Transcriptomic response during wasp parasitism in the *Drosophila-Spiroplasma* interaction

Authors and Contributions

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Abstract

Several facultative bacterial symbionts of insects protect their hosts against natural enemies. *Spiroplasma poulsonii* strain *s*Mel, a male-killing heritable symbiont of *Drosophila melanogaster*, confers protection against some species of parasitic wasps. Several lines of evidence suggest that *Spiroplasma*-encoded ribosome inactivating proteins (RIPs) are involved in the protection mechanism, but the potential contribution of the fly-encoded functions has not been deeply explored. Here we used RNA-seq to evaluate the response of *D. melanogaster* to infection by *Spiroplasma* and parasitism by the *Spiroplasma*-susceptible wasp *Leptopilina heterotoma*, and the *Spiroplasma*-resistant wasp *Ganaspis hookeri*. In the absence of *Spiroplasma* infection, we found evidence of *Drosophila* immune activation by *G. hookeri*, but not by *L. heterotoma*, which in turn negatively influenced functions associated with male gonad development. As expected for a symbiont that kills males, we detected extensive downregulation in the *Spiroplasma*-infected treatments of genes known to have male-biased expression. We detected very few genes whose expression was influenced by the *Spiroplasma*-*L. heterotoma* interaction, and they do not appear to be related to immune response. For most of them, parasitism by *L. heterotoma* (in the absence of *Spiroplasma*) caused an expression change that was at least partly reversed when *Spiroplasma* was also present. It is unclear whether such genes are involved in the *Spiroplasma*-mediated mechanism that leads to wasp death or fly rescue. Nonetheless, the expression pattern of some of these genes, which reportedly undergo expression shifts during the larva-to-pupa transition, is suggestive of an influence of *Spiroplasma* on the development time of *L. heterotoma*-parasitized flies. In addition, we used the RNAseq data and quantitative (q)PCR to evaluate the transcript levels of the *Spiroplasma*-encoded RIP genes. One of the five RIP genes (RIP2) was consistently highly expressed independently of wasp parasitism, in two substrains of *s*Mel. Finally, the RNAseq data revealed evidence consistent with RIP-induced damage in the ribosomal (r)RNA of the *Spiroplasma*-susceptible, but not the *Spiroplasma*-resistant, wasp. We conclude that immune priming is unlikely to contribute to the *Spiroplasma*-mediated protection against wasps, and that the mechanism by which *G. hookeri* resists/tolerates *Spiroplasma* does not involve inhibition of RIP transcription.

63 **1 Introduction**

64 During their life cycle, insects face a large diversity of natural enemies such as
65 predators and parasites, as well as infections by bacteria, fungi, and viruses. Although insects
66 rely on an immune system to overcome these infections (reviewed in Hillyer, 2016), parasites
67 and pathogens have evolved counter defenses. In this arms race, many insects have allied
68 with symbiotic bacteria to fight against parasites. Extensive evidence of such defensive
69 symbioses has been accrued over the last ~17 years (Oliver and Perlman, 2020).

70 Three models of classical ecology can be adapted to explain protection of bacteria
71 against parasites. **Exploitation competition** occurs when the symbiont and the parasite
72 compete for a limiting resource (e.g. *Wolbachia* and vectored-viruses compete for
73 cholesterol;(Caragata et al., 2013). **Apparent competition** can occur when the symbiont
74 activates ("primes") the immunity of the host, and thus indirectly interferes with the parasite
75 (e.g. *Wigglesworthia* in *Glossina* against trypanosomes, (Wang et al., 2009). Finally,
76 **interference competition** can occur when the symbiont produces a compound (e.g. a toxin)
77 that limits the success of the parasite (e.g. *Hamiltonella defensa* in aphid insects (Oliver et al.,
78 2003; Brandt et al., 2017). One or more of these mechanisms can occur in concert, as
79 suggested for the interactions between flies in the genus *Drosophila* and heritable bacteria in
80 the genus *Spiroplasma*, where these endosymbionts protect the host against parasitic wasps or
81 nematodes (Jaenike et al., 2010; Xie et al., 2010).

82 The association between *Drosophila melanogaster* and its naturally occurring heritable
83 bacterium *Spiroplasma poulsonii* (*s*Mel) has emerged as a model system to study the
84 evolutionary ecology and mechanistic bases of both defensive mutualisms (Xie et al., 2010;
85 Ballinger and Perlman, 2017) and reproductive parasitism (i.e, male-killing) (Cheng et al.,
86 2016; Harumoto et al., 2016).

87 The presence of *Spiroplasma* in *Drosophila* larvae prevents the successful development
88 of several species of parasitic wasps (hereafter "*Spiroplasma*-susceptible" wasps) that oviposit
89 on larvae, which leads to enhanced fly survival in some interactions, but not others.
90 Furthermore, several wasp species are unaffected by the presence of *Spiroplasma* in the host,
91 which we hereafter refer to as "resistant" (Mateos et al., 2016). The degree of *Spiroplasma*-
92 mediated protection varies by wasp genotype (Jones and Hurst, 2020b) and is dependent on
93 abiotic factors, e.g. temperature (Corbin et al., 2020)

94 Research into *Spiroplasma*-mediated protection against wasps has revealed that
95 *Spiroplasma*-susceptible wasp embryos manage to hatch into first instars and achieve some
96 growth, which is subsequently stalled (Xie et al., 2010, 2014; Paredes et al., 2016). Evidence
97 consistent with competition for lipids (i.e., exploitative competition) between *Spiroplasma* and
98 the developing wasp has been reported for the wasp *Leptopilina boulardi* (Paredes et al., 2016)
99 Regarding the role of interference competition, the genomes of several *Spiroplasma* strains,
100 including *s*Mel, encode genes with homology to ribosome inactivating proteins (RIP´s,
101 Ballinger and Perlman, 2017).

102 RIP proteins, which are produced by different plants and bacteria (e.g. ricin and Shiga
103 toxin, respectively), cleave a specific adenine present within a highly conserved (i.e., in all
104 eukaryotes) motif of the large ribosomal subunit (28S rRNA), leading to inactivation of the
105 ribosome and inhibition of protein translation (reviewed in Stirpe, 2004).

106 Damage consistent with RIP activity (hereafter referred to as depurination) has been
107 detected in a *Spiroplasma*-susceptible nematode (Hamilton et al., 2015) and in two
108 *Spiroplasma*-susceptible wasps (the larval parasitoids *L. boulardi* and *L. heterotoma*), but not
109 in a *Spiroplasma*-resistant wasp that oviposits on fly pupae (Ballinger and Perlman, 2017).

110 Although the above studies suggest that competition for nutrients and RIP activity are
111 involved in the *Spiroplasma*-mediated mechanism that causes wasp death, they have not
112 demonstrated that the above mechanisms alone or in combination are necessary and sufficient,
113 and alternative mechanisms, including immune priming, have not been ruled out.

114 In response to wasp parasitism, *D. melanogaster* mount an inmune response
115 characterized by proliferation of blood cells also known as hemocytes. Plasmatocytes are the
116 first cells to attach to the foregin egg followed by lamellocytes which form successive layers;
117 both types of hemocytes consolidate around the wasp egg, forming a capsule. The inner cells
118 of the capsule produce melanin and release free radicals into the capsule, killing the wasp
119 (Russo, 1996; Carton et al., 2008). Wasps have evolved a diverse array of strategies that counter
120 the fly-encoded defense (Schlenke et al., 2007; Mortimer et al., 2013). Whether or not
121 *Spiroplasma* contributes to enhancing the fly-encoded defense against wasps has not been
122 extensively investigated. To date only one study has examined the possible influence of
123 *Spiroplasma* on fly-encoded immunity against wasps (Paredes et al., 2016). Their results
124 revealed no effect of *Spiroplasma* on the number of hemocytes in flies parasitized by *L.*
125 *boulardi*. Whether *Spiroplasma* influences this or other aspects of fly-encoded immunity
126 against other wasps has not been examined.

127 Herein, we used an RNA-seq approach to evaluate the transcriptomic response of *D.*
128 *melanogaster* during interactions involving *Spiroplasma* and two wasps that are generalists of
129 the genus *Drosophila*: the *Spiroplasma*-susceptible *L. heterotoma*; and the *Spiroplasma*-
130 resistant *Ganaspis hookeri*. In addition, we evaluated the effect of wasp parasitism on the
131 expression of *Spiroplasma* RIP genes in two closely related substrains of *s*Mel, which have
132 similar genomes (Gerth et al., 2020) but confer different levels of protection against wasps,
133 measured as fecundity of flies surviving a wasp attack (Jones and Hurst, 2020a).

134 **2. Methods**

135 **2.1 Insect and *S. poulsonii* strain**

136 The transcriptomic experiments were performed on *D. melanogaster* flies (strain Canton S),
137 which naturally harbor *Wolbachia* (Riegler et al., 2005). Flies were reared in a Standard
138 cornmeal medium (recipes in Supplementary methods 1) at 25ºC, with a dark: light 12 hour-
139 cycle. Canton S flies were artificially infected with *Spiroplasma poulsonii* strain *s*Mel-BR
140 (original isofemale line "Red42" from Brazil; (Montenegro et al., 2005) via hemolymph
141 transfer (as in Xie et al., 2010) at least 3 generations before initiating the experiment. As *s*Mel-
142 BR is a male-killer, the *Spiroplasma*-infected strain was maintained by addition of
143 *Spiroplasma*-free males (Canton S strain) every generation. Wasps, *Leptopilina heterotoma*
144 strain Lh14 (Schlenke et al., 2007) and the all-female *Ganaspis hookeri* strain G1FL (Mortimer
145 et al., 2013), were reared using second instar Canton S *Spiroplasma*-free larvae. These wasp
146 strains are naturally infected with one or more *Wolbachia* strains (Wey et al., 2020 and Mateos,
147 unpublished data). For the qPCR assays, we used *Wolbachia*-free Oregon R flies to which
148 *s*Mel-BR or *s*Mel-UG (original isofemale line from Uganda, Pool et al., 2006), had been
149 artificially transferred at least 3 generations prior. These flies were maintained by matings with
150 *Spiroplasma*-free Oregon R males under the same environmental conditions as the Canton S
151 background flies, but in a opuntia-banana food medium (recipes in Supplementary methods 1).

