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## ► To cite this version:

Cybèle Prigot-Maurice, Baptiste Lheraud, Samuel Guéritault, Sophie Beltran-Bech, Richard Cordaux, et al.. Investigating Wolbachia symbiont-mediated host protection against a bacterial pathogen using a natural Wolbachia nuclear insert. *Journal of Invertebrate Pathology*, 2023, 197, pp.107893. 10.1016/j.jip.2023.107893 . hal-04240965

**HAL Id: hal-04240965**

**<https://hal.science/hal-04240965>**

Submitted on 13 Oct 2023

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1           **Investigating *Wolbachia* symbiont-mediated host protection**  
2           **against a bacterial pathogen using a natural *Wolbachia* nuclear**  
3           **insert**

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12  
13   **Abstract**

14   *Wolbachia* bacterial endosymbionts provide protection against pathogens in various arthropod  
15   species but the underlying mechanisms remain misunderstood. By using a natural *Wolbachia*  
16   nuclear insert (*f*-element) in the isopod *Armadillidium vulgare*, we explored whether *Wolbachia*  
17   presence is mandatory to observe protection in this species or the presence of its genes is  
18   sufficient. We assessed survival of closely related females carrying or lacking the *f*-element  
19   (and lacking *Wolbachia*) challenged with the bacterial pathogen *Salmonella enterica*. Despite  
20   marginal significant effects, the *f*-element alone did not appear to confer survival benefits to its  
21   host, suggesting that *Wolbachia* presence in cells is crucial for protection.

22  
23   **Keywords**

24   *Wolbachia*, nuclear insert, survival assay, host protection, arthropods

## 29 **Introduction**

30 Symbiotic relationships between eukaryotic hosts and microorganisms oscillate along a  
31 continuum of interactions ranging from parasitism to mutualism (Drew et al., 2021). This is  
32 well exemplified by *Wolbachia*, a maternally inherited bacterial endosymbiont known as a  
33 reproductive parasite of many arthropods (Werren et al., 2008). *Wolbachia* can also be  
34 beneficial to its hosts, for example by improving host survival ability when infected with  
35 pathogens, including viruses, fungi and bacteria (Ye et al., 2013; Pan et al., 2018; Pimentel et  
36 al., 2021). In the isopod *Armadillidium vulgare*, the wVulC *Wolbachia* strain causes  
37 feminization of genetic males into phenotypic females (Cordaux et al., 2004) and it also conveys  
38 host protection against infection by the pathogenic bacterium *Salmonella enterica* (Braquart-  
39 Varnier et al., 2015; Prigot-Maurice et al., 2020). *Wolbachia*-infected individuals exhibit lower  
40 loads of *S. enterica* relative to *Wolbachia*-free individuals, suggesting *Wolbachia* triggers an  
41 increased, beneficial resistance against this pathogen (Braquart-Varnier et al., 2008). Yet,  
42 *Wolbachia*-infected individuals show lower haemocyte density and higher natural sepsis of  
43 other bacteria in the hemolymph (Braquart-Varnier et al., 2008). Such a paradox raises the  
44 question of the mechanisms by which *Wolbachia* confers protection to *A. vulgare* against *S.*  
45 *enterica*.

46 As both *Wolbachia* and *S. enterica* have intracellular lifestyles, the main hypothesis for  
47 *Wolbachia*-mediated host protection is direct competition for resource within the cellular niche  
48 (Brown et al., 2009; McLaren & Callahan, 2020; Pimentel et al., 2021). For example,  
49 *Wolbachia* may divert cholesterol production for its replication, thereby depriving *S. enterica*  
50 of this resource for its own use (Caragata et al., 2013; Huang, 2014; Geoghegan et al., 2017).  
51 Another hypothesis is that *Wolbachia* presence may stimulate host immune response against  
52 other infections (Kambris et al., 2009; Moreira et al., 2009; Pan et al., 2012; Ye et al., 2013;  
53 Prigot-Maurice et al., 2022). In *A. vulgare*, immune related-genes are notably up-regulated in  
54 immune tissues of *Wolbachia* carriers (Chevalier et al., 2012). To distinguish between the two  
55 hypotheses, one possibility would be to experimentally inactivate or remove *Wolbachia* from  
56 hosts with antibiotics, but this method may have sublethal effects on both the host and *S.*  
57 *enterica* (Li et al., 2014).

