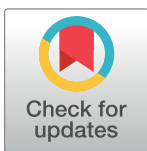RESEARCH ARTICLE

Dual proteomics of *Drosophila melanogaster* hemolymph infected with the heritable endosymbiont *Spiroplasma poulsonii*

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Abstract

Insects are frequently infected with heritable bacterial endosymbionts. Endosymbionts have a dramatic impact on their host physiology and evolution. Their tissue distribution is variable with some species being housed intracellularly, some extracellularly and some having a mixed lifestyle. The impact of extracellular endosymbionts on the biofluids they colonize (*e. g.* insect hemolymph) is however difficult to appreciate because biofluid composition can depend on the contribution of numerous tissues. Here we investigate *Drosophila* hemolymph proteome changes in response to the infection with the endosymbiont *Spiroplasma poulsonii*. *S. poulsonii* inhabits the fly hemolymph and gets vertically transmitted over generations by hijacking the oogenesis in females. Using dual proteomics on infected hemolymph, we uncovered a weak, chronic activation of the Toll immune pathway by *S. poulsonii* that was previously undetected by transcriptomics-based approaches. Using Drosophila genetics, we also identified candidate proteins putatively involved in controlling *S. poulsonii* growth. Last, we also provide a deep proteome of *S. poulsonii*, which, in combination with previously published transcriptomics data, improves our understanding of the post-transcriptional regulations operating in this bacterium.

Introduction

Insects frequently harbor vertically transmitted bacterial symbionts living within their tissues, called endosymbionts [1]. Endosymbionts have a major impact on the host physiology and evolution as they provide ecological benefits such as the ability for the host to thrive on unbalanced diets [2], protection against viruses or parasites [3–6] or tolerance to heat [7]. A peculiar group of insect endosymbionts also directly affects their host reproduction. This group is taxonomically diverse and includes bacteria from the *Wolbachia*, *Spiroplasma*, *Arsenophonus*, *Cardinium* and *Rickettsia* genera [8,9]. Four reproduction-manipulative mechanisms have been unraveled so far, namely cytoplasmic incompatibility, male-killing, parthenogenesis and male feminization [10], all of them leading to an evolutionary drive that favors infected individuals over non-infected ones and promotes the endosymbiont spread into populations. Their ease of

spread coupled with the virus-protecting ability of some species [3,4] make them promising tools to control insect pest populations or to render them refractory to human arboviruses [11]. Reproductive manipulators are however fastidious bacteria that are difficult to manipulate, hence slowing down research on the functional aspects of their interaction with their hosts [12]. Their real impact on host physiology thus remains largely elusive.

Spiroplasma are long helical bacteria belonging to the Mollicutes class, which are devoid of cell wall [13]. They infect a wide range of arthropods and plants where they act as pathogens, commensals or vertically transmitted endosymbionts depending on the species [13]. *S. poulsonii* (hereafter *Spiroplasma*) is a vertically transmitted endosymbiont infecting the fruit fly *Drosophila* [14,15]. It lives free in the host hemolymph where it feeds on circulating lipids [16] and achieves vertical transmission by infecting oocytes [17]. Most strains cause male-killing, that is the death of the male progeny during early embryogenesis through the action of a toxin named Spaid [18]. *Spiroplasma* infection also confers protection to the fly or its larvae against major natural enemies such as parasitoid wasps [19,20] and nematodes [21,22].

Spiroplasma has long been considered as untractable, but recent technical advances such as the development of *in vitro* culture [23] and transformation [24] make it an emergent model for studying the functional aspects of insect-endosymbiont interactions. Some major steps have been made in the understanding of the male killing [25–28] and protection phenotypes [20,22,29] or on the way the bacterial titer was kept under control in the adult hemolymph [16,30]. Such studies, however, relied on single-gene studies and did not capture the full picture of the impact of *Spiroplasma* on its host physiology. We tackled this question using dual-proteomics on fly hemolymph infected by *Spiroplasma*. This non-targeted approach allowed us to get an extensive overview of the end-effect of *Spiroplasma* infection on the fly hemolymph protein composition and to identify previously unsuspected groups of proteins that where over- or under-represented in infected hemolymph. Using *Drosophila* genetics to knock-down the corresponding genes, we identified new putative regulators of the bacterial titer. This work also provides the most comprehensive *Spiroplasma* proteome to date. By comparing this proteome to the existing transcriptomics data, we draw conclusions about *Spiroplasma* post-transcriptional regulations.

Results

1. *Drosophila* hemolymph protein profiling

We investigated the effect of *Spiroplasma* infection on *Drosophila* hemolymph protein content using Liquid Chromatography-tandem Mass Spectrometry (LC-MS/MS) (S1 Fig). To this end, we extracted total hemolymph from uninfected and infected 10 days old females. At this age, *Spiroplasma* is already present at high titers in the hemolymph but does not cause major deleterious phenotypes to the fly [31,32]. Extraction was achieved by puncturing the thorax and drawing out with a microinjector. This procedure induces some tissue damage but recovers very few hemocytes (circulating immune cells).

Peptides were mapped to both the *Drosophila* and *Spiroplasma* predicted proteomes, hence allowing having an overview of i) differentially represented *Drosophila* proteins in infected versus uninfected hemolymph and ii) an in-depth *Spiroplasma* proteome in the infected hemolymph samples. These two datasets will be analyzed in separate sections.

The mapping on *Drosophila* proteome allowed the identification of 909 quantified protein groups (a protein group contains proteins that cannot be distinguished based on the identified peptides). The complete list of quantified *Drosophila* proteins and their fold-change upon *Spiroplasma* infection is available in S1 Table.

The hemolymph extraction process involves tissue damage (*e.g.* the subcuticular epithelium, muscles and fat body) that leads to the release of intracellular proteins in the sample. Hence we first sorted protein groups depending on whether the main protein is predicted to be extracellular or not. Proteins were defined as extracellular if they bore a predicted signal peptide or were annotated with a Gene Ontology (GO) term "extracellular", or intracellular if no signal peptide nor any "extracellular" GO term was predicted. Based on this localization criteria the *Drosophila* dataset could be split in 555 intracellular protein groups representing 61% of the total proteome and 354 extracellular proteins representing the remaining 39% of the total proteome (Fig 1A).

We then compared the relative amounts of protein groups between infected and uninfected hemolymph. Of the intracellular protein groups, 35 were differentially represented in the *Spiroplasma* infected samples, including 8 overrepresented and 27 depleted groups compared to the uninfected control (Fig 1B). A functional GO analysis on the differentially represented protein groups revealed that a significant part of these protein groups were related to the proteasome (subunits α2, 3, 4 and 7 and subunits β3 and 6) and the Toll immune pathway (Fig 1C). The Toll pathway GO enrichment comprises only proteasome subunits, probably because of their involvement in the degradation of Toll pathway intermediates (e.g. Cactus [33]). While differentially abundant intracellular protein groups were mostly underrepresented in *Spiroplasma* infected hemolymph, analysis of the extracellular protein subset revealed an opposite trend. Among the 71 extracellular protein groups having a significantly different abundance, 4 were depleted and 67 were overrepresented in the infected samples compared to the uninfected ones (Fig 1D). Surprisingly, the functional GO annotation highlighted an overrepresentation of serine-endopeptidase molecular function. Serine proteases are involved in the regulation of the melanization and the Toll pathways and indeed the associated GO terms are enriched in the infected samples (Fig 1E).