152 **2.2 RNA-Seq based methods**

153 **2.2.1 Wasp exposure**

154 To examine the effect of the interaction of *Spiroplasma* and wasp on the transcriptome of
155 *Drosophila* (and of *Spiroplasma*), we compared treatments with the presence and absence of
156 *Spiroplasma* (*s*Mel-BR) and one of the two wasp species at two different time points. This
157 experimental design resulted in a combination of twelve treatments; six treatments per time
158 point. For each replicate, parental flies (approximately ten females and ten males) were set up

159 in oviposition vials in the evening for overnight oviposition. Parental flies were removed the
160 next morning. One day later, ~30 second-instar *D. melanogaster* larvae were carefully collected
161 and transferred to a Petri dish (60 mm diameter) containing cornmeal food medium
162 (**Supplementary methods 1**). In replicates assigned to a wasp treatment, 5 male and 6 female
163 wasps of the corresponding species (*L. heterotoma* or *G. hookeri*) were added to the Petri dish,
164 and allowed to oviposit for ~5 h. All female wasps had been previously allowed to oviposit on
165 *D. melanogaster* fly larvae for ~5 hours. The purpose of this "training" is to ensure that the
166 wasps are experienced at oviposition prior to the experiment. All Petri dishes were covered,
167 but a small hole was opened (with a hot needle) to allow for gas exchange, and/or through
168 which wasps could feed on a piece of cotton wool soaked in 1:1 water:honey mix that was
169 placed outside the dish. To collect RNA, larvae were retrieved from each Petri dish at either
170 24h (T1) or 72h (T2) post-wasp attack (PWA) (i.e., one or three days after wasps were removed,
171 respectively). To ensure sufficient material for RNA-seq, 20–30 larvae were collected for the
172 24h time-point, whereas 10–20 for the 72h time point. Both wasp species were embryos at T1
173 and larvae at T2. Fly larvae from the same Petri dish were pooled into a single RNA extraction
174 tube (i.e., a replicate).
175 From each Petri dish in the wasp-exposed treatments, a subsample of fly larvae was used to
176 verify wasp parasitism rate as follows. First, at the time of larvae collection for RNA, five fly
177 larvae per replicate were dissected under the microscope and discarded. If all five larvae
178 contained at least one wasp egg or larva (i.e., 100% wasp oviposition rate), the replicate was
179 retained and processed. If one or more fly larvae did not contain a wasp larva (or embryo), then
180 five additional fly larvae were examined for wasp presence. Only replicates with 90-100%
181 wasp oviposition rate were retained. All collections for RNA were performed in the afternoon-
182 evening, and collected larvae were quickly placed in an empty microtube for processing.

183 **2.2.2 RNA extraction**

184 Collected larvae were either immediately frozen at –80°C or immediately processed for RNA
185 extraction. Preliminary experiments with RNAlater Stabilization Solution revealed that the
186 larvae did not die immediately and appeared to melanize. Total RNA was extracted using the
187 Trizol [invitrogen] method. Each sample consisted of a pool of *Drosophila* larvae that were
188 homogenized by hand with a sterile plastic pestle in Trizol reagent. The Trizol isolation method
189 was performed following the manufacturer's protocol but it was stopped at the 70% ethanol
190 wash step. The total RNA pellet in ethanol was submitted to the Texas AgriLife Genomics and
191 Bioinfomatics Services facility for completion of the RNA isolation procedure, assessment of
192 RNA quality and quantity (using Fragment Analyzer; Agilent, Santa Clara, CA), library
193 preparation, sequencing, and demultiplexing.

194 **2.2.2 Library preparation and sequencing**

195 Total RNA was subjected to removal of ribosomal RNA from eukaryotes and prokaryotes with
196 the RiboZero Epidemiology kit (Illumina, San Diego, CA). The TruSeq stranded kit (Illumina)
197 was then used to prepare the library for sequencing with Illumina (125 bp Single End "HighSeq
198 2400v4 High Output").

199 **2.2.4 Bioinformatic analysis**

200 Quality and presence of adapters was evaluated with FastqC (Andrews S., 2010), followed by
201 a trimming with Trimmomatic v.0.36 (Bolger et al., 2014) using
202 ILLUMINACLIP:/adapters.fasta:2:30:10 LEADING:3 TRAILING:3
203 SLIDINGWINDOW:4:15 MINLEN:36. To examine differential expression of *D.*
204 *melanogaster* genes, trimmed reads were mapped with Hisat2 v.2.0.2-beta (Kim et al., 2015),
205 using --rna-strandness R option. Treatments parasitised by *L. heterotoma* were mapped to an
206 index composed by *D. melanogaster* genome (ensembl version BDGP6) plus *L. heterotoma*

genome, reference VOOK00000000. Treatments parasitised by *G. hookeri* were only mapped to *Drosophila*, because there is not an available genome for *G. hookeri*. The resulting *D. melanogaster* mapped reads were quantified using featureCounts from the Subread package v1.6.2 (Liao et al., 2014), using the following parameters: -s 2, -t exon, -g gene_id. Differential gene expression was done in R (R core Team) with edgeR v 3.24.3 package (Robinson et al., 2009). Genes with counts < 1 cpm for all replicates and treatments under comparison were discarded. Only genes with absolute 2LogFC >= 0.58 and FDR < 0.05 were considered differentially expressed (DE). The Robust parameter robust=TRUE) of edgeR was implemented to minimize false positive DE genes. The gene ids corresponding to the DE genes were loaded (before May 2020) into Flymine, (an integrated database for Drosophila genomics(Lyne et al., 2007) available on the website https://www.flymine.org/flymine/begin.do. This platform outputs gene names plus other information such as Gene Ontology (GO), enrichment of pathways, tissue expression and protein domains. Expression patterns of individual genes were obtained from flybase2.0 (Thurmond et al., 2019) available flybase.org/. Venn diagrams used to detect exclusive genes in the interactions were generated using one web-tool available at http://bioinformatics.psb.ugent.be/webtools/Venn/

To measure *Spiroplasma* gene expression, the trimmed reads from the *Spiroplasma*-infected treatments were subjected to kallisto v.0.43.1 (Bray et al., 2016), using the genome of *S. poulsonii s*Mel-UG as reference GCF_000820525.2). Count tables obtained by kallisto were used to detect DE genes using edgeR. Expression of RIP in transcriptome was obtained from this table using trimmed mean of M-values (TMM) normalized counts. Heatmaps of expression patterns were generated with the R package pheatmap v 1.0.12.

230 **2.2.5 Power analyses for differential expression.**

231 To identify potential limitations on the detection of DE genes, we performed a statistical power
232 analysis with the R package RNASeqPower v.1.22.1 (Hart et al., 2013). Parameters used to run
233 different simulations were inferred from our datasets and are provided in Table S1 and Figure
234 S1.

235 **2.2.6 Analyses of depurination signal in the sarcin-ricin loop (SRL) of the 28S rRNA of**
236 **wasps and flies**

237 RIP toxins remove a specific adenine present in the sarcin-ricin loop (SRL) of the 28S rRNA
238 leaving an abasic site (i.e., the backbone remains intact). When a reverse transcriptase
239 encounters an abasic site, it preferentially adds an adenine in the nascent complementary DNA
240 strand. This property, which results in an incorrect base at the RIP-depurinated site in the cDNA
241 and all subsequent PCR amplification steps, has been used to detect evidence of RIP activity
242 in any procedure that relies on reverse transcription such as RNA-seq or reverse-transcription
243 qPCR (e.g. Hamilton et al., 2015).

244 To examine whether a signal of depurination consistent with RIP activity was detectable in
245 wasp-derived sequences, we mapped RNA-seq data to a reference sequence file comprised of
246 the 28S rRNA sequences of the wasps and of *D. melanogaster* using Bowtie2 v.2.1.054 with
247 default options. Only sequences that mapped to the wasp 28S rRNA were retained (Dataset
248 S1). To visualize and count the shift from A to T (or other bases), the retained reads were
249 mapped again to the 28S rRNA of wasp in Geneious v.11.1.2 (Biomatters Inc., Newark, NJ;
250 "low sensitivity mode"; maximum gap size = 3; iterate up to 25 times, maximum mistmatches
251 per read 2%). The number of reads containing each of the four bases or a gap at the target site
252 was counted by selecting the position at all the reads to be counted, and recording the counts
253 reported by Geneious under the "Nucleotide Statistics" option (gapped reads were excluded
254 from counts). Reads were counted only if they fully covered a specific part of the 28S loop
255 sequence (TACG**A**GAGGAACC). The bold-faced adenine represents the site of RIP

256 depurination. Replicates with fewer than 10 mapped reads were discarded, Table S3 and Figure
257 S10. Statistical analysis was conducted in R v 4.02 (R core Team) using a Bayesian generalized
258 linear model (bayesglm function in "arm" package), this is because of the presence of zeros
259 (no depurination) in some treatments. Using the above strategy, raw sequences were also
260 mapped to the full sequence of the 28S rRNA of D. melanogaster, and depurination was
261 evaluated. Due to the high number of ribosomal sequences that align to the 28S rRNA, only
262 subsets of 1 million of sequences were analyzed (Table S4).Using the above strategy, raw
263 sequences were also mapped to the full sequence of the 28S rRNA of *D. melanogaster,* and
264 depurination was evaluated. Due to the high number of ribosomal sequences that align to the
265 28S rRNA, only subsets of 1M of sequences were analyzed. Table S4

