Wolbachia-Mediated Antiviral Protection in Drosophila Larvae and Adults following Oral Infection

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Understanding viral dynamics in arthropods is of great importance when designing models to describe how viral spread can influence arthropod populations. The endosymbiotic bacterium *Wolbachia* spp., which is present in up to 40% of all insect species, has the ability to alter viral dynamics in both *Drosophila* spp. and mosquitoes, a feature that in mosquitoes may be utilized to limit spread of important arboviruses. To understand the potential effect of *Wolbachia* on viral dynamics in nature, it is important to consider the impact of natural routes of virus infection on *Wolbachia* antiviral effects. Using adult *Drosophila* strains, we show here that *Drosophila-Wolbachia* associations that have previously been shown to confer antiviral protection following systemic viral infection also confer protection against virus-induced mortality following oral exposure to Drosophila C virus in adults. Interestingly, a different pattern was observed when the same fly lines were challenged with the virus when still larvae. Analysis of the four *Drosophila-Wolbachia* associations that were protective in adults indicated that only the w1118-*w*MelPop association conferred protection in larvae following oral delivery of the virus. Analysis of *Wolbachia* density using quantitative PCR (qPCR) showed that a high *Wolbachia* density was congruent with antiviral protection in both adults and larvae. This study indicates that *Wolbachia*-mediated protection may vary between larval and adult stages of a given *Wolbachia*-host combination and that the variations in susceptibility by life stage correspond with *Wolbachia* density. The differences in the outcome of virus infection are likely to influence viral dynamics in *Wolbachia*-infected insect populations in nature and could also have important implications for the transmission of arboviruses in mosquito populations.

Arthropods harbor a wide range of viruses that can be transmitted between individuals or populations of the same species or can bridge the interspecies gap to infect plants or other animals. The outcome of viral infections can be modulated by tripartite interactions between arthropods, viruses, and bacteria (1). One such interaction is the tripartite interaction between insects, viruses, and the endosymbyotic bacterium *Wolbachia pipientis*.

Wolbachia spp. have gained much attention due to the antiviral effects they confer to their host. The impact of *Wolbachia* spp. on virus infection was first described in the *Drosophila melanogaster* host, where it was shown to protect against mortality induced by diverse viruses, including Drosophila C virus (DCV), cricket paralysis virus, and Flock House virus (2, 3). Since that discovery, *Wolbachia*-mediated antiviral effects have been demonstrated in a number of insect hosts and are being investigated as a way of limiting spread of arboviruses (reviewed in references 1, 4, 5, and 6). Notably, *Wolbachia*-mediated antiviral effects have been demonstrated in adult mosquitoes artificially infected with *Wolbachia*; in mosquitoes, *Wolbachia* can interfere with accumulation and transmission of important human pathogens, including dengue and Chikungunya viruses (7–17). While in many cases *Wolbachia* confers antiviral effects to its host organism, in some cases the presence of *Wolbachia* can enhance viral susceptibility (18–23). The impact of the presence of *Wolbachia* on virus infection can include two main effects: (i) interference with viral replication/accumulation, and/or (ii) protection against virus-induced mortality. In mosquitoes, *Wolbachia* interferes with viral replication/accumulation, while in *Drosophila*, *Wolbachia* can interfere with viral replication/accumulation and/or protect flies from virus-induced mortality. In this paper, we focus on the effect of *Wolbachia* on the survival of the host, and we define protection as a reduction/delay in virus-induced mortality.

The mechanisms involved in *Wolbachia*-mediated antiviral effects have not yet been fully elucidated. There is some evidence that microRNAs (24, 25), competition for host-derived resources (26), and elevated reactive oxygen species (27, 28) may influence antiviral effects. *Drosophila-Wolbachia* associations can be subdivided into two groups: protective and nonprotective. The *Drosophila-Wolbachia* pairings CO-*w*Au, DSR-*w*Ri, w1118-*w*Mel, and the overreplicating and life-shortening w1118-*w*MelPop association all show a delay in DCV-induced mortality when DCV is injected into adult flies, while the N7NO-*w*No and DSH-*w*Ha combinations did not (2, 3, 11, 29). A feature that all protective *Wolbachia* strains share is high density within their respective host organism, indicating that high *Wolbachia* density may serve as a prerequisite for antiviral protection (7, 12, 29–34).

