

1 Symbiont-mediated fly survival is independent of defensive symbiont  
2 genotype in the *Drosophila melanogaster*-*Spiroplasma*-wasp interaction

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## 13 Abstract

14 When a parasite attacks an insect, the outcome is commonly modulated by the presence of  
15 defensive heritable symbionts residing within the insect host. Previous studies noted markedly  
16 different strengths of *Spiroplasma*-mediated fly survival following attack by the same strain of  
17 wasp. One difference between the two studies was the strain of *Spiroplasma* used. We therefore  
18 performed a common garden laboratory experiment to assess whether *Spiroplasma*-mediated  
19 protection depends upon the strain of *Spiroplasma*. We perform this analysis using the two  
20 strains of male-killing *Spiroplasma* used previously, and examined response to challenge by two  
21 strains of *Leptopilina bouleari* and two strains of *Leptopilina heterotoma* wasp. We found no  
22 evidence *Spiroplasma* strain affected fly survival following wasp attack. In contrast, analysis of  
23 the overall level of protection, including the fecundity of survivors of wasp attack, did indicate  
24 the two *Spiroplasma* strains tested varied in protective efficiency against three of the four wasp  
25 strains tested. These data highlight the sensitivity of symbiont-mediated protection phenotypes  
26 to laboratory conditions, and the importance of common garden comparison. Our results also  
27 indicate that *Spiroplasma* strains can vary in protective capacity in *Drosophila*, but these  
28 differences may exist in the relative performance of survivors of wasp attack, rather than in  
29 survival of attack per se.

30

## 31 Introduction

32 The outcome of natural enemy attack has traditionally been considered a function of factors  
33 encoded within the genome of the host and infecting parasite. Within this interaction may exist  
34 a degree of specificity whereby a subset of parasite genotypes are able to infect a subset of host  
35 genotypes and, reciprocally, a subset of host genotypes are able to resist a subset of parasite  
36 genotypes (Woolhouse *et al.* 2002; Lambrechts *et al.* 2006). Specificity between host and  
37 parasite genotypes can lead to negative-frequency dependent selection between players and  
38 can contribute to the maintenance of heritable variation for defence and attack factors within a  
39 population (Woolhouse *et al.* 2002; Schmid-Hempel & Ebert 2003).

40 More recently it has been observed that the outcome of natural enemy attack is not solely  
41 determined by host and parasite genotypes, but also by the presence and genotype of defensive  
42 heritable microbial symbionts residing within the host (Brownlie & Johnson 2009; Oliver *et al.*  
43 2009; Ballinger & Perlman 2019). Defensive microbial symbionts have been identified in a wide  
44 range of organisms. For example, microbial symbionts are known to provide protection against  
45 ssRNA viruses (Hedges *et al.* 2008; Teixeira *et al.* 2008), nematodes (Jaenike *et al.* 2010), fungal  
46 pathogens (Scarborough *et al.* 2005; Lukasik *et al.* 2013) and parasitic wasps (Oliver *et al.* 2003;  
47 Xie *et al.* 2010).

48 Recently, studies have described how microbial strain identity can complement host and  
49 parasite genotype as an additional driver of the outcome of a host – parasite interaction. In  
50 aphid systems, this is commonly manifested in symbiont strain  $\times$  host strain  $\times$  enemy strain  
51 interaction terms (Sandrock *et al.* 2010; Schmid *et al.* 2012; Cayetano & Vorburger 2013, 2015;  
52 Parker *et al.* 2017). Beyond the aphid systems, it is known that the strain of infecting *Wolbachia*  
53 is an important source of variation in *Wolbachia*-mediated protection against viruses in  
54 *Drosophila*, associated with different titre achieved by the strains (Osborne *et al.* 2009; Bian *et*  
55 *al.* 2013; Chrostek *et al.* 2013, 2014; Martinez *et al.* 2017). Similarly, in the bumblebee, *Bombus*  
56 *terrestris*, the defensive gut microbiota type is predominantly responsible for resistant

57 phenotypes against the virulent gut trypanosomatid, *Crithidia bombi* (Koch & Schmid-Hempel  
58 2012).

59 The heritable endosymbiont *Spiroplasma*, has been shown to protect *Drosophila* from attack by  
60 nematodes and parasitoid wasps (Jaenike *et al.* 2010; Xie *et al.* 2010, 2014; Mateos *et al.* 2016).  
61 The ability of *Spiroplasma* to protect *Drosophila* is thought to be orchestrated through a  
62 combination of RIP toxin activity (secreted by *Spiroplasma*) and exploitative competition  
63 between *Spiroplasma* and the infecting parasite for lipid stores (Paredes *et al.* 2016; Ballinger &  
64 Perlman 2017). Despite being regarded as an important model system, little is known about the  
65 role of host, symbiont and parasite identity in determining the outcome of the interaction.  
66 Recent work has revealed that the genotype of attacking parasitoid wasp is important for the  
67 degree of protection conferred by *Spiroplasma* (Jones & Hurst 2020). It was observed that  
68 *Spiroplasma* (MSRO-Br strain) conferred protection of 40% against the Lh-Fr and Lh-Mad *L.*  
69 *heterotoma* wasp strains, contrasting with 5% protection against the Lh14 strain. The reasons  
70 underpinning the variation observed is unknown, but intraspecific differences in the toxicity of  
71 wasp venom transferred along with the wasp egg during parasitization may be a factor.

