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September/October 2015

Hamilton, P.T. et al. (2015). Infection dynamics and immune response in a newly described *Drosophila*-trypanosomatid association. *mBio,* 6(5):e01356-15, 1-11. doi:10.1128/mBio.01356-15

RESEARCH ARTICLE

Infection Dynamics and Immune Response in a Newly Described *Drosophila*-Trypanosomatid Association

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ABSTRACT **Trypanosomatid parasites are significant causes of human disease and are ubiquitous in insects. Despite the importance of *Drosophila melanogaster* as a model of infection and immunity and a long awareness that trypanosomatid infection is common in the genus, no trypanosomatid parasites naturally infecting *Drosophila* have been characterized. Here, we establish a new model of trypanosomatid infection in *Drosophila*—*Jaenimonas drosophilae*, gen. et sp. nov. As far as we are aware, this is the first *Drosophila*-parasitic trypanosomatid to be cultured and characterized. Through experimental infections, we find that *Drosophila falleni*, the natural host, is highly susceptible to infection, leading to a substantial decrease in host fecundity. *J. drosophilae* has a broad host range, readily infecting a number of *Drosophila* species, including *D. melanogaster*, with oral infection of *D. melanogaster* larvae resulting in the induction of numerous immune genes. When injected into adult hemolymph, *J. drosophilae* kills *D. melanogaster*, although interestingly, neither the Imd nor the Toll pathway is induced and Imd mutants do not show increased susceptibility to infection. In contrast, mutants deficient in drosocrystallin, a major component of the peritrophic matrix, are more severely infected during oral infection, suggesting that the peritrophic matrix plays an important role in mediating trypanosomatid infection in *Drosophila*. This work demonstrates that the *J. drosophilae-Drosophila* system can be a powerful model to uncover the effects of trypanosomatids in their insect hosts.**

IMPORTANCE **Trypanosomatid parasites are ubiquitous in insects and are significant causes of disease when vectored to humans by blood-feeding insects. In recent decades, *Drosophila* has emerged as the predominant insect model of infection and immunity and is also known to be infected by trypanosomatids at high rates in the wild. Despite this, there has been almost no work on their trypanosomatid parasites, in part because *Drosophila*-specific trypanosomatids have been resistant to culturing. Here, we present the first isolation and detailed characterization of a trypanosomatid from *Drosophila*, finding that it represents a new genus and species, *Jaenimonas drosophilae*. Using this parasite, we conducted a series of experiments that revealed many of the unknown aspects of trypanosomatid infection in *Drosophila*, including host range, transmission biology, dynamics of infection, and host immune response. Taken together, this work establishes *J. drosophilae* as a powerful new opportunity to study trypanosomatid infections in insects.**

Received 6 August 2015 **Accepted** 14 August 2015 **Published** 15 September 2015

Citation Hamilton PT, Votýpka J, Dostálová A, Yurchenko V, Bird NH, Lukeš J, Lemaitre B, Perlman SJ. 2015. Infection dynamics and immune response in a newly described *Drosophila*-trypanosomatid association. mBio 6(5):e01356-15. doi:10.1128/mBio.01356-15.

Editor Keith Gull, University of Oxford

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Trypanosomatids are kinetoplastid parasites that include the causal agents of major human diseases such as African sleeping sickness, American Chagas disease, and diverse leishmaniases. The trypanosomatids causing these diseases have complex dixenous life cycles that alternate between vertebrates and blood-feeding insects, and understanding trypanosomatid interactions with their insect vectors has been a major focus of research (1).

The vast majority of trypanosomatids have simple monoxenous life cycles (2, 3), however, infecting only invertebrate hosts, and monoxenous trypanosomatids are evolutionarily ancient, while the dixenous lifestyle is derived (4). Insects are the most common hosts of monoxenous trypanosomatids, with dipterans (flies) and heteropterans (true bugs) showing particularly high rates of infection. Intriguingly, flies of the genus *Drosophila* are commonly infected by trypanosomatids in the wild (5–8; P. T. Hamilton and S. J. Perlman, unpublished data), with infections first documented over a century ago. However, there has been very little work on *Drosophila*-trypanosomatid interactions, and no trypanosomatids from *Drosophila* hosts have been grown in culture and formally described. Only a few studies have directly examined the effects of trypanosomatid infection on *Drosophila* fitness, and these have used uncharacterized and/or mixed parasites from wild flies (8, 9) or parasites derived from other insect hosts. For example, the only study to examine the immune response of *Drosophila* to trypanosomatids used infections with *Crithidia* parasites of bumblebees and mosquitoes (10). This lack of attention is

