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Wolbachia Endosymbionts Modify *Drosophila* Ovary Protein Levels in a Context-Dependent Manner

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ABSTRACT

Endosymbiosis is a unique form of interaction between organisms, with one organism dwelling inside the other. One of the most widespread endosymbionts is *Wolbachia pipientis*, a maternally transmitted bacterium carried by insects, crustaceans, mites, and filarial nematodes. Although candidate proteins that contribute to maternal transmission have been identified, the molecular basis for maternal *Wolbachia* transmission remains largely unknown. To investigate transmission-related processes in response to *Wolbachia* infection, ovarian proteomes were analyzed from *Wolbachia*-infected *Drosophila melanogaster* and *D. simulans*. Endogenous and variant host-strain combinations were investigated. Significant and differentially abundant ovarian proteins were detected, indicating substantial regulatory changes in response to *Wolbachia*. Variant *Wolbachia* strains were associated with a broader impact on the ovary proteome than endogenous *Wolbachia* strains. The *D. melanogaster* ovarian environment also exhibited a higher level of diversity of proteomic responses to *Wolbachia* than *D. simulans*. Overall, many *Wolbachia*-responsive ovarian proteins detected in this study were consistent with expectations from the experimental literature. This suggests that context-specific changes in protein abundance contribute to *Wolbachia* manipulation of transmission-related mechanisms in oogenesis.

IMPORTANCE

Millions of insect species naturally carry bacterial endosymbionts called *Wolbachia*. *Wolbachia* bacteria are transmitted by females to their offspring through a robust egg-loading mechanism. The molecular basis for *Wolbachia* transmission remains poorly understood at this time, however. This proteomic study identified specific fruit fly ovarian proteins as being upregulated or downregulated in response to *Wolbachia* infection. The majority of these protein responses correlated specifically with the type of host and *Wolbachia* strain involved. This work corroborates previously identified factors and mechanisms while also framing the broader context of ovarian manipulation by *Wolbachia*.

Symbiotic interactions between organisms, ranging from lethal parasitism to indispensable mutualism, frame the foundation of life. Endosymbionts face the same challenges as other microbes, which must replicate well and spread efficiently to be successful. However, the molecular mechanisms that contribute to endosymbiont transmission are not yet well understood. Endosymbiotic *Wolbachia* bacteria provide an excellent system to address this knowledge gap. These alphaproteobacteria of the *Rickettsiales* order are highly successful in nature, infecting filarial nematodes, crustaceans, mites, and over 40% of all insect species, including the well-established model organism *Drosophila melanogaster* (1–4). The presence of *Wolbachia* among this wide range of hosts is due to effective maternal transmission, analogous to mitochondria (1, 5–7). The ovary produces egg chambers, composed of germ line and somatic cells, that mature over 3 to 4 days into completed eggs (8). *Wolbachia* bacteria are loaded into egg chambers through vertical and horizontal transmission (9–14), intracellular replication (15, 16), and achievement of transmission-enhancing localization patterns (17–20). The actin cytoskeleton also contributes to maternal *Wolbachia* transmission by facilitating germ line colonization through an unknown mechanism (21).

With the success of *Wolbachia* being reliant upon maternal germ line cells, it is in the interest of *Wolbachia* to enhance host fecundity (22–24). *Wolbachia* bacteria are thought to achieve this in part by increasing the frequency of germ line stem cell division (11). Other studies indicate that *Wolbachia* bacteria support ovary productivity by enabling proactive management of toxic iron (25–28), suppressing *Sex-lethal* (*Sxl*) mutations (29), and preventing generalized apoptosis in the germ line (11, 30). The specific factors involved in executing these *Wolbachia* impacts on the host germ line are not yet clear. Studies have used a variety of approaches to investigate expression-related host responses to *Wolbachia* (31–42). These analyses of *Wolbachia*-infected cultured cell lines, invertebrate body tissues, and

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intact host organisms to date have yielded a wealth of information. When considering how *Wolbachia* bacteria interact with and manipulate host germ line cells, the implications of this diverse set of findings are unclear, however. This study examines the hypothesis that consensus molecular interactions between *Wolbachia* and the host contribute to maternal *Wolbachia* transmission. The objective of this study was to assess the conservation of *Wolbachia*-host interaction mechanisms through analysis of the ovarian proteome.

MATERIALS AND METHODS

***Wolbachia* strain genotyping.** *Wolbachia* genotyping was performed according to a diagnostic assay based on variable-number tandem-repeat (VNTR) and IS5 markers (43). The profile of fragment sizes matched each sample with a known strain type. These same fragments were amplified from *Drosophila simulans w*Ri as a negative control. To further distinguish *w*Mel^{CS} from the highly similar strain *w*Mel^{Pop}, we used previously outlined diagnostic approaches (44). Sequencing was performed to identify a potential single nucleotide polymorphism (SNP) substitution at position 943,443 in the *w*Mel^{CS} genome. Samples were analyzed on an ABI 3100 genetic analyzer with sequencing analysis and GeneScan software (Applied Biosystems, CA). Octomom copy numbers were determined by quantitative real-time PCR (qRT-PCR) as previously reported (44). PCRs were carried out with Maxima SYBR green/ROX quantitative PCR (qPCR) master mix, using a CFX Connect real-time PCR detection system (Bio-Rad). Data were analyzed with CFX Manager V.3.1. Relative Octomom copy numbers for each *Wolbachia*-infected host combination were calculated by methods reported previously (45).