Wolbachia is estimated to infect 40% of all insects (35); therefore, the effect it exerts on natural viral dynamics could be pronounced. The understanding of natural tripartite *Drosophila*-virus-*Wolbachia* interactions is very limited at present, partially due to a lack of a method for orally delivering the virus. Recently, three methods for oral infection of larvae and adults were described, and these will allow us to study the effects of the

oral route of infection on antiviral protection mechanisms in *Drosophila* (36–38).

To investigate the effects of *Wolbachia* on virus-induced mortality following oral infection, we used DCV, a natural *Drosophila* pathogen and the most widely studied Drosophila virus (39). DCV is a positive-sense RNA virus that belongs to the *Dicistroviridae* family (40). When injected into flies, DCV is pathogenic, causing mortality within 4 to 6 days postinjection (41). Injection of DCV is a useful method to study *Wolbachia*-DCV interactions; however, injection bypasses the fly's natural immune barriers present within the midgut and can cause a differential immune response compared to exposure via virus feeding alone (37). DCV infection by ingestion is less pathogenic than by injection (36, 37) and represents a more natural *Drosophila*-DCV interaction. While *Wolbachia*-mediated protection has been extensively studied in adult flies following a systemic infection, it is not yet clear whether the *Drosophila-Wolbachia* associations that are protective for virus-induced host mortality following viral injection also exhibit a similar protective characteristic following the oral route of infection. Ingestion of infected cadavers is thought to be one of the mechanisms through which DCV transmission occurs naturally within an insect population (42); therefore, *Wolbachia*-mediated antiviral protection following the oral route of infection could have a direct impact on viral transmission and maintenance of the virus within a population.

Understanding the potential of *Wolbachia* to affect viral dynamics in natural populations will be facilitated by insights into the impact of antiviral protection on susceptibility throughout the life cycle of the host, following exposure via a natural route of infection. Both *Drosophila* and mosquitoes are holometabolous insects, as they undergo metamorphosis between the larval and adult stages. A wide range of genes coordinate the disintegration of larval structures, where some larval organs are histolyzed and major new growth takes place, altering the morphology and in some cases pathogen susceptibility (43–45). Pathogen susceptibility is often age or life stage dependent and can have a large effect on population dynamics, viral spread, and maintenance of the virus within the population (45–48). Studies focusing on the antiviral effects of *Wolbachia* have to date been conducted solely on adult flies and mosquitoes, without consideration of other developmental stages.

Here, we investigated the effect of *Wolbachia* on virus-induced mortality of *Drosophila* larvae and adults following oral challenge with DCV. By using four *Drosophila-Wolbachia* associations that have previously been shown to be protective for adult insects following viral injection, we show that the *Drosophila-Wolbachia* associations that are protective against virus-induced mortality following injection are also protective following oral infection of adults. In contrast, *Wolbachia* protection at the adult stages is not indicative of protection at larval stages, as only one out of four *Drosophila-Wolbachia* associations that were protective at the adult stage showed protection at the larval stage.

MATERIALS AND METHODS

Drosophila and Wolbachia. Two *Drosophila melanogaster* and three *Drosophila simulans* fly lines were reared on a standard cornmeal medium at a constant temperature of 25°C with a 12-hour light/dark cycle. Paired populations of flies were used that either contained *Wolbachia* (w1118-*w*Mel, w1118-*w*MelPop, N7NO-*w*No, DSR-*w*Ri, and Co-*w*Au) or had been cured of *Wolbachia* by tetracycline treatment (w1118-T, N7NO-T,

DSR-T, and CO-T); flies were maintained on a standard cornmeal medium for at least five generations before use. Gut flora was reconstituted and normalized across fly lines by using standardized methods (31). Briefly, *Drosophila* embryos were transferred to vials containing 150 μl of a bacterial inoculum, which was prepared by adding 2 g of 10-day-old food containing w1118-*w*MelPop flies to 5 ml of sterile water and strained through a fine sterile mesh to remove larvae and embryos. The newly treated flies were checked for the presence of *Wolbachia* by using PCR, to make sure that no cross-contamination had occurred.

Virus. Plaque-purified DCV isolate EB (49, 50) was propagated and purified from Schneider's *Drosophila* line 2 cells (51), and virus titers were determined based on the 50% tissue culture infective dose (TCID$_{50}$), as described previously (29, 49).

DNA extraction. Thirty 0- to 4-h-old larvae or 10 newly emerged male adult flies were pooled to perform DNA extraction. The flies were homogenized using a pestle in 180 μl of extraction buffer and 20 μl of proteinase K. The DNeasy blood and tissue kit (Qiagen) was used to extract the DNA as per the manufacturer's protocol. Three replicates on independent cohorts were performed for each treatment.