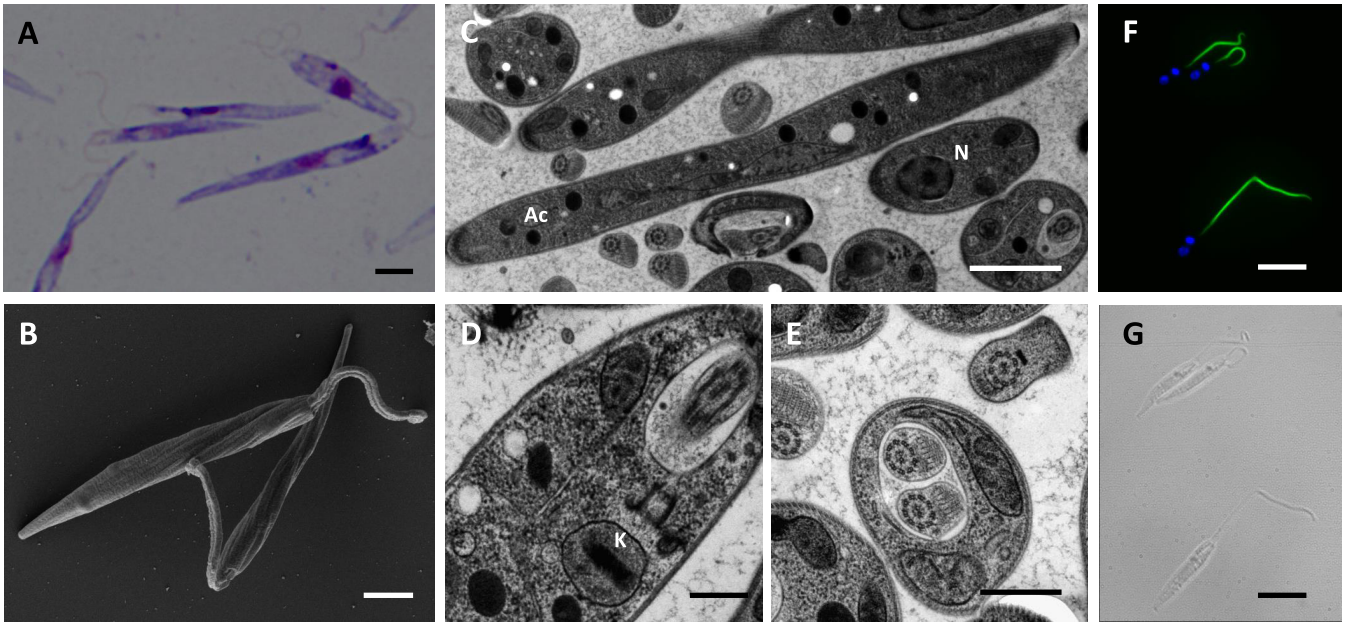

FIG 1 Morphology of *J. drosophilae* sp. nov. in axenic culture (clone dfal-01.02). Shown are images of *J. drosophilae* obtained by light microscopy (Giemsa staining) (A), SEM (B), and HPF TEM (C to E). (C, D) Longitudinal sections reveal typical features of trypanosomatids such as the nucleus (N) and kinetoplast (K), as well as the presence of acidocalcisomes (Ac). (E) Cross section of the cell through the flagellar pocket displaying an extremely well-developed paraflagellar rod supporting flagella even within the pocket. (F, G) *J. drosophilae* stained with mouse monoclonal antibody L8C4 against *Trypanosoma brucei* paraflagellar rod 2 (PFR2) (61). Scale bars are 1 μm (A to C, F, and G) and 500 nm (D and E).

especially surprising given that *Drosophila* has become one of the most important animal models of infection and immunity (11).

Here, we report the successful cultivation and first formal molecular and morphological description of a trypanosomatid parasite naturally infecting a *Drosophila* species. Molecular characterization shows this isolate to be highly divergent from previously described flagellates, representing a new genus and species, which we name *Jaenimonas drosophilae* gen. et sp. nov.

We conducted a series of experiments to investigate the mode of transmission, temporal dynamics of infection, and fitness consequences of *J. drosophilae* in its native host, *Drosophila falleni*. We found that *J. drosophilae* rapidly establishes infections in larvae that persist through the development of the fly, leading to a reduction in host fecundity, and that adult flies readily transmit infections to each other. We also showed that *J. drosophilae* has a wide host range, establishing high-intensity infections in diverse *Drosophila* species.

Having established that *J. drosophilae* readily infects *Drosophila melanogaster*, we took advantage of the wealth of resources and tools associated with this model organism to begin to examine the immune response of *D. melanogaster* to native trypanosomatids. Transcriptomic (RNA sequencing [RNA-seq]) analysis revealed an active immune response in infected larvae. In contrast to work on tsetse fly-trypanosome interactions, we found that mutants deficient in the Imd immune pathway do not show increased susceptibility, although mutants deficient in drosocrystallin, a key component of the *Drosophila* peritrophic matrix (PM), show greater susceptibility to infection. *J. drosophilae* shows substantial promise as a highly tractable model to investigate the interactions of an understudied lineage of parasites in the most established insect model of disease.

RESULTS

Isolation, primary characterization, and subcloning of a new trypanosomatid species. We initially uncovered evidence of an infection in a laboratory line of *D. falleni*; dissections of flies of this line revealed trypanosomatid-like cells localized to the midguts of many specimens. This fly line has presumably been stably infected since its collection in West Hartford, CT, in 2006. This line had been used to maintain a *Drosophila*-parasitic nematode, *Howardula aoronymphium*. We subsequently established a trypanosomatid-infected *D. falleni* line that was free of nematodes. This infection has persisted in lab culture (i.e., vials containing *D. falleni*, where adults transmit the infection to larvae and other adults) for over 2 years. We established axenic cultures of the parasite in brain heart infusion (BHI) medium (12). Laboratory (infected *D. falleni*) and cultured isolates were named dfal-01-lab and dfal-01, respectively. Using the primary culture of dfal-01, we performed successive rounds of clonal isolation to generate a single clonal trypanosomatid line (dfal-01.02).

Morphological and ultrastructural characterization. Light microscopy of dfal-01.02 revealed uniform cells with typical promastigote morphology (Fig. 1A) (3, 13). Importantly, the dfal-01.02 cultured cells were morphologically indistinguishable from those observed *in situ* in *D. falleni* (for detailed size ranges, see the taxonomic description below and the supplemental material).

We next analyzed the dfal-01.02 cells by scanning electron microscopy (SEM) (Fig. 1B) and high-pressure freezing (HPF) electron microscopy (14, 15). SEM confirmed that the dfal-01.02 cells were typical elongated promastigotes with well-developed pellicular ridges (Fig. 1B), though the flagellum was considerably thicker than that of other trypanosomatids (13, 16). HPF transmission electron microscopy (TEM) revealed all of the typical

trypanosomatid features, such as an oval nucleus, basal bodies, glycosomes, and an electron-dense kinetoplast disc within a reticulated mitochondrion rich with tubular cristae (Fig. 1C and D). One peculiarity is an extremely well-developed paraflagellar rod. Notably, it was detectable even within the flagellar pocket (Fig. 1E).

Phylogenetic analysis. We sequenced the small-subunit (SSU; 18S) rRNA, glycosomal glyceraldehyde 3-phosphate dehydrogenase (gGAPDH), and spliced-leader (SL) RNA genes of the laboratory and cultured isolates. The SSU rRNA sequence obtained was 99% similar to one originating from an uncultured trypanosomatid (KC183713) from *Drosophila ananassae* collected in Hawaii (17). For both SSU rRNA and gGAPDH sequences, the next closest homologs in GenBank belonged to *Herpetomonas* and *Strigomonas* species (≥90%). For phylogenetic reconstruction, SSU rRNA and gGAPDH sequences of dfal-01.02 were concatenated and aligned with a set representing major trypanosomatid clades (Fig. 2). The cultured species is distinct from all previously described clades of monoxenous trypanosomatids (3).

