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

Authors and Contributions

Victor M. Higareda-Alvear [1], Mariana Mateos[2]*, Esperanza Martinez-Romero[1], Diego Cortez Quezada [1], Cecilia Tamborindeguy[3].

Affiliations

[1]Center for Genomic Sciences, Universidad Nacional Autónoma de México, Cuernavaca, Morelos, México.

[2]Department of Ecology and Conservation Biology, Texas A&M University, College Station, TX, USA. * corresponding author

[3]Department of Entomology, Texas A&M University, College Station, TX, USA

Key words: Metatranscriptome, Spiroplasma, Parasitic wasp, Protection, Immunity, Drosophila, Toxins.

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.

1 Introduction

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

Three models of classical ecology can be adapted to explain protection of bacteria against parasites. **Exploitation competition** occurs when the symbiont and the parasite compete for a limiting resource (e.g. *Wolbachia* and vectored-viruses compete for cholesterol;(Caragata et al., 2013). **Apparent competition** can occur when the symbiont activates ("primes") the immunity of the host, and thus indirectly interferes with the parasite (e.g. *Wigglesworthia* in *Glossina* against trypanosomes, (Wang et al., 2009). Finally,

interference competition can occur when the symbiont produces a compound (e.g. a toxin) that limits the success of the parasite (e.g. *Hamiltonella defensa* in aphid insects (Oliver et al., 2003; Brandt et al., 2017). One or more of these mechanisms can occur in concert, as suggested for the interactions between flies in the genus *Drosophila* and heritable bacteria in the genus *Spiroplasma*, where these endosymbionts protect the host against parasitic wasps or nematodes (Jaenike et al., 2010; Xie et al., 2010).

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

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

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

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

Damage consistent with RIP activity (hereafter referred to as depurination) has been detected in a *Spiroplasma*-susceptible nematode (Hamilton et al., 2015) and in two *Spiroplasma*-susceptible wasps (the larval parasitoids *L. boulardi* and *L. heterotoma*), but not in a *Spiroplasma*-resistant wasp that oviposits on fly pupae (Ballinger and Perlman, 2017). Although the above studies suggest that competition for nutrients and RIP activity are involved in the *Spiroplasma*-mediated mechanism that causes wasp death, they have not demonstrated that the above mechanisms alone or in combination are necessary and sufficient, and alternative mechanisms, including immune priming, have not been ruled out.

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

124 Herein, we used an RNA-seq approach to evaluate the transcriptomic response of *D.*
125 *melanogaster* during interactions involving *Spiroplasma* and two wasps that are generalists of
126 the genus *Drosophila*: the *Spiroplasma*-susceptible *L. heterotoma*; and the *Spiroplasma*-
127 resistant *Ganaspis hookeri*. In addition, we evaluated the effect of wasp parasitism on the
128 expression of *Spiroplasma* RIP genes in two closely related substrains of *s*Mel, which have
129 similar genomes (Gerth et al., 2020) but confer different levels of protection against wasps,
130 measured as fecundity of flies surviving a wasp attack (Jones and Hurst, 2020a).
131
132 **2. Methods**
133
134 **2.1 Insect and *S. poulsonii* strain**
135
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
153
154

155

156 **2.2 RNA-Seq based methods**

157 **2.2.1 Wasp exposure**

158

159 To examine the effect of the interaction of *Spiroplasma* and wasp on the transcriptome of
160 *Drosophila* (and of *Spiroplasma*), we compared treatments with the presence and absence of
161 *Spiroplasma* (*s*Mel-BR) and one of the two wasp species at two different time points. This
162 experimental design resulted in a combination of twelve treatments; six treatments per time
163 point. For each replicate, parental flies (approximately ten females and ten males) were set up
164 in oviposition vials in the evening for overnight oviposition. Parental flies were removed the
165 next morning. One day later, ~30 second-instar *D. melanogaster* larvae were carefully collected
166 and transferred to a Petri dish (60 mm diameter) containing cornmeal food medium
167 (**Supplementary methods 1**). In replicates assigned to a wasp treatment, 5 male and 6 female
168 wasps of the corresponding species (*L. heterotoma* or *G. hookeri*) were added to the Petri dish,
169 and allowed to oviposit for ~5 h. All female wasps had been previously allowed to oviposit on
170 *D. melanogaster* fly larvae for ~5 hours. The purpose of this "training" is to ensure that the
171 wasps are experienced at oviposition prior to the experiment. All Petri dishes were covered,
172 but a small hole was opened (with a hot needle) to allow for gas exchange, and/or through
173 which wasps could feed on a piece of cotton wool soaked in 1:1 water:honey mix that was
174 placed outside the dish. To collect RNA, larvae were retrieved from each Petri dish at either
175 24h (T1) or 72h (T2) post-wasp attack (PWA) (i.e., one or three days after wasps were removed,
176 respectively). To ensure sufficient material for RNA-seq, 20–30 larvae were collected for the
177 24h time-point, whereas 10–20 for the 72h time point. Both wasp species were embryos at T1
178 and larvae at T2. Fly larvae from the same Petri dish were pooled into a single RNA extraction
179 tube (i.e., a replicate).

180

181 From each Petri dish in the wasp-exposed treatments, a subsample of fly larvae was used to
182 verify wasp parasitism rate as follows. First, at the time of larvae collection for RNA, five fly
183 larvae per replicate were dissected under the microscope and discarded. If all five larvae
184 contained at least one wasp egg or larva (i.e., 100% wasp oviposition rate), the replicate was
185 retained and processed. If one or more fly larvae did not contain a wasp larva (or embryo), then

186 five additional fly larvae were examined for wasp presence. Only replicates with 90-100%
187 wasp oviposition rate were retained. All collections for RNA were performed in the afternoon-
188 evening, and collected larvae were quickly placed in an empty microtube for processing.
189
190 **2.2.2 RNA extraction**
191
192 Collected larvae were either immediately frozen at –80°C or immediately processed for RNA
193 extraction. Preliminary experiments with RNAlater Stabilization Solution revealed that the
194 larvae did not die immediately and appeared to melanize. Total RNA was extracted using the
195 Trizol [invitrogen] method. Each sample consisted of a pool of *Drosophila* larvae that were
196 homogenized by hand with a sterile plastic pestle in Trizol reagent. The Trizol isolation method
197 was performed following the manufacturer's protocol but it was stopped at the 70% ethanol
198 wash step. The total RNA pellet in ethanol was submitted to the Texas AgriLife Genomics and
199 Bioinfomatics Services facility for completion of the RNA isolation procedure, assessment of
200 RNA quality and quantity (using Fragment Analyzer; Agilent, Santa Clara, CA), library
201 preparation, sequencing, and demultiplexing.
202
203 **2.2.2 Library preparation and sequencing**
204
205 Total RNA was subjected to removal of ribosomal RNA from eukaryotes and prokaryotes with
206 the RiboZero Epidemiology kit (Illumina, San Diego, CA). The TruSeq stranded kit (Illumina)
207 was then used to prepare the library for sequencing with Illumina (125 bp Single End "HighSeq
208 2400v4 High Output").
209
210 **2.2.4 Bioinformatic analysis**
211
212 Quality and presence of adapters was evaluated with FastqC (Andrews S., 2010), followed by
213 a trimming with Trimmomatic v.0.36 (Bolger et al., 2014) using
214 ILLUMINACLIP:/adapters.fasta:2:30:10 LEADING:3 TRAILING:3
215 SLIDINGWINDOW:4:15 MINLEN:36. To examine differential expression of *D.*
216 *melanogaster* genes, trimmed reads were mapped with Hisat2 v.2.0.2-beta (Kim et al., 2015),

using --rna-strandness R option. Treatments parasitised by *L. heterotoma* were mapped to an 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.

248

249 **2.2.5 Power analyses for differential expression.**

250

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

255

256 **2.2.6 Analyses of depurination signal in the sarcin-ricin loop (SRL) of the 28S rRNA of**
257 **wasps and flies**

258

259 RIP toxins remove a specific adenine present in the sarcin-ricin loop (SRL) of the 28S rRNA
260 leaving an abasic site (i.e., the backbone remains intact). When a reverse transcriptase
261 encounters an abasic site, it preferentially adds an adenine in the nascent complementary DNA
262 strand. This property, which results in an incorrect base at the RIP-depurinated site in the cDNA
263 and all subsequent PCR amplification steps, has been used to detect evidence of RIP activity
264 in any procedure that relies on reverse transcription such as RNA-seq or reverse-transcription
265 qPCR (e.g. Hamilton et al., 2015).
266 To examine whether a signal of depurination consistent with RIP activity was detectable in
267 wasp-derived sequences, we mapped RNA-seq data to a reference sequence file comprised of
268 the 28S rRNA sequences of the wasps and of *D. melanogaster* using Bowtie2 v.2.1.054 with
269 default options. Only sequences that mapped to the wasp 28S rRNA were retained (Dataset
270 S1). To visualize and count the shift from A to T (or other bases), the retained reads were
271 mapped again to the 28S rRNA of wasp in Geneious v.11.1.2 (Biomatters Inc., Newark, NJ;
272 "low sensitivity mode"; maximum gap size = 3; iterate up to 25 times, maximum mistmatches
273 per read 2%). The number of reads containing each of the four bases or a gap at the target site
274 was counted by selecting the position at all the reads to be counted, and recording the counts
275 reported by Geneious under the "Nucleotide Statistics" option (gapped reads were excluded
276 from counts). Reads were counted only if they fully covered a specific part of the 28S loop
277 sequence (TACG**A**GAGGAACC). The bold-faced adenine represents the site of RIP
278 depurination. Replicates with fewer than 10 mapped reads were discarded, Table S3 and Figure