Fly strains and rearing. The *D. melanogaster* genetic background used for all proteomic analyses was an uninfected *w*;*Sp/Cyo*;*Sb/TM6B* strain. *w*Mel and *w*Mel^{CS} strains described in previous studies were crossed into this line to ensure a uniform genetic background for all experiments (17, 44). The strain of *D. simulans* used as a control was a w^{-} stock that was cured of *Wolbachia* with tetracycline 10 years ago. The *w*Ri *Wolbachia* strain endogenous to *D. simulans* and the *w*Mel *Wolbachia* strain transfected into *D. simulans* (46) were backcrossed into the cured fly stock for six generations to standardize the *D. simulans* genetic background used in this study.

All *Drosophila* populations used in this study were maintained at 25°C on a 12-h light/dark cycle. The flies were housed in bottles containing food that was generated in-house, based upon a modified Bloomington Stock Center recipe for *Drosophila* medium (47, 48). The fly food was prepared in batches consisting of 20 liters water, 337 g yeast, 190 g soy flour, 1,325 g yellow corn meal, 96 g agar, 1.5 liters Karo light corn syrup, and 94 ml propionic acid.

Sample collection for proteomic analysis. Flies were collected from equally aged generations during the first 3 days of eclosion only. Food bottles were cleared, and newly emerged flies were collected after 24 h to ensure equal age among parallel experimental cohorts. Flies were then aged for 2 days in vials and subsequently transferred onto new food for 3 more days. Comparability between samples was maximized by running all procedures in parallel for the flies used in each biological replicate. Samples were prepared from a minimum of 25 flies for each biological replicate for all six *Wolbachia* host-strain combinations. Ovaries were dissected in 0.8 to 1.0 ml of ice-cold lysis buffer (50 mM NH_4HCO_3, 1 mM EDTA, 2 mM sodium vanadate). The uninfected and cured flies were always dissected first, and all dissection equipment (i.e., dissection dish and tweezers) was cleaned with 70% ethanol and rinsed with lysis buffer between dissections for each sample. Immediately after dissection, ovarian samples were imaged on a Leica MZ6 stereomicroscope at a ×10 magnification with a 1.6× zoom. Ovary size and staging were also assessed, and replicates presenting heterogeneous morphology between sample types were discarded. Tissue for which follow-up processing was performed was transferred into 1.5-ml centrifuge tubes, all excess solution was removed, and tissue was flash-frozen. Four biological replicates of each sample type were shipped overnight to the Purdue Proteomics Facility (Bindley Bioscience Center, Discovery Park, West Lafayette, IN) to be analyzed by a shotgun approach referred to as "discovery-based proteomics" (49–51).

Sample digestion. Gel bands were cut into 1-mm pieces and washed to remove the stain with 50:50 acetonitrile (ACN)–25 mM ammonium bicarbonate (ABC) (vol/vol). After washing, the samples were reduced and alkylated. Sequence-grade Lys-C–trypsin (Promega) was used to enzymatically digest the samples. All digestions were carried out with a Barocycler NEP2320 instrument (Pressure BioSciences) at 50°C under 20 kilopounds per square inch for 2 h. Peptides were recovered from gel samples by using 60% ACN–5% trifluoroacetic acid (TFA)–35% purified H_2O with sonication in an ice bath. The supernatant was removed from the gels, and a vacuum centrifuge was used to dry samples. The resulting pellet was resuspended in 10 μl of 97% purified H_2O–3% ACN– 0.1% formic acid (FA). A 5-μl volume was used for nanoscale liquid chromatography-tandem mass spectrometry (NanoLC-MS/MS) analysis.

LC-MS/MS analysis. The samples were analyzed on a Nano Eksigent 425 high-performance liquid chromatography (HPLC) system coupled to a Triple TOF 5600 Plus instrument (ABsciex, Framingham, MA) (52). The gradient was 120 min at 300 nl/min over the cHiPLC-nanoflex system. The trap column was a Nano cHiPLC 200-μm by 0.5-mm ChromXP C_{18}-CL 3-μm 120-Å column, followed by the analytical column, a Nano cHiPLC 75-μm by 15-cm ChromXP C_{18}-CL 5-μm 120-Å column. The sample was injected into the Triple TOF 5600 Plus column through the Nanospray III source. Data acquisition was performed for 50 precursors at 50 ms/scan. Three technical replicates of this analysis were performed for each sample.