2. Immune-related protein enrichment is a consequence of a mild transcriptional activation

Since *Spiroplasma* is devoid of cell wall, it lacks microbe-associated molecular patterns such as peptidoglycan and is not expected to interact with pattern-recognition receptors regulating the fly immune system. This idea was supported by previous work showing that *Spiroplasma* do not trigger a strong level of Toll and Imd pathway, the two main immune pathways in *Drosophila* [31,34]. Previous work also showed that flies defective for the inducible humoral immune response have normal *Spiroplasma* titer, suggesting that immune pathways are not required to control *Spiroplasma* growth [31]. Our observation that several immune-related proteins are more abundant in the hemolymph of infected flies (Fig 2A and S1 Table) led us to investigate further on this point. We first tested if the changes in immune-related protein abundance were a consequence of altered gene expression. We measured the corresponding mRNA levels of several differentially represented proteins (Fig 2B). Immune-related genes were slightly upregulated in infected flies, although their induction levels did not compare with those observed after systemic infection by a pathogenic bacteria [35,36]. These results confirm that *Spiroplasma* does not trigger a strong immune response [31,34], but rather provokes a mild and chronic production of immunity proteins.

3. A majority of enriched hemolymphatic proteins are not involved in *Spiroplasma* growth control

A systemic infection or a genetic induction of the humoral immune response promotes *Spiroplasma* growth in flies [31]. We therefore wondered if the presence of small amounts of
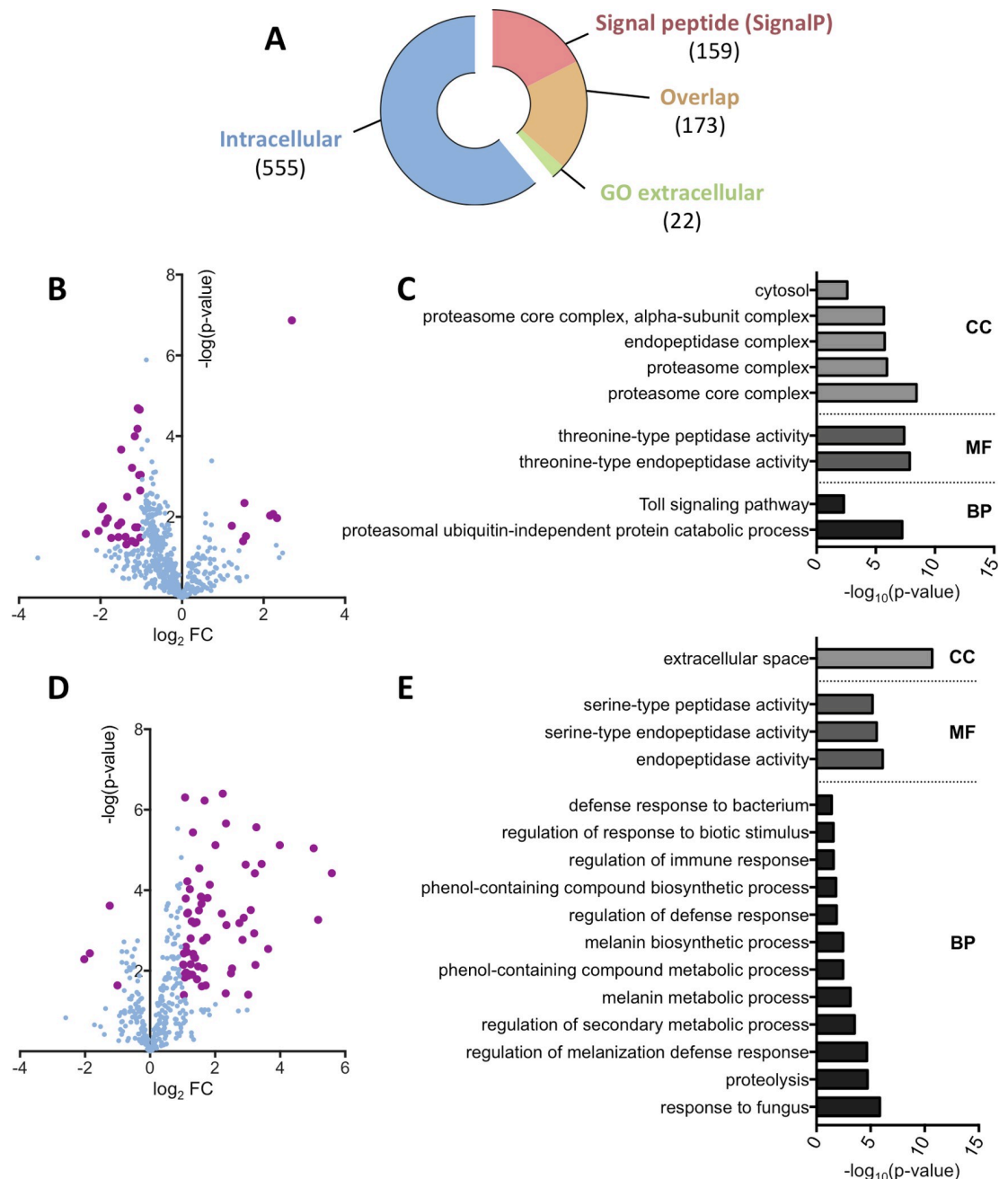
Fig 1. *Drosophila* hemolymph protein profiling upon *Spiroplasma* infection. (A) Representation of the dataset repartition between intracellular and extracellular protein groups. Extracellular groups are detected either by the presence of a predicted signal peptide using the SignalP algorithm or the annotation with a GO term referring to the extracellular space. The overlap category corresponds to protein groups with both a predicted signal peptide and a GO term "extracellular". Numbers in brackets indicate the number of protein groups in each category. (B) Volcano plot of the intracellular protein group subset. The $\log_2$ fold-change indicates the differential representation of the protein group in the infected samples versus the uninfected samples. Bold purple dots indicate significance as defined by an absolute fold change value over 2 or under -2 and a p-value below 0.05. (C) Functional GO annotation of the intracellular protein groups. Only significant (p-value < 0.05) GO terms of hierarchy 4 or less are displayed. CC: Cell Component; MF: Molecular Function; BP: Biological Process. (D) Volcano plot of the extracellular protein group subset. The $\log_2$ fold-change indicates the differential representation of the protein group in the infected samples versus the uninfected samples. Bold purple dots indicate significance as defined by an absolute fold change value over 2 or under -2 and a p-value below 0.05. (E) Functional GO annotation of the extracellular protein groups. Only significant (p-value < 0.05) GO terms of hierarchy 4 or less are displayed. CC: Cell Component; MF: Molecular Function; BP: Biological Process.
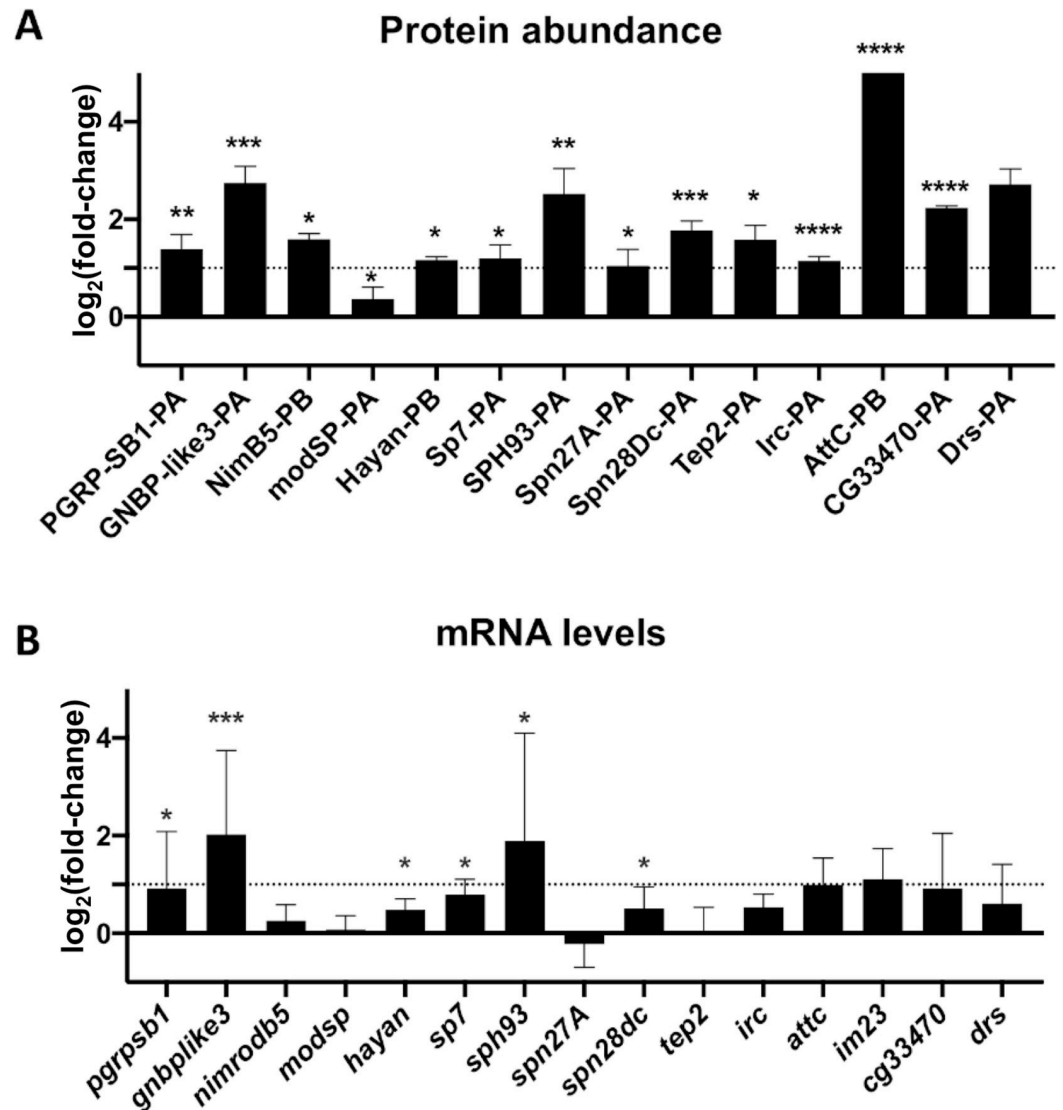
Fig 2. Spiroplasma infection induces a mild transcriptional immune response. (A) Changes in a selection of immune-related protein group abundances quantified by LC-MS/MS. Data are expressed as fold change of the Label-Free Quantification (LFQ) values of *Spiroplasma* infected hemolymph over the uninfected control (log$_2$ scale). *, p<0.05; ** p<0.005, ***; p<0.005, ****, p<0.0005 upon Student t-test. (B) mRNA quantification of a selection of candidate genes in uninfected and *Spiroplasma*-infected flies. Results are expressed as the fold change of target mRNA normalized by *rpL32* mRNA in *Spiroplasma*-infected flies as compared to uninfected flies (log$_2$ scale). *, p<0.05; ** p<0.005, ***; p<0.0005 upon Mann-Whitney test on ΔCt values. Each bar represents the mean +/- standard deviation of a pool of at least 2 independent experiments with 3 biological replicates each.

