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## Deleterious effects of *Wolbachia* on life history and physiological traits of common pill woodlice

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### ABSTRACT

Most of eukaryotic organisms live in close interaction with micro-organisms called symbionts. Symbiotic interactions underpin the evolution of biological complexity, the health of organisms and, ultimately, the proper functioning of ecosystems. While some symbionts confer adaptive benefits on their host (mutualistic symbionts) and others clearly induce costs (parasitic symbionts), a number of micro-organisms are difficult to classify because they have been described as conferring both benefits and costs on their host. This is particularly true of the most widespread animal endosymbiont, *Wolbachia pipiensis*. In this study, we investigated the influence of *Wolbachia* infection on a broad spectrum of ecological and physiological parameters of one of its native hosts, *Armadillidium vulgare*. The aim was to gain as complete a picture as possible of the influence of this endosymbiont on its host. Our results showed that the presence of *Wolbachia* resulted in a decrease in individual reproductive success and survival. Host immune cells density decreased and  $\beta$ -galactosidase activity (ageing biomarker) increased with the presence of *Wolbachia*, suggesting a negative impact of this endosymbiont on woodlice health. While previous studies have shown that *Wolbachia* can have a positive impact on the immunocompetence of *A. vulgare*, here we shed more light on the costs of infection. Our results illustrate the complex dynamics that exist between *Wolbachia* and its arthropod host and therefore offer valuable insights into the intricate interplay of symbiotic relationships in ecological systems.

### 1. Introduction

Living organisms face a myriad of challenges, including exposure to both biotic (e.g., competition and parasitic infections) and abiotic (e.g., temperature and humidity changes) stresses in their environment. Biotic stresses, especially those imposed by symbionts, can induce significant changes in life history traits and physiology of their hosts (Hector et al., 2022; Leonardo and Mondor, 2006; Shapira, 2016). Nevertheless, though certain symbionts provide adaptive advantages, known as mutualistic symbionts, and others undoubtedly impose costs, referred to as parasitic symbionts, a subset of microorganisms poses challenges in classification since they have been described as conferring benefits to their host while negatively impacting certain ecological or physiological traits. The balance between the costs and benefits of the symbiotic interactions is therefore complex and not totally understood (Douglas, 2021, 2014; Prigot-Maurice et al., 2022). For instance, the enterobacteria *Serratia marcescens*, have been shown to impact differently both survival and reproduction in their different hosts, leading to an

ambiguity in the cost-benefit balance of these interactions (Adamo et al., 2001; Laughton et al., 2014). The same outcome is observed in *Culex pipiens* mosquitoes infected with one of the agents responsible for avian malaria, *Plasmodium relictum*. This apicomplexan parasite reduces the fertility of the females but significantly increases their survival rate (Pigeault and Villa, 2018; V  zilier et al., 2012). The benevolence of symbiotic interactions may also vary with the environment. The cost-benefit balance of a symbiotic association may vary with the presence or absence of other organisms in the surrounding habitat. For example, some secondary symbionts known to confer protection to their host against parasite infection can be costly to the host when parasites are not present (Leybourne et al., 2020).

The complexity in assessing the costs and benefits of symbiotic interactions may stem from resource allocation trade-offs, as proposed by life history theory (Cody, 1966; Lema  tre et al., 2015; Stearns, 1992, 1989). An explanation could be that some symbionts manipulate certain traits that are beneficial to them, and due to trade-offs, this manipulation negatively impacts other traits (as suggested by V  zilier et al. 2012).

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Additionally, inconsistencies arise from independent studies that measure costs in some cases and benefits in others, hindering a comprehensive evaluation of the interaction's overall impact. To address this challenge, it is crucial to study the influence of symbiotic interactions on a broad spectrum of ecological and physiological traits of the host, including parameters associated with survival, reproduction, immunity, as well as biomarkers indicative of the host's physiological health.

Among symbionts, the alpha-proteobacterium *Wolbachia*, a vertically transmitted endosymbiont, is widespread in arthropods (Cordaux et al., 2004; Correa and Ballard, 2016; Werren et al., 2008; Zug and Hammerstein, 2012). Extensive research has been conducted on the diverse effects of *Wolbachia* on host reproduction, ranging from obligatory mutualism in nematodes to various reproductive manipulations in other host species (Werren et al. 2008). In the terrestrial isopod *Armadillidium vulgare*, *Wolbachia* induces feminization (Cordaux et al., 2004) and is considered as a reproductive parasite, which entails costs for its host (Braquart-Varnier et al., 2008; Caubet et al., 1998; Juchault and Mocquard, 1989; Moreau et al., 2001; Rigaud and Moreau, 2004; Templé and Richard, 2015; Valette et al., 2017). However, *Wolbachia* also appears to confer certain benefits to its terrestrial isopod host by providing, for example, immune protection against infection by intracellular pathogenic bacteria (Braquart-Varnier et al., 2015; Prigot-Maurice et al., 2021).