Quantitative PCR. The abundance of *Wolbachia* was assessed by quantitative PCR (qPCR) to determine the abundance of the *Wolbachia* surface protein gene (*wsp*) relative to that of either the *D. melanogaster RrpL32* or *D. simulans Act5C* genes. Platinum SYBR green qPCR SuperMix-UDG (Invitrogen) was used per the manufacturer's instruction using the *wsp*-specific primer pair 5′-GCATTTGGTTAYAAAATGGACGA-3′ and 5′-GGAGTGATAGGCATATCTTCAAT-3′ (producing a 185-bp PCR product) (29), *RpL32*-specific primers 5′-GACGCTTCAAGGGAC AGTATCTG-3′ and 5′-AAACGCGGTTCTGCATGAG-3′ (producing a 141-bp PCR product) (49), and *Act5C*-specific primers 5′-GACGAAGA AGTTGCTGCTCTGGTTG-3′ and 5′-TGAGGATACCACGCTTGCTCT GC-3′ (producing a 192-bp PCR product) (30). The Rotor-Gene 6000 thermal cycler (Corbett Life Sciences, Qiagen) was used with the following profile: 95°C for 2 min, followed by 40 cycles of 95°C for 10 s, 52°C for 10 s, and 72°C for 20 s. This was followed by a standard melt analysis to assess the specificity of the amplified product. Two technical replicates (separate qPCRs on the same DNA) were performed for each sample (with a third performed where necessary), and DNA extracted from flies without *Wolbachia* was used as a negative control. Mean normalized *wsp:RpL32* DNA ratios were calculated using qGENE software (52), and statistical analysis included a two-tailed Student's *t* test to compare differences of the means.

Survival bioassay. Virus for larval and adult feeding assays was prepared by injecting flies with either 5,000 infectious units (IU) of DCV or an equivalent volume of phosphate-buffered saline (PBS), which acted as a control. Live flies were collected at 4 days postinjection and stored at −20°C until further use. Thirty PBS- or DCV-injected flies were pooled and homogenized in 300 μl of PBS, and the supernatant was filter sterilized using a Millex GV 22-μm filter (Merck Millipore). Homogenates prepared in this way were used for both adult and larval bioassays. The titers for DCV-injected fly homogenates were measured on four occasions and ranged between 4.4×10^{10} and 2×10^{11} IU/ml.

For adult infections, a modified version of a previously described method was used (37). A mix (250-μl volume) containing 75% fly homogenate (DCV or PBS treated, as described above) and 25% dry yeast was applied to a 1.5- by 1.5-cm filter paper and placed in a vial containing 10 4- to 7-day-old male flies. Flies were incubated with the medium for 24 h at 25°C with high humidity to prevent the food from drying out. Following this period, the flies were transferred to standard cornmeal medium, and mortality was scored daily for 15 days. Three replicates of independent cohorts were performed for each treatment.

Larval infections were performed by spreading DCV- or mock-infected fly homogenates onto petri dishes containing 10 ml of standard cornmeal medium (36). One hundred eggs were collected for each treatment on a wet piece of sterile filter paper and transferred

onto petri dishes containing homogenates from either PBS- or DCV-injected flies. Larvae were maintained on the treatment medium until adult emergence, and they were counted 3 days postemergence. Egg-to-adult survival was determined as the proportion of adults poste-mergence relative to the initial number of eggs at the start of the treatment, and each survival bioassay was replicated 3 times with independent cohorts of insects.

Statistical analysis of the survival bioassay results. We used generalized linear mixed-effects regression (GLMER) models based on a binomial distribution to examine the effect of feeding treatment and coinfection on the mortality of five *D. melanogaster* and *D. simulans* larvae, and we employed the lme4 R package in R 2.15.3 (53) (R Foundation for Statistical Computing, Vienna, Austria). The mortality response, as the binomial count of flies that survived or died for each line, was determined by fitting the feeding treatment (PBS or DCV) and coinfection treatment (absence of Wolbachia [-*wol*] or with a *Wolbachia* strain), as well as the interaction between the two factors. The interaction term compared across the mortality values of each of the feeding treatments across the absence ($-wol$) or presence ($+$ indicated *Wolbachia* strain) of *Wolbachia*. Each model included an experimental replicate as a random factor for analysis of the replicate variance component in each model. For adult survival bioassays, the survival curves were compared using Kaplan-Meier analysis and log rank statistics within the GraphPad Prism program.