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antimicrobial peptides (AMPs) could be beneficial for *Spiroplasma* growth. To test this hypothesis, we measured *Spiroplasma* titer in flies over-expressing different AMP genes [37]. We found that AMP gene overexpression did not increase *Spiroplasma* titer (Fig 3A). Similarly, flies lacking ten AMP coding genes [38] had a *Spiroplasma* titer comparable to that of control flies (Fig 3B). These results suggest that AMPs released during the humoral immune response do not affect *Spiroplasma* growth.

We then tested if other immune-related proteins found more abundant in infected hemolymph could alter *Spiroplasma* growth. We first compared the *Spiroplasma* titers of
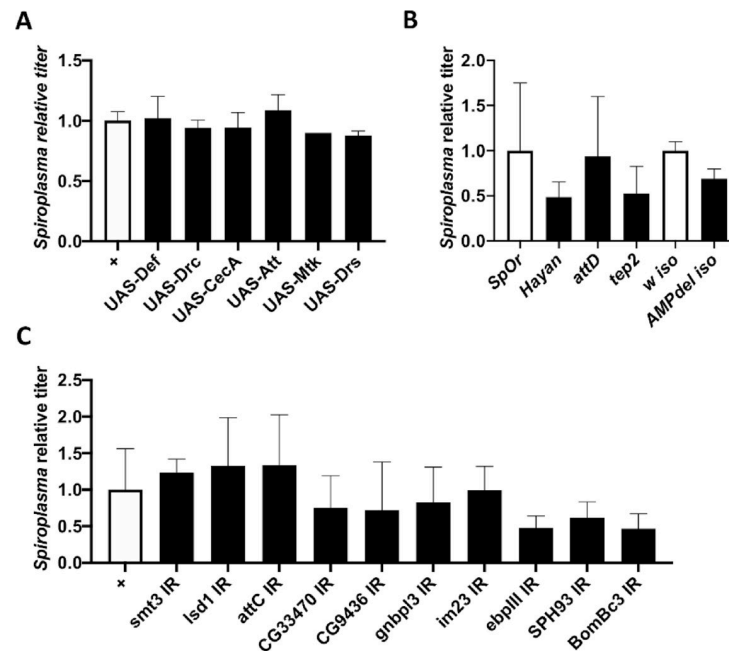
Fig 3. Targeted genetic screening of candidate proteins. (A-C) Quantification of *Spiroplasma* titer in two weeks old flies in various genetic backgounds. Titer is expressed as the fold-change over the appropriate control line. All graphs represent the mean +/- standard deviation of a pool of at least 2 independent experiments with 3 biological replicates each. (A) Control flies (*Act5C-GAL4* driver crossed w^{1118}, white bars) are compared to flies overexpressing a single antimicrobial peptide gene driven by the ubiquitous *Act5C-GAL4* driver (black bars). Titer is expressed as the fold-change over control. (B) Quantification of *Spiroplasma* titer in wild-type flies (*Oregon^R*, white bar) and *hayan*, *attD* and *tep2* null mutants (black). Spiroplasma titer in *AMP10 iso* flies were compared to that of their isogenic wild-type counterpart (w^{1118} iso, white bar). (C) Quantification of the impact of RNAi-mediated knockdown on *Spiroplasma* titer with the *Act5C-GAL4* driver. Titer is expressed as the fold-change over the control (*Act5C-GAL4* driver crossed to w^{1118}).

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control *Oregon^R* flies with that of *hayan*, *attD* and *tep2* mutants. We observed no difference in bacterial titer, indicating that these genes are not required neither detrimental to *Spiroplasma* growth (Fig 3B). We then used an *in vivo* RNAi approach to silence immune genes coding for the most enriched proteins in *Spiroplasma*-infected flies using the ubiquitously expressed *Act5C-GAL4* driver. Silencing these genes did not provoke an increase in *Spiroplasma* titer, further reinforcing the idea that immune proteins do not prevent *Spiroplasma* growth (Fig 3C).