279 S10. Statistical analysis was conducted in R v 4.02 (R core Team) using a Bayesian generalized
280 linear model (bayesglm function in "arm" package), this is because of the presence of zeros
281 (no depurination) in some treatments. Using the above strategy, raw sequences were also
282 mapped to the full sequence of the 28S rRNA of D. melanogaster, and depurination was
283 evaluated. Due to the high number of ribosomal sequences that align to the 28S rRNA, only
284 subsets of 1 million of sequences were analyzed (Table S4).Using the above strategy, raw
285 sequences were also mapped to the full sequence of the 28S rRNA of *D. melanogaster,* and
286 depurination was evaluated. Due to the high number of ribosomal sequences that align to the
287 28S rRNA, only subsets of 1M of sequences were analyzed. Table S4
288
289 **2.3 Quantitative (q)PCR-based Methods**
290 **2.3.1 Expression of *Spiroplasma* Ribosomal inactivation proteins (RIPs).**
291
292 To verify the RIP expression patterns inferred from the transcriptome (see Results) and to
293 examine whether they were consistent between substrains of *s*Mel, we used qPCR on a a new
294 set of treatments. We followed the "Wasp exposure" methodology (described above), but used
295 *Wolbachia*-free *D. melanogaster* (OregonR) harboring the *S. poulsonii* strain *s*Mel-BR or
296 *s*Mel-UG. Five larvae per treatment (parasitized or not by *L. heterotoma* or *G.hookeri*), were
297 collected at 24 and 72h PWA, flash frozen in liquid nitrogen and homogenized by hand with a
298 pestle. Total RNA was extracted with the All prep DNA/RNA mini kit (Qiagen, Germantown,
299 MD). 1ug of total RNA was used to synthesize cDNA using superscript II reverse transcriptase
300 (Invitrogen), following manufacturer's procedures. cDNA was used as template for qPCRs,
301 performed on a CFX96 detection system (Bio-Rad, Hercules, CA). The mix contained 5ul of
302 iTaq Universal SYBR Green Supermix (Bio-Rad), 2.5ul water, 2ul cDNA, and 0.25ul of each
303 primer (stock solution at 10uM). Primer sequences and efficiencies for RIP2, RIP3-5 and rpoB
304 were taken from (Ballinger and Perlman, 2017). For RIP1, we designed and used the following
305 primers Forward: 5'- AATCAGAGGGGCATTAGCTC-3' Reverse 5'-
306 CTTCGCTTGTGGTTCTTGAT-3', efficiency = 0.995. Although (Ballinger and Perlman,
307 2017) reported a primer pair targeted at RIP1, this primer pair matches a fragment of RIP2
308 instead.

309 Relative expression was calculated using efficiency-corrected Ct values using
310 (Ct*(Log(efficiency)/Log(2))) formula. DeltaCt was calculated as Ct-rpoB minus Ct-RIPx
311 (Dataset S2) . We used JMP Pro v.15 (SAS, Cary, NC) to fit a full factorial Generalized
312 Regression (Normal distribution) model. The response variable was delta Ct Value. The
313 independent variables (all fixed and categorical) were: RIP gene ("RIP": RIP1, RIP2, RIP3-5),
314 Wasp Treatment (No wasp, Lh and Gh), *Spiroplasma* strain Brazil, Uganda) Time Point (24h
315 or 72h). Significant effects and interactions were explored with Tukey HSD tests with Least
316 Square Means Estimates (Supplementary file S1).
317

318 **2.4 Data availability**

319
320 All raw reads generated in this project have been submitted to NCBI under the SRA accession
321 numbers PRJNA577145. Count tables for *D. melanogaster* and *Spiroplasma* are in Dataset S3.
322 Command lines used to run bioinformatic analyses are available in Supplementary methods 2.
323 Data generated during this study are available at figshare
324 https://doi.org/10.6084/m9.figshare.c.5104559
325

326 # 3. Results

327
328 To examine the effect of *Spiroplasma* on *D. melanogaster* gene expression under wasp
329 parasitism, we generated twelve-RNA-seq treatments (Figure1), with an average of 47 million
330 quality single-end reads per sample, ~90% of these reads mapped to the *D. melanogaster*
331 genome. A fraction of these reads mapped to ribosomal sequences of the host, which is an
332 indication of incomplete ribodepletion (Table S2).
333
334 The multidimensional scaling (MDS) plot of all treatments at 24h post wasp attack (PWA; T1)
335 did not reveal any particular grouping by treatment (Fig. S2). At the 72h PWA time point (T2),
336 however, the treatments separated at the first dimension by presence/absence of *Spiroplasma*
337 (Fig S3). This plot allowed us to detect two replicates that we deemed outlier and decided to
338 exclude from further analyses (red samples in Fig 1). Replicate S0Lh_4, which lacked

Spiroplasma, grouped with the *Spiroplasma*-infected treatments. We confirmed the absence of *Spiroplasma* because no reads derived from *Spiroplasma* were detected. Nonetheless, we realized that this replicate only contained three larvae individuals, which by chance may have been all female. Concerning replicate S+Lh_1, its expression pattern included upregulation of numerous genes associated with immune response. Consequently, we suspected that this particular replicate likely contained one or more larvae infected by a pathogenic bacterium.

Below we first describe the response of *D. melanogaster* to parasitism by the wasps (*L. heterotoma* or *G. hookeri*) in the absence of *Spiroplasma*, followed by the fly response to the sole presence of *Spiroplasma*. Finally, taking these results into account, we examine the response of *Drosophila* during the *Spiroplasma*-wasp interaction.

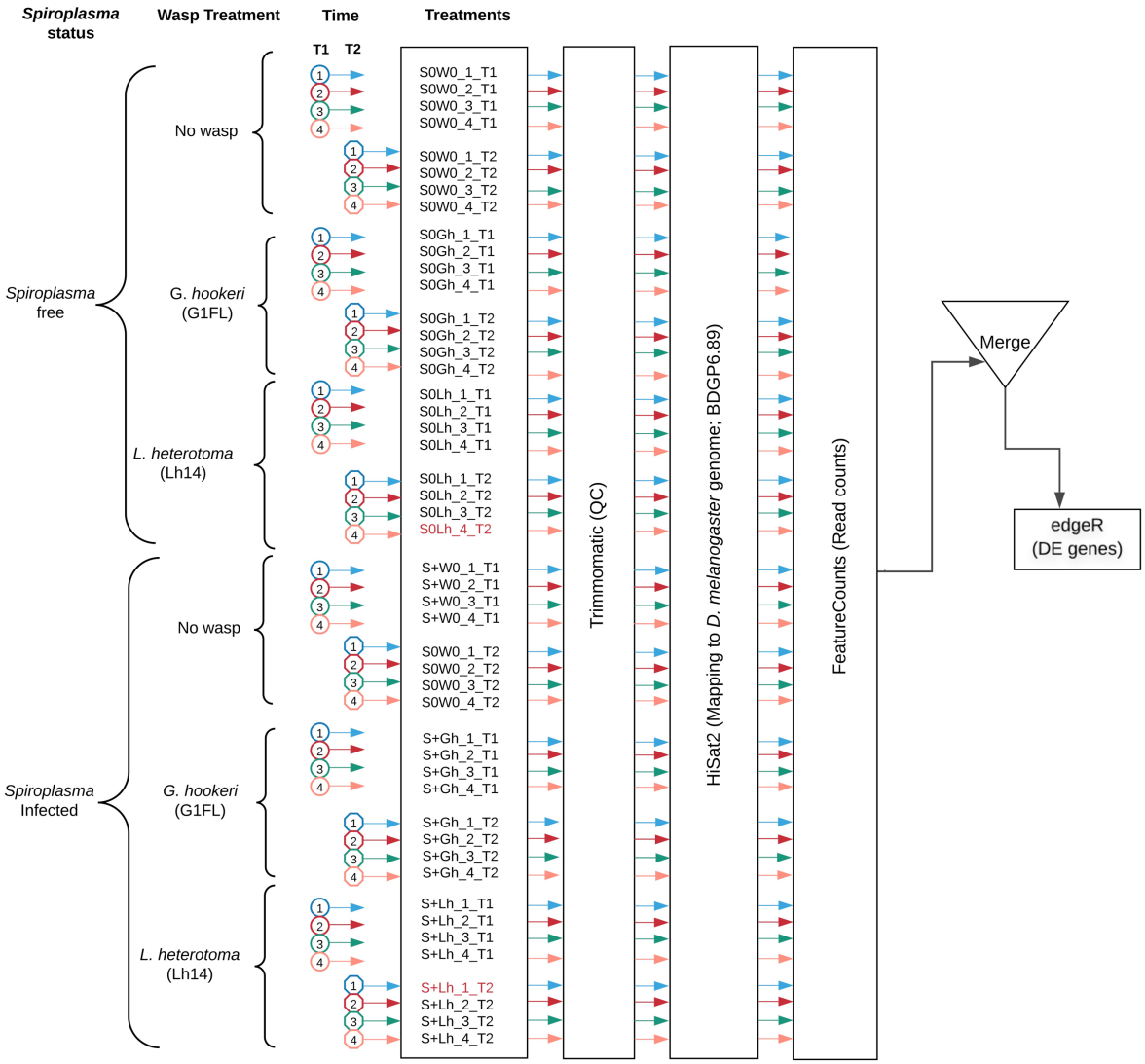
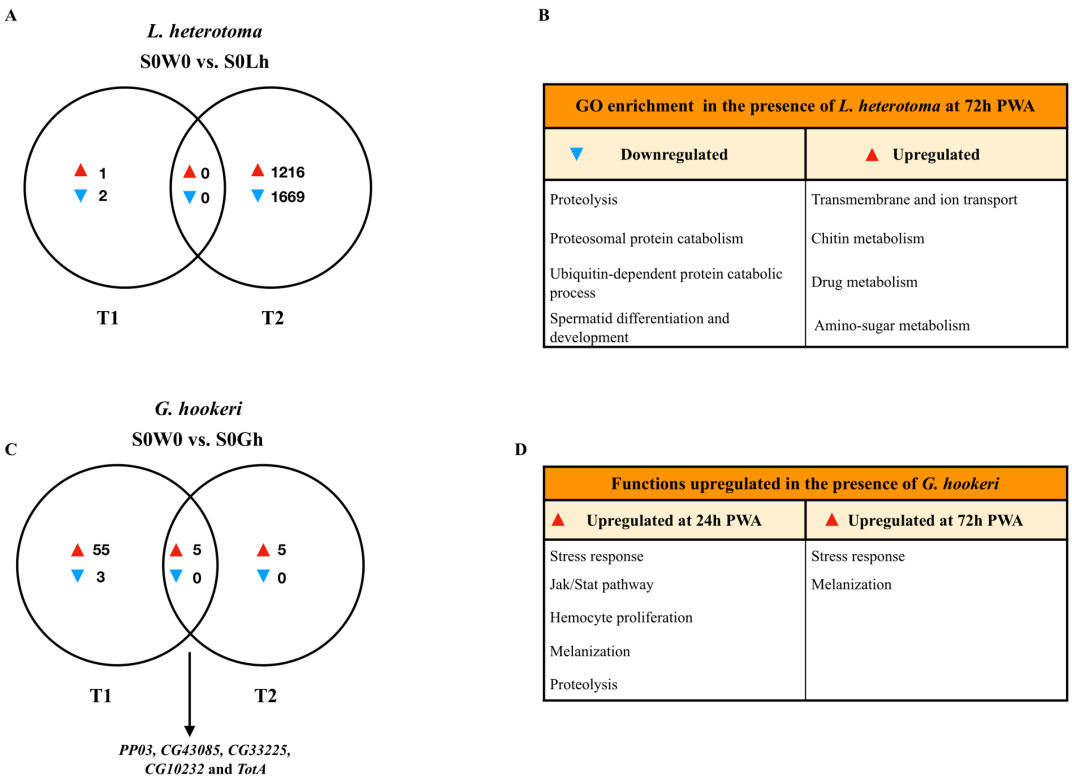


Figure 1. Experimental design and workflow for data analysis. Two samples (S0Lh_4_T2 and S+Lh_1_T2) were removed from the analysis (see text).