Given the accessibility of molecular and cellular biomarkers for assessing individual quality in common pill woodlice (Depeux et al. 2020), this species emerges as a pertinent experimental model for investigating the influence of biotic stress, such as *Wolbachia* infection, on life history and physiological traits. In this study, we specifically examine the impact of *Wolbachia* on key life history traits and selected biomarkers, which were developed for their association with ageing patterns (Depeux et al., 2023, 2020) in 1-year-old woodlice females. Subsequently, we extend our analysis to 2-year-old females to observe the potential evolution of these biomarkers over the organism's lifetime. We hypothesize that *Wolbachia* exert negative effects on these traits, with the possibility of amplification with age, as previously demonstrated with abiotic stressors (thermal and water stresses) that prematurely aged *A. vulgare* (Depeux et al., 2023). Our approach provides a comprehensive framework for understanding the different aspects of the intricate interaction between the most widespread endosymbiont and on of it arthropod host.

## 2. Materials and methods

### 2.1. Biological model – routine breeding

*Armadillidium vulgare* individuals used in this study descended from a natural population coming from Helsingør (Denmark) and brought into the laboratory in 1982. In *A. vulgare* populations infected with *Wolbachia*, the bacterium is not transmitted at 100 %, resulting in the coexistence of both infected and un-infected animals within the same population. From the population collected on the field, two lineages have been maintained in the lab: one un-infected by *Wolbachia* (i.e. 'asymbiotic' population, which we used in this study), derived from un-infected individuals of the initial natural population, and another infected by the *Wolbachia* strain wVulC, which allows us to maintain this strain of bacterium. Over time, the maintenance of these two lineages since the 1980s has led to gradual divergence between the host lineages. Therefore, to accurately compare the effects of this *Wolbachia* strain on its host, we opted to reinfest hosts from the "asymbiotic" lineage in 2012 (see Le Clec'h et al., 2012), allowing us to obtain a new "symbiotic" lineage and to compare hosts sharing the same genetic background, with or without the wVulC strain. In this context, the asymbiotic lineage was naturally un-infected by *Wolbachia* while the symbiotic one was experimentally infected by the feminizing *Wolbachia* strain wVulC. Symbiotic lineage was maintained by crossbreeding infected symbiotic females with un-infected asymbiotic males to maintain the same genetic

background with the asymbiotic lineage. All individuals were maintained under natural photoperiod at 20 °C. Animals from the same clutches were sorted according to their sex (to prevent inbred reproduction) and reared in 10 × 30 cm plastic boxes containing moistened potting mix supplied with *ad libitum* linden leaves and fresh carrot slices. For the investigation of *Wolbachia* infection effects, only females from the two lineages were utilized in this study. This choice is attributed to the scarcity of males in the symbiotic lineage, a result of the feminization effect induced by *Wolbachia*.

### 2.2. Experimental design

The experiment began in January 2020 and involved four groups of virgin females infected or not by *Wolbachia* and aged of 1-year-old (considered as young mature female) or 2-years-old (considered as old mature females). The selected biomarkers (i.e., immune cell parameters, TERT expression and β-galactosidase activity) were compared between the four groups of females. The selected life history traits (i.e., reproductive success and survival) were only compared between 1-year-old virgin females infected or not by *Wolbachia*. The number of 2-years-old females was not sufficient to carry out all the experiments and we decided to focus on the study of *Wolbachia*'s influence on biomarkers. The Table 1 contains the numbers of used animals for the study.

### 2.3. Influence of *Wolbachia* infection on woodlice survival and reproductive success

The influence of *Wolbachia* infection on the survival rate of woodlice was evaluated weekly over a period of 119 days. In parallel, 40 virgin females aged of 1-year-old from both *A. vulgare* lineages were crossed with un-infected and virgin males of the same age. Each breeding pair was placed in a box, at 20 °C, with food provided *ad libitum* and in a photoperiod of 16:8 (L/D) known to stimulate reproduction (Mocquard et al., 1989). We followed all pairs for five months during which each clutch produced was recorded. At the end of this period, the ability to produce a clutch (i.e., the probability that one clutch is produced by a given pair) and the reproductive success of woodlice (i.e., total number of offspring in the first clutch) was compared between infected and un-infected animals. Focusing on the first clutch for the evaluation of reproductive success allows us to confidently compare females at the same developmental stage. While additional clutches can occur (even after a single fertilization), they tend to be more asynchronous and unpredictable in *A. vulgare*, complicating comparisons across different time points. As a result, the first clutch emerges as the most relevant and

**Table 1**  
Number of animals used in each experiment. YW-: young (1-year-old) un-infected females; YW+: young (1-year-old) infected with *Wolbachia* females; OW-: old (2-years-old) un-infected females; OW+: old (2-years-old) infected with *Wolbachia* females; NA non tested; TERT: telomerase reverse transcriptase.