RESULTS

***Wolbachia* protection in adult flies following oral challenge with DCV.** Initially, we tested the protective effects of the *Wolbachia* strain *w*Au in a CO fly background (CO-*w*Au) due to a strong antiviral protection observed previously following systemic DCV infection (29). *Wolbachia*-free CO flies challenged with DCV by oral infection showed 40% mortality within 15 days postfeeding. In contrast, CO-*w*Au flies showed a significant reduction in mortality during the same time period, to 7% (Fig. 1A; Kaplan-Meyer analysis, $P < 0.05$). We investigated an additional three *Drosophila-Wolbachia* associations, DSR-*w*Ri, w1118-*w*Mel, and w1118-*w*MelPop, all of which have previously been shown to confer protection against DCV-induced mortality in adult flies following a systemic infection (3, 29, 49), and the results indicated that all three *Drosophila-Wolbachia* associations conferred protection against DCV-induced mortality following the oral route of infection (Fig. 1B, D, and E; Kaplan-Meyer analysis, $P < 0.05$). Because not all *Drosophila-Wolbachia* associations protect against systemic viral infections, we tested a nonprotective association, N7NO-*w*No, to see whether protection would occur following oral virus challenge (29). Feeding the nonprotective N7NO-*w*No flies with DCV led to a nonsignificant difference in virus-induced mortality compared to *Wolbachia*-free flies (Fig. 1C; Kaplan-Meyer analysis, $P > 0.05$). Taken together, these results indicate that *Wolbachia*-mediated protection against virus-induced mortality in adults infected through the oral route was consistent with what was previously reported following injection of virus.

***Wolbachia* protection in larvae following oral challenge with DCV.** To determine whether exposure to *Wolbachia* sp. protects larvae from virus-induced mortality, we orally challenged CO-*w*Au larvae with DCV. We found that in *Wolbachia*-free flies, lar-vum-to-adult mortality increased from about 37% in mock-infected flies to about 46% in DCV-infected flies and that the presence of *Wolbachia* had no significant effect on DCV-induced mortality (Fig. 2A and Table 1). This suggests that the *Wolbachia* strain *w*Au may not protect its host against DCV-induced mortal-

ity following this route of infection at the larval developmental stages.

As no protection was observed in CO-*w*Au larvae, we then investigated whether the lack of protection was specific to this *Drosophila-Wolbachia* association. We investigated other protective *Drosophila-Wolbachia* associations, DSR-*w*Ri and w1118-*w*Mel, and one nonprotective association, N7NO-*w*No. None of these associations showed a significant difference in DCV-induced mortality between larvae with and without *Wolbachia* (Fig. 2B to D and Table 1), suggesting that the lack of *Wolbachia*-mediated protection at the larval stages is not confined to CO-*w*Au flies.

The *Wolbachia* strain *w*MelPop has a strong protective effect in both adult flies and mosquitoes, so we investigated whether w1118-*w*MelPop larvae exhibited a protective phenotype. In this *Drosophila-Wolbachia* association, there was a statistically significant difference in DCV-induced mortality between flies with and without *Wolbachia* (25% and 37% mortality, respectively) (Fig. 2E and Table 1). Unlike the other *Drosophila-Wolbachia* associations, *w*MelPop provided complete protection against DCV-induced mortality (Fig. 2E). Because the ability to confer antiviral effects is strongly associated with *Wolbachia* density in adult flies and mosquitoes and because *w*MelPop is known to be an overreplicative strain, we investigated whether the observed differences in *Wolbachia* protection were associated with differences in *Wolbachia* densities.

***Wolbachia* densities.** *Wolbachia* densities have previously been determined in adults but not in larvae for different *Drosophila-Wolbachia* associations. Using qPCR, we determined *Wolbachia* densities at both the larval and adult stages for all five *Drosophila-Wolbachia* associations used in this study (Fig. 3). In adults, the densities of the protective *Wolbachia* strains *w*Au, *w*Ri, *w*Mel, and *w*MelPop were significantly higher than that of the nonprotective *w*No strain, providing an association between *Wolbachia* density and protection. In contrast, *Wolbachia* strains *w*Au, *w*Ri, and *w*Mel showed lower abundance at the larval stage than at the adult stage (two-tailed Student's *t* test, $P < 0.05$) (Fig. 3A and B), while the *Wolbachia* strain *w*MelPop showed high densities at both the larval and adult stages (Fig. 3A). *Wolbachia* density in the nonprotective N7NO-*w*No larvae remained lower than in either the *w*Ri- or *w*Au-treated insects at both developmental stages (Fig. 3B), consistent with a lack of protection.