The spliced leader (SL) RNA gene is the most suitable marker for determining relationships between closely related species, subspecies, or populations of insect trypanosomatids (12, 18). Analysis of this gene also confirmed a unique sequence, with its position on the phylogenetic tree broadly correlated with the novel clade inferred from the SSU rRNA marker, with the most similar SL sequences in GenBank also from uncultured trypanosomatids from *D. ananassae* (17). The sequences showed 95% identity, clearly indicating that they represent the same typing unit (TU*173* [5]) and, on the basis of the 90% threshold rule, belong to the same species (19).

TAXONOMY

Class: Kinetoplastea Honigberg 1963 emend. Vickerman 1976.
Subclass: Metakinetoplastina Vickerman 2004.
Order: Trypanosomatida Kent 1880.
Family: Trypanosomatidae Doflein 1901.
Genus: *Jaenimonas* gen. nov., Votýpka and Hamilton 2015.
Generic diagnosis: A well-supported monophyletic group of monoxenous trypanosomatids of invertebrate hosts (Diptera: Drosophilidae) without bacterial endosymbionts. It is defined by a set of unique sequences of the SSU rRNA, gGAPDH, and SL RNA genes. Molecular phylogenetic analyses confirm this genus as a new member of the family Trypanosomatidae that cannot be associated with any valid genus.
Etymology: The generic name honors John Jaenike of the University of Rochester, Rochester, NY, who has made important contributions to the field of host-parasite ecology and evolution, with much of his research focused on natural populations of *Drosophila*. "Monas" (Greek)—monad; third declension (monas); feminine; the word monas is included in many generic names of flagellates.
Species: *Jaenimonas drosophilae* sp. nov. Votýpka and Hamilton 2015 (Fig. 1).
Diagnosis and description: Cultured *J. drosophilae* cells are of the typical promastigote morphology. Cells range from 11.4 to 20.3 μm in length and from 0.9 to 3.1 μm in width, with a flagellum measuring 6.2 to 16.6 μm. The kinetoplast disk varies between 114 and 210 nm in thickness and 310 and 670 nm in diameter. The species is identified by the unique sequences KP260534 (SSU rRNA), KP260535 (gGAPDH), and KP260536 (SL RNA) and belongs to typing unit TU*173*.
Type host: *Drosophila falleni* Wheeler 1960 (Diptera: Drosophilidae). The xenotype (2006/dfal-01) is deposited at the Royal British Columbia Museum.
Site: Intestine (midgut).
Type locality: Vicinity of West Hartford, CT (41°46′04″N, 72°45′14″W).
Type material: Hapantotype (Giemsa-stained slide 2006/Dfal-01/S), axenic cultures of the primary isolate (Dfal-01) and clonal line (Dfal-01.02) are deposited in the research collections of respective institutions in Prague, Ostrava, and Budweis, Czech Republic.
Etymology: The species name is derived from the name of the typical host, a *Drosophila* species.
Remarks: On the basis of the SSU rRNA (KC183713) and SL RNA (KC183707, KC183708, and KC183709) sequences, the environmental samples from *D. ananassae* captured in the vicinity of Captain Cook, HI, also belong to the same species, *J. drosophilae*.

***J. drosophilae* infects *Drosophila* larvae, persisting throughout the development of the fly.** We developed a *per os* larval infection model to assay the effects of *J. drosophilae* in *Drosophila* by exposing newly hatched larvae to a homogenate of *J. drosophilae*-infected *D. falleni* adults in insect Ringer solution. This homogenate was added to fly food (mushroom or banana, depending on the host species) at 1 day postoviposition (dpo). To establish the number of parasites required to initiate infection, we exposed *D. falleni* larvae to a series of dilutions of *J. drosophilae* (from ~62,500 to ~2,300 cells) and found that the majority of the larvae became infected even at the lowest dose, confirming substantial infectivity (see Fig. S1 in the supplemental material). Adding high densities ($>10^5$ trypanosomatids per exposure) of cultured (i.e., axenic) *J. drosophilae* to larval food produced some gut infections, but at low intensities compared to those obtained with fly homogenate. We therefore used fly homogenate in subsequent experiments. Changes in infectivity are not uncommon in cultured trypanosomatids (20, 21), so we were not surprised to find similar effects in *J. drosophilae*, though the reason for this effect here is not known.

Quantification of infections over time revealed rapid acquisition and persistence of infection in *D. falleni*. Flies exposed to *J. drosophilae* at 1 dpo showed high-intensity infections when sampled as larvae at 5 dpo. This was evident qualitatively as masses of parasites visible in the larval midgut and PM upon dissection and also quantitatively from quantitative PCR (qPCR) assays of whole larvae (see Fig. S2 in the supplemental material). qPCR assays monitoring the same fly cohort over time showed that infection persisted and increased modestly as flies developed (see Fig. S2; linear model [LM] of log-transformed genome equivalents [GEs]; $t_{1,45} = 3.64$, $P < 0.001$). High infection rates in all life stages following larval exposure demonstrated persistent infection