		YW-	YW+	OW-	OW+
<b>Life history traits</b>					
Survival	(following during 17 weeks)	30	30	NA	NA
Reproduction	(nb of pairs)	40	41	NA	NA
<b>Physiological traits</b>					
Immune cells (Hemocytes in haemolymph)	Cell density Cell viability Cell size	22	25	14	14
TERT		7 pools of 5 female ovaries	5 pools of 5 female ovaries	7 pools of 5 female ovaries	6 pools of 5 female ovaries
β-galactosidase		21	23	14	14

comparable measure.

## 2.4. Biomarkers

### 2.4.1. Immune cells

As immune cells are free-circulating hemocytes in the animal haemolymph, they can inform about a potential premature biological ageing (Sicard et al., 2010). Indeed, we have previously demonstrated that these immune cells decrease in density and viability while increasing in size with the ageing of *A. vulgare* (Depeux et al. 2020). To study the influence of *Wolbachia* infection on the evolution of these parameters during ageing of *A. vulgare*, we collected 3  $\mu$ L of haemolymph per individual (see Table 1 for details on the number of used females in each tested group) and placed it in 15  $\mu$ L of MAS-EDTA (EDTA 9 mM, Trisodium citrate 27 mM, NaCl 336 mM, Glucose 115 mM, pH 7, (Rodriguez et al. 1995)). We then added 6  $\mu$ L of Trypan Blue at 0.4 % (Invitrogen®) to discriminate live and dead cells. Ten microliters of this solution were put in Invitrogen Countess® counting slide and put in an automated Cell Counter (Invitrogen) to quantify cell density (measured as the number of immune cells per mL of haemolymph), viability (measured as the proportion of live cells) as well as cell size (in  $\mu$ m).

### 2.4.2. TERT expression

The TERT is telomerase reverse transcriptase, an essential subunit of telomerase protein and has been shown to be related to cell survival in humans (Cao et al., 2002). We expect, as previously observed, an age-dependent decrease in TERT gene expression in *A. vulgare* (Depeux et al. 2020). In order to measure TERT expression and compare it between females infected or un-infected by *Wolbachia*, we harvested ovaries from each female and pooled (5 to 7 pools of 5 pairs of ovaries for each group, Table 1) to obtain sufficient quantity of biological material. We chose this tissue because *Wolbachia* is localized in the ovaries and is transmitted through them, thereby feminizing the next generation. In this context, the use of this tissue is particularly relevant. We deposited these tissues in TRIzol (Invitrogen®) for RNA extraction according to the manufacturer's protocol. After cell disintegration using a Vibra Cell 75.185 sonicator (30 % amplitude), the samples were stored at  $-80^{\circ}\text{C}$  until use. We performed reverse transcriptases using a 170 ng RNA solution and the SuperScript™ IV kit (ThermoFisher®). We used the following primers: TERT\_F primer: 5'-AGGGAAAACGATGCACAACC-3' and TERT\_R primer: 5'-GTTTCGCCAAATGTTCCGAAC-3' (Chebbi et al. 2019; Depeux et al. 2020). RT-qPCR was performed using 0.5  $\mu$ L of each primer (10  $\mu$ M), 1.5  $\mu$ L of nuclease-free water, 5  $\mu$ L of SYBRGreen 2X Mix and 2.5  $\mu$ L of cDNA and a thermal cycler with the following programme: 10 min at  $95^{\circ}\text{C}$ , 45 cycles of 10 s at  $95^{\circ}\text{C}$ , 10 s at  $60^{\circ}\text{C}$  and 20 s at  $72^{\circ}\text{C}$ . The expression levels of the target gene were normalised to the expression levels of two previously established reference genes: ribosomal protein L8 (RbL8) and elongation factor 2 (EF2) (Chevalier et al. 2011).