352
353
354
355 **3.1 Response of *D. melanogaster* to *L. heterotoma* parasitism in the absence of**
356 ***Spiroplasma***
357
358 Parasitism by *L. heterotoma* at T1 (24h) post wasp attack (PWA), did not have a large effect
359 on *D. melanogaster* gene expression, as only one gene (*thor*) was upregulated, whilst two
360 genes, *Hml* and *mt:ATPase6*, were downregulated (Figure 2a and Dataset S5**)**. In contrast, at
361 T2 (72h PWA), 1216 genes were up- and 1669 down-regulated (Figure 2a and Dataset S5). Of
362 the 1216 upregulated genes at T2, 810 grouped into 32 GO enriched categories (Figure 2b); of
363 which transport was the most enriched. A pathway analysis revealed that upregulated genes are
364 involved with energy generation pathways, such as lipid metabolism and citric acid cycle
365 (Dataset S5). Of the 1669 downregulated genes, 925 grouped into 25 GO categories, of which
366 proteolysis was the most enriched (Figure 2B and Dataset S5). Unexpectedly, a subset of 67
367 downregulated genes belongs to GO categories related to spermatogenesis.
368
369 **3.2 Response of *D. melanogaster* to *G. hookeri* parasitism in the absence of *Spiroplasma***
370
371 In the presence of the wasp *G. hookeri* (i.e, S0W0 vs. S0Gh), three genes were down- and 60
372 were up-regulated at T1 (Figure 2C)**.** The two downregulated genes are known to be expressed
373 by hemocytes (*Peroxidasin* and *hemolectin*) (Irving et al., 2005), whereas the other is a small
374 nucleolar RNA (*Uhg4*). Only one gene, *hemolectin*, was down-regulated by both *L. heterotoma*
375 and *G. hookeri* at T1 (Dataset S5). The sixty upregulated genes in the presence of *G. hookeri*
376 at T1 include genes that are known to be expressed preferentially by hemocytes such as *hemese*,
377 *ItgaPS4, ItgaPS5*, *Scavenger receptor class C* (*CG3212*), one serpin (*CG6687*), a serine
378 protease (*CG6639*), as well as one gene involved in hemocyte proliferation (*pvf2*). (Irving et
379 al., 2005). One activator of the Jak Stat pathway *upd3* and some effectors of this pathway, *TotA,*
380 *tep1* and *tep2* (Agaisse and Perrimon, 2004), were also upregulated. Prophenoloxidase 3
381 (*PPO3)* and *yellow-f* genes, which are involved in the melanization process (Han et al., 2002;

382 Dudzic et al., 2015), were also upregulated. *PPO3* was highly upregulated (2 LogFC = 9). The
383 complete list of DE genes and GO enrichment is provided in Dataset S4.
384
385 At T2, the presence of *G. hookeri* induced upregulation of ten genes, but no genes were
386 downregulated (Figure 2C and Dataset S5). The upregulated genes included the stress response
387 genes *TotA*, *TotB*, *TotC* and *victoria*, but also *PPO3*; log2FC of these genes ranged 3–10. Five
388 genes were shared between the two time points (Figure 2c). Two of them, *CG10232* and
389 *CG33225* are predicted to be involved in proteolysis, and *CG43085* has no function or
390 prediction assigned. The other two genes were *PPO3* and *TotA*; their log2FC were higher at
391 T1 than at T2. In general, immune functions were upregulated by *G. hookeri* parasitism at both
392 times (Figure 2D).
393



394
395 **Figure 2**. Venn diagrams and enriched functions or Gene Ontology (GO) categories.
396 Differentially expressed genes of *D. melanogaster* at time points T1 and T2 [24h and 72h post
397 wasp attack (PWA), respectively] by (A) and (B) *L. heterotoma* or (C) and (D) *G. hookeri*.

398 Enriched GO categories or functions, if applicable, are reported in (B) and (D). The arrow in
399 panel (C) indicates the five genes in the T1 and T2 intersection.
400
401 **3.3 Response of *D. melanogaster* to *Spiroplasma***
402
403 The sole presence of *Spiroplasma* (i.e., S0W0 vs. S+W0 comparison) at 24h PWA did not
404 reveal any upregulated genes, but 27 were downregulated. Twenty of 27 downregulated genes
405 are reported as preferentially expressed in adult testis (Dataset S4). At 72h PWA, the presence
406 of *Spiroplasma* induced upregulation of 16 and downregulation of 1476 genes. Only
407 downregulated genes (692 of the 1476) were assigned to one or more of 71 GO categories
408 (Dataset S5). Some of these categories are related to the energy generation process such as
409 oxidative phosphorylation, pyruvate metabolic process, and glycolytic process. Among the
410 most enriched categories were male gamete generation and spermatogenesis, which is in
411 agreement with the expected lack of males in S+W0 treatments. In addition, 1333 of the 1476
412 downregulated genes at T2 are classified as preferentially upregulated in fly testis.
413 Furthermore, the cpm values of ~78% of these genes in all replicates of the S+W0 treatment
414 were < 1, implying very low expression levels. Therefore, as expected, a large number of the
415 genes with lower expression in the *Spiroplasma* treatment, including the roX1 and roX2
416 (exclusively expressed in males, and part of the dosage compensation system; reviewed in
417 Lucchesi and Kuroda, 2015), may simply reflect the absence of males.
418
419 **3.4 *Drosophila* gene expression during *Spiroplasma-L. heterotoma* interaction**
420
421 To explore if the presence of *Spiroplasma* influences gene expression of *D. melanogaster*
422 during parasitism by *L. heterotoma*, we adopted the following strategy to identify genes whose
423 expression was specifically influenced by the *Spiroplasma- L. heterotoma* interaction. We used
424 a Venn diagram depicting differentially expressed (DE) genes from the following three
425 pairwise treatment comparisons: S0Lh vs. S+Lh (i.e., effect of *Spiroplasma* in the presence of
426 Lh; green sets in Fig. 3); S0W0 vs. S+W0 (i.e., effect of *Spiroplasma* in the absence of any
427 wasp; blue sets); and S0W0 vs. S0Lh (i.e., effect of Lh in the absence of *Spiroplasma*; red sets).
428 Genes that fell in the exclusive part of the green set were deemed as influenced specifically by

429 the *Spiroplasma-L. heterotoma* interaction. Furthermore, genes that fell in the intersection of
430 the green set with one or both of the blue and red sets, were further evaluated with heatmaps
431 depicting expression levels in four treatments (S0W0, S+W0, S0Lh, S+Lh). This approach
432 aimed to identify genes that had substantially different expression levels in the S+Lh treatment
433 vs. the treatments that only had Lh or only had *Spiroplasma*.
434
435 The S0Lh vs. S+Lh comparison at T1 yielded zero up- and 18 down-regulated genes (exclusive
436 green set in Fig. 3A, Dataset S4). Ten of these genes code for small nucleolar rnas (snoRNA).
437 Five other genes (*fest*, *CG10063*, *SkpC*, *eIF4E3* and *cona*), albeit exclusively downregulated
438 in *Spiroplasma-L. heterotoma* interaction, seem to be influenced by *Spiroplasma* alone, based
439 on the observation that these genes were downregulated by *Spiroplasma* alone at the later time
440 point T2 (Dataset S5). Furthermore, *fest* and *eIF4E3* are upregulated in testis, and thus their
441 downregulation may simply reflect absence of males.
442 The only gene-containing intersection (green+blue), had 19 genes, of which 16 are
443 preferentially expressed in adult testis (Dataset S4). The heatmap of all genes in the intersection
444 (Fig. S4), revealed that most of them had similarly low levels of expression in the two
445 *Spiroplasma*-infected treatments (i.e., S+W0 and S+Lh), compared to the S0W0, which had
446 the highest. In turn, the S0Lh treatment exhibited an intermediate expression level (Fig. S4).
447
448 At T2, 21 genes were up- and 11 down-regulated exclusively in S+Lh treatment compared to
449 S0Lh (exclusive green set in Figure 3B; Dataset S5). Four (*CG18649, Cyp4d14, CG42494* and
450 *CG44215*) out of the 11 genes exclusively downregulated in the S+Lh treatment were
451 exclusively upregulated in S0Lh with respect to S0W0 (i.e., identified with * and labeled
452 "Group 1" in Fig. 3c). Seven (*Br*, *CG32115*, *Fs*, *PLCXD*, *Npc2e*, *phm* and l*ncRNA-CR43828*)
453 of the 21 genes exclusively upregulated in the S+Lh treatment were exclusively down-
454 regulated in S0Lh with respect to S0W0 (i.e., identified with ** and labeled "Group 2" in Fig.
455 3c). Hereafter, we refer to genes in Groups 1 and 2 as "restored" because parasitism by *L.*
456 *heterotoma* (S0Lh) increased and decreased (respectively) their expression with respect to the
457 S0W0 control, but in the presence of *Spiroplasma* plus *L. heterotoma* (i.e. the S+Lh treatment),
458 expression levels appear to return to those observed in the control (S0W0; Fig.3C). In other
459 words, *Spiroplasma* appears to "buffer" or counter the effects caused by the presence of *L.*