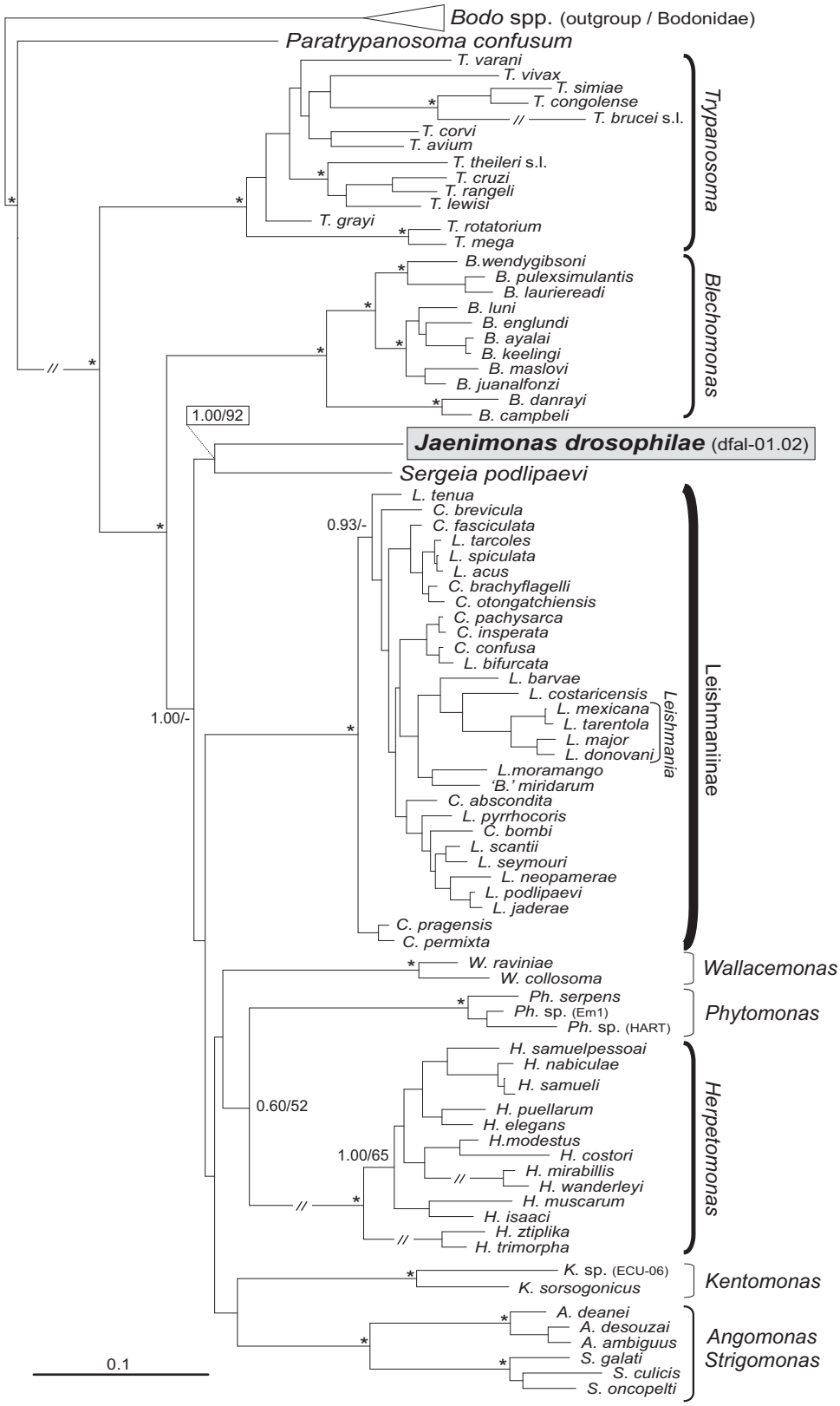

FIG 2 *J. drosophilae* is a novel and deep-branching trypanosomatid lineage. An SSU rRNA-gGAPDH-based Bayesian phylogenetic tree of Trypanosomatidae is shown. Names of species whose sequences were retrieved from GenBank are indicated. Bootstrap values from Bayesian posterior probabilities (5 million generations) and bootstrap percentages for maximum-likelihood analysis (1,000 replicates) are shown at the nodes. Dashes indicate <50% bootstrap support or different topology. Asterisks represent >90% bootstrap support and a Bayesian posterior probability of >0.95. Double-crossed branches are 50% of the original length. The tree was rooted with two sequences of *Bodo saltans*, the closest free-living relative of trypanosomatids. The scale bar shows the number of substitutions per site.

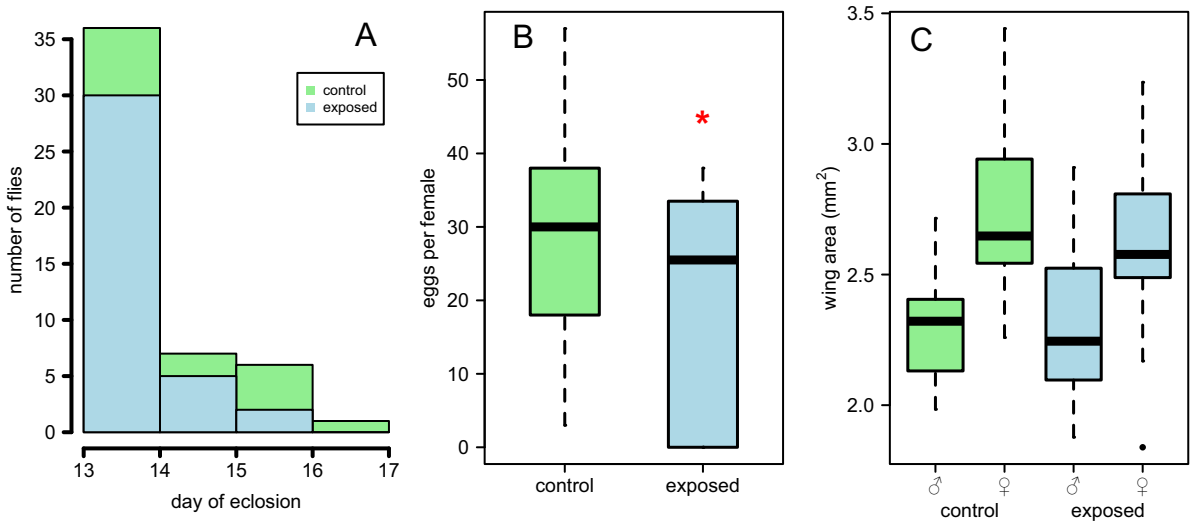

FIG 3 *J. drosophilae* decreases female fly fecundity. The results shown are from oral exposure of *D. falleni* larvae at 1 dpo. Shown are the development time and number of emerging flies (bars overlaid, not stacked) (A), the number of eggs per 7-day-old female (B), and the wing area of 7-day-old males and females (C). Exposed females have significantly fewer eggs than do unexposed controls ($P = 0.015$).

throughout fly development and metamorphosis; the mean infection prevalence overall was 82.5% ± 5.9% (standard error) with no appreciable decrease in the infection frequency as flies developed (binomial generalized LM [GLM] likelihood ratio test [LRT]; $\chi^2 = 0.75$, $P = 0.39$). To confirm *J. drosophilae* persistence through metamorphosis, we dissected pupae that were exposed as larvae and found that, over pupation, infection consistently became sequestered in a structure we identified as the "yellow body" on the basis of its morphology and location within pupae (22).