Proteomic data analysis. Initial data analysis was performed by using PeakView (ABsciex) and Mascot (Matrix Science) for database searches. *D. melanogaster* and *D. simulans* peptide information was compared to information in the respective databases for each host and assigned UniProt identifiers accordingly. All isoform information corresponding to each protein was grouped together for classification as a single protein. To facilitate comparisons of *D. melanogaster* to *D. simulans* proteins, each *D. simulans* protein was assigned the name of its nearest *D. melanogaster* homolog. Intensity-based absolute quantification (iBAQ) of the protein amount (53) was used as a measure of initial protein detection for each sample type. Label-free quantification (LFQ) was performed by using MaxQuant (54) to identify proteins that satisfied a quality scoring function, enabling comparisons of protein quantity between infection conditions. Both iBAQ and LFQ data were recorded from 4 biological and 3 technical replicates for a combined total of 12 replicates per experimental condition. Proteins designated "reliable" were required to have been detected in 2 out of 3 technical replicates and 3 out of 4 biological replicates according to the LFQ data in order to be included in further data analyses. A coefficient of variation (CV) was also calculated for each significant protein by using the average of LFQ scores from all biological replicates. Only proteins exhibiting a CV below 50% were included in the final list of reliable hits. The reliable proteins were analyzed by using a one-way analysis of variance (ANOVA) approach to identify statistically significant proteins, based upon the LFQ scores of each biological replicate.

Differential protein abundance between sample types was determined by creating pairwise ratios of the average protein LFQ scores for each sample type. For *D. melanogaster*, differential abundance comparisons were made between the Dmel *w*Mel/Dmel Uninf, Dmel *w*Mel^{CS}/Dmel Uninf, and Dmel *w*Mel/Dmel *w*Mel^{CS} strains. For *D. simulans*, differential abundance comparisons were made between the Dsim *w*Ri/Dsim Cured, Dsim *w*Mel/Dsim Cured, and Dsim *w*Mel/Dsim *w*Ri strains. Proteins that showed an abundance change of >0.58 ($\log_2$)-fold (equivalent to a 1.5-fold change) were considered to represent differentially abundant proteins. In terms of regular numbers, these thresholds are indicated by a <0.67-fold change or a >1.5-fold change (38).

To assign the significant and differentially abundant proteins to func-

TABLE 1 Genotyping of host-specific *Wolbachia* variants used in this study[a]

Strain	Size of PCR product (kb) (no. of copies)[b]				Fragment size of WD0983 (bp)	Presence of G or A at position 943443	Octomom copy no.
	VNTR-105	VNTR-141	IS*5*-WD0516/7	IS*5*-WD1310			
Published variants							
*w*Mel	1.35 (5)	1.33 (7)	2.49 (+)	0.75 (−)	550	G	1
*w*Mel2	1.35 (5)	1.19 (6)	2.49 (+)	0.75 (−)	550	G	1
*w*Mel3	1.35 (5)	1.33 (7)	1.57 (−)	0.75 (−)	550	G	1
*w*Mel^{CS}	1.25 (4)	1.19 (6)	1.57 (−)	1.67 (+)	550	G	1
*w*Mel^{CS2}	1.35 (5)	1.19 (6)	1.57 (−)	1.67 (+)	550	G	1
*w*Mel^{Pop}	1.25 (4)	1.19 (6)	1.57 (−)	1.67 (+)	550	A	Varies (1–15)
Laboratory strains							
Dmel *w*Mel	1.35	1.33	2.49	0.75	550	G	1
Dmel *w*Mel^{CS}	1.25	1.19	1.57	1.67	550	G	1
Dsim *w*Mel	1.35	1.33	2.49	0.75	550	G	1
Dsim *w*Ri	ND	ND	ND	ND	ND	ND	0

[a] Diagnostic VNTR and insertion sequence element (IS*5*) regions were analyzed as described previously (43). The expected product size for a given variant as well as those determined for laboratory strains are listed. Distinguishing criteria for *w*Mel^{CS} and *w*Mel^{Pop}, including the G-to-A transition at position 943443 and the Octomom copy number, are also shown (44).

[b] Presence or absence is represented by + or −, respectively. ND, not detected.

tional classes, we first retrieved sequence information for each UniProt identification (55). An eggNOG v4.5 sequence search was then performed (56) to assign each protein to 1 of 20 possible orthologous groups. The first orthologous group assigned by eggNOG was selected as the initial functional classification for each of the proteins, followed by refinement of certain classifications in consultation with FlyBase and the scientific literature.

