Somatic *Wolbachia* (Rickettsiales: Rickettsiaceae) Levels in *Culex quinquefasciatus* and *Culex pipiens* (Diptera: Culicidae) and Resistance to West Nile Virus Infection

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ABSTRACT The endosymbiotic bacteria *Wolbachia pipientis* Hertig infects a wide variety of insect species and can increase viral resistance in its host. *Wolbachia* naturally infects *Culex quinquefasciatus* Say and *Culex pipiens* L. mosquitoes, both vectors of West Nile virus (WNV). We recently demonstrated that *Wolbachia* infection of *Cx. quinquefasciatus* laboratory strain Ben95 increases host resistance to WNV infection, reducing vector competence. This observation raised the possibility that *Wolbachia* could impact vector competence in other populations of *Cx. quinquefasciatus* or *Cx. pipiens*. To investigate this possibility, *Wolbachia* densities were measured in Ben95 *Cx. quinquefasciatus* and compared with densities in a newly established colony of *Cx. quinquefasciatus*, and in field-collected and colonized *Cx. pipiens*. *Wolbachia* densities in somatic tissues of Ben95 *Cx. quinquefasciatus* were significantly higher than densities in the other mosquito populations tested. There was also no significant spatiotemporal variation in *Wolbachia* density in the field-collected *Cx. pipiens*, although significant familial differences were observed. Correlating *Wolbachia* densities and vector competence in individual colonized *Cx. pipiens* indicated that the densities of somatic *Wolbachia* observed in the mosquitoes other than Ben95 *Cx. quinquefasciatus* were too low to inhibit WNV infection and reduce vector competence. These results suggest that the high *Wolbachia* densities capable of inducing resistance to WNV in Ben95 *Cx. quinquefasciatus* are not a general characteristic of *Cx. quinquefasciatus* or *Cx. pipiens* mosquitoes and that the impact of *Wolbachia* on vector competence in field populations of *Cx. quinquefasciatus* and *Cx. pipiens*, if any, is likely to be limited to specific populations.

KEY WORDS *Wolbachia*, West Nile virus, *Culex* mosquito

Wolbachia pipientis Hertig is a gram-negative α-proteobacteria in the order Rickettsiales (Dumler et al. 2001, Fitzpatrick et al. 2006, Werren et al. 2008, Bordenstein et al. 2009). It is a highly successful obligate intracellular symbiont that infects 20–40% of terrestrial arthropod species, including insects, arachnids, and crustaceans (Duron et al. 2008, Cordaux et al. 2012, Zug and Hammerstein 2012). *Wolbachia* is vertically transmitted through the egg cytoplasm, and the frequency with which it infects a given species tends to be either at or near fixation, as in the mosquito species *Culex quinquefasciatus* Say and *Culex pipiens* L., or highly variable, as in the fruit fly *Drosophila melanogaster* Meigen (Hoffman et al. 1994, Rasgon and Scott 2003, Hilgenboecker et al. 2008, Sunish et al. 2011, Verspoor and Haddrill 2011). *Wolbachia*'s success at infecting such a large number of species can be attributed to its ability to manipulate its host's reproduction to increase the reproductive success of infected over uninfected females. *Wolbachia* infection can produce a number of different reproductive phenotypes, including cytoplasmic incompatibility, male killing, parthenogenesis, and feminization (Werren et al. 2008). These phenotypes provide a strong drive mechanism with which a *Wolbachia* infection can spread into uninfected populations (Turelli and Hoffman 1991, Vavre and Charlat 2012). To ensure both vertical transmission and access to the germline cells needed to manipulate reproduction, the principal tissues of *Wolbachia* infection are the germline and somatic tissues of the gonads (Hertig and Wolbach 1924, Frydman et al. 2006, Serbus and Sullivan 2007).

Wolbachia also can cause phenotypes that increase host fitness independent of reproductive effects, including metabolic and nutritional provisioning (Brownlie et al. 2009, Hosokawa et al. 2010), increased longevity (Brelsfoard and Dobson 2011), and increased pathogen resistance (Hedges et al. 2008, Teixeira et al. 2008). Such fitness phenotypes helps explain *Wolbachia* persistence in host species that exhibit weak reproductive phenotypes. Increased resistance to viral pathogens, in particular, has received consid-

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erable attention since its initial discovery in *D. melanogaster* and is being exploited as an approach for biocontrol of disease vectors (Iturbe-Ormaetxe et al. 2011, Mcgraw and O'Neill 2013). *Wolbachia*-mediated viral resistance is cell autonomous and positively correlated with *Wolbachia* density (Moreira et al. 2009, Frentiu et al. 2010, Lu et al. 2012, Osborne et al. 2012). In contrast to reproductive phenotypes, the principal tissues important for *Wolbachia*-mediated viral resistance are nongonadal somatic tissues of the body where *Wolbachia* and viral infection co-occur. *Wolbachia* is broadly distributed in the somatic tissues of its host (Min and Benzer 1997, Dobson et al. 1999, Zouache et al. 2009).