We also observed *J. drosophilae* transmission among adult flies by cohousing uninfected flies with those from the stably infected line. After 5 and 7 days, 44% ± 6% of the recipient (uninfected line) flies were found to be infected (71% ± 9% of the flies from the donor line were infected). Only 24% ± 7% of the uninfected-line flies had obviously replicating infections, though, with the remaining new infections evident as only a few free-swimming trypanosomatids in the adult gut, suggesting that adults may be more resistant to acquiring infection than larvae are.

***J. drosophilae* exposure decreases host fecundity.** We measured a number of fitness parameters of *D. falleni*, chosen *a priori*, following larval exposure to *J. drosophilae* or a mock control. These were egg-to-adult survival, egg-to-adult development time, adult wing area (as a proxy for fly size), and adult fecundity (mature eggs in week-old females). Of these, the most affected was female fecundity, with exposed flies having, on average, 34% fewer eggs (Fig. 3B, generalized linear mixed model [GLMM] LRT, $\chi^2_1 = 5.95$, $P = 0.015$). The number of flies surviving to adulthood was also somewhat lower in trypanosomatid-exposed treatments, though not significantly so (Fig. 3A, GLMM, $\chi^2_1 = 3.34$; $P = 0.068$). Neither fly development time nor fly wing size appeared to be affected by exposure (Fig. 3A and C, LMMs; $P > 0.5$).

***J. drosophilae* has a wide host range.** We exposed larvae from three *Drosophila* species to *J. drosophilae*: *D. falleni* and *D. neotestacea* (both subgenus *Drosophila*), which share a mushroom feeding niche and are sympatric in parts of their range (23), and *D. melanogaster* (Oregon-R), which is a distant relative (subgenus *Sophophora*) not known to breed on mushrooms (24). Infection rates and intensities in exposed 5-day-old larvae and 5-day-old adults from the same experimental cohort were quantified by qPCR.

Larvae of all species became infected at high rates (Fig. 4A). The species differed in susceptibility, with *D. falleni* and *D. melanogaster* infected at the highest and lowest rates, respectively (species main effect; $\chi^2_2 = 30.38$, $P < 0.001$). Infections persisted through metamorphosis in all species and were present in 5-day-old adults. In *D. melanogaster*, however, there was a pronounced bottleneck in infection over metamorphosis, with substantially lower infection rates in adults than in larvae (Fig. 4A; binomial GLM LRT; species × stage interaction; $\chi^2_2 = 8.11$, $P = 0.017$). The intensity of infection, as measured by qPCR, did not differ detectably between species (Fig. 4B, $F_{2,46} = 0.26$, $P = 0.79$).

Oral infection with *J. drosophilae* induces an immune response in *D. melanogaster* larvae. To test for immune gene and other transcriptional responses to infection, we conducted RNA sequencing of infected *D. melanogaster* larvae matched with mock-infected controls (see Data S1 in the supplemental material). Tests for differential expression on multiple biological replicates using TopHat and Cufflinks (25) identified 122 genes that responded significantly to infection after multiple test correction (Cuffdiff Q value, <0.05). Of these, 54 were upregulated in response to exposure and 68 were downregulated (see Fig. S3 in the supplemental material).

Among the upregulated genes, Gene Ontology (GO) enrichment analysis with DAVID (26) predominately identified enrichment of GO terms representing defense response, with 8 of the 54 upregulated genes having immune response-related functional annotation (see Table S1 in the supplemental material). Among these were three genes encoding antimicrobial peptides (AMPs), i.e., diptericin B, cecropin A1, and attacin A, that were upregulated as much as 4-fold (for *DptB*) in response to infection. Interestingly, one of the most upregulated genes was *CG11313*, which encodes a serine protease specific to the *melanogaster* subgroup, evolving under positive selection, present in larval hemolymph clots, and also upregulated in response to parasitoid attack (27–

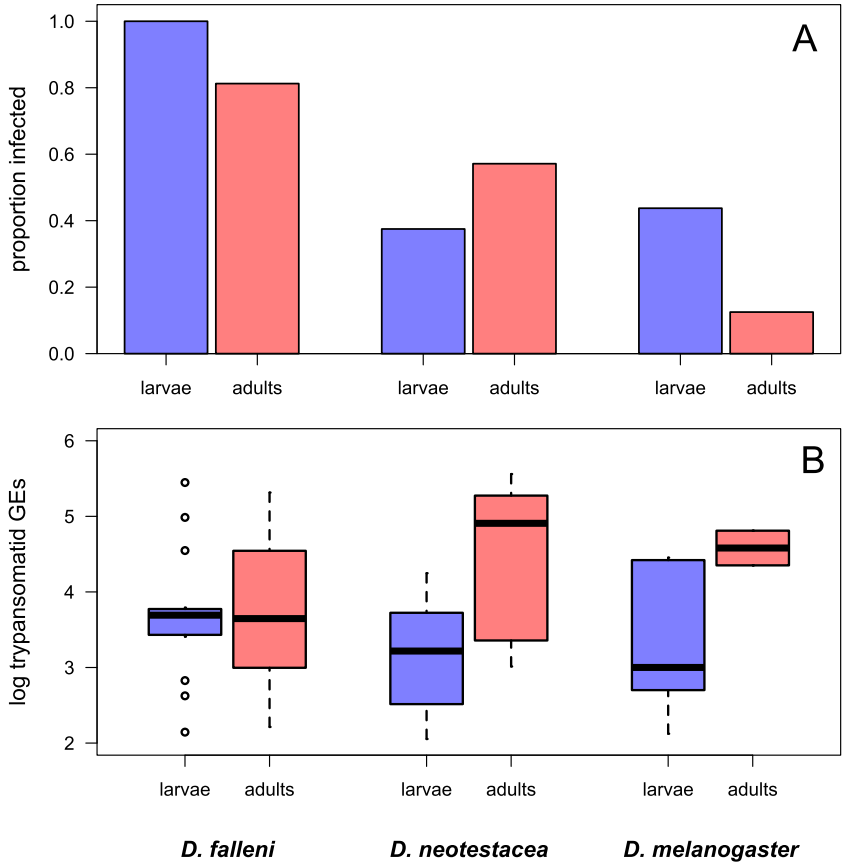

FIG 4 *J. drosophilae* has a broad host range. Shown are infection rates of larvae and flies following exposure to *J. drosophilae* orally at 1 dpo (62,500 cells per replicate of 20 fly eggs; 16 larvae and flies screened per species, $n = 96$) (A) and infection intensities expressed in GEs measured via qPCR (B). Infection rates are significantly higher in larvae ($P < 0.001$) and lowest in *D. melanogaster* adults (species × stage interaction, $P = 0.017$).