266 **2.3 Quantitative (q)PCR-based Methods**

267 **2.3.1 Expression of *Spiroplasma* Ribosomal inactivation proteins (RIPs).**

268 To verify the RIP expression patterns inferred from the transcriptome (see Results) and to
269 examine whether they were consistent between substrains of *s*Mel, we used qPCR on a a new
270 set of treatments. We followed the "Wasp exposure" methodology (described above), but used
271 *Wolbachia*-free *D. melanogaster* (OregonR) harboring the *S. poulsonii* strain *s*Mel-BR or
272 *s*Mel-UG. Five larvae per treatment (parasitized or not by *L. heterotoma* or *G.hookeri*), were
273 collected at 24 and 72h PWA, flash frozen in liquid nitrogen and homogenized by hand with a
274 pestle. Total RNA was extracted with the All prep DNA/RNA mini kit (Qiagen, Germantown,
275 MD). 1ug of total RNA was used to synthesize cDNA using superscript II reverse transcriptase
276 (Invitrogen), following manufacturer's procedures. cDNA was used as template for qPCRs,
277 performed on a CFX96 detection system (Bio-Rad, Hercules, CA). The mix contained 5ul of
278 iTaq Universal SYBR Green Supermix (Bio-Rad), 2.5ul water, 2ul cDNA, and 0.25ul of each
279 primer (stock solution at 10uM). Primer sequences and efficiencies for RIP2, RIP3-5 and rpoB
280 were taken from (Ballinger and Perlman, 2017). For RIP1, we designed and used the following

281 primers Forward: 5'- AATCAGAGGGGCATTAGCTC-3' Reverse 5'-
282 CTTCGCTTGTGGTTCTTGAT-3', efficiency = 0.995. Although (Ballinger and Perlman,
283 2017) reported a primer pair targeted at RIP1, this primer pair matches a fragment of RIP2
284 instead.

285 Relative expression was calculated using efficiency-corrected Ct values using
286 (Ct*(Log(efficiency)/Log(2))) formula. DeltaCt was calculated as Ct-rpoB minus Ct-RIPx
287 (Dataset S2) . We used JMP Pro v.15 (SAS, Cary, NC) to fit a full factorial Generalized
288 Regression (Normal distribution) model. The response variable was delta Ct Value. The
289 independent variables (all fixed and categorical) were: RIP gene ("RIP": RIP1, RIP2, RIP3-5),
290 Wasp Treatment (No wasp, Lh and Gh), *Spiroplasma* strain Brazil, Uganda) Time Point (24h
291 or 72h). Significant effects and interactions were explored with Tukey HSD tests with Least
292 Square Means Estimates (Supplementary file S1).

293 2.4 Data availability

294 All raw reads generated in this project have been submitted to NCBI under the SRA
295 accession numbers PRJNA577145. Count tables for *D. melanogaster* and *Spiroplasma* are in
296 Dataset S3. Command lines used to run bioinformatic analyses are available in
297 Supplementary methods 2. Data generated during this study are available at figshare
298 https://doi.org/10.6084/m9.figshare.c.5104559.v3

299 # 3. Results

300 To examine the effect of *Spiroplasma* on *D. melanogaster* gene expression under wasp
301 parasitism, we generated twelve-RNA-seq treatments (Figure1), with an average of 47 million
302 quality single-end reads per sample, ~90% of these reads mapped to the *D. melanogaster*
303 genome. A fraction of these reads mapped to ribosomal sequences of the host, which is an
304 indication of incomplete ribodepletion (Table S2).

305 The multidimensional scaling (MDS) plot of all treatments at 24h post wasp attack (PWA; T1)
306 did not reveal any particular grouping by treatment (Fig. S2). At the 72h PWA time point (T2),
307 however, the treatments separated at the first dimension by presence/absence of *Spiroplasma*
308 (Fig S3). This plot allowed us to detect two replicates that we deemed outlier and decided to
309 exclude from further analyses (red samples in Fig 1). Replicate S0Lh_4, which lacked
310 *Spiroplasma*, grouped with the *Spiroplasma*-infected treatments. We confirmed the absence of
311 *Spiroplasma* because no reads derived from *Spiroplasma* were detected. Nonetheless, we
312 realized that this replicate only contained three larvae individuals, which by chance may have
313 been all female. Concerning replicate S+Lh_1, its expression pattern included upregulation of
314 numerous genes associated with immune response. Consequently, we suspected that this
315 particular replicate likely contained one or more larvae infected by a pathogenic bacterium.
316 Below we first describe the response of *D. melanogaster* to parasitism by the wasps (*L.*
317 *heterotoma* or *G. hookeri*) in the absence of *Spiroplasma*, followed by the fly response to the
318 sole presence of *Spiroplasma*. Finally, taking these results into account, we examine the
319 response of *Drosophila* during the *Spiroplasma*-wasp interaction.

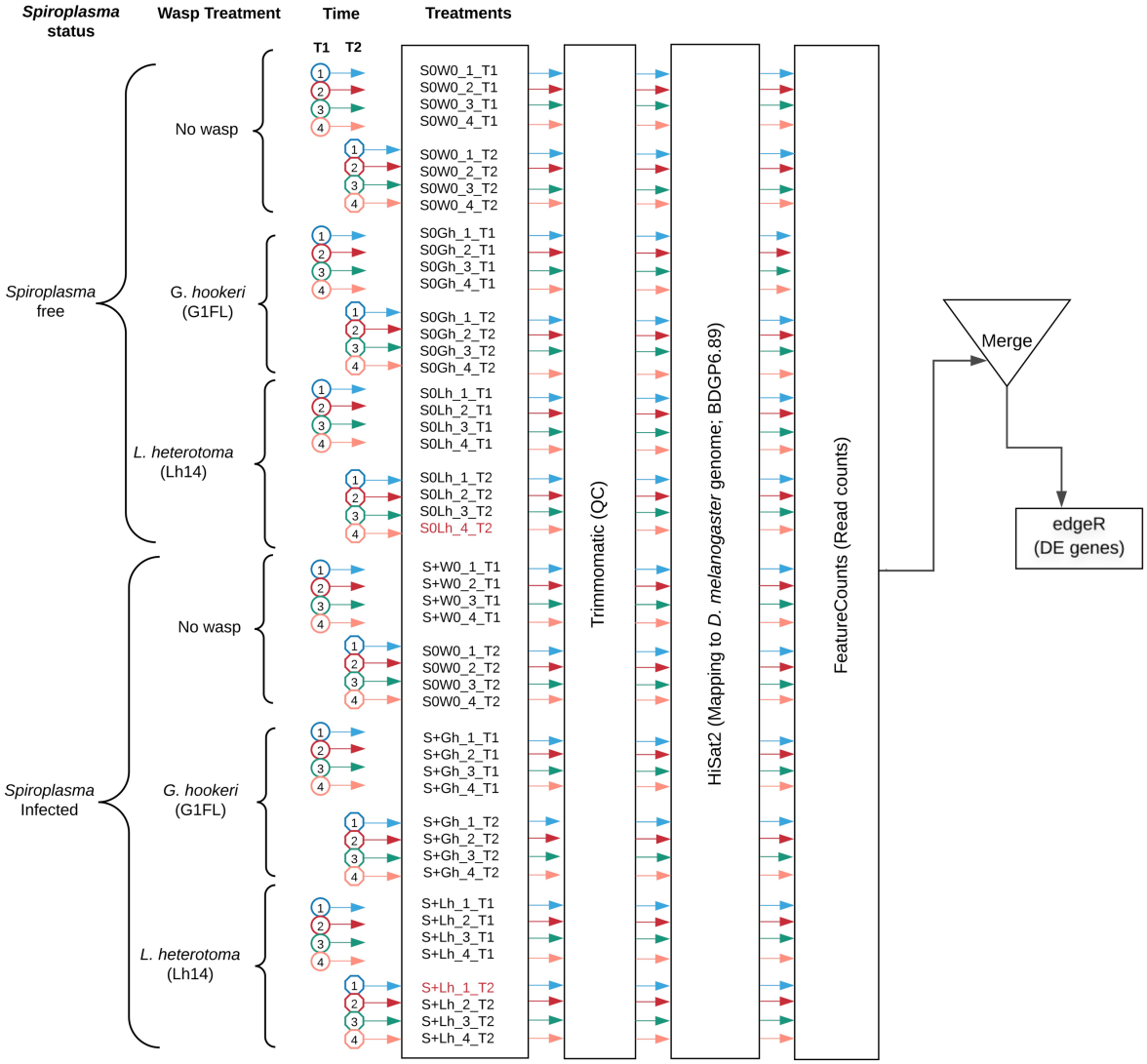


Figure 1. Experimental design and workflow for data analysis. Two samples (S0Lh_4_T2 and S+Lh_1_T2) were removed from the analysis (see text).

3.1 Response of *D. melanogaster* to *L. heterotoma* parasitism in the absence of *Spiroplasma*

Parasitism by *L. heterotoma* at T1 (24h) post wasp attack (PWA), did not have a large effect on *D. melanogaster* gene expression, as only one gene (*thor*) was upregulated, whilst two genes, *Hml* and *mt:ATPase6*, were downregulated (Figure 2a and Dataset S5). In contrast, at T2 (72h PWA), 1216 genes were up- and 1669 down-regulated (Figure 2a and Dataset S5). Of the 1216 upregulated genes at T2, 810 grouped into 32 GO enriched categories (Figure 2b); of

331 which transport was the most enriched. A pathway analysis revealed that upregulated genes are
332 involved with energy generation pathways, such as lipid metabolism and citric acid cycle
333 (Dataset S5). Of the 1669 downregulated genes, 925 grouped into 25 GO categories, of which
334 proteolysis was the most enriched (Figure 2B and Dataset S5). Unexpectedly, a subset of 67
335 downregulated genes belongs to GO categories related to spermatogenesis.