DNA extraction for qPCR. *Wolbachia* titers were assessed by qPCR analysis of six biological replicates from each host-strain combination. All flies were prepared as described above, and all sample types were run in parallel for each replicate. In running each replicate, ovary pairs were dissected from 5 females of each sample type. These pairs were homogenized in 200 μl of 0.1 M Tris HCl, 0.1 M EDTA, and 1% SDS (pH 9.0) and incubated for 30 min at 70°C. Twenty microliters of 3 M sodium acetate was added, and samples were mixed by shaking. After incubation for 30 min on ice, the samples were centrifuged at 14,000 rpm for 15 min at 4°C. Two hundred microliters of the supernatant containing DNA was collected, and DNA was precipitated to a final volume of 50 μl by ethanol precipitation. Briefly, 500 μl of absolute ethanol was added to 200 μl of the supernatant. The sample was gently mixed and kept at −20°C for 1 h. After centrifugation of the sample at 14,000 rpm for 15 min at 4°C, the supernatant was removed carefully, and 1 ml of 70% ethanol was added to the pellet. After 1 min, samples were centrifuged again at 14,000 rpm for 15 min at 4°C. After the supernatant was discarded, the DNA pellet was air dried and resuspended in 50 μl of water. These DNA samples were diluted 1:10 for use in qPCR.

Real-time quantitative PCR analysis. Real-time PCRs were carried out with a CFX96 real-time PCR detection system (Bio-Rad). Each reaction was performed with a 20-μl final volume containing 10 μl of Maxima SYBR green-fluorescein qPCR master mix (Thermo Scientific), 0.5 μl of 5 mM each primer, and 2 μl of diluted DNA. Primers for the *Wolbachia*-specific protein (Wsp) gene were used (44). Wsp plasmid standards ranging from 10^2 to 10^8 copy numbers were used to generate a standard curve for absolute quantification. The thermal cycling protocol for Wsp amplification involved a 50°C incubation for 2 min and then denaturation for 10 min at 95°C, followed by 40 cycles of 95°C for 30s, 57°C for 1 min, and 72°C for 30 s. Melting curves were examined to confirm the specificity of the amplified product. Data were analyzed by using Bio-Rad CFX manager3.1 with default threshold settings. Absolute *Wolbachia* copy numbers were obtained by comparing threshold cycle (C_T) values with a standard curve generated from the plasmid standard.

RESULTS

Each host-strain combination had ovarian proteins that were reliable in abundance. To investigate the impact of *Wolbachia* on maternal transmission, this study focused on analyzing *D. melanogaster* and *D. simulans* ovaries of various infection statuses. *D. melanogaster* stocks that carried the native *w*Mel strain (Dmel *w*Mel) or the virulent *w*Mel^{CS} strain (Dmel *w*Mel^{CS}) (44) were derived from the same genetic background as uninfected control flies (Dmel Uninf). *D. simulans* stocks that carried the native *w*Ri strain (Dsim *w*Ri) or the artificially introduced *w*Mel strain (Dsim *w*Mel) were also generated (46) in the same genetic background as control flies cured with tetracycline (Dsim Cured). The identity of all *Wolbachia* strains was confirmed with diagnostic PCR assays, sequencing, and quantitative real-time PCR as described previously (43, 44, 57). The use of strain-specific markers confirmed that the *D. melanogaster* and *D. simulans* hosts infected with *w*Mel carried the same *w*Mel^{I} strain type (Tables 1 and 2). The other infected *D. melanogaster* line was verified to carry the *w*Mel^{CS} strain and not *w*Mel^{CS2} or *w*Mel^{Pop} variant types (Table 1), based upon the abundance of tandem repeats, the absence of additional Octomom repeats, and the absence of a specific G→A transition found in the *w*Mel^{Pop} strain (44, 58). From this point forward, the confirmed Dmel *w*Mel and Dsim *w*Ri host-strain combinations are collectively referred to as "endogenous," and the Dmel *w*Mel^{CS} and Dsim *w*Mel combinations are referred to as "variant."

To assess the impact of *Wolbachia* on the *Drosophila* ovary proteome, ovaries were dissected from all host-strain combinations and analyzed by label-free LC-MS/MS. Four biological replicates were collected for each sample type, and 3 technical replicates were analyzed per sample, for a total of 12 replicates per sample type. This resulted in the initial identification of 927 proteins from the *D. melanogaster* ovarian samples (see Table S1 in the supplemental material). A total of 853 of these proteins were shared among all host-strain combinations (Fig. 1a). Further analysis determined that 549 of the shared proteins were based upon quality peptides in all *D. melanogaster* sample types (Fig. 1b; see also Table S1 in the supplemental material). In *D. simulans*,

TABLE 2 Quantitative PCR of host-specific *Wolbachia* variants

Gene	Slope	PCR efficiency	Dilution	C_T for indicated host-strain combination			
				Dmel *w*Mel	Dmel *w*Mel^{CS}	Dsim *w*Mel	Dsim *w*Ri
Reference *wsp* gene	−3.108	2.0977	1:10	19.15	21.71	21.46	21.55
	−3.108	2.0977	1:100	22.29	25.05	24.48	24.86
Target WD0513 gene	−3.085	2.1094	1:10	19.21	21.97	21.8	32.97
	−3.085	2.1094	1:100	22.26	24.49	24.53	35.25
Fold change relative to control			1:10	1.00	0.85	0.80	0.00
			1:100	1.00	1.46	0.93	0.00
Mean (SE)				1.00 (±0.0)	1.16 (±0.43)	0.87 (±0.09)	0.00 (±0.00)

834 total proteins were initially identified (see Table S2 in the supplemental material). A total of 762 of these proteins were shared among all *D. simulans* ovary proteomes analyzed (Fig. 1d). A total of 449 of these shared *D. simulans* protein identifications were based upon quality peptide information (Fig. 1e; see also Table S2 in the supplemental material). Taken together, these data indicate that 54 to 59% of the protein identifications initially associated with *D. simulans* and *D. melanogaster* ovarian proteomes were assigned with high confidence. This set of consensus quality proteins is pursued further in the analyses described below.