29). *PPO1* and *PPO2*, which encode key enzymes in the melanization defense response (30), were also both upregulated—unexpectedly, given a lack of obvious melanization response observed during infection, but possibly in response to wounding of the gut epithelium.

Among the genes downregulated in response to infection, there was only one enriched GO term, corresponding to metabolism of aromatic amino acids (see Table S1 and Fig. S2 in the supplemental material). Many genes relating to host nutritional status were also strongly affected by *J. drosophilae*, either up- or downregulated (e.g., *Fbp1*, *Lsp2*, *CG5966*), which is not surprising, since we might expect heavy gut infections to affect host nutrition and metabolism.

Imd-deficient *D. melanogaster* flies are resistant to *J. drosophilae* infection. The Imd pathway is the major regulator of the intestinal immune response to oral bacterial infection in *Drosophila* (31). The upregulation of larval AMPs associated with the Imd pathway (above) prompted us to assay whether Imd-deficient flies are more susceptible to the parasite. We challenged larvae deficient in the transcription factor Relish—which mediates the Imd pathway response (32)—with parasites and screened adult flies for infection. The proportion of infected Relish-deficient flies did not differ from that of wild-type control lines (Fig. 5A, GLM; $\chi^2 = 2.66$, $P = 0.45$). Thus, while *J. drosophilae* induced the expression of several AMPs in larvae, the Imd pathway does not appear to constitute a major component of resistance to the parasite.

The PM serves as a barrier to *J. drosophilae* infection in the intestine. The PM has been implicated as an important barrier to trypanosomatid establishment in dipterans (33). To assess its importance in *Drosophila* resistance to *J. drosophilae*, we infected a line deficient in the drosocrystallin protein (encoded by *dcy*). Drosocrystallin is a component of the PM in adult flies, and its deletion results in a reduction of the width of the PM, an increase in its permeability, and increased susceptibility to bacterial infection (34). Drosocrystallin-deficient flies had substantially higher rates of infection than control *dcy* rescue flies, in terms of adult infection levels following larval exposure (Fig. 5A, $\chi^2 = 4.74$, $P = 0.029$), suggesting a role for an intact PM as a barrier to the establishment and/or persistence of *J. drosophilae* in the midgut.

Systemic infection with *J. drosophilae* kills *D. melanogaster* but does not induce a pronounced immune response. We explored the impact of *J. drosophilae* on the host immune response by directly injecting the parasite into the *D. melanogaster* body cavity. qPCR confirmed that *J. drosophilae* survives and proliferates in the host hemolymph, multiplying ~5× by 8 days after injection (Fig. 5B; $P = 0.028$). *J. drosophilae* injection caused a very high mortality rate, albeit with a late onset consistent with slow replication within the host (Fig. 5C; $P < 0.001$, log rank test). This effect, however, varied with the host genotype, with some strains being more or less susceptible (representative Oregon-R strain shown).