DISCUSSION

The importance of the route of pathogen entry on the outcome of infection has been well-documented following bacterial infections in *Drosophila*. Injecting bacteria into the hemocoel induces a systemic immune response (54–57), while oral infections often lead to localized immune induction in the gut, often making the bacteria less pathogenic (58–60). A recent paper that showed the involvement of the Toll immune pathway in mediating resistance to oral viral infection with DCV, Flock House virus, cricket paralysis virus, or Nora virus, however, showed no influence of the viral exposure pathway following a systemic bacterial infection (37), indicating that the route of viral entry can have an effect on the host's response to viral infection.

We used a natural route of DCV infection, oral feeding, to investigate the effect of *Wolbachia* on protection against virus-induced mortality, in order to determine whether *Wolbachia*-mediated protection is confined to systemic viral infections in *Dro-
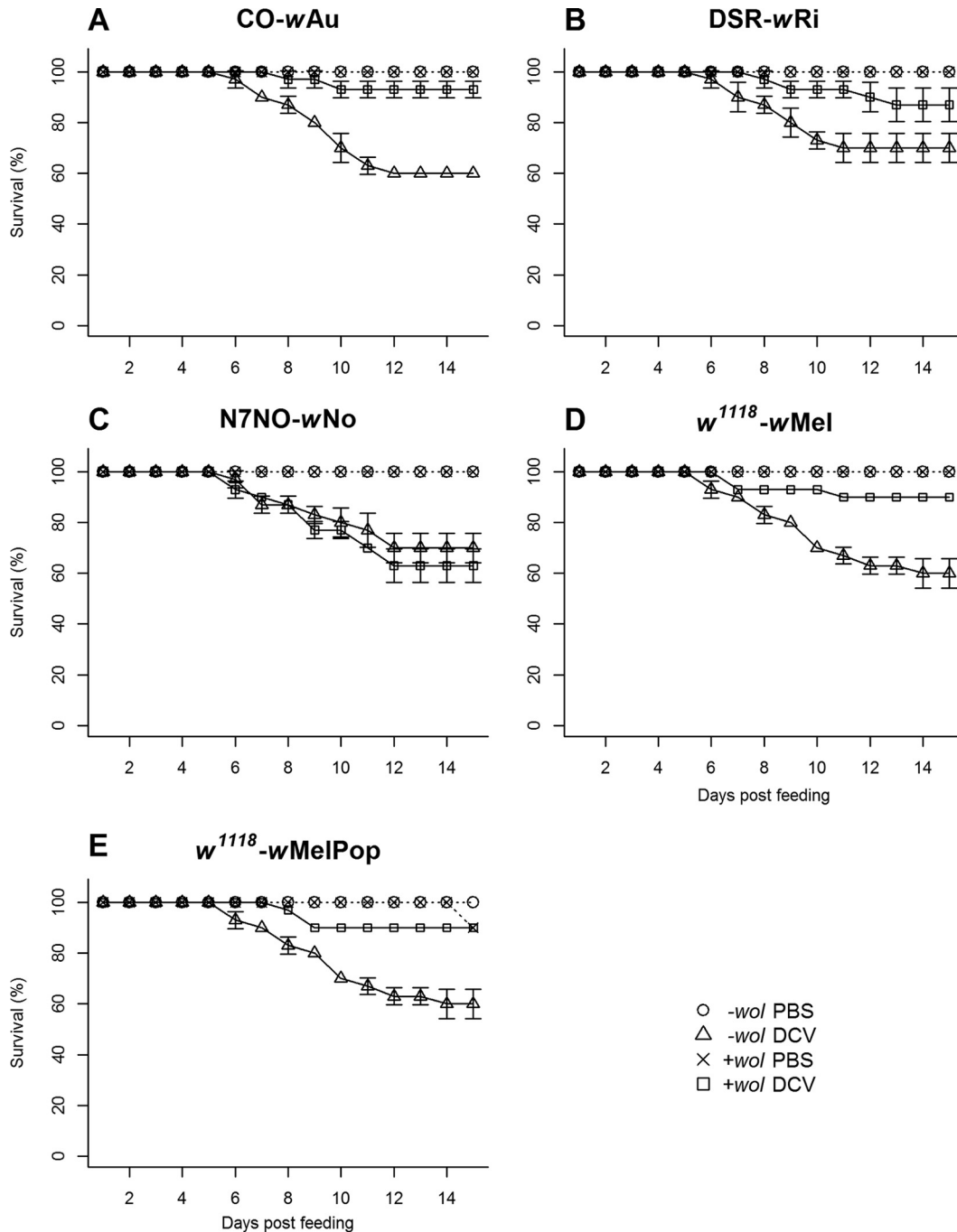
FIG 1 Survival of adult flies following oral challenge with DCV. Each fly strain contained a *Wolbachia* strain (+*wol*) or was tetracycline treated to remove *Wolbachia* (−*wol*). Adult flies were exposed to either homogenates from DCV-infected or mock-infected (PBS) flies for 24 h before being transferred to vials containing standard cornmeal medium. Survival of flies is shown from 3 biological replicates of 10 flies (or from 1 replicate of 10 flies for PBS controls). Statistically significant differences in survival were determined by using the log rank test on Kaplan-Meier curves.