58 The *A. vulgare* system allows an original, alternative strategy to be used to control for  
59 intracellular bacterial competition, as some lines possess a nuclear insert of the nearly complete  
60 *Wolbachia* genome known as the *f*-element, while lacking cytoplasmic *Wolbachia* bacteria

61 (Leclercq et al., 2016; Cordaux and Gilbert 2017). The *f*-element results from the recent  
62 horizontal transfer of a feminizing *Wolbachia* genome closely related to *wVulC*, as indicated  
63 by the 99.7% nucleotide identity between the *f*-element and *wVulC* genomic sequences  
64 (Leclercq et al., 2016). Moreover, at least some *Wolbachia*-derived genes probably continue to  
65 be expressed because the *f*-element induces feminization in some lineages, as *wVulC*  
66 *Wolbachia* symbionts do (Leclercq et al., 2016). In this context, we aimed at testing whether  
67 the *f*-element alone can confer a protective effect to *A. vulgare* against *S. enterica* or whether  
68 *Wolbachia* presence is mandatory to observe such protection.

69

## 70 **Materials and Methods**

71 To control for genetic background, we performed controlled crosses which resulted in closely  
72 related individuals carrying either the *f*-element (F-females) or not (NOF-females). A detailed  
73 description of the crossing scheme and methods is provided in supplementary information. As  
74 opposed to *Wolbachia*-infected lineages, in which all non-*Wolbachia* carriers are males,  
75 siblings differing by the presence of the *f*-element can be of the same sex.

76 We then used 196 one year +/- 3-month-old virgin females (89 F-females and 107 NOF-  
77 females) in a survival assay to *S. enterica* infection. Specifically, we applied three different  
78 treatments: (1) females not injected at all (17 F-females and 20 NOF-females) to control natural  
79 mortality during experiment, (2) females injected with sterile PBS (20 F-females and 26 NOF-  
80 females) to control **the injection effect**, and (3) females injected with  $8.10^5 \pm 10^3$  *Salmonella*  
81 *enterica typhimurium* in 100 nL of sterile PBS (52 F-females and 61 NOF-females). The *S.*  
82 *enterica* strain, culture, dosage, solution compositions and injection method are described in  
83 Prigot-Maurice et al. (2019). The survival assay was performed in five runs, each containing F-  
84 females and NOF-females (see supplementary information for details). Following injections,  
85 we maintained females in individual boxes with wet soil and rehydrated linden leaves. We  
86 monitored their survival rate daily for 7 days (Prigot-Maurice et al., 2019, 2020).

87 Statistical analyses were performed with R software v.4.1.0 (R Core Team, 2022). As no  
88 mortality was observed in the non-injected and PBS injected females, we only compared the  
89 survival rate of *S. enterica* injected F-females and NOF-females with a mixed effect Cox  
90 proportional hazard regression. **This model was built with *survival* (Therneau and Grambsch,**  
91 **2000) and *coxme* packages (Therneau et al., 2003) and tested with Wald  $\chi^2$  using the *car***

92 **package** (Bates et al., 2015). This model included the type of females as categorical fixed effect  
93 and two random factors: the run to account for experimental variability (Harrisson et al., 2018)  
94 and the family to which each female belongs to account for genetic variability. The hazard ratio  
95 (HR) estimating the instantaneous relative risk of death at any given time point (Sashegy &  
96 Ferry, 2017) was obtained by using NOF-females as reference group and statistically tested by  
97 the Log-Rank test (*survival package*). Rscript and dataset are available on Mendeley Data  
98 repository platform

99 ([https://data.mendeley.com/datasets/ndcmv55yyx/draft?a=dd3a1b26-e5c1-4725-87d4-](https://data.mendeley.com/datasets/ndcmv55yyx/draft?a=dd3a1b26-e5c1-4725-87d4-61660df9ef15)  
100 [61660df9ef15](https://data.mendeley.com/datasets/ndcmv55yyx/draft?a=dd3a1b26-e5c1-4725-87d4-61660df9ef15)).