We measured immune induction in systemic infections

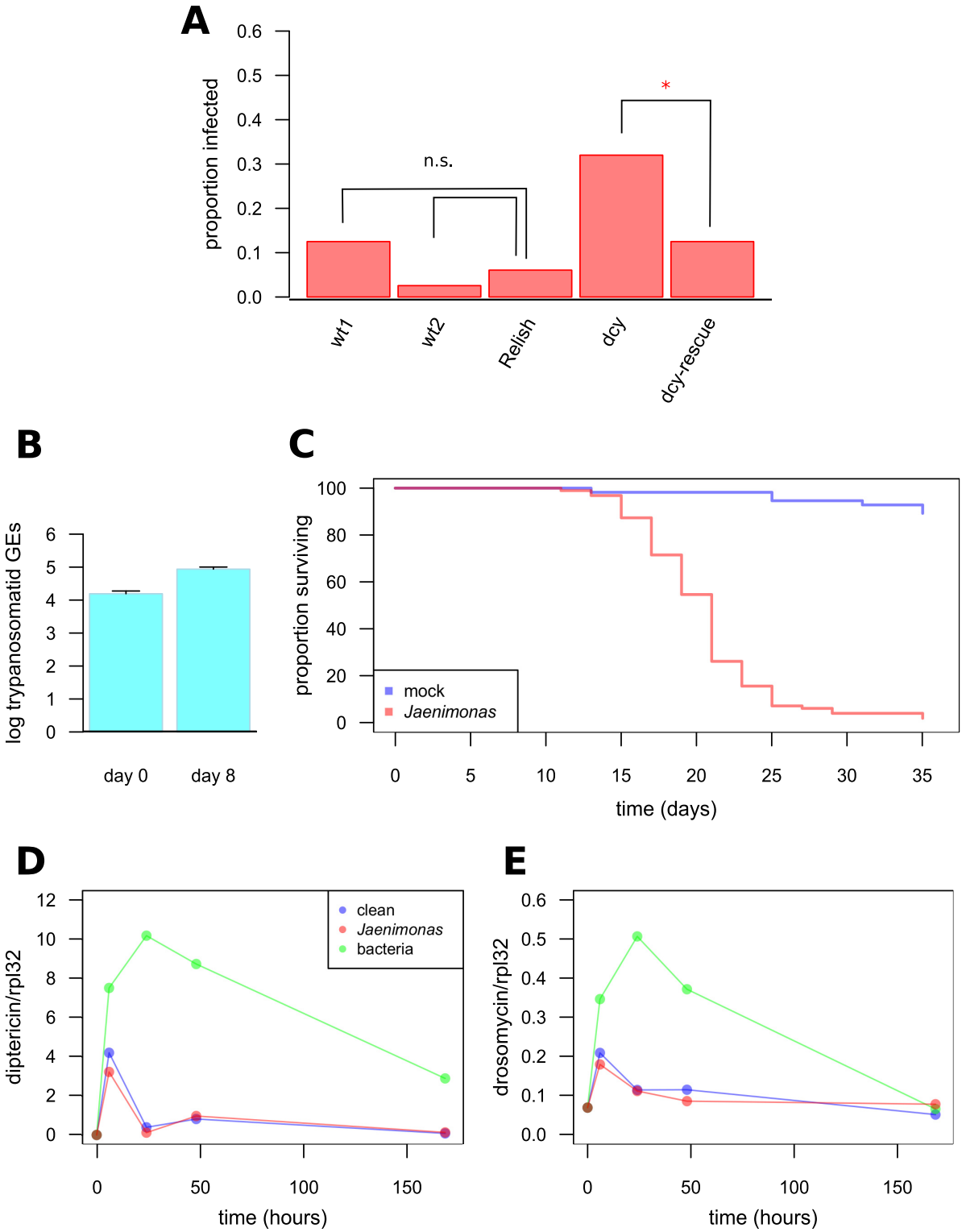

FIG 5 *D. melanogaster* immune response to *J. drosophilae*. (A) Infection rates of adults following larval oral exposure to *J. drosophilae*. Drosocrystallin (*dcy*) knockout flies are significantly more infected than the control line (dcy-rescue; $\chi^2 = 4.74$, $P = 0.029$), but the proportion of infected $Relish^{E20}$ mutant flies is not different from that of the control lines (wt1, white 1118; wt2, Oregon-R; $P \gg 0.05$, not significant; $n = 146$). (B) Intensity of *J. drosophilae* infection of *D. melanogaster* after injection of parasites into the hemolymph of adult flies. Intensity is significantly higher on day 8 ($P = 0.028$). (C) Parasites cause a high mortality rate when injected (Oregon-R; log rank test, $P < 0.001$). (D, E) Normalized induction of diptericin (D) and drosomycin (E) expression after *J. drosophilae* or bacteria were injected into the hemolymph of adult *D. melanogaster* flies.

through the expression of diptericin and drosomycin, two antimicrobial peptides commonly used as readouts for activation of the Imd and Toll pathways. Interestingly, there was no observable upregulation of either AMP above the background level, even upon the injection of large doses of parasites (~14,000 parasites/fly; Fig. 5D and E), suggesting that though variation in resistance appears to exist, it is not clearly linked to Imd and Toll pathway competence.

DISCUSSION

Although it has been known for over a century that trypanosomatids are pervasive parasites of *Drosophila*, they have received scant attention. To date, no *Drosophila*-specific trypanosomatids have been formally described, save some early morphological descriptions that appear to be apocryphal because of the notorious difficulty of identifying trypanosomatids without molecular markers (e.g., *Trypanosoma drosophilae* in reference 5), and most aspects of trypanosomatid biology within *Drosophila* hosts remain unknown. These include the diversity, systematics, and effects of these parasites in their hosts, which is remarkable, given the intense study that the genus receives as a model of infection and immunity.

Here, we begin to address this deficiency by describing a new trypanosomatid genus and species from *Drosophila*. Though it was isolated from *D. falleni*, we find that *J. drosophilae* has a broad host range, easily infecting the distantly related species *D. melanogaster*. Previous molecular screenings of a wide range of wild *Drosophila* flies have found sequences of *J. drosophilae* in *D. ananassae* collected in Hawaii (17), thus confirming *J. drosophilae* to be geographically widespread and with a broad host range, infecting both *Sophophora* and *Drosophila* subgenera.

Oral infection of larval *Drosophila* demonstrated that infection persists throughout the life of the fly, including through metamorphosis, when the fly gut undergoes a nearly complete rearrangement that can also purge it of dominant microbes (35). Early work on *Crithidia* infection in mosquitoes demonstrated that some infections persist from larval mosquitoes to adults (36), and early accounts of *Drosophila* trypanosomatids suggest that this is common (5, 6, 37). Interestingly, the main observed difference between the *Drosophila* species we challenged was persistence of the infection after pupation, as *D. melanogaster*, but not *D. falleni* or *D. neotestacea*, adults were less infected than larvae. Whether this is due to differential immune response or specific structural rearrangements during pupation is unclear, although it is interesting that there was a clear effect of the integrity of the PM on rates of infection of adult *D. melanogaster*.

We observed *J. drosophilae* transmission to both larvae and adults, as well as between adult flies, but suspect that transmission to larvae is an especially important component of the epidemiology of these parasites. Characteristics of the life history of *Drosophila* suggest that larval infections would be important; many *Drosophila* species breed and reach high larval densities on ephemeral or patchy food resources (24), with a substantial opportunity for density-dependent pathogen transmission from adults to larvae or among larvae. Indeed, many *Drosophila*-specific nematode parasites and parasitoid wasps have life cycles that rely entirely on transmission to larvae (23, 38), suggesting that many *Drosophila* parasites commonly exploit larval aggregation.

Though trypanosomatids have mostly been considered relatively benign in their insect hosts (3), few direct experiments have been performed so far to test this. In fact, we found a pronounced negative effect of *J. drosophilae* exposure in the laboratory, where it led to an ~1/3 reduction in female fecundity. Ebbert et al. (9) also found costs of trypanosomatid infection in larval *D. melanogaster*—larvae that were fed infected adult carcasses took longer to pupate. In many cases, trypanosomatid virulence in insects is also context dependent; the virulence of *Crithidia bombi* in bumblebees, for instance, increases under times of nutritional or environmental stress (39). It is therefore likely that the effect we see under laboratory conditions is conservative.