336 **3.2 Response of *D. melanogaster* to *G. hookeri* parasitism in the absence of *Spiroplasma***

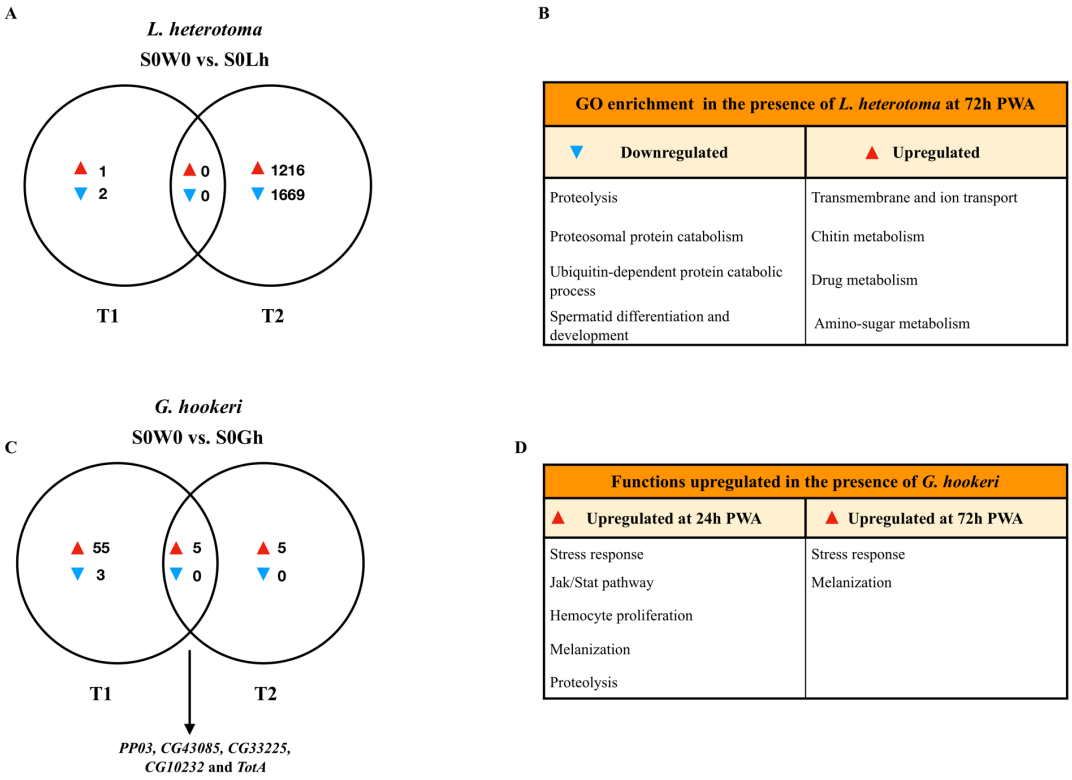
337 In the presence of the wasp *G. hookeri* (i.e, S0W0 vs. S0Gh), three genes were down- and 60
338 were up-regulated at T1 (Figure 2C)**.** The two downregulated genes are known to be expressed
339 by hemocytes (*Peroxidasin* and *hemolectin*) (Irving et al., 2005), whereas the other is a small
340 nucleolar RNA (*Uhg4*). Only one gene, *hemolectin*, was down-regulated by both *L. heterotoma*
341 and *G. hookeri* at T1 (Dataset S5). The sixty upregulated genes in the presence of *G. hookeri*
342 at T1 include genes that are known to be expressed preferentially by hemocytes such as *hemese*,
343 *ItgaPS4, ItgaPS5*, *Scavenger receptor class C* (*CG3212*), one serpin (*CG6687*), a serine
344 protease (*CG6639*), as well as one gene involved in hemocyte proliferation (*pvf2*). (Irving et
345 al., 2005). One activator of the Jak Stat pathway *upd3* and some effectors of this pathway, *TotA,*
346 *tep1* and *tep2* (Agaisse and Perrimon, 2004), were also upregulated. Prophenoloxidase 3
347 (*PPO3)* and *yellow-f* genes, which are involved in the melanization process (Han et al., 2002;
348 Dudzic et al., 2015)**,** were also upregulated. *PPO3* was highly upregulated (2 LogFC = 9). The
349 complete list of DE genes and GO enrichment is provided in Dataset S4.

350 At T2, the presence of *G. hookeri* induced upregulation of ten genes, but no genes were
351 downregulated **(**Figure 2C and Dataset S5**)**. The upregulated genes included the stress response
352 genes *TotA*, *TotB*, *TotC* and *victoria*, but also *PPO3*; log2FC of these genes ranged 3–10. Five
353 genes were shared between the two time points (Figure 2c). Two of them, *CG10232* and
354 *CG33225* are predicted to be involved in proteolysis, and *CG43085* has no function or
355 prediction assigned. The other two genes were *PPO3* and *TotA*; their log2FC were higher at

356 T1 than at T2. In general, immune functions were upregulated by *G. hookeri* parasitism at both

357 times (Figure 2D).

358



359

360 **Figure 2**. Venn diagrams and enriched functions or Gene Ontology (GO) categories.

361 Differentially expressed genes of *D. melanogaster* at time points T1 and T2 [24h and 72h post

362 wasp attack (PWA), respectively] by (A) and (B) *L. heterotoma* or (C) and (D) *G. hookeri*.

363 Enriched GO categories or functions, if applicable, are reported in (B) and (D). The arrow in

364 panel (C) indicates the five genes in the T1 and T2 intersection.

365

366 **3.3 Response of *D. melanogaster* to *Spiroplasma***

367 The sole presence of *Spiroplasma* (i.e., S0W0 vs. S+W0 comparison) at 24h PWA did not

368 reveal any upregulated genes, but 27 were downregulated. Twenty of 27 downregulated genes

369 are reported as preferentially expressed in adult testis (Dataset S4). At 72h PWA, the presence

370 of *Spiroplasma* induced upregulation of 16 and downregulation of 1476 genes. Only

371 downregulated genes (692 of the 1476) were assigned to one or more of 71 GO categories
372 (Dataset S5). Some of these categories are related to the energy generation process such as
373 oxidative phosphorylation, pyruvate metabolic process, and glycolytic process. Among the
374 most enriched categories were male gamete generation and spermatogenesis, which is in
375 agreement with the expected lack of males in S+W0 treatments. In addition, 1333 of the 1476
376 downregulated genes at T2 are classified as preferentially upregulated in fly testis.
377 Furthermore, the cpm values of ~78% of these genes in all replicates of the S+W0 treatment
378 were < 1, implying very low expression levels. Therefore, as expected, a large number of the
379 genes with lower expression in the *Spiroplasma* treatment, including the roX1 and roX2
380 (exclusively expressed in males, and part of the dosage compensation system; reviewed in
381 Lucchesi and Kuroda, 2015), may simply reflect the absence of males.

382 **3.4 *Drosophila* gene expression during *Spiroplasma-L. heterotoma* interaction**

383 To explore if the presence of *Spiroplasma* influences gene expression of *D. melanogaster*
384 during parasitism by *L. heterotoma*, we adopted the following strategy to identify genes whose
385 expression was specifically influenced by the *Spiroplasma- L. heterotoma* interaction. We used
386 a Venn diagram depicting differentially expressed (DE) genes from the following three
387 pairwise treatment comparisons: S0Lh vs. S+Lh (i.e., effect of *Spiroplasma* in the presence of
388 Lh; green sets in Fig. 3); S0W0 vs. S+W0 (i.e., effect of *Spiroplasma* in the absence of any
389 wasp; blue sets); and S0W0 vs. S0Lh (i.e., effect of Lh in the absence of *Spiroplasma*; red sets).
390 Genes that fell in the exclusive part of the green set were deemed as influenced specifically by
391 the *Spiroplasma-L. heterotoma* interaction. Furthermore, genes that fell in the intersection of
392 the green set with one or both of the blue and red sets, were further evaluated with heatmaps
393 depicting expression levels in four treatments (S0W0, S+W0, S0Lh, S+Lh). This approach
394 aimed to identify genes that had substantially different expression levels in the S+Lh treatment
395 vs. the treatments that only had Lh or only had *Spiroplasma*.

396 The S0Lh vs. S+Lh comparison at T1 yielded zero up- and 18 down-regulated genes (exclusive
397 green set in Fig. 3A, Dataset S4). Ten of these genes code for small nucleolar rnas (snoRNA).
398 Five other genes (*fest*, *CG10063*, *SkpC*, *eIF4E3* and *cona*), albeit exclusively downregulated
399 in *Spiroplasma-L. heterotoma* interaction, seem to be influenced by *Spiroplasma* alone, based
400 on the observation that these genes were downregulated by *Spiroplasma* alone at the later time
401 point T2 (Dataset S5). Furthermore, *fest* and *eIF4E3* are upregulated in testis, and thus their
402 downregulation may simply reflect absence of males.

403 The only gene-containing intersection (green+blue), had 19 genes, of which 16 are
404 preferentially expressed in adult testis (Dataset S4). The heatmap of all genes in the intersection
405 (Fig. S4), revealed that most of them had similarly low levels of expression in the two
406 *Spiroplasma*-infected treatments (i.e., S+W0 and S+Lh), compared to the S0W0, which had
407 the highest. In turn, the S0Lh treatment exhibited an intermediate expression level (Fig. S4).