### 2.4.3. $\beta$ -galactosidase activity

The  $\beta$ -galactosidase activity is commonly used as a biomarker of cellular senescence (Lee et al., 2006). This enzyme functions as a hydrolase, converting polysaccharides into monosaccharides. Its lysosomal activity becomes elevated as cells undergo senescence (Dimri et al., 1995; Itahana et al., 2007). This increase in activity has been observed in senescent cells across a variety of organisms, from humans (Gary & Kindell, 2005) to honeybees (Hsieh and Hsu, 2011) and we previously showed that its activity increases with age in *A. vulgare* (Depeux et al., 2020). To investigate the effect of *Wolbachia* infection on  $\beta$ -galactosidase activity, we dissected and removed the nerve cord of one- and two-years-old females after having collected haemolymph for assessing the immune parameters (see above). We chose the nerve cord tissue because it is not renewed during molting and is therefore relevant candidate to measure level of  $\beta$ -galactosidase activity and optimize the chances of detecting an effect of *Wolbachia* on senescence, if it exists. Nerve cords

were placed individually in a tube containing 300  $\mu$ L of lysis buffer 1X (CHAPS 5 mM, Citric acid 40 mM, Sodium Phosphate 40 mM, Benzamidine 0.5 mM, PMSF 0.25 mM, pH = 6) (Gary & Kindell, 2005). We centrifuged the sample at 15 000g for 30 min at  $4^{\circ}\text{C}$  and then we collected and kept the supernatant at  $-80^{\circ}\text{C}$ . We quantified the protein concentration thanks to the BCA Assay and we homogenized all samples at the 0.025 mg/mL protein concentration. Then, 125  $\mu$ L of these protein extracts were added to 125  $\mu$ L of reactive 4-methylumbelliferyl-D-galactopyranoside (MUG) solution. The synthesis of the fluorescent 4-methylumbelliferone (4-MU), the result of the contact of MUG reactive with  $\beta$ -galactosidase, was measured using the multimode microplate reader Mithras LB940 133 HTS III, Berthold; excitation filter: 120 nm, emission filter 460 nm, for 120 min. We performed two technical replicates for each sample and used the average of these two replicates as a measure of enzyme activity.

## 2.5. Statistical analyses

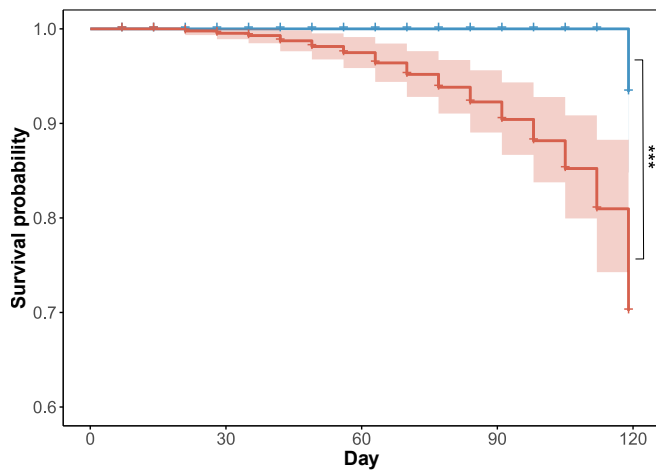
All statistical analyses were performed with R (v. 4.1.3). The sample sizes included in each analysis and the different statistical models built to analyze the data are described in the supplementary material (Table S1). Generalized linear models (glm), with an error structure appropriate to each response variable (see Table S1), were used to test for difference in biomarkers (i.e., parameters associated to immune cells, TERT and  $\beta$ -galactosidase activity,) according to woodlouse infection status (i.e., infected or un-infected by *Wolbachia*). Woodlouse age (i.e., one- or two-years-old) was also added as explanatory variable in all analyses relating to biomarkers. The influence of *Wolbachia* infection on woodlouse reproductive parameters (i.e., the probability that a given pair produces at least one brood and, when the females have laid eggs, the number of offspring born from the first brood) were analyzed using glm with an error structure appropriate to each response variable (see Table S1). Woodlouse survival rate was analyzed using Cox proportional hazards regression model (coxph, survival package (Therneau, 2024) with infection status fitted as explanatory variable. Maximal models, including all higher-order interactions, were simplified by sequentially eliminating non-significant interactions and terms to establish a minimal model (Crawley, 2012). The significance of the explanatory variables was established using a Chi-square or F test according to the error structure. The significant Chi-square or F values given in the text are for the minimal model, whereas non-significant values correspond to those obtained before the deletion of the variable from the model (Crawley, 2012). The figures were created using the ggplot2 package (Wickham, 2016).