The next phase of analysis focused on identifying which consensus quality proteins were reliably detected during oogenesis for each host-strain combination. This required protein detection in at least 2 out of 3 technical replicates per biological sample and at least 3 out of 4 biological samples of each sample type. The coefficient of variation (Gini coefficient) was also calculated for each quality protein hit, and proteins with a CV below 50% were selected as described previously (59). These rigorous criteria defined 316 proteins as being "reliable" among all the *D. melanogaster* ovarian proteomes analyzed (Fig. 1c; see also Table S1 in the supplemental material). A total of 279 proteins were reliably detected within all ovarian proteomes of *D. simulans* (Fig. 1f; see also Table S2 in the supplemental material). All quantitative analyses of the ovarian proteomes described below focus on these reliable proteins.

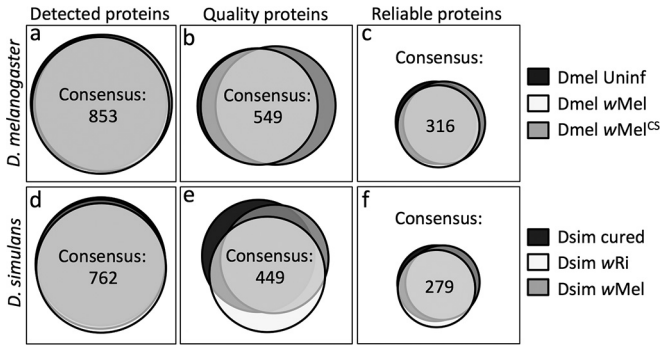

FIG 1 Systematic identification of reliable ovarian proteins shared within each host type. Venn diagrams represent the total number of proteins and the extent of content overlap between each sample type. (a to c) These data are indicated for *D. melanogaster* at the level of detection (a), quality peptide identification (b), and reliability (c). (d to f) The *D. simulans* samples had slightly fewer proteins represented overall in the categories of detection (d), quality peptide identification (e), and reliability (f). Proteins detected in *Wolbachia*-free samples are shown in black. Endogenous *Wolbachia*-host combinations are shown in white. Variant *Wolbachia*-host combinations are shown in gray.

Most *Wolbachia*-associated proteomic changes are restricted to a given host type. To identify ovarian proteins that exhibit significant abundance changes in *Wolbachia*-infected tissue, comparisons between sample types were performed by using ANOVA. For *D. melanogaster*, this analysis revealed 61 host proteins whose abundance changed significantly under one or more of the *Wolbachia*-infected conditions (see Table S3 in the supplemental material) ($P < 0.05$). The *Wolbachia* surface protein, Wsp, was also identified in Dmel *w*Mel and Dmel *w*Mel^{CS} samples only. For *D. simulans*, ANOVA identified 49 host proteins that exhibited significantly altered abundance in one or both *Wolbachia*-infected samples (see Table S4 in the supplemental material) ($P < 0.05$). These ovarian proteins are referred to here as "significant proteins."

To address the overall functional implications of the group of significant proteins, each protein was assigned to a functional class, based on information from the eggNOG v4.5 program and the *Drosophila* literature. This analysis grouped the significant proteins into 15 functional classes (Fig. 2a and c). Six functional classes were specific to either *D. melanogaster* or *D. simulans* and represented ≤10% of the total proteins. The remaining 9 functional classes were shared between host types. Translation-related proteins were highly represented, comprising up to half of the significant proteins overall. The other shared functional classes were carbohydrate transport and metabolism; chromatin structure and dynamics; cytoskeleton and cell motility; energy conversion; lipid transport and metabolism; protein modification, folding, and turnover; RNA binding, processing, and modification; and signal transduction (Fig. 2a and c). This implicates a diverse subset of ovarian cellular processes as being responsive to *Wolbachia*.