Among the most differentially represented proteins in *Spiroplasma* infected hemolymph, we also detected several proteins of unknown function. To test their role in *Spiroplasma* growth control, we used the same RNAi-mediated knockdown approach and quantified *Spiroplasma* titer in these flies. Most of the RNAi lines tested showed no change in *Spiroplasma* titer as compared to control lines (S2 Fig). Two RNAi lines targeting *CG18067* and *CG14762*, respectively, showed a slight yet not significant reduction in *Spiroplasma* titer. These results suggest that all the tested genes do not facilitate *Spiroplasma* growth nor control its titer. Instead, these proteins are likely to be induced as a consequence of *Spiroplasma* infection and may participate in the host adaptation to the bacteria. A mutant line was available for only one of the most enriched uncharacterized proteins (*CG15293*). We found that this mutation leads to a significant increase in bacteria titer (S2 Fig). This gene codes for a 37 kDa secreted protein with no known function. Our results raised the hypothesis that *CG15293* participates in the control of *Spiroplasma* titer *in vivo*.

4. Transcriptome-proteome correlation in *Spiroplasma poulsonii*

The proteomics analysis of infected hemolymph samples also allowed the detection and quantification of *Spiroplasma* proteins. With a total of 503 proteins, this is the most comprehensive *Spiroplasma* proteome to date. The complete list of *Spiroplasma* quantified proteins is available in S2 Table.

We took advantage of a previously published transcriptomics dataset of *Spiroplasma* [23] to compare the expression level of *Spiroplasma* genes to their corresponding protein abundance by building a linear model of the proteomics signal as a function of the transcript level (Fig 4). The two measures are poorly correlated (Kendall's τ = 0.40). Analyzing the normalized residuals of the model also allowed us to identify proteins that are particularly discrepant with the model, that is with absolute standardized residuals greater than 2. This approach uncovered 27 proteins, of which 11 have a significantly higher abundance and 16 a lower abundance in the proteome than what was predicted from the transcriptomics data (S3 Table). Over-represented proteins
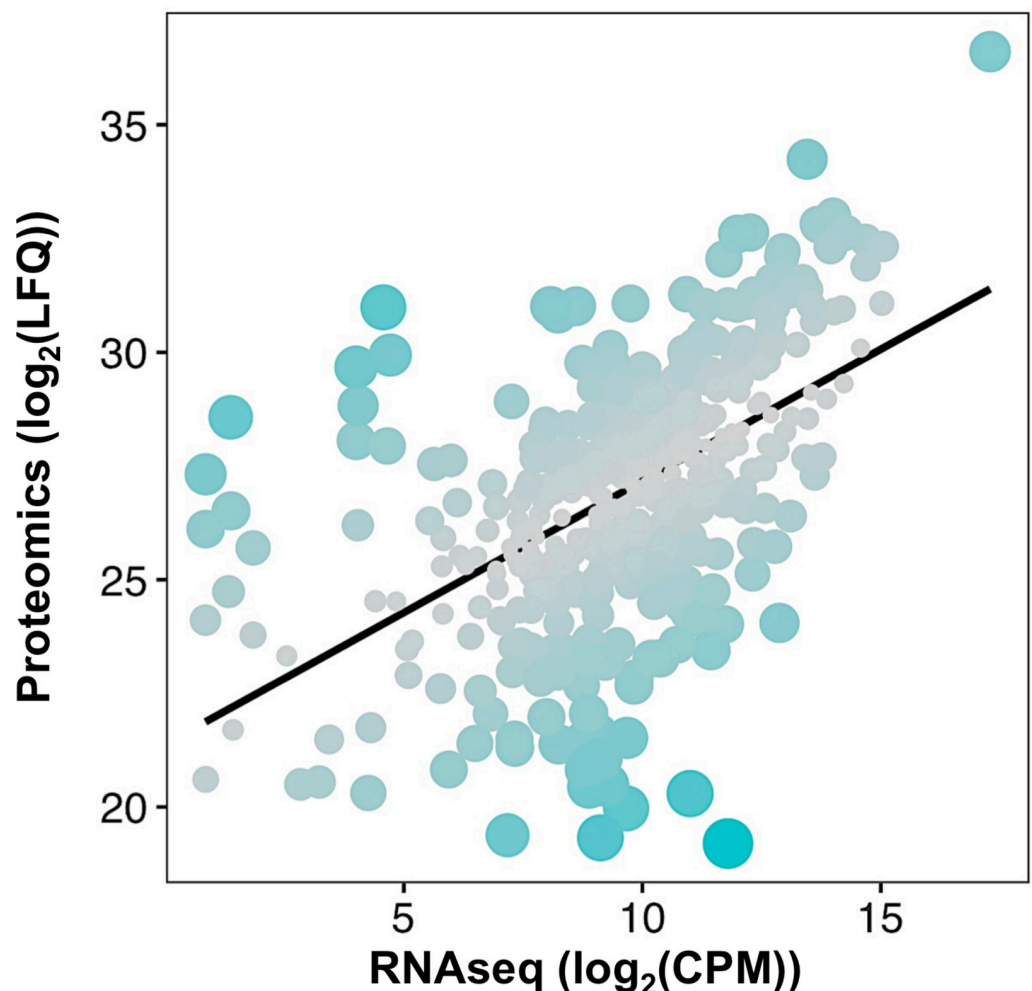
Fig 4. *Spiroplasma* transcription-translation correlation. Each dot represents a protein positioned according to its $\log_2$(LFQ; Label-free Quantification) value in the proteome versus its $\log_2$(CPM; Count Per Million) in the transcriptomics dataset from [23]. The black line represents the linear model $\log_2$(LFQ) = 0.57892 x $\log_2$(CPM) + 21.38325, with an adjusted R^2 = 0.2909. Dot size and color are adjusted to the residuals of the model, with bigger bluer dots indicating a higher residual hence a stronger deviation from the linear model for the considered protein.

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with a reliable annotation include the membrane lectin Spiralin B, the cytoskeletal protein Fibrilin, the glucose transporter Crr, the potassium channel KtrA, a ferritin-like protein Ftn, the chromosome partitioning protein ParA and the DNA polymerase subunit DnaN. Under-represented proteins include the transporters SteT and YdcV, the serine/threonine phosphatase Stp, the helicase PcrA, the ATP synthase subunit AtpH, the GMP reductase GuaC and the tRNA-(guanine-N(7)-)-methyltransferase TrmB. Other proteins have no predicted function.

Discussion

This study provides an extensive characterization of the proteome of *Spiroplasma*-infected *Drosophila* hemolymph. This dual-proteomics approach provides a deep proteome of *Spiroplasma* in its natural environment and pinpoints host proteins which abundance is altered by the presence of the bacteria. The power of *Drosophila* genetics allowed us to test the involvement of these candidate genes in regulating endosymbiosis. A targeted genetic screen revealed that most of the differentially abundant proteins upon *Spiroplasma* infection do not participate in the control of symbiont growth but may rather be involved in host adaptation to *Spiroplasma*.