## 3. Results

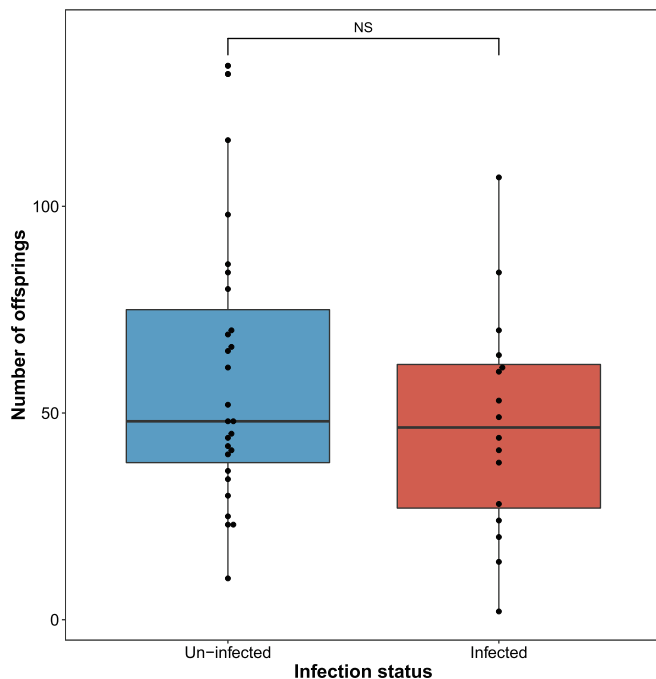
### 3.1. Life history traits

As mentioned above, the influence of *Wolbachia* infection on host life history traits was only studied in one-year old females. Survival was strongly impacted by infection by *Wolbachia* (model 1,  $\chi^2_1 = 38.624$ ,  $p < 0.001$ ). The mortality risk was more than fifteen times higher in infected individuals than in un-infected ones (hazard ratio = 18.68, 95 %CI [4.501;77.498], Fig. 1).

The proportion of females having offspring during the experiment was significantly higher in un-infected females than in infected individuals (infected females: 0.675, 95 %CI [0.523; 0.827], un-infected females: 0.390, 95 %CI [0.234; 0.546], model 2,  $\chi^2_1 = 6.688$ ,  $p = 0.009$ , estimate =  $-1.177$ , 95 %CI [ $-2.113$ ;  $-0.281$ ]). However, the number of offspring in egg-laying females did not vary significantly with infection status (model 3,  $\chi^2_1 = 1.551$ ,  $p = 0.213$ , estimate =  $-0.224$ , 95 %CI [ $-0.589$ ;  $0.126$ ], Fig. 2).



**Fig. 1. Survival probability.** Survival curves comparing *Wolbachia*-infected (red) and un-infected (blue) *A. vulgare* females over a 120-day period. The semi-transparent red colour corresponds to the 95 % confidence interval. \*\*\*  $p < 0.001$ . (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



**Fig. 2. Reproduction parameters.** Graphics comparing the number of offspring between *Wolbachia*-infected (red) and un-infected (blue) *A. vulgare* females. NS: non-significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

### 3.2. Biomarkers

Immune cell density did not vary significantly as the woodlice aged (model 4,  $F = 0.776$ ,  $p = 0.381$ ). On the other hand, we found that infected woodlice had a significantly lower circulating immune cell density than un-infected individuals (model 4,  $F = 13.43$ ,  $p < 0.001$ , estimate =  $-0.835$ , 95 %CI [ $-1.280$ ;  $-0.388$ ], Fig. 3A). Immune cell viability was not affected by age (model 5,  $\chi^2_1 = 3.695$ ,  $p = 0.055$ , estimate =  $-0.294$ , 95 %CI [ $-0.593$ ;  $0.006$ ]) but was significantly different between infected and un-infected individuals (model 5,  $\chi^2_1 = 4.969$ ,  $p = 0.025$ , estimate =  $0.348$ , 95 %CI [ $0.042$ ;  $0.659$ ], Fig. 3B). Immune cell viability was significantly higher in *Wolbachia*-infected

animals than in un-infected individuals (infected: 64.36 %, un-infected: 59.88 %). Immune cell size was not affected by both age and infection status (model 6, age:  $F = 0.213$ ,  $p = 0.646$ , estimate =  $-0.001$ , 95 %CI [ $-0.053$ ;  $0.327$ ], infection status:  $F = 0.001$ ,  $p = 0.998$ , estimate =  $0.001$ , 95 %CI [ $-0.041$ ;  $0.041$ ]).

The TERT gene expression did not vary according to both woodlouse age and infection status (model 7, age:  $F = 0.002$ ,  $p = 0.963$ , estimate =  $-0.002$ , 95 %CI [ $-0.076$ ;  $0.073$ ], infection status:  $F = 0.750$ ,  $p = 0.386$ , estimate =  $0.038$ , 95 %CI [ $-0.107$ ;  $0.044$ ], Fig. 3C).