The similarity of *D. melanogaster* and *D. simulans* ovarian responses to *Wolbachia* was further assessed in terms of overlap between consensus significant proteins. As *D. simulans* annotation is less extensive than that of *D. melanogaster*, all *D. simulans* proteins were named as per the closest *D. melanogaster* homologs to facilitate this comparison. Out of 95 total significant proteins, this analysis identified 15 significant proteins as being shared between *D. melanogaster* and *D. simulans* ovarian proteomes (see Tables S3 and S4 in the supplemental material). These proteins were glycogen phosphorylase, the ATP synthase delta subunit, retinoid- and fatty acid-binding glycoprotein, heat shock proteins 26 and 27, the hnRNP protein Squid, and 9 different ribosomal proteins (see Tables S3 and S4 in the supplemental material). Thus, a

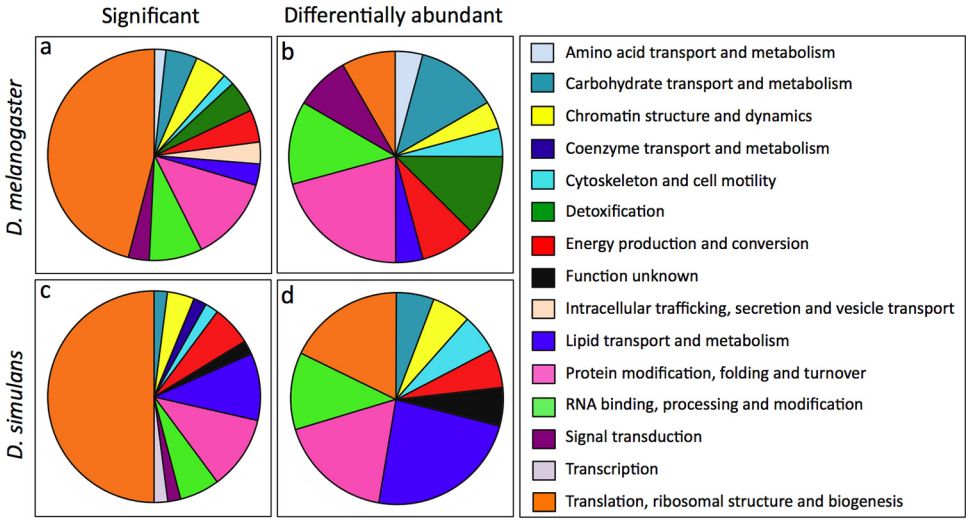

FIG 2 Functional classification of significant and differentially abundant ovarian proteins. The proportional representation of each class is shown for 62 significant *D. melanogaster* proteins (a), 25 differentially abundant *D. melanogaster* proteins (b), 49 significant *D. simulans* proteins (c), and 17 differentially abundant *D. simulans* proteins (d). Each class is distinguished by a different color, as indicated by the key on the right.

limited redundancy of individual proteins was evident among the significant ovarian proteins of *D. melanogaster* and *D. simulans*.

To assess the putative functional impact of *Wolbachia*-responsive significant proteins, the magnitude of protein abundance changes was examined. As in previous proteomics studies, differential abundance on the order of a ≥1.5-fold change is predicted to indicate functional upregulation. Conversely, a ≤0.667-fold change is predicted to indicate functional downregulation (38, 60–62). Comparisons of the significant protein data yielded 25 differentially abundant proteins in *D. melanogaster*, representing 11 functional classes (Fig. 2b and Table 3). Seventeen differentially abundant proteins were detected in *D. simulans*, comprised of 9 functional classes (Fig. 2d and Table 4). The 8 classes of differentially abundant proteins shared between host types were carbohydrate transport and metabolism; chromatin structure and dynamics; cytoskeleton and cell motility; energy production and conversion; lipid transport and metabolism; protein modification, folding, and turnover; RNA binding, processing, and modification; and translation, ribosomal structure, and biogenesis (Fig. 2b and d). This suggests that the differentially abundant proteins represent a distinct subset of significant proteins. The differential abundance data also indicated that the composition of each shared functional class is largely organism specific. The few differentially abundant proteins that were shared between hosts were glycogen phosphorylase, the ATP synthase delta subunit, and heat shock proteins 26 and 27.

Differential protein abundance patterns associated with host and *Wolbachia* types. To further define the impact of specific *Wolbachia* strains on the host ovary proteome, host-strain combinations were examined in terms of the commonalities that they share. One issue was to determine the extent of overlap between proteomic responses to endogenous and variant *Wolbachia* infections. In *D. melanogaster*, comparison of Dmel *w*Mel to Dmel Uninf yielded 3 differentially abundant proteins, whereas comparison of Dmel *w*Mel^{CS} to Dmel Uninf yielded 12 (Table 3). In *D. simulans*, comparison of Dsim *w*Ri and Dsim Cured revealed 6 differentially abundant proteins, while comparison of Dsim *w*Mel to Dmel Cured identified 13 (Table 4). This suggests that infections by variant *Wolbachia* strains had a more robust impact than infections by endogenous *Wolbachia* strains on ovarian proteomic responses at the level of differential abundance.