Spiroplasma is devoid of cell wall, hence devoid of peptidoglycan, which is the main immune elicitor for the insect immune system [39]. This led to the assumption that *Spiroplasma* was undetectable by the canonical innate immune pathways. Furthermore, the elicitation of the fly immune system (by an infection or using genetics) has no impact on *Spiroplasma* titer, suggesting that the bacteria are not only invisible by also resistant to the fly immune effectors [31]. Flies that over-express AMPs and the *ΔAMP10* mutant line have normal bacterial titer that confirms the host immune system does not affect *Spiroplasma* growth. However, numerous immune-related proteins (mostly associated to the Toll pathway) were enriched in *Spiroplasma* infected hemolymph, including receptors or putative receptors (GNBP1, GNBP-like3), signal transduction intermediates (Spätzle-Processing Enzyme and Hayan) and effectors or putative effectors (Attacin, Bomanin Bc3 and CG33470). The *Spiroplasma* titer was not altered in several fly lines carrying loss-of function alleles of these genes, indicating that the immune elicitation is a consequence of the infection but not a control mechanism. It is worth noting that the expression levels of the genes were extremely low as compared to a fully-fledged immune response against pathogenic bacteria [35]. Such low induction of the immune response has been reported in flies experiencing stress, such as cold or heat stress [40,41]. Therefore, the mild induction of immune proteins in infected hemolymph may be an unspecific stress response associated to the presence of bacteria. Another attractive hypothesis would be that proteases released by *Spiroplasma* could trigger the soluble sensor Persephone and activate the Toll pathway in a peptidoglycan-independent fashion [42,43].

Another uncovering of this study is the depletion of proteasome components in the hemolymph upon *Spiroplasma* infection. As the ubiquitin-proteasome system is a major degradation pathway for intracellular proteins [44], the components that we detected in the hemolymph are presumably released from cells that broke upon hemolymph collection (epithelial, fat or muscular cells, but also possibly hemocytes). However, functionally active 20S proteasome units have been discovered circulating in extracellular fluids in mammals, including serum [45] hence we cannot exclude the existence of circulating proteasome units in *Drosophila* hemolymph. Host ubiquitin-proteasome systems have long been suspected to be a key element in symbiotic homeostasis. It is upregulated in cells harboring intracellular symbionts in weevils, presumably to increase protein turnover and provide free amino-acids to the bacteria [46]. *In vitro* work on cell cultures infected by the facultative endosymbiont *Wolbachia* also revealed that it induces the host proteasome presumably also to support its own growth [47–49]. Remarkably, one proteasome subunit was detected as enriched upon *Spiroplasma*

infection in the aphid *Aphis citricidus* when the insect was feeding on a suboptimum but not on an optimum host plant, suggesting an interaction between symbiosis, nutrition and the proteasome-ubiquitin degradation system [50]. In the case of *Drosophila-Spiroplasma* interaction, the depletion of proteasome subunits upon infection could thus be a titer regulation mechanism: by decreasing its proteolysis, the host would limit the release of amino acids in the extracellular space that would be usable by *Spiroplasma.*

Our approach also produced an in-depth *Spiroplasma* proteome on total proteins regardless of their cell localization. This gives a quantitatively unbiased overview of each protein abundance which was not achieved by previous data based on detergent extractions [30]. Such quantitative approach allowed us to make a comparison between the transcript and protein levels on about one fourth of the total number of predicted coding sequences [23]. Although the correlation between transcriptomics and proteomics data greatly varies among models, tissues and experimental set-ups [51], our data indicate a rather low correlation in the case of *Spiroplasma.* The correlations between transcript and protein levels depends on the interaction between transcript stability and protein stability [52]. It also depends on the intrinsic chemical properties of each transcript that affect the ribosome binding or the translation speed, for example the Shine-Dalgarno sequence in prokaryotes [53] or more importantly the codon adaptation index of the coding sequence [54]. An explanation for the overall lack of correlation between transcripts and proteins regardless of the model could be that selection operates at the protein level, hence changes in mRNA levels would be offset by post-transcriptional mechanisms to maintain stable protein levels over evolutionary times [55]. This hypothesis entails that genes having a low mRNA level compared to their protein levels are likely to be under strong selective pressure to maintain high protein abundancy through post-transcriptional regulations. Intriguingly, this is the case for *S. poulsonii* Spiralin B, a protein homologous to the *S. citri* Spiralin A, a membrane lectin suspected to be essential for insect to plant transmission [56,57]. Spiralin B has been identified as a putative virulence factor in *S. poulsonii* as it is upregulated when the bacteria are in the fly hemolymph compared to *in vitro* culture [23] and could be involved in oocyte infection for vertical transmission. Similarly, the Crr glucose transporter has an unexpectedly high protein/mRNA ratio, possibly in connection with its role in bacteria survival in the hemolymph. *Spiroplasma* has a pseudogenized transporter for trehalose, the most abundant sugar in the hemolymph [30]. This could have been selected over host-symbiont coevolution to prevent *Spiroplasma* overgrowth, hence increasing the stability of the interaction by limiting the metabolic cost of harboring the bacteria. Maintaining high Crr levels could thus be an offset response to maintain bacteria proliferation despite trehalose inaccessibility. Last, the ferritin-like protein Ftn has also a bias towards high protein abundancy, suggesting that iron could be a key metabolite in *Spiroplasma-Drosophila* symbiotic homeostasis.

All tissues bathed by hemolymph contribute to its composition. As a consequence, studying the impact of symbionts on this biofluid is unapproachable by transcriptomic methods only. Dual proteomics proved to be an efficient approach to overcome this hurdle and gain novel insights into the *Drosophila-Spiroplasma* symbiosis. This method is also promising for the study of other symbioses, particularly those where symbionts inhabit biofluids rather than cells.

Material and methods

Spiroplasma and *Drosophila* stocks

Flies were kept at 25°C on cornmeal medium (35.28 g of cornmeal, 35.28 g of inactivated yeast, 3.72 g of agar, 36 ml of fruits juice, 2.9 ml of propionic acid and 15.9 ml of Moldex for 600 ml of medium). The complete list of fly stocks is available in S4 Table.

Spiroplasma poulsonii strain Uganda-1 [58] was used for all infections. Fly stocks were infected as previously described [31]. Briefly, 9 nL of *Spiroplasma*-infected hemolymph was injected in their thorax. The progeny of these flies was collected after 5–7 days using male killing as a proxy to assess the infection (100% female progeny). Flies from at least the 3rd generation post-injection were used experimentally.

Hemolymph extraction procedure

Embryos were collected from conventionally reared flies and dechorionated using a bleach-based previously published method [59] to ensure that they were devoid of horizontally transmitted pathogens. One generation was then left to develop in conventional rearing conditions to allow for gut microbiota recontamination. Hemolymph was extracted from 10 days-old flies from the second generation after bleach treatment using a Nanoject II (Drummond) as previously described [16]. 1 μL of hemolymph was collected for each sample and frozen at -80˚C until further use. Hemolymph was then diluted 10 times with PBS containing Protease Inhibitor Cocktail 1X (Roche). 1 μl was used for protein quantification with the Pierce BCA Protein Assay Kit (Thermofisher). The remaining 9 μl were mixed with SDS (0.2% final), DTT (2.5mM final) and PMSF (10μM final). Aliquots of 15 μg were used for proteomics analysis.