101

## 102 **Results and discussion**

103 To test if the *f*-element confers protection against *S. enterica*, we compared survival of F-  
104 females and NOF-females following experimental injection of *S. enterica*. No mortality was  
105 observed in the control treatments, including non-injected females and females injected with  
106 sterile PBS (Fig. 1). **By contrast, females injected with *S. enterica* showed reduced survival at**  
107 **7 days of monitoring**, with survival rates of 42% (22/52) for F-females and 20% for NOF-  
108 females (12/61) (Fig. 1). Statistical analysis showed no significant difference at the 5% risk in  
109 survival ability between F-females and NOF-females when infected with *S. enterica* ( $X^2 = 3.58$ ,  
110  $df = 1$ ,  $p = 0.058$ ), although F-females survived approximately twice as well as NOF-females.  
111 The instantaneous relative risk of death of F-females was significantly lower (38%) than that

112 of NOF-females (HR = 0.62, CI: [0.39;0.98]; Log-Rank test: p = 0.039), indicating that F-  
 113 females have less risk to die at each time point compared to NOF-females.

114

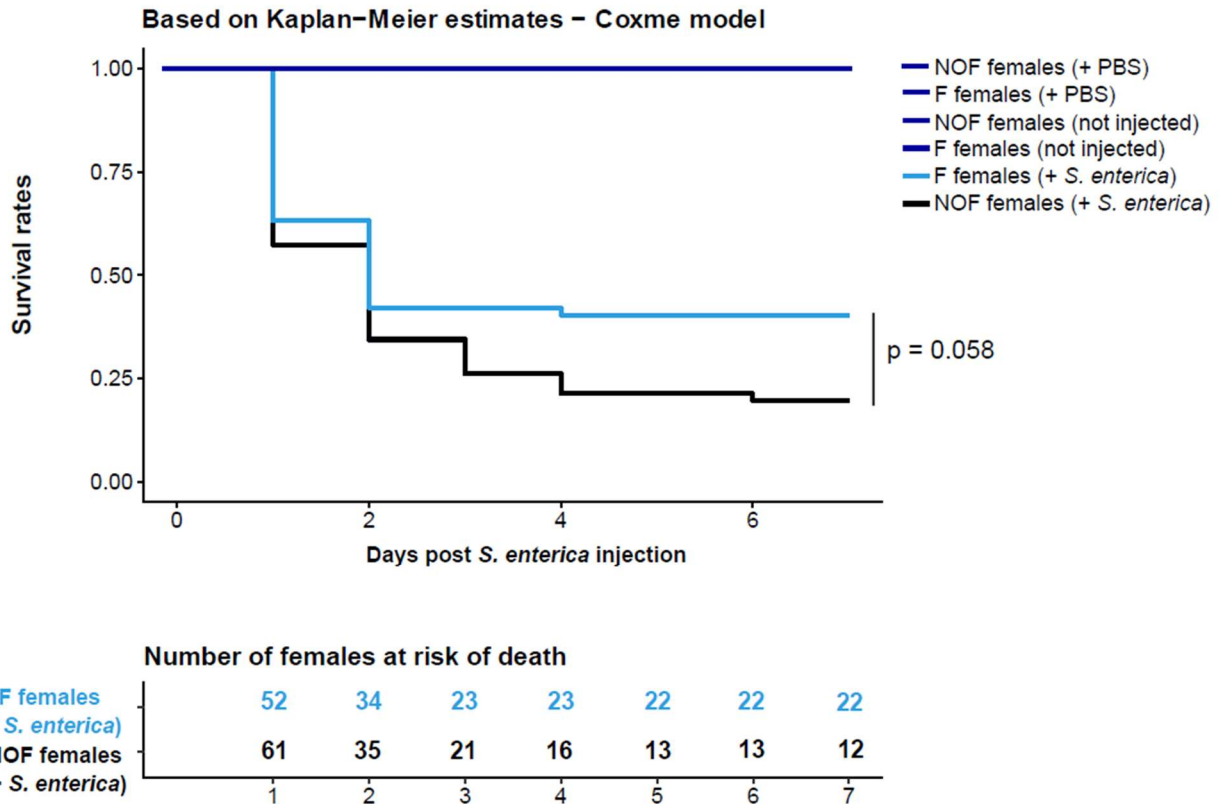


Fig. 1: Survival of *A. vulgare* females over 7 days after one injection of *S. enterica*. F-females: females carrying the *f*-element; NOF-females: females lacking the *f*-element. The number of females at risk of death shows the remaining number of females still accounted for that had not experienced event of death for each day following *S. enterica* injection.

115 Our results did not show that the *f*-element alone provides significant survival benefit to *A.*  
 116 *vulgare* females when infected with the pathogenic bacterium *S. enterica* (at the 5% risk). As  
 117 the *f*-element is a nuclear insert of the *Wolbachia* genome, the physical presence of *Wolbachia*  
 118 symbionts within host cells seems to be required to confer protection against *S. enterica* host  
 119 lethality. This conclusion is in line with other studies, demonstrating that *Wolbachia* removal  
 120 from *Drosophila* and *Aedes aegypti* symbiotic hosts results in concomitant removal of  
 121 protection, for both antiviral and antibacterial effects (Hedges et al., 2008; Moreira et al., 2009;  
 122 Chrostek et al., 2013; Ye et al., 2013). It is well known that *Wolbachia* bacteria exert strong  
 123 modifications on physiological and morphological parameters of hosting cells (Brennan et al.,  
 124 2008; Frientu et al., 2010; White et al., 2017; Nainu et al., 2019). Such a cellular stress caused  