72 A more general understanding of how symbiont and parasite genotypes are likely to interact is  
73 essential for predicting the dynamics of symbionts in natural populations. In this study, we  
74 determine whether parasite genotype  $\times$  symbiont genotype interactions exist within the  
75 *Spiroplasma-Drosophila melanogaster* system. Most studies concerning *Spiroplasma*-mediated  
76 protection have reported the outcome of experiment in which a single symbiont strain defends  
77 against a single enemy strain. Analysis across these studies indicates that the strain of  
78 *Spiroplasma* may be an important component of *Spiroplasma*-mediated protection. For instance,  
79 survival of flies exposed to the Lb17 strain of the specialist parasitoid wasp *L. boulandi* was  
80 recorded at 5% in *D. melanogaster* infected with the MSRO-Br strain (Xie *et al.* 2014), and 50%  
81 in *D. melanogaster* infected with the MSRO-Ug *Spiroplasma* strain (Paredes *et al.* 2016). One  
82 interpretation of these results is that the *Spiroplasma* strains differ in protective capacity in *D.*

83 *melanogaster*. However, analysis of these two strains within a common experimental design  
84 (controlling for potential lab practice, wasp strain and fly strain differences) is required to  
85 determine the precise importance of symbiont strain in determining the outcome of the  
86 parasite-host interaction.

87 We here present an analysis of the capacity of MSRO-Br and MSRO-Ug to defend *D. melanogaster*  
88 against wasp attack. This analysis is performed for two strains of the specialist parasitoid *L.*  
89 *boulardi*, and two strains of the generalist *L. heterotoma*. We compare survival following wasp  
90 attack, mirroring previous studies, and additionally estimate overall protection combining fly  
91 survival data with data on the fertility of flies that survived wasp attack to establish a protective  
92 index for each wasp strain by *Spiroplasma* strain combination.

93

## 94 Materials and methods

### 95 Strains and maintenance

96 Two strains of *Spiroplasma* were used in this study. The first, Red 42, was originally collected in  
97 Campinas, São Paulo State, Brazil in 1997 (Montenegro *et al.* 2000) and later transinfected and  
98 maintained in the laboratory on a Canton-S background. The second *Spiroplasma* strain was  
99 collected from Namulonge, Uganda in 2005 (Pool *et al.* 2006) which was later transferred and  
100 maintained in the laboratory on an Oregon-R background. It should be noted that all larvae from  
101 the *Spiroplasma* infected treatments are female due to the high efficiency of male-killing.  
102 However, there does not appear to be any differences in survival between the sexes against  
103 parasitoid wasp attack (Xie *et al.* 2014). All flies were maintained on ASG corn meal agar vials  
104 (10 g agarose, 85 g sugar, 60 g maize meal, 40 g autolysed yeast in a total volume of 1 L, to  
105 which 25 mL 10% Nipagin was added) at 25 °C on a 12:12 light:dark cycle.

106 The *L. bouleari* strains used were the NSRef strain, established from an initial female collected in  
107 Gotheron, near Valence, France (Varaldi *et al.* 2003), and the Lb17 strain, initially collected in  
108 Winters, California in 2002 (Schlenke *et al.* 2007). The *L. heterotoma* strains used were the  
109 inbred Lh14 strain also collected in Winters, California in 2002 (Schlenke *et al.* 2007) and the  
110 Lh-Mad strain established from a single female collected in Madeira, Portugal in March 2017  
111 (Jones & Hurst 2020). The wasp stocks were all maintained on second instar Oregon-R larvae at  
112 25°C on a 12:12 light:dark cycle. After emergence, wasps were maintained on grape agar vials  
113 supplemented with a flug moistened with honey water and allowed to mature and mate for 7  
114 days prior to exposure to *D. melanogaster* L2 larvae.

### 115 Artificial infection of *Spiroplasma*

116 The *Spiroplasma* strains (MSRO-Br and MSRO-Ug) were artificially transferred into a common  
117 host background (Canton-S) to remove any effect of host nuclear background on the level of  
118 protection conferred. Canton-S stocks carry the naturally occurring *Wolbachia* strain wMel.  
119 *Wolbachia* has been shown to provide a weak positive effect on fly larva-to-adult survival and a

120 negative effect on wasp success in flies attacked against *L. heterotoma* (Lh14 strain) (Xie *et al.*  
121 2014). Artificial infections were carried out as described by Nakayama *et al.* (2015).  
122 Hemolymph was extracted from the thorax of *Spiroplasma*-infected *D. melanogaster* and mixed  
123 with sterile PBS. Virgin female Canton-S were artificially injected in the abdomen with 0.1-0.2  $\mu$ l  
124 of PBS-hemolymph, using a hydraulic positive-pressure microinjection apparatus (Model IM-6,  
125 Narushige Ltd, Tokyo, Japan).