408 At T2, 21 genes were up- and 11 down-regulated exclusively in S+Lh treatment compared to
409 S0Lh (exclusive green set in Figure 3B; Dataset S5). Four (*CG18649, Cyp4d14, CG42494* and
410 *CG44215*) out of the 11 genes exclusively downregulated in the S+Lh treatment were
411 exclusively upregulated in S0Lh with respect to S0W0 (i.e., identified with * and labeled
412 "Group 1" in Fig. 3c). Seven (*Br*, *CG32115*, *Fs*, *PLCXD*, *Npc2e*, *phm* and l*ncRNA-CR43828*)
413 of the 21 genes exclusively upregulated in the S+Lh treatment were exclusively down-
414 regulated in S0Lh with respect to S0W0 (i.e., identified with ** and labeled "Group 2" in Fig.
415 3c). Hereafter, we refer to genes in Groups 1 and 2 as "restored" because parasitism by *L.*
416 *heterotoma* (S0Lh) increased and decreased (respectively) their expression with respect to the
417 S0W0 control, but in the presence of *Spiroplasma* plus *L. heterotoma* (i.e. the S+Lh treatment),
418 expression levels appear to return to those observed in the control (S0W0; Fig.3C). In other
419 words, *Spiroplasma* appears to "buffer" or counter the effects caused by the presence of *L.*
420 *heterotoma*. Similarly, *Fbp2*, at the blue+green intersection, exhibits a similar expression

421 pattern to genes in Group 2 (Fig. 3C). *TotC* (in the green+red intersection) also exhibited a
422 peculiar expression pattern (Fig. 3C); it was upregulated by *L. heterotoma* parasitism (S0Lh),
423 but its expression was highest in the S+Lh treatment.

424 The rest of the DE genes exclusive to *Spiroplasma-L. heterotoma* interaction were 14 up- and
425 7 down-regulated (Fig. 3B green set and Dataset S5). These genes did not group into any GO
426 or specific pathway. Among the upregulated genes, there were *Amylase proximal*,
427 *anachronism*, and the antimicrobial peptide *Attacin-C* (*AttC*; FC= 1.5), which was the only
428 gene associated with immune response. Downregulated genes included two snoRNA
429 (*CR34643* and *CR34560*), *HemK1* and a long non-coding RNA (lncRNA:CR32661) (Dataset
430 S5).

431 The heatmaps of the red+blue+green (386 genes) and of the blue+green (143 genes)
432 intersections at T2 (Fig. 3B and Fig S5-6) revealed the following general pattern that was also
433 observed for the 19 downregulated genes in the blue+green intersection of T1 (see above): the
434 two *Spiroplasma* treatments (S+W0 and S+Lh) had the lowest expression levels; the S0W0
435 treatment had the highest; and the S0Lh treatment was intermediate.

436

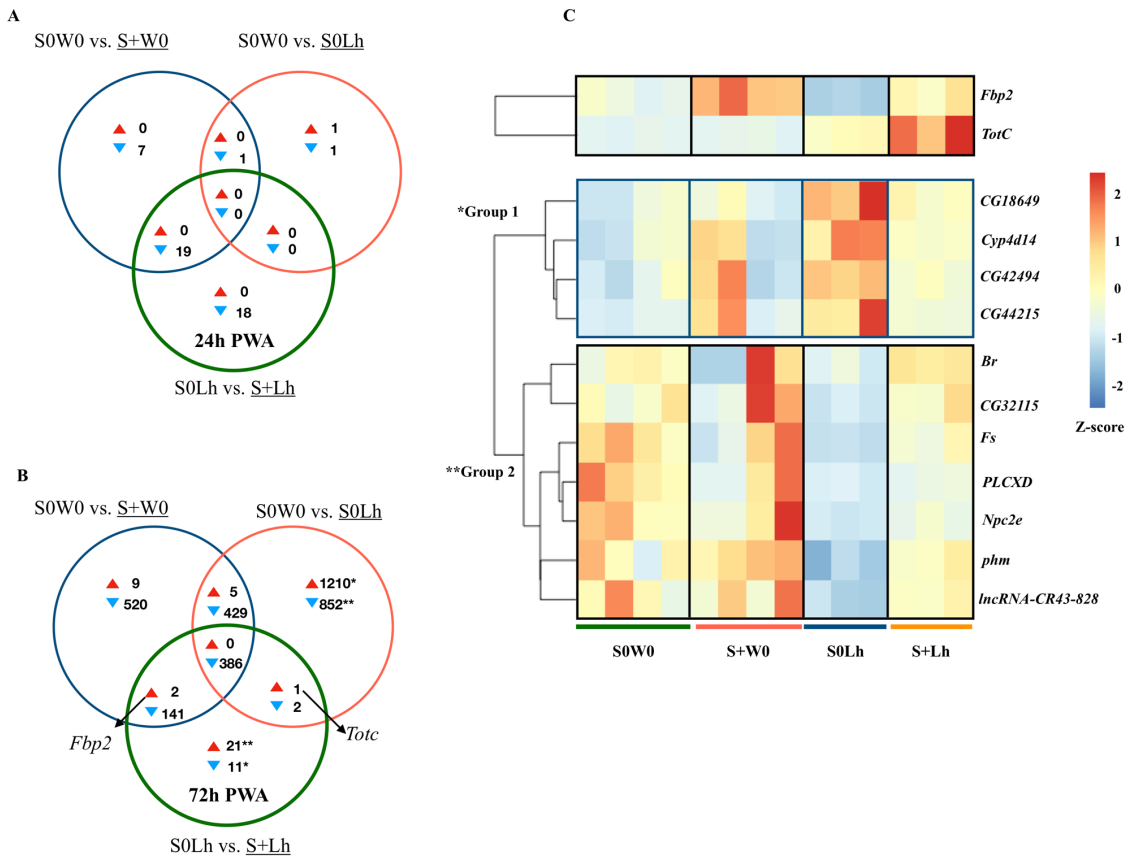


Figure 3. Patterns of differential expression (DE) in the context of the interaction of *Spiroplasma* and the wasp *L. heterotoma* (Lh14). Unique and shared number of DE genes in each of three treatment comparisons. (A) Time-point 1 (T1): 24h post-wasp attack (PWA). (B) Time-point 2 (T2): 72h PWA. Red- and blue-filled triangles indicate up- and downregulation, respectively, in the treatment that is underlined. (C) Heatmap of genes with peculiar expression pattern; genes in Group 1 were upregulated in the presence of only Lh and "restored" in the presence of *Spiroplasma* and Lh. Genes in Group 2 were downregulated in the presence of only Lh and "restored" in the presence of *Spiroplasma* and Lh (see text for explanation regarding TotC and Fbp2). Z-score of edgeR TMM-normalized values are represented.

3.5 *Drosophila* gene expression during *Spiroplasma-G. hookeri* interaction

To identify fly genes specifically influenced by the *Spiroplasma-G. hookeri* interaction, we adopted the same Venn diagram plus heatmaps strategy as with the *Spiroplasma-L. heterotoma* interaction, except that the *L. heterotoma* treatments were replaced with *G. hookeri* treatments (see Fig. 4). Excluding genes induced by the sole presence of *Spiroplasma* or *G. hookeri*,

452 resulted in eight and 21 exclusively up- and down-regulated (respectively) genes at T1
453 (exclusive green set, Fig 4A, Dataset S4). The eight upregulated genes were: four snoRNA
454 (*CR33662*, *CR34611*, *CR34616,* and *CR34631*), one small nuclear RNA (*CR32162*), lysozyme
455 E, diphthamide methyltransferase (*Dph5*) and mitochondrial ribosomal protein S14 (*mRpS14)*.
456 Among the 21 downregulated genes were *DnaJ-1* (whose product is a cofactor of heat shock
457 proteins), *starvin* (which acts as co-chaperone of Hsp70 proteins), *nervana 3* (which codes for
458 one subunit of a sodium-potassium pump), *nanos* (which encodes a ribosomal RNA-binding
459 protein), and one long non-coding RNA (*CR31400*).

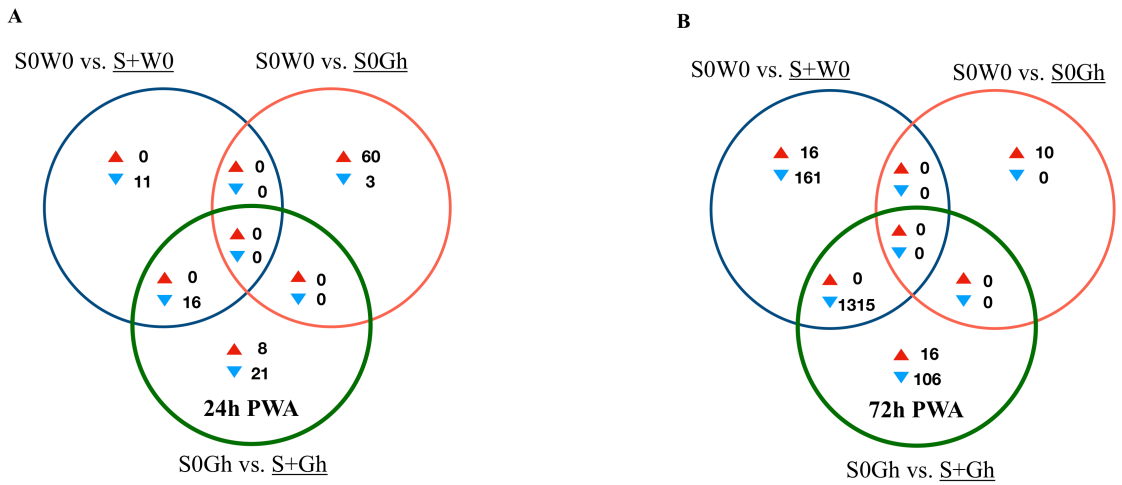
460 At T1, only one intersection (green+blue) contained genes; all downregulated (n = 16; Fig.
461 4A). A heatmap of expression levels of these 16 genes (Fig. S7) reveals that the two
462 *Spiroplasma* treatments (S+Gh and S+W0) have similarly low expression levels, whereas the
463 two treatments lacking *Spiroplasma* (S0W0 and S0Gh) have similarly high expression levels;
464 implying that there is no influence of *G. hookeri* on expression of these genes. Three of the
465 downregulated genes are involved with male functions (*msl-2, roX1* and *roX2*), one gene
466 (*blanks*) is highly expressed in adult testis, and three other genes (*RpL22-like*, *RpS5b* and
467 *RpS19b*) code for ribosomal proteins (Dataset S4).