460 *heterotoma*. Similarly, *Fbp2*, at the blue+green intersection, exhibits a similar expression
461 pattern to genes in Group 2 (Fig. 3C). *TotC* (in the green+red intersection) also exhibited a
462 peculiar expression pattern (Fig. 3C); it was upregulated by *L. heterotoma* parasitism (S0Lh),
463 but its expression was highest in the S+Lh treatment.
464
465 The rest of the DE genes exclusive to *Spiroplasma-L. heterotoma* interaction were 14 up- and
466 7 down-regulated (Fig. 3B green set and Dataset S5). These genes did not group into any GO
467 or specific pathway. Among the upregulated genes, there were *Amylase proximal*,
468 *anachronism*, and the antimicrobial peptide *Attacin-C* (*AttC*; FC= 1.5), which was the only
469 gene associated with immune response. Downregulated genes included two snoRNA
470 (*CR34643* and *CR34560*), *HemK1* and a long non-coding RNA (lncRNA:CR32661) (Dataset
471 S5).
472 The heatmaps of the red+blue+green (386 genes) and of the blue+green (143 genes)
473 intersections at T2 (Fig. 3B and Fig S5-6) revealed the following general pattern that was also
474 observed for the 19 downregulated genes in the blue+green intersection of T1 (see above): the
475 two *Spiroplasma* treatments (S+W0 and S+Lh) had the lowest expression levels; the S0W0
476 treatment had the highest; and the S0Lh treatment was intermediate.
477
478
479
480
481
482
483
484
485
486
487
488
489
490

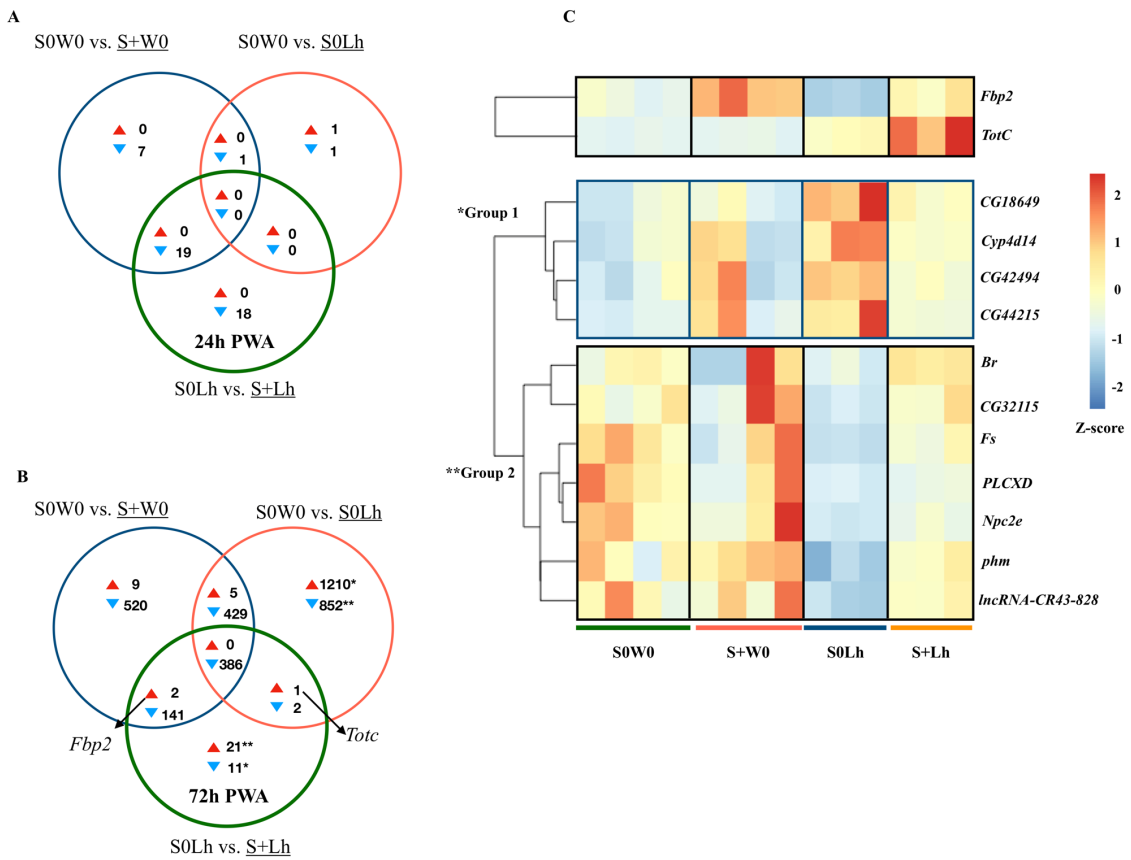


Figure 3. Patterns of differential expression (DE) in the context of the interaction of Spiroplasma and the wasp L. heterotoma (Lh14). Unique and shared number of DE genes in each of three treatment comparisons. (A) Time-point 1 (T1): 24h post-wasp attack (PWA). (B) Time-point 2 (T2): 72h PWA. Red- and 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.

507

508 **3.5 *Drosophila* gene expression during *Spiroplasma-G. hookeri* interaction**

509

510 To identify fly genes specifically influenced by the *Spiroplasma-G. hookeri* interaction, we
511 adopted the same Venn diagram plus heatmaps strategy as with the *Spiroplasma-L. heterotoma*
512 interaction, except that the *L. heterotoma* treatments were replaced with *G. hookeri* treatments
513 (see Fig. 4). Excluding genes induced by the sole presence of *Spiroplasma* or *G. hookeri*,
514 resulted in eight and 21 exclusively up- and down-regulated (respectively) genes at T1
515 (exclusive green set, Fig 4A, Dataset S4). The eight upregulated genes were: four snoRNA
516 (*CR33662*, *CR34611*, *CR34616,* and *CR34631*), one small nuclear RNA (*CR32162*), lysozyme
517 E, diphthamide methyltransferase (*Dph5*) and mitochondrial ribosomal protein S14 (*mRpS14)*.
518 Among the 21 downregulated genes were *DnaJ-1* (whose product is a cofactor of heat shock
519 proteins), *starvin* (which acts as co-chaperone of Hsp70 proteins), *nervana 3* (which codes for
520 one subunit of a sodium-potassium pump), *nanos* (which encodes a ribosomal RNA-binding
521 protein), and one long non-coding RNA (*CR31400*).

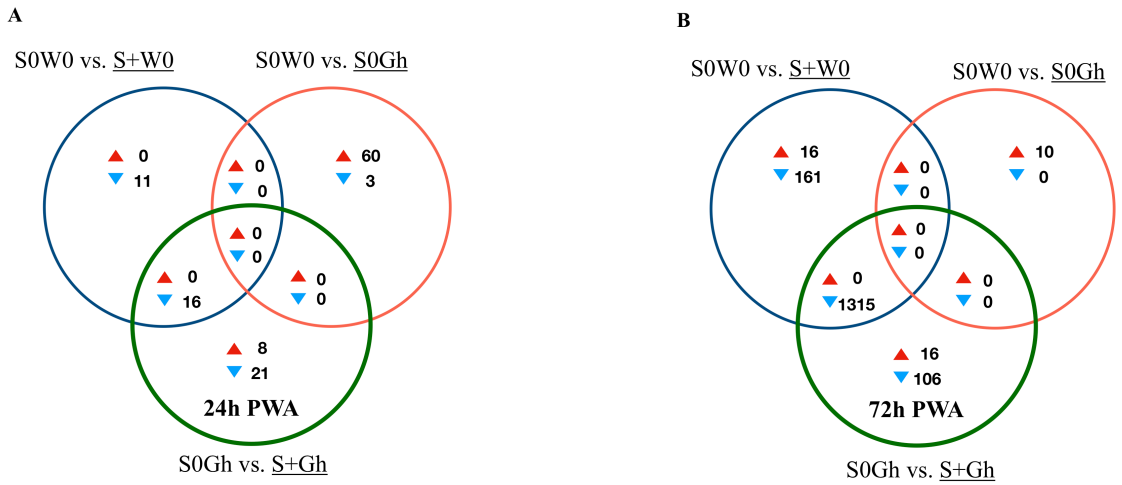
522

523 At T1, only one intersection (green+blue) contained genes; all downregulated (n = 16; Fig.
524 4A). A heatmap of expression levels of these 16 genes (Fig. S7) reveals that the two
525 *Spiroplasma* treatments (S+Gh and S+W0) have similarly low expression levels, whereas the
526 two treatments lacking *Spiroplasma* (S0W0 and S0Gh) have similarly high expression levels;
527 implying that there is no influence of *G. hookeri* on expression of these genes. Three of the
528 downregulated genes are involved with male functions (*msl-2, roX1* and *roX2*), one gene
529 (*blanks*) is highly expressed in adult testis, and three other genes (*RpL22-like*, *RpS5b* and
530 *RpS19b*) code for ribosomal proteins (Dataset S4).

531

532 At T2, 16 and 106 genes were exclusively up- and down-regulated (respectively) in the S+Gh
533 treatment (exclusive green set in Fig. 4B, Dataset S5). The 16 upregulated genes included three
534 mitochondrially-encoded genes (*ND1, CoIII*, and *Co*I), *necrotic* (which is negative regulator
535 of the Toll pathway; Green et al., 2000), *dumpy* (which is involved in wing development), and
536 one multidrug resistance gene (*Mdr50*). The 106 exclusively downregulated genes did not
537 group in any GO category, making it difficult to link these genes to informative biological

538 functions. The expression values to several of these genes in the S+Gh treatment is similar to
539 S+W0 treatment (Fig S8.). This observation, along with the report that 74 of these genes are
540 highly expressed in adult testis (Dataset S5), suggests that observed expression patterns are
541 likely influenced solely by the presence of *Spiroplasma.* The heatmap of the 16 exclusively
542 upregulated genes (Fig S9), also indicates a possible influence of only *Spiroplasma*. These
543 genes showed a trend of higher expression levels in the S+W0 treatment relative to the S0W0
544 treatment, but this difference was not significant, possibly due to the high variation among
545 S+W0 replicates.
546
547 Only one intersection at T2 (green+blue sets) contained genes (n = 1315; all down-regulated;
548 Fig. 4B). These genes are associated with spermatid differentiation/development functions and
549 1214 of these 1315 are predominantly expressed in the adult testis (Dataset S5). Thus, their
550 lower expression in the presence of *Spiroplasma* (with or without Gh; see Fig S10) is likely
551 simply the result of an absence of males in these treatments.
552



553
554 **Figure 4**. Patterns of differential expression (DE) in the context of the interaction of
555 *Spiroplasma* and the wasp *G. hookeri* (G1FL). Unique and shared number of DE genes in each
556 of three treatment comparisons. (A) Time-point 1 (T1): 24h post-wasp attack (PWA). (B)
557 Time-point 2 (T2): 72h PWA. Red and blue filled triangles indicate up- and downregulation,
558 respectively, in the treatment that is underlined.
559
560

