time points. See materials and methods for details on priming treatments.

1

<i>Comparison</i>	<i>Gehan-Breslow-Wilcoxon</i>	<i>Log-rank</i>
<i>Scorpions</i>		
Naive - Bacteria vs Naive - PBS	$z = -7.8, p < 0.001$	$z = 4.1, p < 0.001$
PBS - Bacteria vs PBS - PBS	$z = -8.3, p < 0.001$	$z = 4.3, p < 0.001$
LPS - Bacteria vs LPS - PBS	$z = -7.8, p < 0.001$	$z = 4.1, p < 0.001$
LPS - Bacteria vs Naive - Bacteria	$z = 1.9, p = 0.05$	$z = -3.65, p = 0.03$
PBS - Bacteria vs Naive - Bacteria	$z = 1.9, p = 0.11$	$z = -3.65, p = 0.07$
PBS - Bacteria vs LPS - Bacteria	$z = 0.38, p = 0.69$	$z = -0.63, p = 0.53$
Naive - PBS vs PBS - PBS	$z = 0, p > 0.05$	$z = 0.01, p = 0.99$
Naive - PBS vs LPS - PBS	$z = -0.02, p = 0.98$	$z = -0.03, p = 0.98$
PBS - PBS vs LPS - PBS	$z = -0.01, p = 0.98$	$z = -0.03, p = 0.98$
<i>Spiders</i>		
Naive - Bacteria vs Naive - PBS	$z = -4.7, p < 0.0001$	$z = -4.06, p < 0.0001$
PBS - Bacteria vs PBS - PBS	$z = -4.0, p < 0.0001$	$z = -3.7, p < 0.001$
LPS - Bacteria vs LPS - PBS	$z = -3.4, p < 0.001$	$z = 3.4, p < 0.001$
LPS - Bacteria vs Naive - Bacteria	$z = -0.75, p = 0.44$	$z = 0.27, p = 0.79$
PBS - Bacteria vs Naive - Bacteria	$z = -0.06, p = 0.95$	$z = -0.12, p = 0.9$
PBS - Bacteria vs LPS - Bacteria	$z = -0.80, p = 0.42$	$z = 0.41, p = 0.68$
Naive - PBS vs PBS - PBS	$z = 0.53, p = 0.59$	$z = 0.61, p = 0.54$
Naive - PBS vs LPS - PBS	$z = -0.91, p = 0.36$	$z = -0.78, p = 0.44$
PBS - PBS vs LPS - PBS	$z = -0.34, p = 0.73$	$z = -0.19, p = 0.85$

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