sophila. By examining *Wolbachia*-mediated protection in adult flies across four *Drosophila-Wolbachia* associations that had previously been shown to be protective following systemic bacterial infection, we found that oral DCV infections led to a reduction in virus-induced mortality in adult flies infected with *Wolbachia* compared to *Wolbachia*-free flies (Fig. 1). These findings are consistent with a recently published report (37) and support the idea

that *Wolbachia*-mediated protection extends beyond systemic viral infections and could be used in future experiments to better understand the effects of *Wolbachia* on viral dynamics in natural insect populations.

While in adults the *Wolbachia*-mediated reduction in virus-induced mortality is comparable between systemically and orally infected flies, the same is not always true in larvae. Of the four
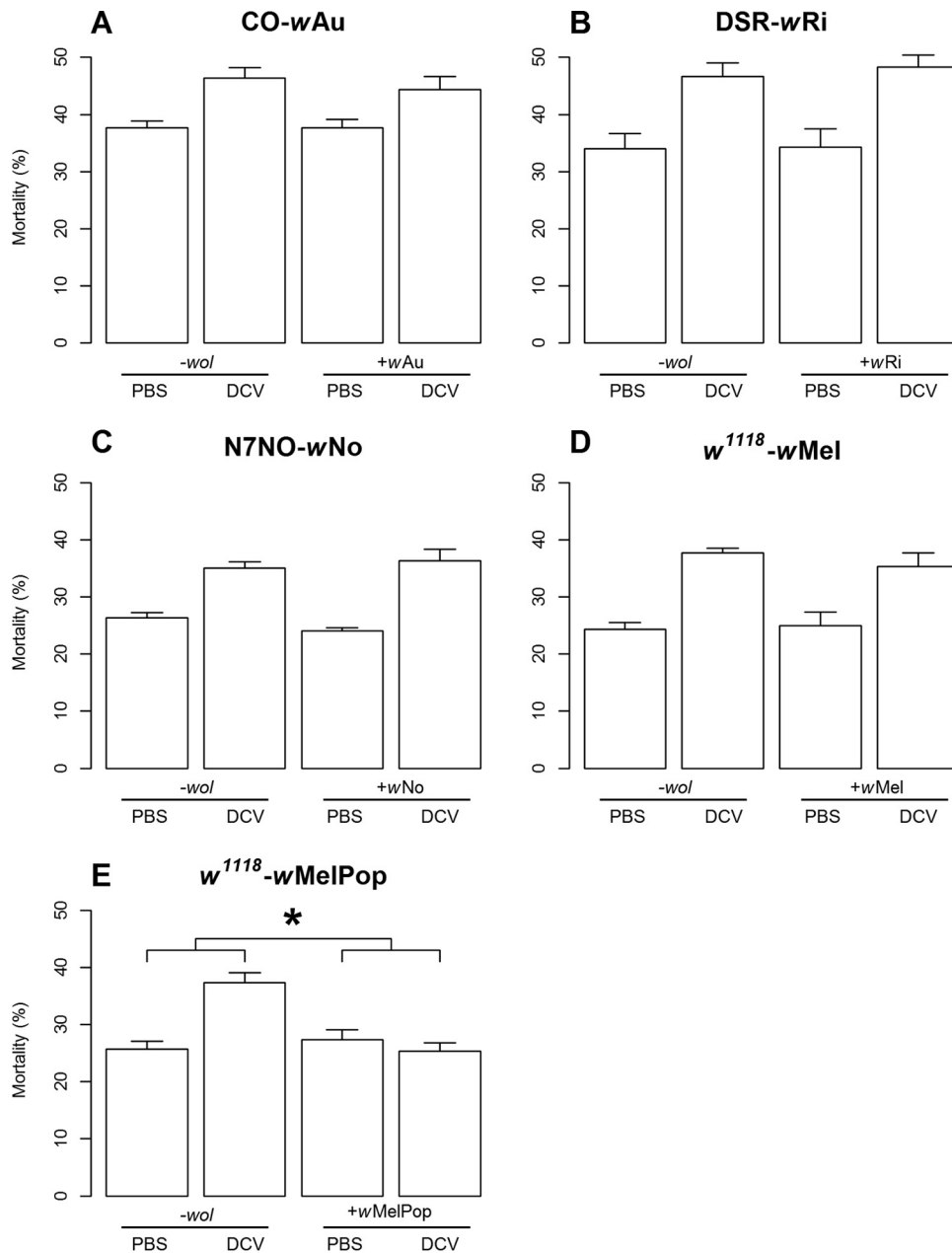
FIG 2 The impact of *Wolbachia* on virus-induced mortality in DCV-infected *Drosophila* larvae. Each fly line was exposed to a different *Wolbachia* strain or was tetracycline treated to remove *Wolbachia* (−wol), as indicated on the x axis. Larvae were exposed to homogenates from either DCV-infected or mock-infected (PBS) flies. Graphs display means and standard errors from three replicates with 100 individuals per fly strain. *, significant interaction ($P < 0.05$) between the feeding treatment and presence or absence of *Wolbachia* for mortality.