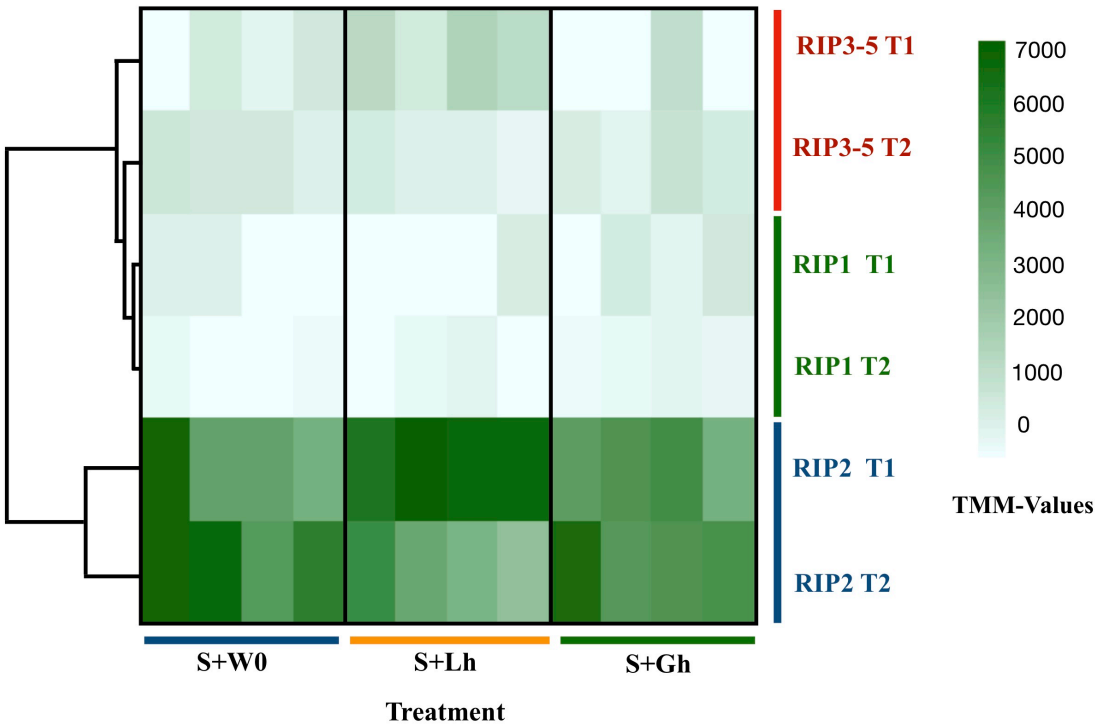
561

562 **3.6 Expression of Spiroplasma RIP proteins and evidence ribosomal damage**

563

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

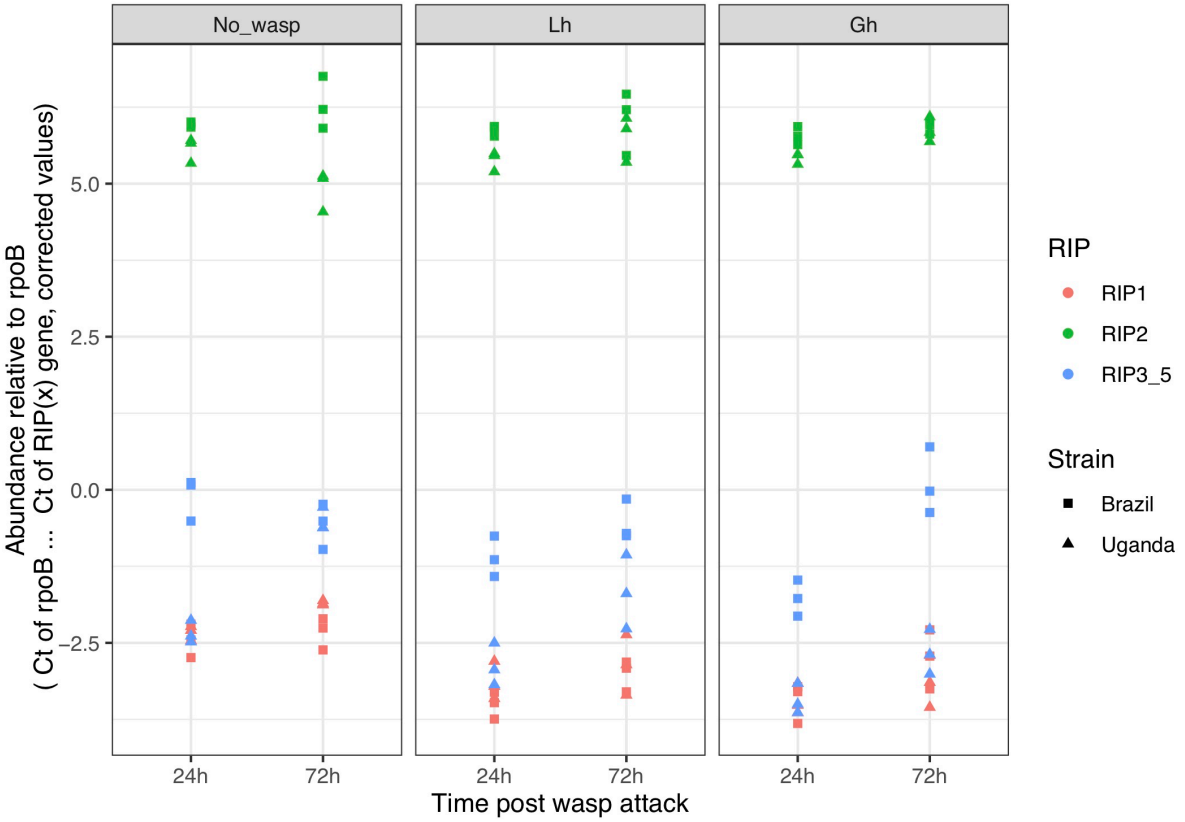
572



573

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

579 To corroborate the observed patterns of RIP gene expression based on the RNA-seq data, RT-
580 qPCR assays were conducted using both the Uganda (UG) and Brazil (BR) *Spiroplasma* sMel
581 strains, and the two wasps, *G. hookeri* and *L. heterotoma*. Consistent with the RNA-seq results,
582 the gene encoding RIP2 was the most highly expressed of the RIP genes in both *Spiroplasma*
583 strains regardless of the presence or absence of wasp (Fig 6, dataset S2). The full output of the
584 statistical analyses is provided in Supplementary file 1. Wasp depurination in the 28S rRNA
585 was significant (X^2=128.58, df=1, P< 2.2 e-16) in the context of *L. heterotoma* in presence of
586 *Spiroplasma* at T2 (Figure S11 and Table S3). No depurination was detected in *G. hookeri* or
587 fly sequences (Figure S11 and Table S3-4).
588
589



590
591

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

597

598 **4 Discussion**

599 **4.1 The adopted ribodepletion procedure is not an ideal strategy for dual**

600 **transcriptomics in the *Drosophila-Spiroplasma* system**

601

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

616

617 **4.2 Wasp parasitism in the absence of *Spiroplasma***

618

619 The two wasp species used in this study, *L. heterotoma* and *G. hookeri* belong to the same
620 family (Figitidae), but their parasitism strategies are quite different (Schlenke et al., 2007;
621 Mortimer et al., 2013). Our fly transcriptome analysis also revealed differences in the effects
622 of these wasps. Whereas *G. hookeri* appears to activate an immune response in the host, *L.*
623 *heterotoma* does not, but in turn induces genes related to energy production. Increasing energy
624 could be a strategy of the wasp to obtain more resources from the host. One the other hand, it
625 may reflect energy investment for a fly function such as immune response, which is
626 energetically expensive (Schlenke et al., 2007; Dolezal et al., 2019). If it indeed reflects
627 investment in immune functions, these have not been detected (this study; Schlenke et al.,

628 2007) and are unsuccessful, as the fly generally does not survive attacks by *L. heterotoma* (Xie
629 et al., 2014; Jones and Hurst, 2020b).
630
631 The *L. heterotoma*-induced downregulation of genes related to spermatogenesis; a
632 phenomenon not induced by *G. hookeri*, is an interesting contrast between the two wasps. In
633 accordance with our results, (Schlenke et al., 2007) reported downregulation of genes involved
634 with gonad development during parasitism by *L. heterotoma* and *L. boulardi*, albeit at earlier
635 stages. These two independent observations suggest that *L. heterotoma* might induce castration
636 of male hosts. Xie et al., 2011, reported that *Drosophila hydei* males (infected with a non-
637 male-killing strain of *Spiroplasma*) that survived parasitism by *L. heterotoma* (Lh14) had
638 extremely reduced fecundity, but the experiments could not rule out causes other than
639 castration. The phenomenon of male castration has only been reported in parasitoids of the
640 family Braconidae, where the polydnavirus present in the wasp venom induces testis
641 degradation (reviewed in Beckage and Gelman, 2004).
642
643 This study is the second one to use a genome-wide approach to evaluate gene expression of *D.*
644 *melanogaster* in response to *L. heterotoma*. The first one was conducted by Schlenke et al.,
645 2007 and employed microarrays at time points earlier than 24h PWA. In their latest time point
646 (22–24h PWA), Schlenke et al., 2007 found 37 DE genes ($P < 0.01$, $-0.5 > FC > 1.0$; fold-
647 changes for genes DE at a $P < 0.05$ were not reported). At a similar time point (our T1), we
648 detect only one DE gene (*thor*), which was not detected by . Schlenke et al., 2007. Differences
649 between our results and those of Schlenke et al., 2007 may be attributable to the different
650 methodology (e.g. microarrays vs. RNAseq, different software pipelines, and cutoff
651 parameters). Other potential differences could be related to *Drosophila* genotypes or
652 *Wolbachia* infection status, or other experimental conditions or their interactions. In spite of
653 all these variables, both studies provide evidence that parasitism by *L. heterotoma* does not
654 induce an immune response in *D. melanogaster*.
655
656 In a different scenario, parasitism by *G. hookeri* induced the Jak/Stat pathway, one of the
657 immune pathways of *D. melanogaster*. Effectors of this pathway (*Tot* and *Tep* genes) were
658 highly upregulated. Tep proteins play an immune role in the mosquito *Anopheles gambiae*,