 125 by *Wolbachia* physical presence would prevent any other pathogen from taking up residence in

126 the same cells (Lindsey et al., 2018). Hence, despite the presence of the *f*-element in the cell  
127 nucleus, absence of *Wolbachia* in the cytoplasm may allow *S. enterica* to replicate in host cells,  
128 thus maintaining its pathogenic activity. If the protective effect of *Wolbachia* against pathogens  
129 confers an evolutionary benefit to maintain the symbiosis with its host (Lindsey et al., 2018), it  
130 would follow that *Wolbachia* presence is mandatory to induce protection. However, the absence  
131 of a significant survival benefit when females carried the *f*-element does not demonstrate that  
132 the *f*-element is not involved in protection, especially as we observed marginally significant  
133 effects.

134

## 135 **Conclusion**

136 Without strictly excluding a protective role conferred by the *f*-element, our results leave the  
137 possibility that the physical presence of *Wolbachia* in the host cells, not just the presence of  
138 some of its genes, helps to confer protection against *S. enterica*. The protection mediated by  
139 *Wolbachia* may require specific genes that are not present – or not expressed – in the *f*-element.  
140 Alternatively, this protection may be related to important physiological or immunological  
141 changes in hosts due to *Wolbachia* presence, such as an up-regulation of the basal immunity or  
142 an immune priming effect (Pan et al., 2012; Pan et al., 2018; Prigot-Maurice et al., 2022), that  
143 cannot only be triggered by the *f*-element nuclear inclusion in the host. By contributing to the  
144 understanding of the mechanisms that may explain the protection conferred by *Wolbachia*, our  
145 results open new research horizons on the genetic aspect of the symbiotic interaction between  
146 *Wolbachia* and its hosts.

147

## 148 **Declaration of competing interest**

149 None.

## 150 **Acknowledgements**

151 We thank Alexandra Lafitte and Hélène Paulhac for maintenance of *A. vulgare* lines in the  
152 EBI breeding facility.

## 153 **Funding**

154 This work was partly funded by Agence Nationale de la Recherche grant ANR-20-CE02-004  
155 (SymChroSex) to JP and intramural funds from the French Ministry of Higher Education and  
156 Research, the University of Poitiers and the French National Centre for Scientific Research.

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