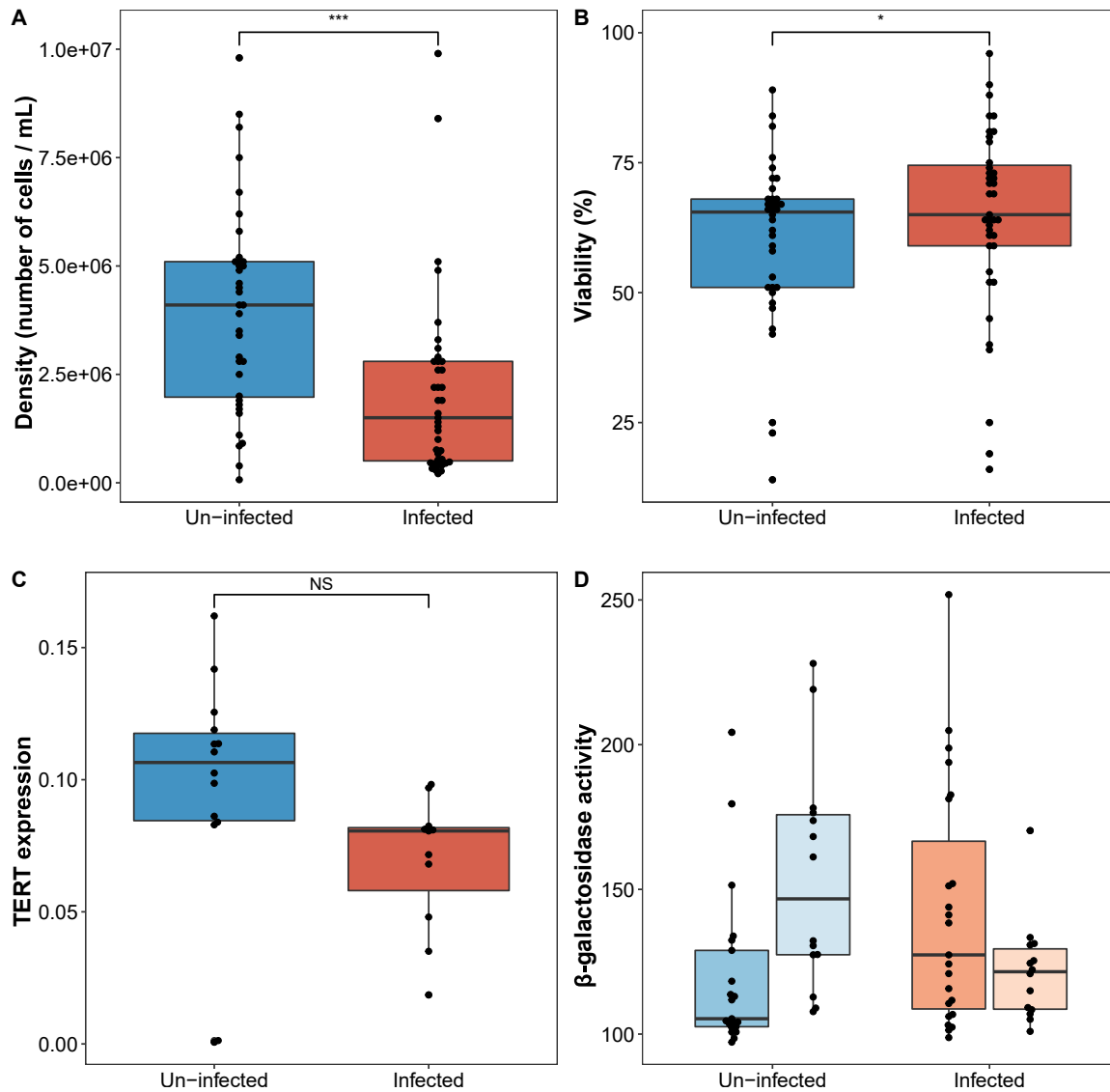
Significant interaction between age and infection status was observed regarding the  $\beta$ -galactosidase activity (model 8,  $F = 10.935$ ,  $p = 0.001$ , estimate =  $-54.564$ , 95 %CI [ $-86.587$ ;  $-22.54$ ]). In un-infected individuals,  $\beta$ -galactosidase activity increased with age (model 9,  $F = 9.105$ ,  $p = 0.005$ , estimate =  $34.171$ , 95 %CI [ $11.975$ ;  $56.366$ ], Fig. 3D), whereas it did not vary significantly in infected animals (model 10,  $F = 3.013$ ,  $p = 0.091$ , estimate =  $-20.393$ , 95 %CI [ $-43.419$ ;  $2.633$ ], Fig. 3D). The interaction can also be interpreted in another way. In young animals,  $\beta$ -galactosidase activity was higher in *Wolbachia*-infected individuals than in un-infected females (model 11,  $F = 4.349$ ,  $p = 0.037$ , estimate =  $22.571$ , 95 %CI [ $1.391$ ;  $43.753$ ]). Conversely, in older females,  $\beta$ -galactosidase activity was lower in infected than in un-infected animals (model 12,  $F = 7.382$ ,  $p = 0.006$ , estimate =  $-31.991$ , 95 %CI [ $-54.381$ ;  $-9.6022$ ]).