468 At T2, 16 and 106 genes were exclusively up- and down-regulated (respectively) in the S+Gh
469 treatment (exclusive green set in Fig. 4B, Dataset S5). The 16 upregulated genes included three
470 mitochondrially-encoded genes (*ND1, CoIII*, and *Co*I), *necrotic* (which is negative regulator
471 of the Toll pathway; Green et al., 2000), *dumpy* (which is involved in wing development), and
472 one multidrug resistance gene (*Mdr50*). The 106 exclusively downregulated genes did not
473 group in any GO category, making it difficult to link these genes to informative biological
474 functions. The expression values to several of these genes in the S+Gh treatment is similar to
475 S+W0 treatment (Fig S8.). This observation, along with the report that 74 of these genes are
476 highly expressed in adult testis (Dataset S5), suggests that observed expression patterns are

477 likely influenced solely by the presence of *Spiroplasma.* The heatmap of the 16 exclusively
478 upregulated genes (Fig S9), also indicates a possible influence of only *Spiroplasma*. These
479 genes showed a trend of higher expression levels in the S+W0 treatment relative to the S0W0
480 treatment, but this difference was not significant, possibly due to the high variation among
481 S+W0 replicates.

482 Only one intersection at T2 (green+blue sets) contained genes (n = 1315; all down-regulated;
483 Fig. 4B). These genes are associated with spermatid differentiation/development functions and
484 1214 of these 1315 are predominantly expressed in the adult testis (Dataset S5). Thus, their
485 lower expression in the presence of *Spiroplasma* (with or without Gh; see Fig S10) is likely
486 simply the result of an absence of males in these treatments.

487



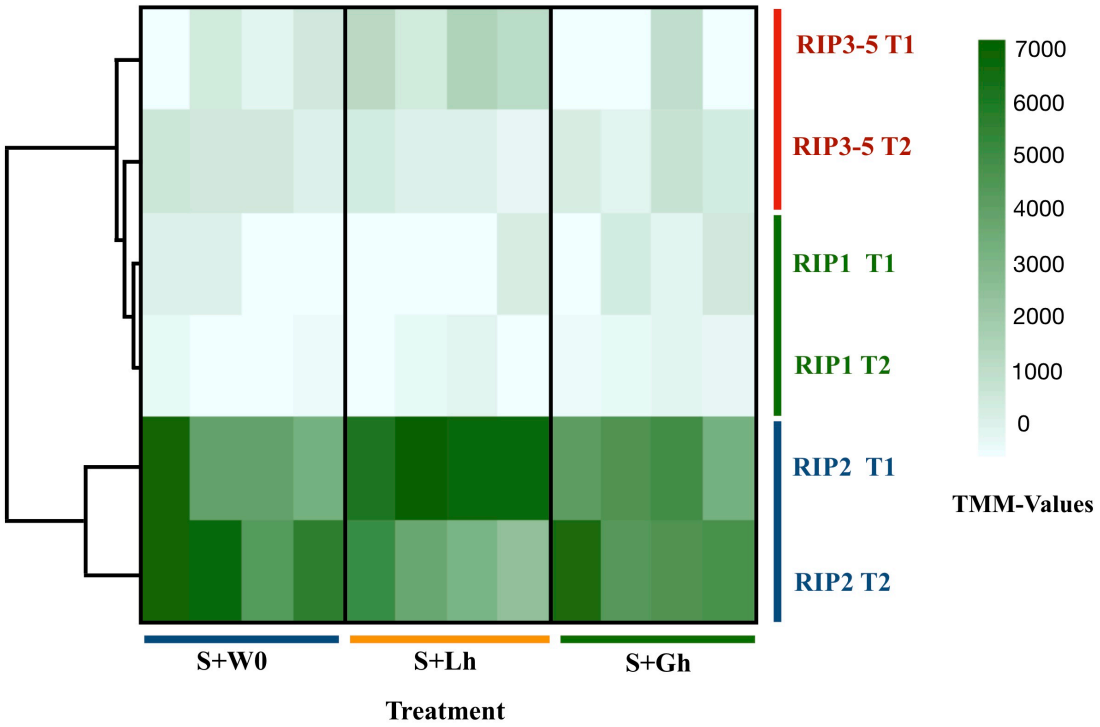
488

489 **Figure 4**. Patterns of differential expression (DE) in the context of the interaction of
490 *Spiroplasma* and the wasp *G. hookeri* (G1FL). Unique and shared number of DE genes in each
491 of three treatment comparisons. (A) Time-point 1 (T1): 24h post-wasp attack (PWA). (B)
492 Time-point 2 (T2): 72h PWA. Red and blue filled triangles indicate up- and downregulation,
493 respectively, in the treatment that is underlined.

494 **3.6 Expression of *Spiroplasma* RIP proteins and evidence ribosomal damage**

495 The total number of reads mapped to *Spiroplasma* excluding ribosomal sequences, was very
496 low (range = 1080-17,826 per replicate, Table S2). No DE genes were detected, but this could
497 be a reflection of lack of power. For differential gene expression analyses in bacteria, a
498 minimum of four-five million reads per replicate has been recommended. (Haas et al., 2012)
499 Due to the relevance of RIP genes in *Spiroplasma* (Ballinger and Perlman, 2017), we examined
500 the read counts of the five RIP genes encoded in the *s*Mel genome, which revealed that the
501 RIP2 gene encoding was the most highly expressed at both time points and in all treatments
502 (Figure 5)**.**

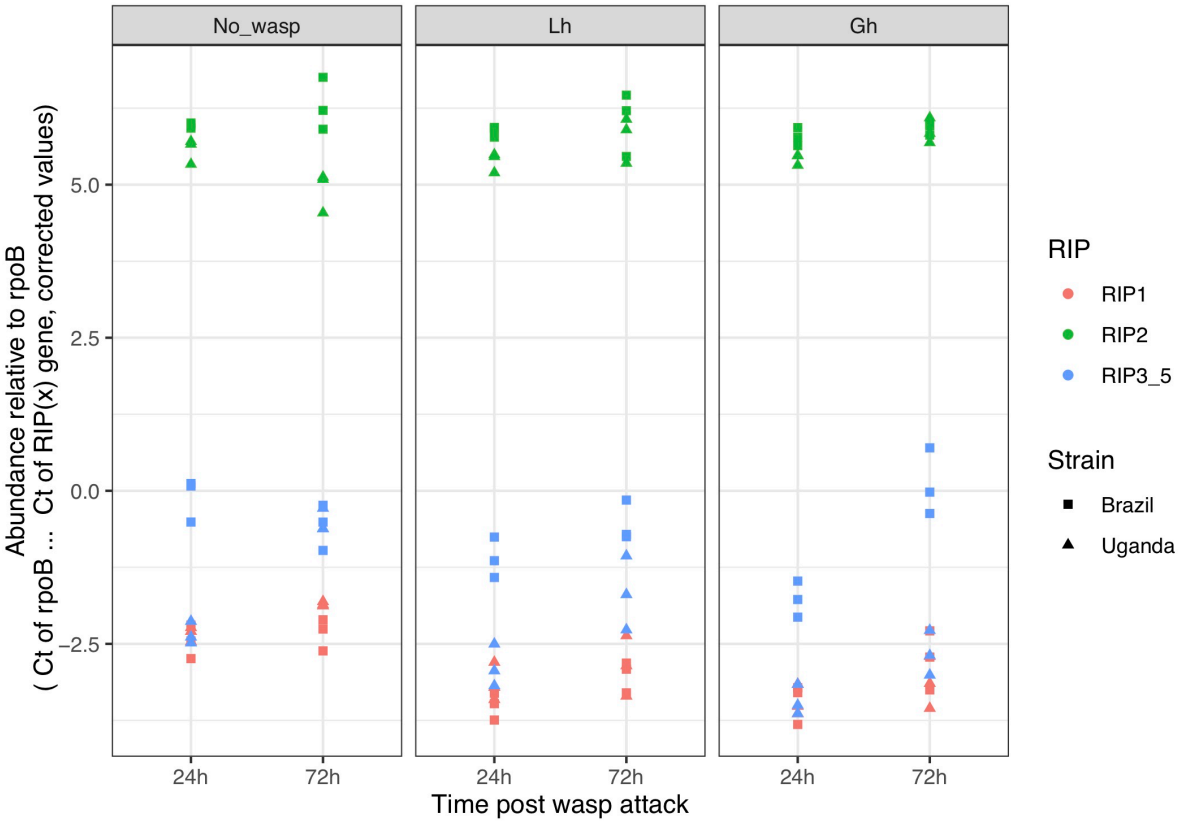
503



504

505 **Figure 5**. Expression levels of the five ribosome inactivating protein (RIP) genes encoded by
506 Spiroplasma genome, in absence of parasitism (S+W0), in parasitism by *L. heterotoma* (S+Lh)
507 or *G. hookeri* (S0Gh), at 24h (T1) or 72h (T2) post-wasp attack (PWA). Values of TMM edgeR
508 normalized counts are shown. Because the RIP3, RIP4, and RIP5 genes are identical at the
509 nucleotide level, their collective expression levels were reported under the label "RIP3-5".