#### 126 Confirmation of *Spiroplasma* infection status

127 Three weeks post injection, the infection status of the artificially infected flies was confirmed via  
128 *Spiroplasma*-specific PCR. DNA extraction was carried out using the Wizard® Genomic DNA  
129 Purification Kit (Promega). To this end, each injected mother was taken and macerated in 150  
130  $\mu$ l of Nuclei Lysis Solution and incubated at 65 °C for 30 min. After incubation, 50  $\mu$ l of Protein  
131 Precipitation Solution was added to each sample and then placed on ice for 5 min. Samples were  
132 then centrifuged for a further 4 min at 16,000 x g and the supernatant was transferred into a  
133 new tube containing 150  $\mu$ l of isopropanol. Samples were centrifuged for 2 min at 16,000 x g  
134 and the supernatant discarded. 150  $\mu$ l of 70% ethanol was added to each sample and  
135 centrifuged for 1 min at 16,000 x g. The supernatant was discarded. Pellets were dried before  
136 re-suspending in 25  $\mu$ l of molecular grade water at 4 °C overnight before use in subsequent PCR  
137 assays.

138 PCR amplifications were conducted using *Spiroplasma* specific primers, SpoulF (5'-GCT TAA CTC  
139 CAG TTC GCC-3') and SpoulR (5'-CCT GTC TCA ATG TTA ACC TC-3') (Montenegro *et al.* 2005).  
140 Each reaction was carried out in 15  $\mu$ l volume containing 7.5  $\mu$ l of GoTaq® Hot Start Green  
141 Master Mix (Promega), 0.5  $\mu$ l each of the forward and reverse primer, 5.5  $\mu$ l of Molecular Grade  
142 Water and 1  $\mu$ l of DNA. All reactions were conducted alongside the positive and negative  
143 controls. This included a PCR negative control containing the PCR reaction mixture only  
144 (excluding DNA template). The PCR thermal program consisted of an initial denature of 5 min at  
145 95 °C, followed by 35 cycles of 15 s at 94 °C, 1 min at 55 °C and 40 s at 72 °C. The PCR products

146 were electrophoresed in a 1.5% agarose gel at 155 V for 15 min and the products were  
147 visualised. Offspring sex ratio of infected mothers were also checked to determine *Spiroplasma*  
148 efficiency. Only mothers which were infected with *Spiroplasma* and produced all female broods  
149 were used to create new lines.

150 To confirm the *Spiroplasma* strain status of each artificially injected line of *Drosophila*  
151 *melanogaster*, sequencing was performed on 5 individual flies from each strain. To this end, the  
152 DNA of 5 flies from each *Spiroplasma* strain line were extracted using the Wizard® Genomic  
153 DNA Purification Kit following the methodology from above. PCR amplifications were conducted  
154 using *Spiroplasma* specific primers, Spiro\_MSRO\_diff\_F (5'-TAC GAC CAA TGG CTT GTC CC-3' and  
155 Spiro\_MSRO\_diff\_R (5'- CTG GCA TTG CTT TTT CCC CA-3'). The PCR thermal program consisted  
156 of an initial denature of 2 min at 94 °C, followed by 35 cycles of 15 s at 94 °C, 1 min at 56 °C and  
157 40 s at 72 °C. To prepare the PCR reaction for sequencing, PCR products underwent an ExoSAP  
158 digest clean up to remove excess primers. To this end, 5 µl of PCR product was added to a  
159 mixture containing 0.2 µl Shrimp alkaline phosphatase, 0.05 µl of Exonuclease I, 0.7 µl 10X RX  
160 Buffer and 1.05 µl of molecular grade water. Samples were then incubated for 45 min at 37 °C  
161 followed by 15 min at 80 °C and sent for Sanger sequencing. The *Spiroplasma* strain status of the  
162 MSRO-Br and MSRO-Ug line were confirmed by the presence of a Guanine and Thymine  
163 respectively in position 414193, coding for a type III pantothenate kinase. The expected  
164 amplicon size is 509bp. Transinfected fly lines were passaged for >10 generations before  
165 experiments were conducted.

#### 166 Wasp attack assay

167 To ensure efficient vertical transmission of *Spiroplasma*, infected females were aged to at  
168 least ten days prior to egg laying. Flies were allowed to mate in cages and lay eggs on a grape  
169 Petri dish painted with live yeast for 24 h. Grape Petri dishes were incubated for a further 24  
170 h to allow larvae to hatch. First instar larvae were picked from the grape plate into the  
171 experimental vials at 30 larvae per vial. A fully factorial design was created for each of the



172 four wasp strains described which included *Spiroplasma* strain (MSRO-Br, MSRO-Ug and  
173 uninfected control) and wasp (presence or absence). Five experienced, mated female wasps  
174 were transferred into the wasp treatment vials. Adult wasps were allowed to parasitise for 2  
175 days before being removed. All vials were maintained at 25 °C on a 12:12 light:dark cycle. For  
176 each vial, the number of puparia, emerging flies and emerging wasps were recorded.  
177 Experiments using *L. boulandi* and *L. heterotoma* were conducted in separate blocks, one  
178 week apart.