Another issue to address was the extent of bacterial versus host influence on the ovarian proteomic responses to *Wolbachia*. To assess the consistency of responses associated with a single *Wolbachia* strain, ovarian responses to *w*Mel were tracked across host types. This analysis indicated that distinctive proteomic responses were evident in the natural *D. melanogaster* host compared to the ectopic *D. simulans* host (Tables 3 and 4). The similarity of host responses to multiple *Wolbachia* strains was also investigated. Direct comparison of Dmel *w*Mel^{CS} to Dmel *w*Mel identified 11 additional differentially abundant proteins, including the *Wolbachia* surface protein (Wsp) (Table 3). Most of these hits were due to oppositely directed protein abundance shifts under each *Wolbachia* infection condition. Direct comparison of Dsim *w*Mel to Dmel *w*Ri identified 8 differentially abundant proteins as well. However, nearly all these shifts were redundant with shifts already identified in comparisons between infected and uninfected *D. simulans* ovaries (Table 4). This suggests that ovarian proteomic responses to different *Wolbachia* strains were milder and more diversified in *D. melanogaster* than in *D. simulans*, where all-or-nothing responses were predominant.

Previous studies showed that high-titer *Wolbachia* infections exert the most extensive impact on host physiological processes (44, 63–65). This precedent raises questions about the role of *Wolbachia* titer in specifying *Wolbachia*-associated changes in the ovary proteome. Real-time quantitative PCR was performed to assess ovarian *Wolbachia* abundance. The data indicated that Dmel *w*Mel^{CS} ovaries carried only 51% of the *Wolbachia* titer detected in Dmel *w*Mel ovaries ($P = 0.047$) ($n = 60$ ovaries per condition) (see Fig. S1 in the supplemental material). Ovarian *Wolbachia* titers detected in Dsim *w*Ri and Dsim *w*Mel ovaries were not significantly different from each other or from those in Dmel *w*Mel ovaries (see Fig. S1 in the supplemental material). This does not support a role for elevated *Wolbachia* titers as a

TABLE 3 Differentially abundant proteins identified through comparison of *D. melanogaster* ovarian proteomes[a]

Functional classification	Protein	Relative abundance		
		*w*Mel/Uninf	*w*Mel^{CS}/Uninf	*w*Mel^{CS}/wMel
Amino acid transport and metabolism	Eip55E	1.271	**1.587**	1.248
Carbohydrate transport and metabolism	Aldolase	0.919	**0.630**	0.685
	Glycogen phosphorylase	0.839	1.447	**1.724**
	Succinyl coenzyme A synthetase α subunit	NA	NA	**0.591**
Chromatin structure and dynamics	Vig2	0.868	1.330	**1.533**
Cytoskeleton and cell motility	Ciboulot	0.858	1.380	**1.608**
Detoxification	Glutathione *S*-transferase D1	1.028	**1.626**	**1.581**
	Peroxinectin-like	0.889	**1.704**	**1.917**
	Transferrin 1	**2.000**	NA	NA
Energy production and conversion	ATP synthase, δ subunit	0.888	1.367	**1.540**
	Isocitrate dehydrogenase	0.776	1.314	**1.693**
Lipid transport and metabolism	Jabba	0.801	1.298	**1.621**
Protein modification, folding, and turnover	Cysteine proteinase 1	1.042	**1.603**	**1.539**
	Heat shock protein 26	0.951	**1.558**	**1.639**
	Heat shock protein 27	1.087	**1.525**	1.403
	Hsc/Hsp70-interacting protein related	0.828	1.266	**1.529**
	Regulatory particle non-ATPase 6	1.098	**1.578**	1.437
RNA binding, processing, and modification	Hoi-polloi	1.214	0.795	**0.655**
	Modulo	1.035	0.687	**0.663**
	Rm62	0.999	**0.644**	**0.644**
Signal transduction	14-3-3ζ	**0.611**	**0.555**	0.909
	Terribly reduced optic lobes	1.154	**0.647**	**0.561**
Translation, ribosomal structure, and biogenesis	Ribosomal protein S27	0.989	**0.645**	**0.652**
	Seryl-tRNA synthetase	**0.618**	NA	NA
Wolbachia protein	*Wolbachia* surface protein	NA	NA	**0.632**

[a] Relative abundance represents a ratio of average LFQ scores for each sample type: Dmel *w*Mel/Dmel Uninf, Dmel *w*Mel^{CS}/Dmel Uninf, and Dmel *w*Mel^{CS}/Dmel *w*Mel. Ratios indicating protein up- or downregulation are shown in boldface type. NA, not applicable.

determinant of ovarian proteomic responses but alternatively favors consideration of molecular and cellular mechanisms intrinsic to each scenario.

DISCUSSION

In applying a proteomic approach to ovarian responses to *Wolbachia*, a central consideration is whether the data set substantiates current knowledge of infection. Based upon previous work, one expectation is that variant host-strain combinations should exhibit stress indicators (36, 40, 66, 67). Notably, the variant Dmel *w*Mel^{CS} and Dsim *w*Mel combinations in this study exhibited depletion of dozens of ribosomal constituents, consistent with overall downregulation (68–70). Upregulation of heat shock and detoxification proteins was also seen, consistent with a stress response (71–73). Ovaries from the Dsim *w*Mel combination have also been shown to exhibit extensive chromatin structuring defects in nurse cells, analogous to *squid* mutant organisms (16, 74). The downregulation of the Squid protein observed here informs the basis for this response.