Drosophila-*Wolbachia* associations that showed protection following DCV infection in adults, only the w1118-*w*MelPop flies showed protection against DCV-induced mortality during the larval stages (Fig. 2E). These results suggest that *Wolbachia*-mediated protection may vary between different life stages of the same *Drosophila*-*Wolbachia* association, although it is possible that the amounts of virus ingested by larvae and adults are different. Since *Wolbachia* density has previously been shown to be important for mediating antiviral effects, we measured *Wolbachia* density in adults and found that there was congruence between *Wolbachia* density and protection against DCV-induced mortality following

the oral route of infection. Similarly to adults, *Wolbachia*-protection in larvae was associated with *Wolbachia* density, however interestingly high *Wolbachia* density was only observed in w1118-*w*MelPop larvae, which was also the only association to show protection against DCV-induced mortality at the larval stages. The *w*MelPop strain causes a life-shortening phenotype and is present in relatively high densities in both mosquitoes and *Drosophila* (7, 61–63). The relatively high density and the life-shortening effects of the *w*MelPop strain have been reported to be due to the high copy number of 8 *Wolbachia* genes referred as the Octomom region (31, 62). It remains to be seen whether other strains will be

TABLE 1 Analysis of mortality of *Drosophila* larvae in response to DCV in feed with or without *Wolbachia*[a]

| *Drosophila* line and basis of analysis	Parameter estimate	SE	Z score	P value
CO				
Intercept	−0.147	0.116	−1.269	0.204
Feeding treatments	−0.357	0.166	−2.147	<0.05
Wolbachia treatments	−0.081	0.164	−0.492	0.623
Treatment interaction	−0.081	0.235	0.343	0.731
Replicate variance component	0	0		
DSR				
Intercept	−0.134	0.116	−1.154	0.249
Feeding treatments	−0.53	0.168	−3.152	<0.01
Wolbachia treatments	0.067	0.164	0.409	0.683
Treatment interaction	−0.052	0.237	−0.219	0.827
Replicate variance component	2.4×10^{-11}	4.8×10^{-6}		
N7NO				
Intercept	−0.619	0.121	−5.114	<0.0001
Feeding treatments	−0.41	0.178	−2.296	<0.05
Wolbachia treatments	0.058	0.17	0.341	0.733
Treatment interaction	−0.182	0.254	−0.717	0.474
Replicate variance component	9.7×10^{-15}	9.8×10^{-8}		
w1118				
Intercept	−0.518	0.119	−4.339	<0.0001
Feeding treatments	−0.545	0.178	−3.062	<0.01
Wolbachia treatments	−0.563	0.179	−3.154	<0.01
Treatment interaction	0.649	0.257	2.522	<0.05
Replicate variance component	5.9×10^{-12}	2.4×10^{-6}		
w1118				
Intercept	−0.504	0.119	−4.228	<0.0001
Feeding treatments	−0.631	0.18	−3.51	<0.001
Wolbachia treatments	−0.101	0.17	−0.594	0.553
Treatment interaction	0.137	0.254	0.537	0.591
Replicate variance component	0	0		