### 179 Measuring female fecundity

180 *Spiroplasma* infected flies that survive wasp attack generally have a lower fecundity than  
181 *Spiroplasma* infected flies which were not exposed to wasps (Xie *et al.* 2011; Jones & Hurst,  
182 2020). To determine whether the wasp attacked survivors were differentially impacted by  
183 *Spiroplasma* strain the average daily emerged offspring of *Spiroplasma* infected survivors  
184 (“Exposed”) and *Spiroplasma* infected flies which did not undergo wasp attack (“Unexposed”)   
185 was measured for the MSRO-Br and MSRO-Ug strains. The *Spiroplasma* uninfected wasp  
186 attacked group was not included due to the extremely low number of flies which emerged,  
187 which were also likely to have avoided wasp attack all together. After emergence, flies from the  
188 wasp attack assay were stored in vials containing sugar yeast medium (20 g agarose, 100 g  
189 sugar, 100 g autolysed yeast in a total volume of 1 L, to which 30 mL 10% Nipagin w/v  
190 propionic acid was added) at mixed ages. A week after emergence commenced, a subset of flies  
191 from each of the *Spiroplasma* treatments were placed into an ASG vial with two Canton-S males  
192 with a single yeast ball and allowed to mate. Approximately 25 replicates per treatment were  
193 created. Flies were transferred onto fresh ASG vials each day for five days. Flies were given two  
194 weeks to emerge to ensure every fly had emerged before counting. Female fecundity was  
195 measured as the average number of offspring produced over four days (day 2-5).

### 196 Statistical analysis

197 All statistical analyses were performed using the statistical software R, version 3.5.0 (R Core  
198 Team 2018). Fly and wasp survival data were analysed by fitting a generalized linear model

199 with binomial distributions. In all cases, a fully saturated model including all factors and their  
200 interaction was reduced to a minimum adequate model through step-wise simplification. Non-  
201 significant factors are reported as the output of the model comparisons. The effect of significant  
202 independent variables are reported from the analysis of the minimum adequate model using the  
203 'car' package.

204 To produce a composite measure of protection, a Protective Index (PI) was calculated by  
205 comparing the survival and fecundity of *Spiroplasma*-infected flies in the presence/absence of a  
206 given strain of wasp. The PI was calculated as the ratio of  $p(\text{survival}) \times p(\text{fertile}) \times \text{fecundity}$  of  
207 fertile individuals for attacked vs unattacked *Spiroplasma*-infected flies and reflects the benefit  
208 of *Spiroplasma* in the face of wasp attack. Credible intervals for PI were calculated through  
209 simulation. By assuming prior probability distributions for each parameter (Survival probability  
210 = beta distribution; Fertility probability = beta distribution; Fecundity = normal distribution),  
211 the 'rbeta' and 'rnorm' functions were used to calculate 95% credible intervals for PI. The  
212 simulation data was also used to establish the posterior probability of PI differing between  
213 attacking wasp strains.

214

## 215 Results

### 216 Fly survival and wasp success

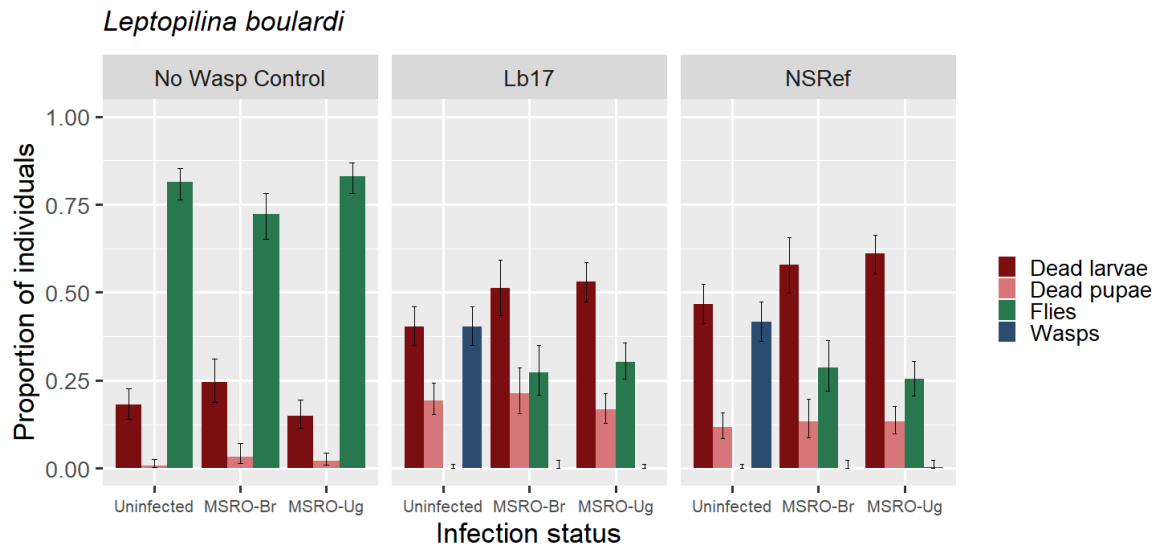
#### 217 *Leptopilina bouleari* experiment