659 against the parasite *Plasmodium berghei* (Blandin et al., 2004).The function of *Tot* genes is
660 unknown, but they belong to a family of eight genes that are activated by stressful conditions
661 such as bacterial challenge, high temperatures, mechanical pressure, or UV radiation (Ekengren
662 and Hultmark, 2001). Parasitism of *D. melanogaster* by the wasps *L. boulardi* (Schlenke et al.,
663 2007) and *Asobara tabida* (Salazar-Jaramillo et al., 2017) also induce the up-regulation of *Tep*
664 and *Tot* genes, suggesting that upregulation of these genes could be a common response against
665 wasp parasitism. Another potential signal of immune activation against *G. hookeri* is the
666 induction of genes encoding serine proteases, because these enzymes are involved in triggering
667 immune response in insects (reviewed in Alvarado et al., 2020). The melanization cascade,
668 another feature of *D. melanogaster* immune response, also appears to be activated by *G.*
669 *hookeri*, as two components of this cascade (*PPO3* and *yellow-f*) were up-regulated. Finally
670 the upregulation of genes that are known to be expressed preferentially in hemocytes (i.e.,
671 *PPO3*, *ItgaPS4 and he*), or involved in hemocyte proliferation (i.e., *pvf2*) implies that
672 hemocytes are activated by *G. hookeri* parasitism. In accordance with this, Mortimer et al.,
673 2013 showed that the number of lamellocytes increases during parasitism by *G. hookeri*. Even
674 though *D. melanogaster* appears to mount an immune response against *G. hookeri*, the response
675 is inadequate, as ~100% of flies parasitized by this wasp die (Mortimer et al., 2013; Mateos et
676 al., 2016). The reason likely lies in that the venom of this wasp contains a calcium ATPase that
677 prevents activation of plasmatocytes; a necessary early step of the encapsulation process
678 (Mortimer et al., 2013).
679
680 **4.3 Wasp parasitism in the presence of *Spiroplasma***
681
682 The primary goal of this study was to identify *Drosophila* fly-encoded functions
683 relevant to the *Spiroplasma*-wasp interaction, as such functions could be involved in the
684 mechanism that leads to the death of certain wasps, including *L. heterotoma*. Our analyses
685 revealed several genes whose expression appears to be influenced specifically by the
686 *Spiroplasma-L. heterotoma* interaction. In the context of the *Spiroplasma*-susceptible wasp (*L.*
687 *heterotoma*), eight and 32 genes were exclusively DE at T1 and T2, respectively. None of these
688 genes, with exception of attacin-C (*AttC*), were associated with an immune function in *D.*
689 *melanogaster*. We thus infer that the *Spiroplasma*-mediated wasp-killing mechanism is not

690 strongly influenced by host-encoded immunity, at least at a level detectable by our experiments.
691 Consistent with our findings, an RNA-seq analysis of *Spiroplasma* protection against
692 nematodes in *D. neotestacea* did not detect changes in the host's immune response (Hamilton
693 et al., 2014). Similarly, Paredes et al., 2016 did not detect an increased cellular response, based
694 on the number of hemocytes, during the *Spiroplasma-L. boulardi* interaction. The lack of
695 detectable influence of *Spiroplasma* on host-encoded immunity is consistent with evidence that
696 *Spiroplasma* is not detected as an intruder by the fly, due to the lack of cell wall, where the
697 typical bacterial immune elicitors are found (Hurst et al., 2003; Herren and Lemaitre, 2011).
698
699 In the context of *Spiroplasma-L. heterotoma* interaction, we identified a set of twelve genes
700 whose expression patterns appear to be "restored", as well as two genes (TotC and Fbp2) whose
701 expression patterns are likely influenced by the interaction between *Spiroplasma* and *L.*
702 *heterotoma*. Furthermore, these genes did not exhibit such an expression pattern in the context
703 of the *Spiroplasma*-resistant wasp *G. hookeri*. These genes could contribute to the wasp death
704 and/or fly survival (i.e., a *Spiroplasma*-mediated protection mechanism), but also could simply
705 reflect a side effect of the *Spiroplasma-Lh* interaction. In Group 1 of the restored genes, only
706 two had annotations, one of which is predicted to have chitin-binding activity (*CG42494*)
707 whereas the other has a cytochrome P450 domain (*Cyp4d14*); fold changes of these genes were
708 low (< 2). Cytochrome P450 are a large family of proteins that is involved in detoxification,
709 but also in developmental processes (Chung et al., 2009). For both of these genes, expression
710 levels are reported to go down between the L3 and pupa transition. Therefore, if the S0Lh
711 treatment were developmentally delayed with respect to the S+Lh treatment (see discussion
712 below), our observed expression patterns may simply be a consequence of development time
713 differences between the treatments, rather than a cause of wasp death or enhanced fly survival.
714 Furthermore, if the detoxification function of Cyp4d14 were relevant to the *Spiroplasma*-
715 mediated protection mechanism, we would expect higher expression in the S+Lh than in the
716 S0Lh treatment, which is contrary to our observations. Experimental down-regulation of these
717 and the other Group 1 genes, in the absence of *Spiroplasma*, could help determine whether they
718 have any role in the *Spiroplasma*-mediated protection against wasps.
719

720 Genes in Group 2 exhibit a substantial increase in expression in wild type flies during the larva-
721 to-pupa transition according to flybase (Thurmond et al., 2019). Therefore, our observed
722 expression patterns could simply reflect slight differences in fly development time, where *L.*
723 *heterotoma* slows down host development, but presence of *Spiroplasma* "restores" the
724 development time to that of the unparasitized host. In partial support of this hypothesis, larvae
725 parasitized by *L. hetetoroma* and *L. boulardi* pupate ~2 days later than the unparasitized
726 controls (Schlenke et al., 2007). Whether *Spiroplasma* counteracts the wasp-induced delay in
727 development remains to be determined.
728 Two genes (*Fbp2* and *TotC*), were not part of the restored genes, but their expression patterns
729 and functions allow us to speculate about their possible roles in the *Spiroplasma*-mediated
730 protection. We acknowledge that the *s*Mel-*L. heterotoma* (strain Lh14) interaction, albeit
731 leading to wasp death, seldom results in fly surviva(Xie et al., 2014; Paredes et al., 2016; Jones
732 and Hurst, 2020b). Given its possible function as a sulfur-storage protein (Meghlaoui and
733 Veuille, 1997), increased expression of *Fbp2* might reflect a greater availability of nutrients
734 for the fly, which might enhance its tolerance to insults from the wasp. Similarly, based on its
735 association to "response to stress", increased expression of *TotC* may contribute to increasing
736 the fly's tolerance to the wasp parasitism. In addition, *TotA*, another stress responsive gene,
737 was upregulated but with an FDR value slightly above our cutoff (0.053). Experimental over-
738 expression of these, and the Group 2 "restored" genes, in the absence of *Spiroplasma*, could be
739 used to test whether they influence the outcome of parasitism by *L. heterotoma* and other
740 wasps, including those against which *s*Mel confers stronger rescue (e.g. *L. boulardi* or *L.*
741 *victoriae*; Mateos et al., 2016; Paredes et al., 2016; Jones and Hurst, 2020a)
742
743 **4.4. RIP expression**
744
745 Concerning the hypothesis that a *Spiroplasma*-encoded toxin contributes to killing of
746 *Spiroplasma*-susceptible wasps, we found evidence that neither wasp influences expression of
747 RIP genes, but RIP2 gene was highly expressed in contrast to RIP1 or RIP3-5. In a previous
748 report in the absence of parasitism, RIP2 was also found to be the most highly expressed of the
749 RIP genes throughout the fly life cycle (only substrain Uganda was examined; Garcia-Arraez
750 et al., 2019)). The only context where relatively lower expression of RIP2 and RIP1 in *s*Mel

751 has been reported is in the case of in vitro culture versus fly hemolymph (Masson et al.,
752 2018),implying that expression of these genes can be regulated. The expression of RIP genes
753 in context of other tolerant/resistant wasps had not been determined.
754
755 Our study also reveals that both the *s*Mel-UG and *s*Mel-BR sub-strains of *S. poulsonii* express
756 their RIP genes at similar levels in the presence or absence of *L. heterotoma* and *G. hookeri*.
757 Although differences in the genomes of substrains *s*Mel-Ug and *s*Mel-BR have been reported
758 (Gerth et al., 2020) our observations suggest that at least for RIP expression patterns the two
759 strains are very similar. Based on expression levels alone, it appears that *s*Mel RIP2 might have
760 a stronger role in wasp death than the other RIP genes. However, it is intriguing that the genome
761 of sHy (the poulsonii-clade native *Spiroplasma* of *D. hydei)* does not harbor a gene with high
762 homology to sMel RIP2. Instead, its genome encodes a gene with high homology to sMel RIP1,
763 as well as putative RIP-encoding genes with homology to genes in *Spiroplasma* strains other
764 than *D. melanogaster* (Gerth et al., 2020), sHy is known to kill *L. heterotoma*, and enhance
765 survival of its host *D. hydei* (Xie et al., 2010). It is thus possible that more than one of the RIP
766 genes in the *Spiroplasma* strains that kill *L. heterotoma*, contributes to wasp killing.
767
768 Detection of signals of ribosomal depurination in the *Spiroplasma*-susceptible (*L. heterotoma*)
769 but not in the *Spiroplasma*-resistant (*G. hookeri*) wasp is consistent with the hypothesis that
770 RIP-induced depurination contributes to wasp death. Ballinger and Perlman, 2017 , using a
771 more direct approach to evaluate depurination (i.e., qPCR), reported evidence of *Spiroplasma*
772 (strain sMel-UG)-induced depurination in *L. heterotoma*, as well as in *L. boulardi* (another
773 *Spiroplasma*-susceptible wasp), but not in the *Spiroplasma*-resistant pupal ectoparasitic wasp
774 (*Pachycrepoideus vindemmiae*). Ballinger and Perlman, 2017. hypothesized that *P.*
775 *vindemmiae* resistance to *Spiroplasma* (and depurination) may stem from the fact that it is not
776 immersed in *Spiroplasma*-layden hemolymph during development. This explanation, however,
777 would not apply to *G. hookeri*, which is a larval endo-parasitoid that spends the initial stages
778 of development in the host hemocoel. Whether RIP-induced depurination is necessary and
779 sufficient to kill susceptible wasps has not been determined. Ideally, the effect of RIP on the
780 wasp would be tested in the absence of *Spiroplasma*, or in "knocked-out" mutants in these
781 genes. In addition, the target cells and the mechanism of entry of *Spiroplasma* RIPs has not

been determined. It is unclear whether *Spiroplasma* must colonize wasp tissues in order to deliver RIP or if toxins can be acquired during wasp feeding; both RIP1 and RIP2 proteins have been detected in the fly hemolymph (see Garcia-Arraez et al., 2019). It is also possible that other *Spiroplasma*-encoded putative virulence factors such as chitinase (*ChiD*) or glycerol-3-phosphate oxidase (*glpO*) could contribute to wasp death (Masson et al., 2018). Presence of *glpO* in *S. taiwanense* is proposed to be the cause of its pathogenicity to mosquitoes (Lo and Kuo, 2017).