[a] Generalized linear mixed-effects regression (GLMER) analysis of the percent mortality in five *Drosophila* lines in response to feeding treatment with PBS or DCV. Each *Drosophila* line was either coinfected with a *Wolbachia* strain (*w*Au, *w*Ri, *w*No, *w*MelPop, or *w*Mel) or was not exposed to *Wolbachia* (−*wol*). Treatment interaction is a comparison of percent mortality across all combinations of feeding treatment (DCV or PBS) and the presence/absence of *Wolbachia*.

protective in larvae and what controls the differences in density between larvae and adults. The finding that *Wolbachia*-protection correlates with *Wolbachia* density is consistent with previous findings in adult flies following a systemic infection (29–32). Gradually reducing *Wolbachia* density in both *Drosophila* adults and mosquito cell culture using tetracycline leads to a dose-dependent loss of antiviral protection (12, 30).

Wolbachia-mediated antiviral protection is not limited to *Drosophila*, and since *Wolbachia* infects up to 40% of all arthropod species (35) it may be important to consider the impact of life-stage susceptibility on arthropod population dynamics and viral transmission. Similarly to *Drosophila*, mosquitoes also undergo metamorphosis, a change that can result in life-stage-dependent

differences in viral susceptibility. Mosquitoes are known to form natural associations with *Wolbachia*; however, it is artificial *Wolbachia* transinfections that have shown promise as a tool for limiting spread of human pathogenic viruses (5, 6). Commonly, there is a focus on transmission of arboviruses that occurs between mosquitoes and human hosts. While this horizontal transmission is responsible for the major health concerns in humans, vertical transmission of arboviruses within mosquito populations can affect the maintenance of the virus within the population (64, 65). Viruses such as dengue virus and Chikungunya virus can be vertically transmitted from an infected adult female to its offspring. Dengue virus can spread vertically under both natural (66–69) and laboratory conditions (70–72). Furthermore, transovarially infected female mosquitoes can transmit dengue virus orally (73). Chikungunya is also capable of vertical transmission under laboratory conditions, which would suggest that similar transmission is possible in nature (64).

Various models have been applied to try to understand the impact of *Wolbachia* on the transmission of dengue virus in its mosquito host (74–76). These models do not consider the effects of vertical transmission on the maintenance of dengue virus within a population, which has been suggested to be an important factor affecting the ability of the virus to persist within the population in rural areas with low human population densities (65). Furthermore, vertical transmission could allow the survival of ar-
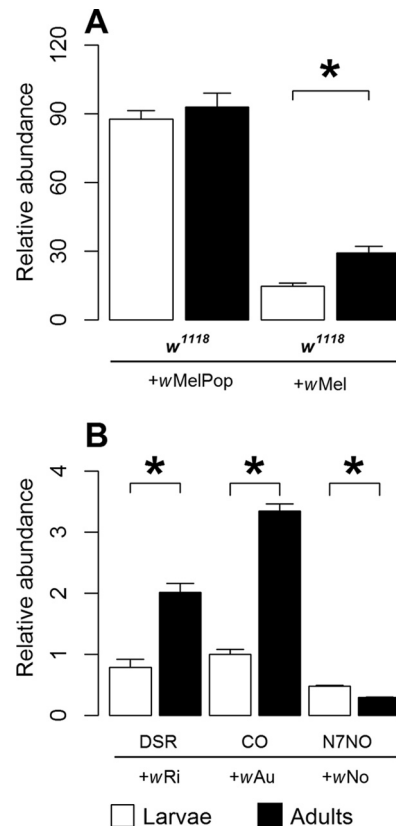
FIG 3 The densities of six different *Wolbachia* strains during larval and adult stages of development. (A) Relative abundance of the *Wolbachia* surface protein gene (*wsp*) in *D. melanogaster*, using *RpL32* as the reference gene. (B) Relative abundance of the *wsp* gene in *D. simulans*, using *Act5C* as the reference gene.

boviruses during adverse climatic conditions and has been suggested to be an important mechanism of maintenance of the virus during interepidemic periods (64). Given the importance of vertical transmission on virus dynamics and the possible life-stage-dependent variations in *Wolbachia*-mediated protection, it is important to consider the impact of *Wolbachia* antiviral protection, or the lack of thereof, on the maintenance of a virus within a population. Understanding the impact of *Wolbachia* antiviral protection at different insect life stages is likely to be an important consideration when designing programs to minimize the spread of insect-borne viruses.

ACKNOWLEDGMENTS

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