## 4. Discussion

Our study investigated the impact of *Wolbachia* infection on various immune parameters and life history traits in the woodlouse *A. vulgare*. The results present a nuanced understanding of how *Wolbachia* infection and the age factors interplay to influence host immune function and reproductive success.

### 4.1. Life history traits

*Wolbachia* infection had a profound impact on both the survival rate and the reproductive success of woodlice, which aligns with the pathogen's typically detrimental effects on host fitness (Anderson and May, 1978; Dallas et al., 2016). A decrease in survival rate was previously reported in *A. vulgare* infected by *Wolbachia* wVulC (Braquart-Varnier et al., 2008) but also in other *Wolbachia*-infected arthropod species as *Aedes aegypti* (Ross et al., 2020) or *Drosophila melanogaster* (Serga et al., 2021) infected by *Wolbachia* wMel. This decrease might reveal the cost induced by *Wolbachia* on its hosts. The reduction in host longevity could also be due to manipulation of the parasite, with a reallocation of the host's energy resources towards another key life history trait involved in *Wolbachia* transmission, namely reproduction. Since *Wolbachia* is transmitted maternally, it could be adaptive to manipulate the hosts so that it produces more offspring, potentially to the detriment of survival. However, as with the survival rate, the reproductive success of *A. vulgare* was partly negatively affected by *Wolbachia* wVulC infection. The proportion of infected females that laid eggs during the monitoring was around a third lower than that of un-infected females. Given the disadvantages of reducing the fitness of their host (i.e., fitness alignment), it would be expected that *Wolbachia* does not have a negative impact on host's reproduction. But previous studies have also observed negative effects of *Wolbachia* infection on host fertility and/or fecundity, whether in the context of a natural *Wolbachia* host association (de Almeida et al., 2011; Lau et al., 2021; Maciel de Freitas, R. et al., 2024; Rigaud et al., 1999) or following experimental infection (e.g., injection of *Wolbachia* into naturally un-infected hosts (Pigeault et al., 2014; Rigaud et al., 2001)). However, in our study, the fact that the proportion of females that produced offspring was lower among infected females was not necessarily due to an inability to reproduce. It could be explained by a delay in the timing of egg-laying. Future studies should now investigate the effect of *Wolbachia* infection on lifetime reproductive success and not only on current reproductive success.



**Fig. 3. Physiological traits.** Box plots comparing *Wolbachia*-infected (red) and un-infected (blue) *A. vulgare* females across various physiological traits: A. Hemocyte density (number of cells per mL), B. Percentage of hemocyte viability, C. TERT expression, and D.  $\beta$ -galactosidase activity. In panel D, light blue and light red indicate two years old animals. NS: non-significant; \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

## 4.2. Physiological traits

### 4.2.1. Immune cell parameters

Regarding the profile of immune cells, our study demonstrates that *Wolbachia* had a negative impact on the cellular concentration, no effect on the immune cell size and a positive effect on the immune cell viability. Several studies supported the observation that *Wolbachia*-infected animals experience a decrease in the concentration of their immune cells (Braquart-Varnier et al., 2008; Sicard et al., 2010). We hypothesise that the presence of *Wolbachia* within immune cells can disrupt their function and lead to early cell death and/or disrupt their production in hematopoietic organs, leading to a scenario where fewer immune cells are observed in *Wolbachia*-infected animals. However, the size of immune cells remained unaffected by both age and infection status. This consistency in cell size might indicate that *Wolbachia* does not interfere with the maturation or morphological characteristics of immune cells, even though it affects their quantity. The viability of immune cells was significantly higher in *Wolbachia*-infected animals compared to un-infected ones. This counterintuitive finding suggests that while *Wolbachia* decreases immune cell density, it may enhance the

functional quality or survival of the remaining cells. This phenomenon could be a strategic manipulation used by *Wolbachia* to maintain a minimally effective immune system that does not completely compromise the host's health, thus ensuring its own persistence and transmission. Another hypothesis is that *Wolbachia* may sustain the viability of the colonized hemocytes to ensure its own survival. Indeed, if the hemocytes die, some *Wolbachia* would perish along with them. Consequently, un-infected hemocytes may undergo natural cell death due to the host's limited energy resources, leading to a reduced total hemocyte density in infected hosts. However, the subset of hemocytes infected by *Wolbachia* (approximately 35–50 %, as reported in (Chevalier et al., 2011)) might be kept alive longer in presence of the bacteria. This hypothesis could be tested by measuring the change in the proportion of infected hemocytes with the age of the woodlice, predicting an increase in this proportion if our hypothesis holds true.