218 In the absence of *L. bouleari* wasps, *Spiroplasma* strain had a significant effect on fly larva-to-  
219 adult *D. melanogaster* survival ( $\chi^2 = 7.74$ , d.f. = 1,  $P = 0.005$ ). The mean survival of MSRO-Br  
220 infected and MSRO-Ug infected *D. melanogaster* was 72.2% and 83%, respectively (Fig. 1A). In  
221 the presence of *L. bouleari* wasps, there was no significant effect of wasp strain ( $\chi^2 = 0.281$ , d.f. =  
222 1,  $P = 0.596$ ), *Spiroplasma* strain ( $\chi^2 = 0.0008$ , d.f. = 1,  $P = 0.977$ ), nor a significant interaction  
223 between wasp strain and *Spiroplasma* strain on larva-to-adult survival of *D. melanogaster* ( $\chi^2 =$   
224 0.284, d.f. = 1,  $P = 0.594$ ) (Fig. 1A). There was no significant effect of wasp strain on wasp  
225 success ( $\chi^2 = 0.121$ , d.f. = 1,  $P = 0.728$ ) (Fig. 1A), and wasps were observed only in the absence of  
226 *Spiroplasma*.

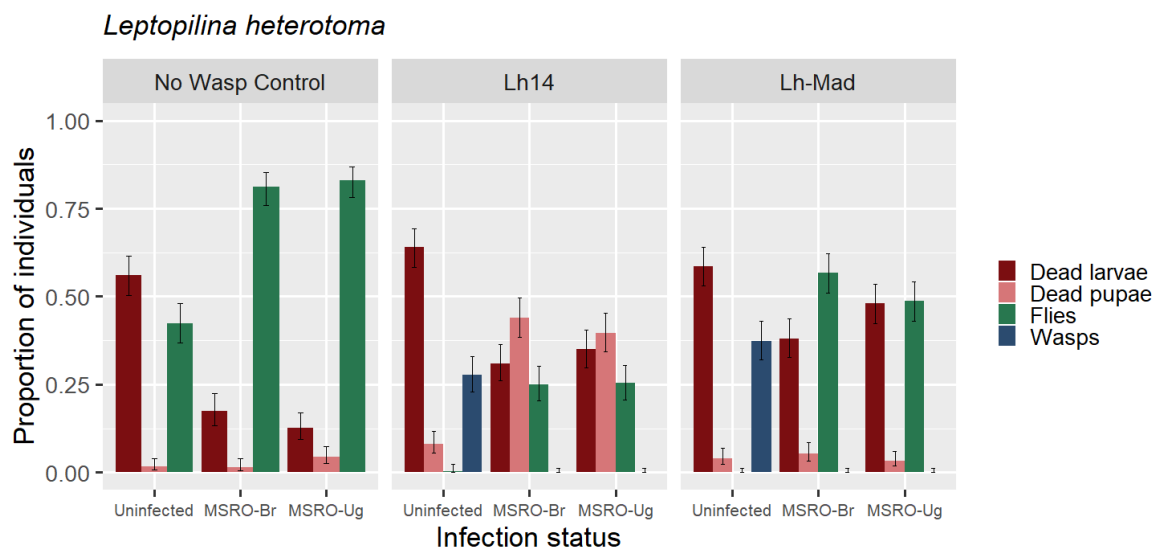
#### 227 *Leptopilina heterotoma* experiment

228 In the absence of *L. heterotoma* wasps, there was no significant effect of *Spiroplasma* strain on  
229 fly larva-to-adult survival ( $\chi^2 = 0.345$ , d.f. = 1,  $P = 0.557$ ). The mean survival of uninfected,  
230 MSRO-Br infected and MSRO-Ug infected *D. melanogaster* was 81.1% and 83%, respectively  
231 (Fig. 1B). In the presence of *L. heterotoma* wasps, there was a significant effect of wasp strain on  
232 fly larva-to-adult survival of *D. melanogaster* ( $\chi^2 = 34.21$ , d.f. = 1,  $P < 0.001$ ). Fly larva-to-adult  
233 survival of *Spiroplasma*-infected *D. melanogaster* attacked by the Lh-Mad strain of *L. heterotoma*  
234 was approximately double that observed for flies attacked by the Lh14 strain of *L. heterotoma*  
235 (Fig. 1B). Similar to the *L. bouleari* experiment, there was no significant effect of *Spiroplasma*  
236 strain ( $\chi^2 = 0.740$ , d.f. = 1,  $P = 0.390$ ), nor a significant interaction between wasp strain and  
237 *Spiroplasma* strain ( $\chi^2 = 0.674$ , d.f. = 1,  $P = 0.412$ ) on larva-to-adult survival of *D. melanogaster*  
238 (Fig. 1B). There was a significant effect of wasp strain on wasp success ( $\chi^2 = 4.805$ , d.f. = 1,  $P =$   
239 0.028) (Fig. 1B). The average wasp success of the Lh14 and Lh-Mad wasp strains were 27.7%  
240 and 37.3% respectively. Wasps only emerged in the absence of *Spiroplasma*, with both symbiont  
241 strains preventing development of both wasp strains.