Another expectation is that *Wolbachia* should strategically enhance ovarian survival and proliferation mechanisms to maximize transmission. The findings of this study corroborate the involvement of known factors while also identifying new candidate contributors. The upregulation of the iron-sequestering protein transferrin 1 is in agreement with previous reports that *Wolbachia* bacteria protect the germ line from iron-associated toxicity (25–28). An increased abundance of the retinoid- and fatty acid-binding protein, indicated to have heme-binding activity, may help to protect the germ line from oxidative stress as well (75). Upregulation of the Sxl effector protein, Female-specific independent of transformer, opens a speculative route for *Wolbachia* modulation of *Sxl*-induced germ line lethality (29, 76, 77). The downregulation of the cell division suppressor 14-3-3 zeta is also consistent with enhanced germ line stem cell division rates observed for *Wolbachia*-infected organisms (11, 78).

It is further expected that *Wolbachia* bacteria drive modifications of the ovarian environment that support *Wolbachia* persistence. Some evidence from this study supports that prediction. From a nutritional standpoint, an elevated abundance of proteases and proteasome subunits is consistent with the possibility of

TABLE 4 Differentially abundant proteins identified through comparison of *D. simulans* ovarian proteomes[a]

Functional classification	Protein	Relative abundance		
		*w*Ri/Cured	*w*Mel/Cured	*w*Mel/*w*Ri
Carbohydrate transport and metabolism	Glycogen phosphorylase	1.097	**1.884**	**1.717**
Chromatin structure and dynamics	Histone H4	0.913	**0.645**	0.706
Cytoskeleton and cell motility	Tropomyosin 2	1.081	**1.701**	**1.573**
Energy production and conversion	ATP synthase, δ subunit	**1.687**	1.120	**0.664**
Function unknown	Female-specific independent of transformer	0.785	**3.359**	**4.277**
Lipid transport and metabolism	CG3902 (acyl-CoA dehydrogenase activity)	**1.651**	**1.664**	0.974
	Retinoid- and fatty acid-binding glycoprotein	**2.175**	**2.385**	1.008
	Yolk protein 1	1.407	**1.990**	1.414
	Yolk protein 2	1.400	**1.693**	1.209
Protein modification, folding, and turnover	Heat shock protein 26	1.193	**1.753**	1.469
	Heat shock protein 27	**0.631**	1.299	**2.058**
	Tripeptidyl-peptidase II	0.701	1.230	**1.755**
RNA binding, processing, and modification	Fibrillarin	1.089	**0.488**	**0.448**
	Squid	**0.569**	0.677	1.191
Translation, ribosomal structure, and biogenesis	Ribosomal protein L32	0.957	**0.651**	**0.654**
	Ribosomal protein L34b	**0.654**	**0.593**	0.906
	Ribosomal protein S25	0.962	**0.629**	0.680

[a] Relative abundance represents a ratio of average LFQ scores for each sample type: Dsim *w*Ri/Dsim Cured, Dsim *w*Mel/Dsim Cured, and Dsim *w*Mel/Dsim *w*Ri. Ratios that indicate up- or downregulation are indicated in boldface type. CoA, coenzyme A.

increased amino acid availability for *Wolbachia* (79). The upregulation of glycogen phosphorylase complements recent work using *Brugia malayi* nematodes, which indicated that *Wolbachia* bacteria induce the upregulation of glycolytic enzymes (80). An increased local availability of pyruvate is hypothesized to benefit *Wolbachia* (81). It was also recently shown that filamentous actin is important for stabilizing *Wolbachia* colonization of the host germ line (21). *Wolbachia*-associated upregulation of tropomyosin, a microfilament-stabilizing protein, is consistent with that model (82). Taken together, these data support the study outcomes as being representative while also associating the potential use of these transmission-enhancing mechanisms with new host-strain combinations.

It is notable that very few proteins were detected as being significant or differentially abundant across all sample types analyzed in this study. Data sets from previous *Wolbachia*-omics studies exhibit a wide range of *Wolbachia*-responsive host expression changes, indicating that contextual influences are substantial (31–42) (see Table S5 in the supplemental material). Analagous to those prior studies, our study provides substantial evidence of context-dependent responses to *Wolbachia* infection. Infections with endogenous *Wolbachia* strains had little effect on the host proteome compared to infections with variant *Wolbachia* strains, in agreement with data from previous work on heterologous symbiont infections of cnidarians (83). Ovarian proteomic responses to *Wolbachia* also correlated poorly with *Wolbachia* titers, paralleling results from a previous fecundity study (84). This argues against the conservation of *Wolbachia*-ovary interactions in terms of specific protein abundance shifts. A combination of effects may contribute to this outcome, including technical limitations of the assay (51) as well as *Wolbachia* adaptation (85), modification of *Wolbachia* population structure (85), and/or selection (86). Regardless, the finite physical constraints of transmission inherently favor *Wolbachia* manipulation of the most functionally advantageous processes. Context-specific regulation of consensus ovarian mechanisms may contribute substantially to the achievement of this goal.

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