Exposure of *D. melanogaster* larvae to *J. drosophilae* induced a modest but clear change in host gene expression, with a number of immune response-related transcripts upregulated (see Fig. S3 in the supplemental material), including three AMPs. Induction of AMPs has also been demonstrated in tsetse flies and bumblebees exposed to trypanosomatids (1, 40, 41), and AMPs have been shown to have trypanocidal effects in these systems (42, 43). AMP levels in our *J. drosophilae* exposures were lower (i.e., ~4-fold for *DptB*), however, than those induced by pathogenic gut bacteria (31), and this may be due to localization of the response to the gut (we measured gene expression in whole larvae). It is also possible that the induced AMPs could be an indirect byproduct of other gut microbes affected by and/or interacting with *J. drosophilae*. A number of recent studies have found that gut bacteria have important effects on immune responses to trypanosomatids (44, 45). Relatively low but significant increases in AMP expression are also consistent with an early study that examined *D. melanogaster* immune responses to oral exposure to mosquito and bumblebee trypanosomatid parasites (10).

Interestingly, mutant Imd-deficient *D. melanogaster* did not show higher levels of *J. drosophilae* infection, further suggesting that the AMP induction observed in our larval transcriptome may not be a direct consequence of *J. drosophilae* infection. This also appears very different from tsetse flies, in which the Imd pathway appears to play an important role in limiting trypanosome infections (40). Of course, there are many crucial differences between tsetse fly-*Trypanosoma* and *Drosophila*-*J. drosophilae* interactions, including the importance of a blood meal in trypanosome transmission and the presence of *Wigglesworthia* bacteria, obligate nutritional symbionts of tsetse flies that also play a critical role in immunity to parasites (46).

In contrast to Imd mutants, flies deficient in drosocrystallin were more permissive to *J. drosophilae*. An intact PM thus appears to be an important component of the *Drosophila* defense against trypanosomatids, similar to what has been shown for bacterial enteric infections (34). The PM has also been implicated in resistance to trypanosomatids in other dipterans, where it can act as a physical barrier, as has been suggested for *Leishmania* development in sand flies (33), or it could modulate host immune responses, as proposed for tsetse flies (47). Our results favor the former scenario, but further study is necessary to identify the precise role of the PM in resistance to *J. drosophilae*.

When injected into host hemolymph, *J. drosophilae* proliferated, causing a high mortality level, but we did not observe an upregulation of either diptericin or drosomycin, a readout for the Imd or Toll pathway, respectively. This is in contrast to the upregulation of diptericin, drosomycin, and drosocin observed when *Crithidia* parasites from mosquitoes and bumblebees were injected into *D. melanogaster* (10). Still, induction of AMPs in that study was lower than that provoked by injection of bacteria (11),

and the authors did not observe a clear negative relationship between AMP induction and the parasite-induced mortality rate. Thus, our results suggest that *J. drosophilae* is not recognized by the fly immune system when injected—at least the Toll and Imd pathways. This might be because, as a eukaryote, it lacks easy determinants of recognition.

In sum, this is the first of an apparently diverse and ubiquitous lineage of parasites from *Drosophila* to be characterized and allows the exploitation of a rich body of knowledge and experimental tools to study insect-trypanosomatid interactions, particularly monoxenous infections. In addition to beginning to unravel the ecology and dynamics of *Drosophila*-trypanosomatid associations, the infection of *D. melanogaster* makes *J. drosophilae* an excellent model for understanding insect immunity to trypanosomatids more generally.

MATERIALS AND METHODS

For details, see Text S1 in the supplemental material. In brief, flies were maintained as described in reference 48 or on grape juice agar. Trypanosomatids were isolated from dissected fly guts (19, 49, 50) in BHI medium as described in reference 12, with the pH adjusted to 6.5 in subsequent passages (see Text S1 in the supplemental material). Clonal isolates were obtained through the limiting-dilution method (51), with modification. Imaging was performed as previously described (14–16, 18). The primers used for DNA amplification included S762 and S763 (52) (SSU rRNA), M200 and M201 (15) (gGAPDH), and M167 and M168 (12) (SL RNA). Alignments for phylogenetic analysis were generated in Kalign and trimmed in BioEdit, and analysis was done in MrBayes (3.2.2) and PhyML with model optimization in ModelTest (53–57).

For fitness, within-host dynamics, and host range experiments, larvae were exposed to a homogenate of ground *J. drosophilae*-infected *D. falleni* or a uninfected control line at 1 dpo. DNA extractions were performed as described in reference 48. qPCR analysis of *J. drosophilae* used an assay based on the SSU rRNA gene, with infection intensity reported in GEs based on a standard curve, designed using Primer3 (58).

For RNA-seq of *D. melanogaster* larvae, we extracted RNA from pooled samples (eight larvae per sample) in three replicates per condition (six single-end libraries, one lane of 100-bp Illumina HiSeq 2000). Library construction and sequencing were done by Genome Quebec, producing ~29 million raw reads per library.

Functional immunity challenges of *D. melanogaster* used the lines shown in Fig. 5. For *J. drosophilae* injections, 13.8 nl of pelleted cells suspended in phosphate-buffered saline (PBS) (~14,000 parasites) were injected into the adult thorax with a Nanoject (Drummond Scientific). Bacterial injections used overnight cultures of *Micrococcus luteus* and *Escherichia coli* (mixed in a 1:1 ratio; optical density at 600 nm, 5), while clean-injury controls used PBS. Reverse transcription-qPCR of AMPs used samples of 15 pooled females following the protocols in reference 30.

R v.3.1.2, including the lme4 package (59, 60), was used for statistical analyses, primarily using GLMs or GLMMs appropriate to individual response variables.

RNA-seq data accession number. The raw RNA-seq reads obtained in this study have been deposited in the NCBI Sequence Read Archive under PRJNA277742.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01356-15/-/DCSupplemental.

Data S1, CSV file, 1.6 MB.
Text S1, DOC file, 0.2 MB.
Figure S1, EPS file, 0.04 MB.
Figure S2, EPS file, 0.05 MB.
Figure S3, TIF file, 0.1 MB.
Table S1, DOCX file, 0.1 MB.

ACKNOWLEDGMENTS

We thank two anonymous reviewers for comments that improved the manuscript.

This work was funded by an NSERC Discovery grant to S.P., and P.H. was supported by an NSERC scholarship. This work was also supported by the Czech Grant Agency (no. 14/23986S) and the Praemium Academiae award to J.L. V.Y. received financial support from the Moravskoslezsky' Kraj research initiative. A.D. was supported by a Sciex fellowship. This work was also supported by a Sinergia grant from the Swiss National Science Foundation to S.P. and B.L.

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