A



B



242

243 **Figure 1:** Proportion of dead larvae (red), dead pupae (pink), emerging flies (green) and  
244 emerging wasps (blue) for *Spiroplasma*-infected (MSRO-Br and MSRO-Ug strains) and  
245 uninfected *Drosophila melanogaster* attacked by A) *L. boulardi* (Lb17 and NSRef strains) and B)  
246 *L. heterotoma* (Lh14 strain and Lh-Mad strains). Error bars represent 95% binomial confidence  
247 intervals.

248 Overall protection index

249 Despite finding no difference between the survival of flies infected with MSRO-Br and MSRO-Ug  
250 against each of the four wasp strains tested, previous work has shown that it is also important  
251 to consider, in combination with survival, the fertility of wasp-attacked flies compared to non-  
252 attacked controls to produce a complete model of protection (Xie et al., 2011; Jones & Hurst  
253 2020). Taking into account the survival, proportion of adults fertile, and the fecundity of wasp  
254 attack survivors, compared to unexposed *Spiroplasma*-infected controls, a protection index (PI)  
255 was calculated as the product of fly survival x p(fertile) x fecundity of exposed vs unexposed  
256 *Spiroplasma*-infected flies (Table 1). This metric assumes complete mortality from wasps in the  
257 absence of *Spiroplasma*, which is approximately true as <1% of individuals tested survived wasp  
258 attack. Against the Lb17, NSRef and Lh-Mad strains of wasp, the posterior probability that the  
259 protection index for MSRO-Br is greater than the protection index for MSRO-Ug is >0.97 (Table  
260 2). However, against the Lh14 strain of wasp, the posterior probability that the protection index  
261 for MSRO-Br is greater than the protection index for MSRO-Ug is 0.44 (Table 2).

262 **Table 1:** The overall protection conferred by MSRO-Br and MSRO-Ug *Spiroplasma* strains against a) *Leptopilina bouleari* (Lb17 and NSRef strains)  
 263 and b) *Leptopilina heterotoma* (Lh14 and Lh-Mad strains) in *Drosophila melanogaster*. Exposed S- = wasp attacked *Spiroplasma*-uninfected flies;  
 264 Exposed S+ wasp attacked *Spiroplasma*-infected flies; Unexposed S+ *Spiroplasma*-infected flies not attacked. Protective Index is calculated as  
 265  $[p(\text{survival}) \times p(\text{fertile}) \times \text{fecundity of fertile individuals}]$  of exposed vs unexposed individuals with credible intervals calculated as given in methods.

a) *Leptopilina bouleari*

<i>Spiroplasma</i> strain	Treatment	Fly Survival (binomial 95% CI intervals (lower, upper))	Proportion fertile (binomial 95% CI intervals (lower, upper))	Fecundity measure $\pm$ SE	Estimated protective index (95% Credible interval (lower, upper))
MSRO-Br	Lb17 exposed S+	0.27 (0.20, 0.35)	0.96 (0.75, 0.99)	15.8 $\pm$ 1.31	0.37 (0.25, 0.55)
	NSRef exposed S+	0.29 (0.22, 0.36)	1.00 (0.86, 1.00)	15.6 $\pm$ 1.45	
	Unexposed control S+	0.72 (0.65, 0.78)	0.92 (0.72, 0.98)	16.9 $\pm$ 1.47	
MSRO-Ug	Lb17 exposed S+	0.30 (0.25, 0.36)	1.0 (0.85, 1.0)	11.0 $\pm$ 1.77	0.30 (0.14, 0.32)
	NSRef exposed S+	0.25 (0.21, 0.30)	0.88 (0.68, 0.96)	14.3 $\pm$ 1.58	
	Unexposed control S+	0.83 (0.78, 0.87)	0.96 (0.776, 0.99)	19.4 $\pm$ 1.49	

b) *Leptopilina heterotoma*

<i>Spiroplasma</i> strain	Treatment	Fly Survival (binomial 95% CI intervals (lower, upper))	Proportion fertile (binomial 95% CI intervals (lower, upper))	Fecundity measure $\pm$ SE	Estimated protective index (95% Credible interval (lower, upper))
MSRO-Br	Lh14 exposed S+	0.25 (0.20, 0.30)	0.79 (0.59, 0.91)	13.2 $\pm$ 1.75	0.24 (0.15, 0.39)
	Lh-Mad exposed S+	0.57 (0.51, 0.62)	0.91 (0.71, 0.98)	14.0 $\pm$ 1.58	0.68 (0.54, 1.16)
	Unexposed control S+	0.81 (0.76, 0.85)	0.92 (0.71, 0.98)	14.3 $\pm$ 1.72	
MSRO-Ug	Lh14 exposed S+	0.25 (0.20, 0.31)	1.0 (0.82, 1)	12.8 $\pm$ 1.14	0.25 (0.18, 0.36)
	Lh-Mad exposed S+	0.49 (0.43, 0.54)	0.91 (0.70, 0.98)	11.0 $\pm$ 1.34	0.39 (0.26, 0.56)
	Unexposed control S+	0.83 (0.78, 0.87)	1.0 (0.82, 1)	15.3 $\pm$ 1.58	