Proteomics sample preparation and LC-MS/MS

Sample preparation and data analysis was carried out at the Proteomics Core Facility of EPFL. Each sample was digested by Filter Aided Sample Preparation (FASP) [60] with minor modifications. Dithiothreitol (DTT) was replaced by Tris (2-carboxyethyl)phosphine (TCEP) as reducing agent and iodoacetamide by chloracetamide as alkylating agent. A proteolytic digestion was performed using Endoproteinase Lys-C and Trypsin. Peptides were desalted on C18 StageTips [61] and dried down by vacuum centrifugation. For LC-MS/MS analysis, peptides were resuspended and separated by reversed-phase chromatography on a Dionex Ultimate 3000 RSLC nanoUPLC system in-line connected with an Orbitrap Q Exactive HF Mass Spectrometer (Thermo Fischer Scientific). Database search was performed using MaxQuant 1.5.1.2 [62] against a concatenated database consisting of the Ensemble *Drosophila melanogaster* protein database (BDGP5.25) and a custom *Spiroplama poulsonii* proteome predicted from the reference genome (Genbank accession number JTLV00000000.2). Carbamidomethylation was set as fixed modification, whereas oxidation (M) and acetylation (Protein N-term) were considered as variable modifications. Label-free quantification was performed by MaxQuant using the standard settings. Hits were considered significant when the fold-change between infected and uninfected hemolymph was >2 or <-2 and the FDR<0.05.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD024340.

Spiroplasma quantification

Spiroplasma quantification was performed by qPCR as previously described [32]. Briefly, the DNA of pools of 5 whole flies was extracted and the copy number of a single-copy bacterial gene (*dnaA* or *dnaK*) was quantified and normalized by that of the host gene *rsp17*. Primers sequences are available in S4 Table. Each experiment has been repeated 2 or 3 independent times with at least 3 biological replicates each. Data were analyzed by one-way ANOVA.

RT-qPCR

Gene expression measurements were performed by RT-qPCR as previously described [63,64]. Briefly, 10 whole flies were crushed and their RNA extracted with the Trizol method. Reverse transcription was carried out using a PrimeScript RT kit (Takara) and a mix of random hexamers and oligo-dTs. qPCR was performed on a QuantStudio 3 (Applied Biosystems) with PowerUp SYBR Green Master Mix using primer sequences available in S4 Table. The expression of the target gene was normalized by that of the housekeeping gene *rp49* (*rpL32*) using the 2-$\Delta\Delta$CT method [65].

Each experiment has been repeated 2 or 3 independent times with at least 3 biological replicates each. Data were analyzed by Mann-Whitney tests.

Supporting information

S1 Fig. Diagram of the experimental procedure for *Drosophila*-*Spiroplasma* dual proteomics.
(TIF)

S2 Fig. (A) Quantification of *Spiroplasma* titer in two weeks old flies. Titer is expressed as the fold-change of *Act5C-GAL4>UAS-RNAi* over *Act5C-GAL4> w¹¹¹⁸*. Bars represent the mean +/- standard deviation of a pool of at least 2 independent experiments. (B) Quantification of *Spiroplasma* titer in *CG15293* mutant flies compared to control *yw* flies. Bars represent the mean +/- standard deviation of a pool of at least 3 independent experiments. ***; $p < 0.0005$ upon Mann-Whitney test on $\Delta\Delta$Ct values.
(TIF)

S1 Table. *Drosophila* genes quantified by LC-MS/MS.
(CSV)

S2 Table. *Spiroplasma* genes quantified by LC-MS/MS.
(CSV)

S3 Table. *Spiroplasma* genes outlying the linear model between mRNA and protein levels.
(CSV)

S4 Table.
(XLSX)

Acknowledgments

Author Contributions

Conceptualization: Florent Masson, Samuel Rommelaere.

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Methodology: Florent Masson, Samuel Rommelaere, Fanny Schüpfer.

Supervision: Bruno Lemaitre.

Validation: Florent Masson, Samuel Rommelaere.

Writing – original draft: Florent Masson, Samuel Rommelaere.

Writing – review & editing: Florent Masson, Samuel Rommelaere.

References

1. Douglas AE. Multiorganismal Insects: Diversity and Function of Resident Microorganisms. Annu Rev Entomol. 2015; 60: 17–34. https://doi.org/10.1146/annurev-ento-010814-020822 PMID: 25341109

2. Douglas AE. How multi-partner endosymbioses function. Nat Rev Microbiol. Nature Publishing Group; 2016; 14: 731–743. https://doi.org/10.1038/nrmicro.2016.151 PMID: 27795568

3. Teixeira L, Ferreira A, Ashburner M. The bacterial symbiont Wolbachia induces resistance to RNA viral infections in Drosophila melanogaster. PLoS Biol. 2009/02/19. 2008; 6: e2. https://doi.org/10.1371/journal.pbio.1000002 PMID: 19222304

4. Hedges LM, Brownlie JC, O'Neill SL, Johnson KN. Wolbachia and virus protection in insects. Science (80-). 2008/11/01. 2008; 322: 702. https://doi.org/10.1126/science.1162418 PMID: 18974344

5. Oliver KM, Russell JA, Moran NA, Hunter MS. Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. Proc Natl Acad Sci U S A. 2003/02/04. 2003; 100: 1803–1807. https://doi.org/10.1073/pnas.0335320100 PMID: 12563031

6. Scarborough CL, Ferrari J, Godfray HC. Aphid protected from pathogen by endosymbiont. Science (80-). 2005/12/17. 2005; 310: 1781. https://doi.org/10.1126/science.1120180 PMID: 16357252

7. Montllor CB, Maxmen A, Purcell AH. Facultative bacterial endosymbionts benefit pea aphids Acyrthosiphon pisum under heat stress. Ecol Entomol. 2002; 27: 189–195. https://doi.org/10.1046/j.1365-2311.2002.00393.x

8. Medina P, Russell SL, Corbett-Detig R. Deep data mining reveals variable abundance and distribution of microbial reproductive manipulators within and among diverse host species. bioRxiv. 2019; 679837. https://doi.org/10.1101/679837

9. Duron O, Bouchon D, Boutin S, Bellamy L, Zhou L, Engelstadter J, et al. The diversity of reproductive parasites among arthropods: Wolbachia do not walk alone. BMC Biol. 2008/06/26. 2008; 6: 27. https://doi.org/10.1186/1741-7007-6-27 PMID: 18577218

10. Werren JH, Baldo L, Clark ME. Wolbachia: master manipulators of invertebrate biology. Nat Rev Microbiol. 2008; 6: 741–751. https://doi.org/10.1038/nrmicro1969 PMID: 18794912

11. Hoffmann AA, Montgomery BL, Popovici J, Iturbe-Ormaetxe I, Johnson PH, Muzzi F, et al. Successful establishment of Wolbachia in Aedes populations to suppress dengue transmission. Nature. 2011; 476: 454. https://doi.org/10.1038/nature10356 PMID: 21866160

12. Masson F, Lemaitre B. Growing Ungrowable Bacteria: Overview and Perspectives on Insect Symbiont Culturability. Microbiol Mol Biol Rev. 2020; 84: e00089–20. https://doi.org/10.1128/MMBR.00089-20 PMID: 33177190

13. Gasparich GE. Spiroplasmas: evolution, adaptation and diversity. Front Biosci. 2002; 7: 619–640.

14. Haselkorn TS. The Spiroplasma heritable bacterial endosymbiont of Drosophila. Fly (Austin). 2010; 4: 80–87. https://doi.org/10.4161/fly.4.1.10883 PMID: 20081357

15. Mateos M, Castrezana SJ, Nankivell BJ, Estes AM, Markow TA, Moran NA. Heritable Endosymbionts of Drosophila. Genetics. 2006; 174: 363–376. https://doi.org/10.1534/genetics.106.058818 PMID: 16783009