510 To corroborate the observed patterns of RIP gene expression based on the RNA-seq data, RT-
511 qPCR assays were conducted using both the Uganda (UG) and Brazil (BR) *Spiroplasma* sMel
512 strains, and the two wasps, *G. hookeri* and *L. heterotoma*. Consistent with the RNA-seq results,
513 the gene encoding RIP2 was the most highly expressed of the RIP genes in both *Spiroplasma*
514 strains regardless of the presence or absence of wasp (Fig 6, dataset S2). The full output of the
515 statistical analyses is provided in Supplementary file 1. Wasp depurination in the 28S rRNA
516 was significant (X^2=128.58, df=1, P< 2.2 e-16) in the context of *L. heterotoma* in presence of
517 *Spiroplasma* at T2 (Figure S11 and Table S3). No depurination was detected in *G. hookeri* or
518 fly sequences (Figure S11 and Table S3-4).
519



520
521 **Figure 6**. RIP (ribosome inactivating protein) gene expression in *D. melanogaster* larvae in
522 the absence of wasp (No_wasp) or parasitised by *L. heterotoma* (Lh) or *G. hookeri* (Gh) at 24
523 or 72h post-wasp attack (PWA). Two different S. poulsonii strains were evaluated: sMel-BR
524 and sMel-UG. RIP2 was consistently expressed at a significantly higher level than RIP1 and
525 RIP3-5 in all wasp treatments, time points, and Spiroplasma strains (Tukey HSD P < 0.05).

526 **4 Discussion**

527 **4.1 The adopted ribodepletion procedure is not an ideal strategy for dual**
528 **transcriptomics in the *Drosophila-Spiroplasma* system**

529 In an attempt to recover mRNA reads from both host and symbiont (also known as "dual
530 transcriptome"), we generated RNA-seq libraries avoiding poly-A-tail enrichment step,
531 typically used for eukaryotic mRNA analyses. Instead, our strategy aimed at removal of
532 ribosomal RNA from both host and symbiont by using the RiboZero Epidemiology kit, which
533 is expected to deplete ribosomal RNA from both bacteria and eukaryotes. A previous
534 application of a similar ribodepletion kit, reported removal of > 90% of the rRNA sequences
535 of *D. ananassae* (Kumar et al., 2012). In contrast, in our study the reads that mapped to rRNA
536 genes comprised 3.4–78% of the total reads mapped to *D. melanogaster*, indicating a variable
537 and ineffective degree of ribosomal RNA depletion. Furthermore, by evaluating the pattern of
538 reads mapped to the 28S rRNA gene, we found that ribodepletion effectiveness varied by
539 region of the gene (see Figure S11). We suggest that future applications of ribodepletion in *D.*
540 *melanogaster* consider including additional probes to capture such regions with higher
541 efficiency. Even if more effective depletion of fly rRNA had been achieved, it is possible that
542 our sequencing effort still would have been inadequate for DE analyses of *Spiroplasma*.

543 **4.2 Wasp parasitism in the absence of *Spiroplasma***

544 The two wasp species used in this study, *L. heterotoma* and *G. hookeri* belong to the same
545 family (Figitidae), but their parasitism strategies are quite different (Schlenke et al., 2007;
546 Mortimer et al., 2013). Our fly transcriptome analysis also revealed differences in the effects
547 of these wasps. Whereas *G. hookeri* appears to activate an immune response in the host, *L.*
548 *heterotoma* does not, but in turn induces genes related to energy production. Increasing energy
549 could be a strategy of the wasp to obtain more resources from the host. One the other hand, it
550 may reflect energy investment for a fly function such as immune response, which is

551 energetically expensive (Schlenke et al., 2007; Dolezal et al., 2019). If it indeed reflects
552 investment in immune functions, these have not been detected (this study; Schlenke et al.,
553 2007) and are unsuccessful, as the fly generally does not survive attacks by *L. heterotoma* (Xie
554 et al., 2014; Jones and Hurst, 2020b).

555 The *L. heterotoma*-induced downregulation of genes related to spermatogenesis; a
556 phenomenon not induced by *G. hookeri*, is an interesting contrast between the two wasps. In
557 accordance with our results, (Schlenke et al., 2007) reported downregulation of genes involved
558 with gonad development during parasitism by *L. heterotoma* and *L. boulardi*, albeit at earlier
559 stages. These two independent observations suggest that *L. heterotoma* might induce castration
560 of male hosts. Xie et al., 2011, reported that *Drosophila hydei* males (infected with a non-
561 male-killing strain of *Spiroplasma*) that survived parasitism by *L. heterotoma* (Lh14) had
562 extremely reduced fecundity, but the experiments could not rule out causes other than
563 castration. The phenomenon of male castration has only been reported in parasitoids of the
564 family Braconidae, where the polydnavirus present in the wasp venom induces testis
565 degradation (reviewed in Beckage and Gelman, 2004).

566 This study is the second one to use a genome-wide approach to evaluate gene expression of *D.*
567 *melanogaster* in response to *L. heterotoma*. The first one was conducted by Schlenke et al.,
568 2007 and employed microarrays at time points earlier than 24h PWA. In their latest time point
569 (22–24h PWA), Schlenke et al., 2007 found 37 DE genes ($P < 0.01$, $-0.5 > FC > 1.0$; fold-
570 changes for genes DE at a $P < 0.05$ were not reported). At a similar time point (our T1), we
571 detect only one DE gene (*thor*), which was not detected by . Schlenke et al., 2007. Differences
572 between our results and those of Schlenke et al., 2007 may be attributable to the different
573 methodology (e.g. microarrays vs. RNAseq, different software pipelines, and cutoff
574 parameters). Other potential differences could be related to *Drosophila* genotypes or
575 *Wolbachia* infection status, or other experimental conditions or their interactions. In spite of

576 all these variables, both studies provide evidence that parasitism by *L. heterotoma* does not
577 induce an immune response in *D. melanogaster*.

578 In a different scenario, parasitism by *G. hookeri* induced the Jak/Stat pathway, one of the
579 immune pathways of *D. melanogaster*. Effectors of this pathway (*Tot* and *Tep* genes) were
580 highly upregulated. Tep proteins play an immune role in the mosquito *Anopheles gambiae*,
581 against the parasite *Plasmodium berghei* (Blandin et al., 2004).The function of *Tot* genes is
582 unknown, but they belong to a family of eight genes that are activated by stressful conditions
583 such as bacterial challenge, high temperatures, mechanical pressure, or UV radiation (Ekengren
584 and Hultmark, 2001). Parasitism of *D. melanogaster* by the wasps *L. boulardi* (Schlenke et al.,
585 2007) and *Asobara tabida* (Salazar-Jaramillo et al., 2017) also induce the up-regulation of *Tep*
586 and *Tot* genes, suggesting that upregulation of these genes could be a common response against
587 wasp parasitism. Another potential signal of immune activation against *G. hookeri* is the
588 induction of genes encoding serine proteases, because these enzymes are involved in triggering
589 immune response in insects (reviewed in Alvarado et al., 2020). The melanization cascade,
590 another feature of *D. melanogaster* immune response, also appears to be activated by *G.*
591 *hookeri*, as two components of this cascade (*PPO3* and *yellow-f*) were up-regulated. Finally
592 the upregulation of genes that are known to be expressed preferentially in hemocytes (i.e.,
593 *PPO3*, *ItgaPS4 and he*), or involved in hemocyte proliferation (i.e., *pvf2*) implies that
594 hemocytes are activated by *G. hookeri* parasitism. In accordance with this, Mortimer et al.,
595 2013 showed that the number of lamellocytes increases during parasitism by *G. hookeri*. Even
596 though *D. melanogaster* appears to mount an immune response against *G. hookeri*, the response
597 is inadequate, as ~100% of flies parasitized by this wasp die (Mortimer et al., 2013; Mateos et
598 al., 2016). The reason likely lies in that the venom of this wasp contains a calcium ATPase that
599 prevents activation of plasmatocytes; a necessary early step of the encapsulation process
600 (Mortimer et al., 2013).

601 **4.3 Wasp parasitism in the presence of *Spiroplasma***

602 The primary goal of this study was to identify *Drosophila* fly-encoded functions
603 relevant to the *Spiroplasma*-wasp interaction, as such functions could be involved in the
604 mechanism that leads to the death of certain wasps, including *L. heterotoma*. Our analyses
605 revealed several genes whose expression appears to be influenced specifically by the
606 *Spiroplasma-L. heterotoma* interaction. In the context of the *Spiroplasma*-susceptible wasp (*L.*
607 *heterotoma*), eight and 32 genes were exclusively DE at T1 and T2, respectively. None of these
608 genes, with exception of attacin-C (*AttC*), were associated with an immune function in *D.*
609 *melanogaster*. We thus infer that the *Spiroplasma*-mediated wasp-killing mechanism is not
610 strongly influenced by host-encoded immunity, at least at a level detectable by our experiments.
611 Consistent with our findings, an RNA-seq analysis of *Spiroplasma* protection against
612 nematodes in *D. neotestacea* did not detect changes in the host's immune response (Hamilton
613 et al., 2014). Similarly, Paredes et al., 2016 did not detect an increased cellular response, based
614 on the number of hemocytes, during the *Spiroplasma-L. boulardi* interaction. The lack of
615 detectable influence of *Spiroplasma* on host-encoded immunity is consistent with evidence that
616 *Spiroplasma* is not detected as an intruder by the fly, due to the lack of cell wall, where the
617 typical bacterial immune elicitors are found (Hurst et al., 2003; Herren and Lemaitre, 2011).