266 **Table 2:** The posterior probability that the estimated protective index for MSRO-Br is greater than the MSRO-Ug for each wasp strain tested.  
 267

Wasp strain	Estimated protective index (95% Credible interval (lower, upper))		Posterior probability (EPI MSRO-Br > EPI MSRO-Ug)
	MSRO-Br	MSRO-Ug	
<i>Leptopilina bouleardi</i>			
Lb17	0.37 (0.25, 0.55)	0.30 (0.14, 0.32)	0.97
NSRef	0.40 (0.27, 0.59)	0.20 (0.14, 0.30)	0.99
<i>Leptopilina heterotoma</i>			
Lh14	0.24 (0.15, 0.39)	0.25 (0.18, 0.36)	0.44
Lh-Mad	0.68 (0.54, 1.16)	0.39 (0.26, 0.56)	0.99



## 268 Discussion

269 Defensive symbionts can contribute to the outcome of a host-parasite interaction. Previous  
270 studies in aphids have shown that the strain of symbiont is an important determinant of  
271 symbiont-mediated protection across multiple model systems (Schmid *et al.* 2012; Cayetano &  
272 Vorburger 2013, 2015; Parker *et al.* 2017). However, whether strains of the *Drosophila*  
273 defensive symbiont, *Spiroplasma poulsonii*, vary in their capacity for protection is unknown.  
274 The contrasting levels of fly survival observed between two previous studies on the  
275 *Drosophila-Spiroplasma-L. boulandi* interaction suggested that the strain of *Spiroplasma* may be  
276 an important determinant of protection capacity in *Drosophila* (Xie *et al.* 2014; Paredes *et al.*  
277 2016). We therefore performed an experiment to determine whether the strength of  
278 *Spiroplasma*-mediated protection depended on the strain of infecting *Spiroplasma* using two  
279 known strains of MSRO *Spiroplasma* (MSRO-Br and MSRO-Ug). We found no evidence that the  
280 strength of *Spiroplasma*-mediated fly survival differed between the MSRO-Br and MSRO-Ug  
281 strains against any of the four *Leptopilina* wasp strains tested. However, the overall protective  
282 index, including the fecundity of survivors of wasp attack, did vary between the two  
283 *Spiroplasma* strains for three of the attacking wasp strains.

284 The strain of *Spiroplasma* did not alter the strength of *Spiroplasma*-mediated fly survival in *D.*  
285 *melanogaster* in our experiment. This result raises the question as to why fly survival following  
286 attack differed between the two previous independent studies. Fly survival against the  
287 parasitoid wasp, *L. boulandi* (strain Lb17) was observed to vary from 5% with MSRO-Br (Xie *et*  
288 *al.* 2014), to 50% with MSRO-Ug (Paredes *et al.* 2016). Comparisons across studies indicate  
289 that the strength of symbiont-mediated fly survival appear to be highly variable across  
290 laboratory studies. In this study, we found survival of 30% against the *L. boulandi* (Lb17  
291 strain), yet Paredes *et al.* (2016) found survival of 50% against the same wasp strain despite  
292 using the same fly strain. Similarly, we found survival of 25% against the Lh14 strain of *L.*  
293 *heterotoma*, despite survival of <8% observed in previous studies (Xie *et al.* 2014, Jones &  
294 Hurst 2020).

295 The variability in *Spiroplasma*-mediated survival observed across studies may be the result of  
296 variability in wasp success. Whilst wasp attack rate was very high in all cases (with very low fly  
297 survival in uninfected controls), wasp success was highly variable across the studies and  
298 correlated to some extent with fly survival. Specifically, against the Lb17 wasp strain, Xie *et al.*  
299 (2014) found high wasp success of ~70% and low fly survival of ~5%. In contrast, this study  
300 observed reduced wasp success of ~40% and increased fly survival of ~30%. Thus, the  
301 variability in *Spiroplasma*-mediated fly survival across studies could be associated with  
302 condition of the attacking wasps. Associated with this, it is notable that larval-to-pupa survival  
303 following attack is lower in our studies than previously observed, and this may potentially  
304 explain differences in wasp survival. These studies may highlight the sensitivity of symbiont-  
305 mediated protection to husbandry conditions of both fly and wasp.