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

Conclusions

We detected a small number of fly genes whose expression was influenced by the interaction between *Spiroplasma* and the susceptible wasp *L. heterotoma*. Based on the annotations of these genes, our results suggest that immune priming does not contribute to the *Spiroplasma*-mediated wasp killing mechanism. Furthermore, based on the reported expression patterns of several of these genes during fly development, it is possible that our results reflect *Spiroplasma*-mediated changes in development time of Lh-parasitized flies, which has yet to be verified. In accordance with previous studies, we detected evidence consistent with ribosomal damage in the susceptible but not the resistant wasp. In addition, our transcriptome and qPCR results indicated that RIP2 is the most highly expressed of the five *s*Mel-encoded RIP genes in both the BR and UG substrains, and that its expression levels were independent

813 of wasp parasitism. We recommend that future studies examine whether any of the sMel-
814 encoded RIPs are necessary and sufficient for wasp killing. The observation that the
815 *Spiroplasma*-resistant wasp *G. hookeri* did not influence RIP transcript levels raises interesting
816 questions about its mechanism of resistance or tolerance to *Spiroplasma*.
817
818 **Acknowledgements**
819 Texas Agrilife Genomics and Bioinformatics Services for library preparation, sequencing, and
820 advice on handling of RNA. Luis E Servín-Garcidueñas critically reviewed the manuscript.
821 Renato D. La Torre Ramirez for help with qPCR standard curve. Alfredo Hernández, Víctor
822 del Moral and Romualdo Zayas for the provided computing support.
823 All bioinformatic analyses were performed on CCG-UNAM servers. Victor Manuel Higareda
824 Alvear is a doctoral student at Programa de Doctorado en Ciencias Biomédicas of the
825 Universidad Nacional Autónoma de México (UNAM), and was supported by Consejo Nacional
826 de Ciencia y Tecnología (CONACyT, CVU 446829).
827
828
829 **Funding**
830 Genomics Seed Grant to MM from Texas Agrilife Genomics and Bioinformatics Services,
831 and CONACyT and Center for Genomic Sciences for sabbatical support to MM.
832 EMR has support from received support for this research from PAPIIT IN207718 from
833 UNAM. VMHA had support from CONACyT and PAEP for PhD and for internship at Texas
834 A&M University.
835
836
837
838
839
840
841
842
843

844

845 **References**

846

847 Agaisse, H., and Perrimon, N. (2004). The roles of JAK/STAT signaling in Drosophila
848 immune responses. *Immunol. Rev.* 198, 72–82. doi:10.1111/j.0105-2896.2004.0133.x.
849 Alvarado, G., Holland, S. R., DePerez-Rasmussen, J., Jarvis, B. A., Telander, T., Wagner, N.,
850 et al. (2020). Bioinformatic analysis suggests potential mechanisms underlying
851 parasitoid venom evolution and function. *Genomics* 112, 1096–1104.
852 doi:10.1016/j.ygeno.2019.06.022.
853 Andrews, S. (2010). FastQC: A Quality Control Tool for High Throughput Sequence Data
854 [Online]. Available online at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc/
855 Ballinger, M. J., and Perlman, S. J. (2017). Generality of toxins in defensive symbiosis:
856 Ribosome-inactivating proteins and defense against parasitic wasps in Drosophila. *PLoS*
857 *Pathog.* 13, 1–19. doi:10.1371/journal.ppat.1006431.
858 Beckage, N. E., and Gelman, D. B. (2004). Wasp Parasitoid Disruption of Host
859 Development: Implications for New Biologically Based Strategies for Insect Control.
860 *Annu. Rev. Entomol.* 49, 299–330. doi:10.1146/annurev.ento.49.061802.123324.
861 Blandin, S., Shiao, S. H., Moita, L. F., Janse, C. J., Waters, A. P., Kafatos, F. C., et al. (2004).
862 Complement-like protein TEP1 is a determinant of vectorial capacity in the malaria
863 vector Anopheles gambiae. *Cell* 116, 661–670. doi:10.1016/S0092-8674(04)00173-4.
864 Bolger, A. M., Lohse, M., and Usadel, B. (2014). Trimmomatic: A flexible trimmer for
865 Illumina sequence data. *Bioinformatics* 30, 2114–2120.
866 doi:10.1093/bioinformatics/btu170.
867 Brandt, J. W., Chevignon, G., Oliver, K. M., and Strand, M. R. (2017). Culture of an aphid
868 heritable symbiont demonstrates its direct role in defence against parasitoids. *Proc. R.*
869 *Soc. B Biol. Sci.* 284. doi:10.1098/rspb.2017.1925.
870 Bray, N. L., Pimentel, H., Melsted, P., and Pachter, L. (2016). Near-optimal probabilistic
871 RNA-seq quantification. *Nat. Biotechnol.* 34, 525–527. doi:10.1038/nbt.3519.
872 Caragata, E. P., Rancés, E., Hedges, L. M., Gofton, A. W., Johnson, K. N., O'Neill, S. L., et
873 al. (2013). Dietary Cholesterol Modulates Pathogen Blocking by Wolbachia. *PLoS*
874 *Pathog.* 9. doi:10.1371/journal.ppat.1003459.
875 Carton, Y., Poirié, M., and Nappi, A. J. (2008). Insect immune resistance to parasitoids.
876 *Insect Sci.* 15, 67–87. doi:10.1111/j.1744-7917.2008.00188.x.
877 Cheng, B., Kuppanda, N., Aldrich, J. C., Akbari, O. S., and Ferree, P. M. (2016). Male-
878 killing Spiroplasma alters behavior of the dosage compensation complex during
879 drosophila melanogaster embryogenesis. *Curr. Biol.* 26, 1339–1345.
880 doi:10.1016/j.cub.2016.03.050.
881 Chung, H., Sztal, T., Pasricha, S., Sridhar, M., Batterham, P., and Daborn, P. J. (2009).
882 Characterization of Drosophila melanogaster cytochrome P450 genes. *Proc. Natl. Acad.*
883 *Sci. U. S. A.* 106, 5731–5736. doi:10.1073/pnas.0812141106.
884 Corbin, C., Jones, J. E., Chrostek, E., Fenton, A., and Hurst, G. D. D. (2020). Thermal
885 sensitivity of the Spiroplasma-Drosophila hydei protective symbiosis: The best of
886 climes, the worst of climes. *bioRxiv*. doi:10.1101/2020.04.30.070938.
887 Dolezal, T., Krejcova, G., Bajgar, A., Nedbalova, P., and Strasser, P. (2019). Molecular
888 regulations of metabolism during immune response in insects. *Insect Biochem. Mol.*
889 *Biol.* 109, 31–42. doi:10.1016/j.ibmb.2019.04.005.
890 Dudzic, J. P., Kondo, S., Ueda, R., Bergman, C. M., and Lemaitre, B. (2015). Drosophila
891 innate immunity: regional and functional specialization of prophenoloxidases. *BMC*

892 *Biol.* 13, 81. doi:10.1186/s12915-015-0193-6.
893 Ekengren, S., and Hultmark, D. (2001). A family of Turandot-related genes in the humoral
894 stress response of Drosophila. *Biochem. Biophys. Res. Commun.* 284, 998–1003.
895 doi:10.1006/bbrc.2001.5067.
896 Garcia-Arraez, M. G., Masson, F., Escobar, J. C. P., and Lemaitre, B. (2019). Functional
897 analysis of RIP toxins from the Drosophila endosymbiont Spiroplasma poulsonii. *BMC*
898 *Microbiol.* 19, 1–10. doi:10.1186/s12866-019-1410-1.
899 Gatehouse, A. M. R., Barbieri, L., Stirpe, F., and Croy, R. R. D. (1990). Effects of ribosome
900 inactivating proteins on insect development – differences between Lepidoptera and
901 Coleoptera. *Entomol. Exp. Appl.* 54, 43–51. doi:10.1111/j.1570-7458.1990.tb01310.x.
902 Gerth, M., Humberto., M.-M., Ramirez, P., Masson, F., Griffin, J., Aramayo, R., et al. (2020).
903 Rapid molecular evolution of Spiroplasma symbionts of Drosophila. *bioRxiv*.
904 doi:10.1101/2020.06.23.165548.
905 Green, C., Levashina, E., McKimmie, C., Dafforn, T., Reichhart, J. M., and Gubb, D. (2000).
906 The necrotic gene in Drosophila corresponds to one of a cluster of three serpin
907 transcripts mapping at 43A1.2. *Genetics* 156, 1117–1127.
908 Haas, B. J., Chin, M., Nusbaum, C., Birren, B. W., and Livny, J. (2012). How deep is deep
909 enough for RNA-Seq profiling of bacterial transcriptomes? *BMC Genomics* 13, 734.
910 doi:10.1186/1471-2164-13-734.
911 Hamilton, P. T., Leong, J. S., Koop, B. F., and Perlman, S. J. (2014). Transcriptional
912 responses in a Drosophila defensive symbiosis. *Mol. Ecol.* 23, 1558–1570.
913 doi:10.1111/mec.12603.
914 Hamilton, P. T., Peng, F., Boulanger, M. J., and Perlman, S. J. (2015). A ribosome-
915 inactivating protein in a Drosophila defensive symbiont. *Proc Natl Acad Sci U S A*,
916 1518648113-. doi:10.1073/pnas.1518648113.
917 Han, Q., Fang, J., Ding, H., Johnson, J. K., Christensen, B. M., and Li, J. (2002).
918 Identification of Drosophila melanogaster yellow-f and yellow-f2 proteins as
919 dopachrome-conversion enzymes. *Biochem. J.* 368, 333–340. doi:10.1042/BJ20020272.
920 Hart, S. N., Therneau, T. M., Zhang, Y., Poland, G. A., and Kocher, J. P. (2013). Calculating
921 sample size estimates for RNA sequencing data. *J. Comput. Biol.* 20, 970–978.
922 doi:10.1089/cmb.2012.0283.
923 Harumoto, T., Anbutsu, H., Lemaitre, B., and Fukatsu, T. (2016). Male-killing symbiont
924 damages host's dosage-compensated sex chromosome to induce embryonic apoptosis.
925 *Nat. Commun.* 7, 12781. doi:10.1038/ncomms12781.
926 Herren, J. K., and Lemaitre, B. (2011). Spiroplasma and host immunity: Activation of
927 humoral immune responses increases endosymbiont load and susceptibility to certain
928 Gram-negative bacterial pathogens in Drosophila melanogaster. *Cell. Microbiol.* 13,
929 1385–1396. doi:10.1111/j.1462-5822.2011.01627.x.
930 Hillyer, J. F. (2016). Insect immunology and hematopoiesis. *Dev. Comp. Immunol.* 58, 102–
931 118. doi:10.1016/j.dci.2015.12.006.
932 Hurst, G. D. D., Anbutsu, H., Kutsukake, M., and Fukatsu, T. (2003). Hidden from the host:
933 Spiroplasma bacteria infecting Drosophila do not cause an immune response, but are
934 suppressed by ectopic immune activation. *Insect Mol. Biol.* 12, 93–97.
935 doi:10.1046/j.1365-2583.2003.00380.x.
936 Irving, P., Ubeda, J. M., Doucet, D., Troxler, L., Lagueux, M., Zachary, D., et al. (2005).
937 New insights into Drosophila larval haemocyte functions through genome-wide analysis.
938 *Cell. Microbiol.* 7, 335–350. doi:10.1111/j.1462-5822.2004.00462.x.
939 Jaenike, J., Unckless, R., Cockburn, S. N., Boelio, L. M., and Perlman, S. J. (2010).
940 Adaptation via symbiosis: Recent spread of a drosophila defensive symbiont. *Science*
941 *(80-.).* 329, 212–215. doi:10.1126/science.1188235.