16. Herren JK, Paredes JC, Schüpfer F, Arafah K, Bulet P, Lemaitre B. Insect endosymbiont proliferation is limited by lipid availability. Elife. 2014; 3: e02964. https://doi.org/10.7554/eLife.02964 PMID: 25027439

17. Herren JK, Paredes JC, Schüpfer F, Lemaitre B. Vertical Transmission of a Drosophila Endosymbiont Via Cooption of the Yolk Transport and Internalization Machinery. MBio. 2013; 4 (2): e00532–12. https://doi.org/10.1128/mBio.00532-12 PMID: 23462112

18. Harumoto T, Lemaitre B. Male-killing toxin in a bacterial symbiont of Drosophila. Nature. Springer US; 2018; 557: 252–255. https://doi.org/10.1038/s41586-018-0086-2 PMID: 29720654

19. Xie J, Butler S, Sanchez G, Mateos M. Male killing Spiroplasma protects Drosophila melanogaster against two parasitoid wasps. Heredity; 2014; 112: 399–408. https://doi.org/10.1038/hdy.2013.118 PMID: 24281548

20. Ballinger MJ, Perlman SJ. Generality of toxins in defensive symbiosis: Ribosome-inactivating proteins and defense against parasitic wasps in Drosophila. Hurst G, editor. PLoS Pathog; 2017; 13: e1006431. https://doi.org/10.1371/journal.ppat.1006431 PMID: 28683136

21. Jaenike J, Unckless R, Cockburn SN, Boelio LM, Perlman SJ. Adaptation via symbiosis: recent spread of a Drosophila defensive symbiont. Science 2010; 329: 212–215. https://doi.org/10.1126/science.1188235 PMID: 20616278

22. Hamilton PT, Peng F, Boulanger MJ, Perlman SJ. A ribosome-inactivating protein in a Drosophila defensive symbiont. Proc Natl Acad Sci U S A. National Academy of Sciences; 2016; 113: 350–355. https://doi.org/10.1073/pnas.1518648113 PMID: 26712000

23. Masson F, Calderon Copete S, Schüpfer F, Garcia-Arraez G, Lemaitre B. In Vitro Culture of the Insect Endosymbiont Spiroplasma poulsonii Highlights Bacterial Genes Involved in Host-Symbiont Interaction. MBio. 2018; 9(2): e00024–18. https://doi.org/10.1128/mBio.00024-18 PMID: 29559567

24. Masson F, Schüpfer F, Jollivet C, Lemaitre B. Transformation of the Drosophila sex-manipulative endosymbiont Spiroplasma poulsonii and persisting hurdles for functional genetics studies. Appl Environ Microbiol. 2020; AEM.00835-20. https://doi.org/10.1128/AEM.00835-20 PMID: 32444468

25. Veneti Z, Bentley JK, Koana T, Braig HR, Hurst GDD. A Functional Dosage Compensation Complex Required for Male Killing in Drosophila. Science 2005; 307: 1461–1463. https://doi.org/10.1126/science.1107182 PMID: 15746426

26. Harumoto T, Anbutsu H, Fukatsu T. Male-killing Spiroplasma induces sex-specific cell death via host apoptotic pathway. PLoS Pathog. 2014; 10: e1003956. https://doi.org/10.1371/journal.ppat.1003956 PMID: 24550732

27. Harumoto T, Anbutsu H, Lemaitre B, Fukatsu T. Male-killing symbiont damages host's dosage-compensated sex chromosome to induce embryonic apoptosis. Nat Commun. Nature Publishing Group; 2016; 7: 12781. https://doi.org/10.1038/ncomms12781 PMID: 27650264

28. Harumoto T, Fukatsu T, Lemaitre B. Common and unique strategies of male killing evolved in two distinct Drosophila symbionts. Proc R Soc B Biol Sci. 2018;285. https://doi.org/10.1098/rspb.2017.2167 PMID: 29563258

29. Paredes JC, Herren JK, Schüpfer F, Lemaitre B. The Role of Lipid Competition for Endosymbiont-Mediated Protection against Parasitoid Wasps in Drosophila. MBio. 2016; 7(4): e01006–16. https://doi.org/10.1128/mBio.01006-16 PMID: 27406568

30. Paredes JC, Herren JK, Schüpfer F, Marin R, Claverol S, Kuo C-H, et al. Genome sequence of the Drosophila melanogaster male-killing Spiroplasma strain MSRO endosymbiont. MBio. 2015; 6(2): e02437–14. https://doi.org/10.1128/mBio.02437-14 PMID: 25827421

31. Herren JK, Lemaitre B. Spiroplasma and host immunity: Activation of humoral immune responses increases endosymbiont load and susceptibility to certain Gram-negative bacterial pathogens in Drosophila melanogaster. Cell Microbiol. 2011; 13: 1385–1396. https://doi.org/10.1111/j.1462-5822.2011.01627.x PMID: 21740495

32. Masson F, Calderon-Copete S, Schüpfer F, Vigneron A, Rommelaere S, Garcia-Arraez MG, et al. Blind killing of both male and female Drosophila embryos by a natural variant of the endosymbiotic bacterium Spiroplasma poulsonii. Cell Microbiol. John Wiley & Sons, Ltd; 2020; 22(5): e13156. https://doi.org/10.1111/cmi.13156 PMID: 31912942

33. Palombella VJ, Rando OJ, Goldberg AL, Maniatis T. The ubiquitin-proteasome pathway is required for processing the NF-kappa B1 precursor protein and the activation of NF-kappa B. Cell. United States; 1994; 78: 773–785. https://doi.org/10.1016/s0092-8674(94)90482-0 PMID: 8087845

34. Hurst GDD, Anbutsu H, Kutsukake M, Fukatsu T. Hidden from the host: Spiroplasma bacteria infecting Drosophila do not cause an immune response, but are suppressed by ectopic immune activation. Insect Mol Biol. 2003; 12: 93–97. https://doi.org/10.1046/j.1365-2583.2003.00380.x PMID: 12542640

35. De Gregorio E, Spellman PT, Rubin GM, Lemaitre B. Genome-wide analysis of the Drosophila immune response by using oligonucleotide microarrays. Proc Nat Acad Sci. 2001; 98: 12590–12595. https://doi.org/10.1073/pnas.221458698 PMID: 11606746

36. Troha K, Im JH, Revah J, Lazzaro BP, Buchon N. Comparative transcriptomics reveals CrebA as a novel regulator of infection tolerance in D. melanogaster. PLoS Pathog. 2018; 14: e1006847. https://doi.org/10.1371/journal.ppat.1006847 PMID: 29394281

37. Tzou P, Reichhart J-M, Lemaitre B. Constitutive expression of a single antimicrobial peptide can restore wild-type resistance to infection in immunodeficient Drosophila mutants. Proc Natl Acad Sci U S A. 2002; 99: 2152–2157. https://doi.org/10.1073/pnas.042411999 PMID: 11854512

38. Hanson MA, Dostálová A, Ceroni C, Poidevin M, Kondo S, Lemaitre B. Synergy and remarkable specificity of antimicrobial peptides in vivo using a systematic knockout approach. Elife. eLife Sciences Publications, Ltd; 2019; 8: e44341. https://doi.org/10.7554/eLife.44341 PMID: 30803481

39. Lemaitre B, Hoffmann J. The host defense of Drosophila melanogaster. Annu Rev Immunol. 2007/01/05. 2007; 25: 697–743. https://doi.org/10.1146/annurev.immunol.25.022106.141615 PMID: 17201680