### 4.2.2. Gene expression and enzyme activity

TERT is a subunit of telomerase, an enzyme that prevents telomere reduction. Over time, TERT decreases, leading to an inability to prevent telomere reduction, which triggers the ageing process (Chiu and Harley,

1997). In our previous study (Depeux et al. 2020), we observed a trend of decreasing TERT expression in female ovaries across ages. In the present one, the TERT expression did not exhibit a statistically significant difference with age, nor between infected and un-infected females. The absence of a significant difference with age may be attributed to the use of pooled female ovaries in the present study, potentially magnifying variability in the biomarker's values. If this hypothesis holds true for age, it may also apply to the effect of *Wolbachia* infection. Consequently, we cannot draw definitive conclusions about the impact of *Wolbachia* infection on this ageing biomarker. An individual-based approach, particularly targeting this TERT expression biomarker, could provide a means to test this hypothesis. However, implementing such an approach in our present experiment was impractical due to the substantial quantity of protein extracts required for 1-year-old females, coupled with the necessity to follow the same protocol for 2-year-old females for meaningful comparisons. Another plausible explanation could be that TERT expression in the ovaries of *A. vulgare* did not exhibit sufficiently pronounced differences in mature individuals (Depeux et al. 2020) to detect variations between infected and un-infected females.

Concerning  $\beta$ -galactosidase activity, our study indicated that 1-year-old infected females have higher  $\beta$ -galactosidase activity than 1-year-old un-infected animals. An increase in  $\beta$ -galactosidase activity indirectly indicates an ageing process (Depeux et al., 2020), suggesting premature ageing in *A. vulgare* females infected with *Wolbachia*. However, although known to be associated with senescence (Depeux et al., 2020; Lee et al., 2006; Valieva et al., 2022), this increase in  $\beta$ -galactosidase activity could also illustrate a stress associated with the presence of the bacteria (Zerofsky et al., 2005). Nevertheless, we observed that at the age of 2-years-old, infected females exhibit lower  $\beta$ -galactosidase activity than un-infected ones. This could result from the selective disappearance of the weakest infected females, leaving room for the most resistant infected animals with low level of  $\beta$ -galactosidase activity. This phenomenon has also been observed in a study on other animals in urban environments, where individuals with short telomeres disappeared early in life, leaving only those with the longest telomeres surviving into adulthood (Salmón et al., 2017).

#### 4.3. Conclusion

Our study on *A. vulgare* unveils the intricacies of interactions between *Wolbachia* and one of its arthropod hosts. Faced with *Wolbachia* infection, *A. vulgare* females appear to experience alterations in their reproductive and survival capacities, confirmed by the physiological analyses, aligning with the presumed negative impact of *Wolbachia* on their physiology. Despite recent findings indicating enhanced pathogen resistance in *Wolbachia*-infected females (Prigot-Maurice et al., 2022; Prigot-Maurice et al., 2021), it seems that in the absence of an immune challenge, the cost of *Wolbachia* outweighs its benefits. Our results therefore strengthen the knowledge that *Wolbachia* in *A. vulgare* is parasite of reproduction. This endosymbiont, in addition to be detrimental to the host at individual level, is also detrimental at population level (Moreau and Rigaud, 2000), yet is maintained or selected in the host population. This selection is the result of a distortion of the sex ratio, which gives infected females an advantage compared with un-infected ones, due to the excess of females they produce, which outweighs the detrimental effect of this endosymbiont on their physical condition. These female-biased sex ratios are maintaining the symbionts in common pill woodlice populations despite the negative effect on fitness reported in this study.

#### CRedit authorship contribution statement

**Charlotte Depeux:** Supervision, Data curation, Conceptualization. **Angèle Branger:** Methodology, Formal analysis, Data curation. **Hélène Paulhac:** Methodology. **Romain Pigeault:** Writing – review & editing, Validation, Methodology, Formal analysis. **Sophie Beltran-Bech:**

Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

#### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

#### Data accessibility statement

The data supporting the conclusions of this article are available on the Figshare data repository (<https://doi.org/10.6084/m9.figshare.26048731.v1>).

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jip.2024.108187>.

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