942 Jones, J. E., and Hurst, G. D. D. (2020a). Symbiont-mediated fly survival is independent of
943 defensive symbiont genotype in the Drosophila melanogaster-Spiroplasma-wasp
944 interaction. *bioRxiv*, 1–24. doi:10.1101/2020.07.05.154906.
945 Jones, J. E., and Hurst, G. D. D. (2020b). Symbiont-mediated protection varies with wasp
946 genotype in the Drosophila melanogaster–Spiroplasma interaction. *Heredity (Edinb).*
947 124, 592–602. doi:10.1038/s41437-019-0291-2.
948 Kim, D., Langmead, B., and Salzberg, S. L. (2015). HISAT: A fast spliced aligner with low
949 memory requirements. *Nat. Methods* 12, 357–360. doi:10.1038/nmeth.3317.
950 Kumar, N., Creasy, T., Sun, Y., Flowers, M., Tallon, L. J., and Dunning Hotopp, J. C. (2012).
951 Efficient subtraction of insect rRNA prior to transcriptome analysis of Wolbachia-
952 Drosophila lateral gene transfer. *BMC Res. Notes* 5, 1. doi:10.1186/1756-0500-5-230.
953 Liao, Y., Smyth, G. K., and Shi, W. (2014). FeatureCounts: An efficient general purpose
954 program for assigning sequence reads to genomic features. *Bioinformatics* 30, 923–930.
955 doi:10.1093/bioinformatics/btt656.
956 Lo, W. S., and Kuo, C. H. (2017). Horizontal Acquisition and Transcriptional Integration of
957 Novel Genes in Mosquito-Associated Spiroplasma. *Genome Biol. Evol.* 9, 3246–3259.
958 doi:10.1093/gbe/evx244.
959 Lucchesi, J. C., and Kuroda, M. I. (2015). Dosage compensation in drosophila. *Cold Spring*
960 *Harb. Perspect. Biol.* 7, 1–21. doi:10.1101/cshperspect.a019398.
961 Lyne, R., Smith, R., Rutherford, K., Wakeling, M., Varley, A., Guillier, F., et al. (2007).
962 FlyMine: An integrated database for Drosophila and Anopheles genomics. *Genome Biol.*
963 8. doi:10.1186/gb-2007-8-7-r129.
964 Masson, F., Copete, S. C., Schüpfer, F., Garcia-Arraez, G., and Lemaitre, B. (2018). In Vitro
965 culture of the insect Endosymbiont Spiroplasma poulsonii highlights bacterial genes
966 involved in host- symbiont interaction. *MBio* 9, 1–11. doi:10.1128/mBio.00024-18.
967 Mateos, M., Winter, L., Winter, C., Higareda-Alvear, V. M., Martinez-Romero, E., and Xie,
968 J. (2016). Independent origins of resistance or susceptibility of parasitic wasps to a
969 defensive symbiont. *Ecol. Evol.*, 2679–2687. doi:10.1002/ece3.2085.
970 Meghlaoui, G. K., and Veuille, M. (1997). Selection and methionine accumulation in the fat
971 body protein 2 gene (FBP2), a duplicate of the Drosophila alcohol dehydrogenase
972 (ADH) gene. *J. Mol. Evol.* 44, 23–32. doi:10.1007/PL00006118.
973 Montenegro, H., Solferini, V. N., Klaczko, L. B., and Hurst, G. D. D. (2005). Male-killing
974 Spiroplasma naturally infecting Drosophila melanogaster. *Insect Mol. Biol.* 14, 281–287.
975 doi:10.1111/j.1365-2583.2005.00558.x.
976 Mortimer, N. T., Goecks, J., Kacsoh, B. Z., Mobley, J. A., and Bowersock, G. J. (2013).
977 Parasitoid wasp venom SERCA regulates Drosophila calcium levels and inhibits cellular
978 immunity. 110, 9427–9432. doi:10.1073/pnas.1222351110.
979 Oliver, K. M., and Perlman, S. J. (2020). *Toxin-mediated protection against natural enemies*
980 *by insect defensive symbionts*. 1st ed. , eds. K. Oliver and Russel J. Elsevier Ltd.
981 doi:10.1016/bs.aiip.2020.03.005.
982 Oliver, K. M., Russell, J. A., Morant, N. A., and Hunter, M. S. (2003). Facultative bacterial
983 symbionts in aphids confer resistance to parasitic wasps. *Proc. Natl. Acad. Sci. U. S. A.*
984 100, 1803–1807. doi:10.1073/pnas.0335320100.
985 Paredes, J. C., Herren, J. K., Schüpfer, F., and Lemaitre, B. (2016). The Role of Lipid
986 Competition for Endosymbiont-Mediated Protection against Parasitoid Wasps in
987 *Drosophila*. *MBio* 7, e01006-16. doi:10.1128/mBio.01006-16.
988 Pool, J. E., Wong, A., and Aquadro, C. F. (2006). Finding of male-killing Spiroplasma
989 infecting Drosophila melanogaster in Africa implies transatlantic migration of this
990 endosymbiont. *Heredity (Edinb).* 97, 27–32. doi:10.1038/sj.hdy.6800830.
991 Riegler, M., Sidhu, M., Miller, W. J., and O'Neill, S. L. (2005). Evidence for a global

992 Wolbachia replacement in Drosophila melanogaster. *Curr. Biol.* 15, 1428–1433.
993 doi:10.1016/j.cub.2005.06.069.
994 Robinson, M. D., McCarthy, D. J., and Smyth, G. K. (2009). edgeR: A Bioconductor package
995 for differential expression analysis of digital gene expression data. *Bioinformatics* 26,
996 139–140. doi:10.1093/bioinformatics/btp616.
997 Russo, J. (1996). Insect immunity: Early events in the encapsulation process of parasitoid
998 (Leptopilina boulardi) eggs in resistant and susceptible strains of Drosophila.
999 *Parasitology* 112, 135–142. doi:10.1017/s0031182000065173.
1000 Salazar-Jaramillo, L., Jalvingh, K. M., de Haan, A., Kraaijeveld, K., Buermans, H., and
1001 Wertheim, B. (2017). Inter- and intra-species variation in genome-wide gene expression
1002 of Drosophila in response to parasitoid wasp attack. *BMC Genomics* 18, 1–14.
1003 doi:10.1186/s12864-017-3697-3.
1004 Schlenke, T. A., Morales, J., Govind, S., and Clark, A. G. (2007). Contrasting infection
1005 strategies in generalist and specialist wasp parasitoids of Drosophila melanogaster. *PLoS*
1006 *Pathog.* 3, 1486–1501. doi:10.1371/journal.ppat.0030158.
1007 Stirpe, F. (2004). Ribosome-inactivating proteins. *Toxicon* 44, 371–383.
1008 doi:10.1016/j.toxicon.2004.05.004.
1009 Thurmond, J., Goodman, J. L., Strelets, V. B., Attrill, H., Gramates, L. S., Marygold, S. J., et
1010 al. (2019). FlyBase 2.0: The next generation. *Nucleic Acids Res.* 47, D759–D765.
1011 doi:10.1093/nar/gky1003.
1012 Wang, J., Wu, Y., Yang, G., and Aksoy, S. (2009). Interactions between mutualist
1013 Wigglesworthia and tsetse peptidoglycan recognition protein (PGRP-LB) influence
1014 trypanosome transmission. *Proc. Natl. Acad. Sci. U. S. A.* 106, 12133–12138.
1015 doi:10.1073/pnas.0901226106.
1016 Wey, B., Heavner, M. E., Wittmeyer, K. T., Briese, T., Hopper, K. R., and Govind, S. (2020).
1017 Immune suppressive extracellular vesicle proteins of Leptopilina heterotoma are
1018 encoded in the wasp genome. *G3 Genes, Genomes, Genet.* 10, 1–12.
1019 doi:10.1534/g3.119.400349.
1020 Xie, J., Butler, S., Sanchez, G., and Mateos, M. (2014). Male killing Spiroplasma protects
1021 Drosophila melanogaster against two parasitoid wasps. *Heredity (Edinb).* 112, 399–408.
1022 doi:10.1038/hdy.2013.118.
1023 Xie, J., Tiner, B., Vilchez, I., and Mateos, M. (2011). Effect of the Drosophila endosymbiont
1024 Spiroplasma on parasitoid wasp development and on the reproductive fitness of wasp-
1025 attacked fly survivors. *Evol. Ecol.* 25, 1065–1079. doi:10.1007/s10682-010-9453-7.
1026 Xie, J., Vilchez, I., and Mateos, M. (2010). Spiroplasma bacteria enhance survival of
1027 Drosophila hydei attacked by the parasitic wasp Leptopilina heterotoma. *PLoS One* 5.
1028 doi:10.1371/journal.pone.0012149.
1029
1030
1031
1032