40. MacMillan HA, Knee JM, Dennis AB, Udaka H, Marshall KE, Merritt TJS, et al. Cold acclimation wholly reorganizes the Drosophila melanogaster transcriptome and metabolome. Sci Rep. 2016; 6: 28999. https://doi.org/10.1038/srep28999 PMID: 27357258

41. Telonis-Scott M, van Heerwaarden B, Johnson TK, Hoffmann AA, Sgrò CM. New Levels of Transcriptome Complexity at Upper Thermal Limits in Wild Drosophila Revealed by Exon Expression Analysis. Genetics. 2013; 195: 809–830. https://doi.org/10.1534/genetics.113.156224 PMID: 24002645

42. Issa N, Guillaumot N, Lauret E, Matt N, Schaeffer-Reiss C, Van Dorsselaer A, et al. The Circulating Protease Persephone Is an Immune Sensor for Microbial Proteolytic Activities Upstream of the Drosophila Toll Pathway. Mol Cell. Cell Press; 2018; 69: 539–550.e6. https://doi.org/10.1016/j.molcel.2018.01.029 PMID: 29452635

43. Gottar M, Gobert V, Matskevich AA, Reichhart J-M, Wang C, Butt TM, et al. Dual detection of fungal infections in Drosophila via recognition of glucans and sensing of virulence factors. Cell. 2006; 127: 1425–1437. https://doi.org/10.1016/j.cell.2006.10.046 PMID: 17190605

44. Kleiger G, Mayor T. Perilous journey: a tour of the ubiquitin-proteasome system. Trends Cell Biol. 2014; 24: 352–359. https://doi.org/10.1016/j.tcb.2013.12.003 PMID: 24457024

45. Sixt SU, Dahlmann B. Extracellular, circulating proteasomes and ubiquitin—Incidence and relevance. Biochim Biophys Acta—Mol Basis Dis. 2008; 1782: 817–823. https://doi.org/10.1016/j.bbadis.2008.06.005.

46. Heddi A, Vallier A, Anselme C, Xin H, Rahbe Y, Wackers F. Molecular and cellular profiles of insect bacteriocytes: mutualism and harm at the initial evolutionary step of symbiogenesis. Cell Microbiol. 2005/01/22. 2005; 7: 293–305. doi:CMI461 [pii] https://doi.org/10.1111/j.1462-5822.2004.00461.x PMID: 15659072

47. Fallon AM, Witthuhn BA. Proteasome activity in a naïve mosquito cell line infected with Wolbachia pipientis wAlbB. In Vitro Cell Dev Biol Anim. 2009; 45: 460–466. https://doi.org/10.1007/s11626-009-9193-6 PMID: 19296184

48. White PM, Serbus LR, Debec A, Codina A, Bray W, Guichet A, et al. Reliance of Wolbachia on High Rates of Host Proteolysis Revealed by a Genome-Wide RNAi Screen of Drosophila Cells. Genetics. 2017; 205: 1473–1488. https://doi.org/10.1534/genetics.116.198903 PMID: 28159754

49. He Z, Zheng Y, Yu W-J, Fang Y, Mao B, Wang Y-F. How do Wolbachia modify the Drosophila ovary? New evidences support the "titration-restitution" model for the mechanisms of Wolbachia-induced CI. BMC Genomics. 2019; 20: 608. https://doi.org/10.1186/s12864-019-5977-6 PMID: 31340757

50. Guidolin AS, Cataldi TR, Labate CA, Francis F, Cônsoli FL. Spiroplasma affects host aphid proteomics feeding on two nutritional resources. Sci Rep. 2018; 8: 2466. https://doi.org/10.1038/s41598-018-20497-9 PMID: 29410456

51. Haider S, Pal R. Integrated analysis of transcriptomic and proteomic data. Curr Genomics. 2013; 14: 91–110. https://doi.org/10.2174/1389202911314020003 PMID: 24082820

52. Schwanhäusser B, Busse D, Li N, Dittmar G, Schuchhardt J, Wolf J, et al. Global quantification of mammalian gene expression control. Nature. 2011; 473: 337–342. https://doi.org/10.1038/nature10098 PMID: 21593866

53. Bingham AH, Ponnambalam S, Chan B, Busby S. Mutations that reduce expression from the P2 promoter of the Escherichia coli galactose operon. Gene. 1986; 41: 67–74. https://doi.org/10.1016/0378-1119(86)90268-4 PMID: 3516794

54. Lithwick G, Margalit H. Hierarchy of sequence-dependent features associated with prokaryotic translation. Genome Res. 2003; 13: 2665–2673. https://doi.org/10.1101/gr.1485203 PMID: 14656971

55. Schrimpf SP, Weiss M, Reiter L, Ahrens CH, Jovanovic M, Malmström J, et al. Comparative Functional Analysis of the Caenorhabditis elegans and Drosophila melanogaster Proteomes. PLOS Biol. 2009; 7: e1000048. https://doi.org/10.1371/journal.pbio.1000048 PMID: 19260763

56. Killiny N, Castroviejo M, Saillard C. Spiroplasma citri Spiralin acts in vitro as a lectin binding to glycoproteins from its insect vector Circulifer haematoceps. Phytopathology. 2005; 95: 541–548. https://doi.org/10.1094/PHYTO-95-0541 PMID: 18943320

57. Duret S, Berho N, Danet JL, Garnier M, Renaudin J. Spiralin is not essential for helicity, motility, or pathogenicity but is required for efficient transmission of Spiroplasma citri by its leafhopper vector Circulifer haematoceps. Appl Environ Microbiol. 2003; 69: 6225–6234. https://doi.org/10.1128/aem.69.10.6225-6234.2003 PMID: 14532084

58. Pool JE, Wong a, Aquadro CF. Finding of male-killing Spiroplasma infecting Drosophila melanogaster in Africa implies transatlantic migration of this endosymbiont. Heredity. 2006; 97: 27–32. https://doi.org/10.1038/sj.hdy.6800830 PMID: 16685282

59. Broderick N a., Buchon N, Lemaitre B. Microbiota-induced changes in Drosophila melanogaster host gene expression and gut morphology. MBio. 2014; 5: 1–13. https://doi.org/10.1128/mBio.01117-14 PMID: 24865556

60. Wiśniewski JR, Zougman A, Nagaraj N, Mann M. Universal sample preparation method for proteome analysis. Nat Methods. 2009; 6: 359–362. https://doi.org/10.1038/nmeth.1322 PMID: 19377485

61. Rappsilber J, Mann M, Ishihama Y. Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. Nat Protoc. 2007; 2: 1896–1906. https://doi.org/10.1038/nprot.2007.261 PMID: 17703201

62. Cox J, Mann M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat Biotechnol. 2008; 26: 1367–1372. https://doi.org/10.1038/nbt.1511 PMID: 19029910

63. Romeo Y, Lemaitre B. Drosophila immunity: methods for monitoring the activity of Toll and Imd signaling pathways. Methods Mol Biol. United States; 2008; 415: 379–394. https://doi.org/10.1007/978-1-59745-570-1_22 PMID: 18370166

64. Iatsenko I, Marra A, Boquete J-P, Peña J, Lemaitre B. Iron sequestration by transferrin 1 mediates nutritional immunity in Drosophila melanogaster. Proc Natl Acad Sci. 2020; 117: 7317 LP-7325. https://doi.org/10.1073/pnas.1914830117 PMID: 32188787

65. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 2001; 29: e45–e45. https://doi.org/10.1093/nar/29.9.e45 PMID: 11328886