618 In the context of *Spiroplasma-L. heterotoma* interaction, we identified a set of twelve genes
619 whose expression patterns appear to be "restored", as well as two genes (TotC and Fbp2) whose
620 expression patterns are likely influenced by the interaction between *Spiroplasma* and *L.*
621 *heterotoma*. Furthermore, these genes did not exhibit such an expression pattern in the context
622 of the *Spiroplasma*-resistant wasp *G. hookeri*. These genes could contribute to the wasp death
623 and/or fly survival (i.e., a *Spiroplasma*-mediated protection mechanism), but also could simply
624 reflect a side effect of the *Spiroplasma-Lh* interaction. In Group 1 of the restored genes, only
625 two had annotations, one of which is predicted to have chitin-binding activity (*CG42494*)

626 whereas the other has a cytochrome P450 domain (*Cyp4d14*); fold changes of these genes were
627 low (< 2). Cytochrome P450 are a large family of proteins that is involved in detoxification,
628 but also in developmental processes (Chung et al., 2009). For both of these genes, expression
629 levels are reported to go down between the L3 and pupa transition. Therefore, if the S0Lh
630 treatment were developmentally delayed with respect to the S+Lh treatment (see discussion
631 below), our observed expression patterns may simply be a consequence of development time
632 differences between the treatments, rather than a cause of wasp death or enhanced fly survival.
633 Furthermore, if the detoxification function of Cyp4d14 were relevant to the *Spiroplasma*-
634 mediated protection mechanism, we would expect higher expression in the S+Lh than in the
635 S0Lh treatment, which is contrary to our observations. Experimental down-regulation of these
636 and the other Group 1 genes, in the absence of *Spiroplasma*, could help determine whether they
637 have any role in the *Spiroplasma*-mediated protection against wasps.

638 Genes in Group 2 exhibit a substantial increase in expression in wild type flies during the larva-
639 to-pupa transition according to flybase (Thurmond et al., 2019). Therefore, our observed
640 expression patterns could simply reflect slight differences in fly development time, where *L.*
641 *heterotoma* slows down host development, but presence of *Spiroplasma* "restores" the
642 development time to that of the unparasitized host. In partial support of this hypothesis, larvae
643 parasitized by *L. hetetoroma* and *L. boulardi* pupate ~2 days later than the unparasitized
644 controls (Schlenke et al., 2007). Whether *Spiroplasma* counteracts the wasp-induced delay in
645 development remains to be determined.
646 Two genes (*Fbp2* and *TotC*), were not part of the restored genes, but their expression patterns
647 and functions allow us to speculate about their possible roles in the *Spiroplasma*-mediated
648 protection. We acknowledge that the *s*Mel-*L. heterotoma* (strain Lh14) interaction, albeit
649 leading to wasp death, seldom results in fly surviva(Xie et al., 2014; Paredes et al., 2016; Jones
650 and Hurst, 2020b). Given its possible function as a sulfur-storage protein (Meghlaoui and

651 Veuille, 1997), increased expression of *Fbp2* might reflect a greater availability of nutrients
652 for the fly, which might enhance its tolerance to insults from the wasp. Similarly, based on its
653 association to "response to stress", increased expression of *TotC* may contribute to increasing
654 the fly's tolerance to the wasp parasitism. In addition, *TotA*, another stress responsive gene,
655 was upregulated but with an FDR value slightly above our cutoff (0.053). Experimental over-
656 expression of these, and the Group 2 "restored" genes, in the absence of *Spiroplasma*, could be
657 used to test whether they influence the outcome of parasitism by *L. heterotoma* and other
658 wasps, including those against which *s*Mel confers stronger rescue (e.g. *L. boulardi* or *L.*
659 *victoriae*; Mateos et al., 2016; Paredes et al., 2016; Jones and Hurst, 2020a)

660 **4.4. RIP expression**

661 Concerning the hypothesis that a *Spiroplasma*-encoded toxin contributes to killing of
662 *Spiroplasma*-susceptible wasps, we found evidence that neither wasp influences expression
663 of RIP genes, but RIP2 gene was highly expressed in contrast to RIP1 or RIP3-5. In a
664 previous report in the absence of parasitism, RIP2 was also found to be the most highly
665 expressed of the RIP genes throughout the fly life cycle (only substrain Uganda was
666 examined; Garcia-Arraez et al., 2019)). The only context where relatively lower expression
667 of RIP2 and RIP1 in *s*Mel has been reported is in the case of in vitro culture versus fly
668 hemolymph (Masson et al., 2018),implying that expression of these genes can be regulated.
669 The expression of RIP genes in context of other tolerant/resistant wasps had not been
670 determined.

671 Our study also reveals that both the *s*Mel-UG and *s*Mel-BR sub-strains of *S. poulsonii* express
672 their RIP genes at similar levels in the presence or absence of *L. heterotoma* and *G. hookeri*.
673 Although differences in the genomes of substrains *s*Mel-Ug and *s*Mel-BR have been reported
674 (Gerth et al., 2020) our observations suggest that at least for RIP expression patterns the two

675 strains are very similar. Based on expression levels alone, it appears that *s*Mel RIP2 might have
676 a stronger role in wasp death than the other RIP genes. However, it is intriguing that the genome
677 of sHy (the poulsonii-clade native *Spiroplasma* of *D. hydei)* does not harbor a gene with high
678 homology to sMel RIP2. Instead, its genome encodes a gene with high homology to sMel RIP1,
679 as well as putative RIP-encoding genes with homology to genes in *Spiroplasma* strains other
680 than *D. melanogaster* (Gerth et al., 2020), sHy is known to kill *L. heterotoma*, and enhance
681 survival of its host *D. hydei* (Xie et al., 2010). It is thus possible that more than one of the RIP
682 genes in the *Spiroplasma* strains that kill *L. heterotoma*, contributes to wasp killing.

683 Detection of signals of ribosomal depurination in the *Spiroplasma*-susceptible (*L. heterotoma*)
684 but not in the *Spiroplasma*-resistant (*G. hookeri*) wasp is consistent with the hypothesis that
685 RIP-induced depurination contributes to wasp death. Ballinger and Perlman, 2017 , using a
686 more direct approach to evaluate depurination (i.e., qPCR), reported evidence of *Spiroplasma*
687 (strain sMel-UG)-induced depurination in *L. heterotoma*, as well as in *L. boulardi* (another
688 *Spiroplasma*-susceptible wasp), but not in the *Spiroplasma*-resistant pupal ectoparasitic wasp
689 (*Pachycrepoideus vindemmiae*). Ballinger and Perlman, 2017. hypothesized that *P.*
690 *vindemmiae* resistance to *Spiroplasma* (and depurination) may stem from the fact that it is not
691 immersed in *Spiroplasma*-layden hemolymph during development. This explanation, however,
692 would not apply to *G. hookeri*, which is a larval endo-parasitoid that spends the initial stages
693 of development in the host hemocoel. Whether RIP-induced depurination is necessary and
694 sufficient to kill susceptible wasps has not been determined. Ideally, the effect of RIP on the
695 wasp would be tested in the absence of *Spiroplasma*, or in "knocked-out" mutants in these
696 genes. In addition, the target cells and the mechanism of entry of *Spiroplasma* RIPs has not
697 been determined. It is unclear whether *Spiroplasma* must colonize wasp tissues in order to
698 deliver RIP or if toxins can be acquired during wasp feeding; both RIP1 and RIP2 proteins
699 have been detected in the fly hemolymph (see Garcia-Arraez et al., 2019). It is also possible

700 that other *Spiroplasma*-encoded putative virulence factors such as chitinase (*ChiD*) or glycerol-
701 3-phosphate oxidase (*glpO*) could contribute to wasp death (Masson et al., 2018). Presence of
702 *glpO* in *S. taiwanense* is proposed to be the cause of its pathogenicity to mosquitoes (Lo and
703 Kuo, 2017).

704 Assuming RIP is an important factor in wasp killing, the apparent lack of ribosome
705 depurination of G. hookeri, along with the unaltered expression of RIP genes, suggest that this
706 wasp avoids RIP-induced damage by interfering with translation of RIP mRNA, or by
707 inactivating RIP. Resistance to RIP toxicity has been reported in Lepidoptera, and has been
708 attributed to serine protease-mediated hydrolysis in the digestive tract (Gatehouse et al., 1990).
709 As suggested by Ballinger and Perlman, 2017 for *P. vindemmiae*, it is possible that conditions
710 in the gut of *G. hookeri* inactivate ingested RIPs. Alternatively, RIP proteins may be active but
711 unable to enter *G. hookeri* cells, or unable to reach the appropriate cellular compartments to
712 damage ribosomes. It is unlikely that wasp ribosomes that come into contact with RIPs are
713 immune to depurination because of the extremely conserved eukaryotic motif targeted by these
714 proteins.

715 **Conclusions**

716 We detected a small number of fly genes whose expression was influenced by the interaction
717 between *Spiroplasma* and the susceptible wasp *L. heterotoma*. Based on the annotations of
718 these genes, our results suggest that immune priming does not contribute to the *Spiroplasma*-
719 mediated wasp killing mechanism. Furthermore, based on the reported expression patterns of
720 several of these genes during fly development, it is possible that our results reflect
721 *Spiroplasma*-mediated changes in development time of Lh-parasitized flies, which has yet to
722 be verified. In accordance with previous studies, we detected evidence consistent with
723 ribosomal damage in the susceptible but not the resistant wasp. In addition, our transcriptome

724 and qPCR results indicated that RIP2 is the most highly expressed of the five *s*Mel-encoded
725 RIP genes in both the BR and UG substrains, and that its expression levels were independent
726 of wasp parasitism. We recommend that future studies examine whether any of the sMel-
727 encoded RIPs are necessary and sufficient for wasp killing. The observation that the
728 *Spiroplasma*-resistant wasp *G. hookeri* did not influence RIP transcript levels raises interesting
729 questions about its mechanism of resistance or tolerance to *Spiroplasma*.

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751 **References**

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