306 From several studies, it has been demonstrated that symbiont-mediated survival against  
307 natural enemies can be highly sensitive to particular environmental conditions. Temperature  
308 is one environmental factor known to impact the strength of symbiont-mediated protection  
309 (Corbin *et al.* 2017). For example, in the pea aphid, higher temperatures can negatively impact  
310 *H. defensa*-mediated survival against *Aphidius ervi* (Doremus *et al.* 2018). Similarly, heat shock  
311 also negatively impacts X-type-mediated survival against *A. ervi* wasps in the pea aphid  
312 (Heyworth & Ferrari 2016). Another possibility, raised by studies of the strength of CI and  
313 male-killing exhibited by *Wolbachia*, is that protection strength is influenced by parental  
314 *Spiroplasma* titre (Dyer *et al.* 2005; Layton *et al.* 2019). It is notable that both thermal  
315 environment and age at reproduction are known to affect *S. poulsonii* titre and male-killing  
316 strength in *D. melanogaster* (Anbutsu & Fukatsu 2003; Montenegro & Klaczko 2004; Anbutsu *et*  
317 *al.* 2008). Finally, wasp husbandry and attack protocols may vary. Wasp attack success is  
318 thought to be higher when wasps are previously conditioning before assays and may also be  
319 impacted by the arena in which attack occurs. Wasps attack fly larvae at the surface of the food,  
320 and the surface area available for attack, and indeed the medium in which the larvae are  
321 feeding, may impact success. The variable strength of protection afforded by symbionts across

322 laboratories may be due to unmeasured differences in stock maintenance/ambient  
323 environmental conditions and reinforce the need for common-laboratory experiments when  
324 comparing outcomes.

325 Our experiment nevertheless did indicate differences in protection associated with *Spiroplasma*  
326 strain, but these were reflected in the overall phenotype, including the survival and fecundity of  
327 wasp-attack survivors. Surviving flies infected with the MSRO-Br strain of *Spiroplasma* had an  
328 overall higher protective index against the NSRef, Lb17 and Lh-Mad strains of wasp compared  
329 to flies infected with the MSRO-Ug strain. The reasons as to why fly survivors infected with  
330 MSRO-Ug had a lower protective index compared to MSRO-Br survivors remains unclear. One  
331 possible factor which cannot be ruled out from this study is the effect of *Wolbachia*. Although  
332 from the results it does not appear that *Wolbachia* is having an effect on fly survival, it may be  
333 possible that the presence of *Wolbachia* is differentially impacting the fertility of wasp-attacked  
334 survivors among the MSRO-Br and MSRO-Ug strains tested. Another factor which is difficult to  
335 determine is the possibility that a proportion of flies in the *Spiroplasma* treatments were not  
336 attacked. Although fly emergence from the *Spiroplasma* negative controls suggests that all  
337 larvae were successfully parasitized, this does not exclude the possibility that not all larvae  
338 were parasitized in the *Spiroplasma* positive treatments, although past work found no evidence  
339 for discrimination by wasps (Xie *et al.* 2010, Jones & Hurst, 2020). However, the result that  
340 there was no difference in the overall protection between wasp-attacked survivors infected  
341 with MSRO-Br and MSRO-Ug against the Lh14 strain of wasp indicates that the reasons for this  
342 difference may be a consequence of wasp strain.

343 This study clearly demonstrates two important features of protection. First, there is a need for  
344 common-laboratory experiments to compare levels of protection, as this phenotype has both  
345 genetic and environmental drivers. Second, there is a clear distinction between symbiont-  
346 mediated survival and symbiont-mediated protection within defensive symbiont studies.  
347 Symbiont-mediated protection is often measured as the relative survival of an infected-

348 individual compared to an uninfected individual when faced with natural enemy attack.  
349 However, symbiont-mediated protection is not only the ability of an infected-host to survive,  
350 but also the relative capacity it has to reproduce compared to un-attacked comparators. Despite  
351 finding no evidence that fly survival differed between the two strains of *Spiroplasma* against all  
352 four wasp strains tested, differences between *Spiroplasma* strains were observed on the overall  
353 strength of symbiont-mediated protection. Assessment of the relative survival and reproductive  
354 ability of un-attacked vs. attacked survivors is essential for revealing the true protective  
355 capacity of a defensive symbiont.

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## 365 Conflict of interest

366 The authors declare no conflicts of interest.

## 367 Data accessibility

368 Data generated and analysed during this study are available at figshare  
369 (<https://doi.org/10.6084/m9.figshare.c.4856790.v1>).

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488 Supplementary material

489 **Table S1:** Replicate identity and number.

Experiment	Figure	Replicate identity	Treatment	Number of replicates					
				Wasp strain					
				Lb17	NSRef	Control	Lh14	Lh-Mad	Control
Survival	1	Vial of 30 larvae	Uninfected	10	10	10	10	10	10
			MSRO-Br	5	5	6	10	10	9
			MSRO-Ug	10	10	10	10	10	10
Proportion fertile	2	Single female fly	MSRO-Br	23	24	24	24	23	24
			MSRO-Ug	23	24	24	19	22	19
Number of daughters produced	3	Single female fly	MSRO-Br	22	24	22	19	21	22
			MSRO-Ug	23	21	23	19	20	19

490