It is common for *Wolbachia* densities to vary in different *Wolbachia*:host strain combinations and to be positively correlated with the strength of viral resistance (Osborne et al. 2009, 2012; Bian et al. 2010; Mousson et al. 2012). Both *Wolbachia* and the host can contribute to determining strain-specific differences in *Wolbachia* density (see Jaenike 2009 for review). For example, different strains of *Wolbachia* replicate to different levels when coinfecting the same host, illustrating a role for each *Wolbachia* strain in determining its own level of replication (Ijichi et al. 2002, Mouton et al. 2003, Dutton and Sinkins 2004). Alternatively, the same *Wolbachia* strain can replicate to different levels in different host strains or different host species, illustrating that the host can also play a role in determining *Wolbachia* replication levels (Berticat et al. 2002, Mcgraw et al. 2002, Ikeda et al. 2003, Kondo et al. 2005, Mouton et al. 2007).

Wolbachia densities can also vary dramatically between individual field-collected adults from a single host population. *Wolbachia* density has been reported to vary as much as 20,000-fold between individual *Drosophila innubila* Spencer (Unckless et al. 2009) and as much as 178,000-fold between individual *Aedes albopictus* Skuse (Ahantarig et al. 2008). The cause for such large differences in *Wolbachia* density is unknown. Under laboratory conditions, *Wolbachia* density varies in response to physiological and environmental variables. For example, *Wolbachia* density in adults can change with age (Duron et al. 2007, Tortosa et al. 2010), and with different temperatures and growth conditions during development (Dutton and Sinkins 2004, Mouton et al. 2006, Wiwatanaratanabutr and Kittayapong 2009, Bordenstein and Bordenstein 2011). However, changes in *Wolbachia* density caused by environmental variables, at least tested under laboratory conditions, are usually relatively small, and sometimes nonexistent (see Correa and Ballard 2012), so it is unclear the extent to which such variables impact *Wolbachia* density in the field and whether the effect of such variables are sufficient to explain the large differences in *Wolbachia* density that have been reported for some host species.

Wolbachia naturally infects a number of mosquito species that are important vectors of human pathogens, including *Ae. albopictus*, an important vector of dengue virus (DENV; Wright and Barr 1980, Sinkins et al. 1995), and *Cx. quinquefasciatus* and *Cx. pipiens*, both important vectors of West Nile virus (WNV; Hertig and Wolbach 1924, Rasgon and Scott 2003). Although determination of vector competence in mosquitoes is clearly complex (Tabachnick 2013), the presence of *Wolbachia* in these mosquito species raises the possibility that native *Wolbachia* infections could impact vector competence by modulating the mosquito's susceptibility to viral infection. Although *Wolbachia*-induced viral resistance in native hosts has been studied most in species of *Drosophila*, where viral resistance can be quite strong, recent evidence suggests that *Wolbachia* also can induce viral resistance in native mosquito hosts and reduce their ability to transmit virus. Native *Wolbachia* infections of some populations of *Ae. albopictus* reduce the mosquito's ability to transmit DENV (Mousson et al. 2012), and we recently demonstrated that native *Wolbachia* infections of *Cx. quinquefasciatus* can reduce the mosquito's ability to transmit WNV (Glaser and Meola 2010).

The native *Wolbachia* infection in a long-term laboratory colony of *Cx. quinquefasciatus* (Ben95) inhibited WNV infection and significantly reduced the frequency with which the mosquitoes transmitted virus (Glaser and Meola 2010). This observation raised the possibility that *Wolbachia* could impact vector competence in field populations of *Cx. quinquefasciatus* such that variation in *Wolbachia* density could contribute to variation in vector competence between individual mosquitoes or between different *Cx. quinquefasciatus* populations. It is unknown, however, whether the observation of *Wolbachia*-induced resistance to WNV infection in the Ben95 colony of *Cx. quinquefasciatus* can be extrapolated to other *Cx. quinquefasciatus* populations, or to other *Wolbachia*-infected *Culex* species, such as *Cx. pipiens.* To address this question, we measured *Wolbachia* density in the somatic tissues of Ben95 *Cx. quinquefasciatus* and compared it with densities in a colony of *Cx. quinquefasciatus* newly collected from the field and maintained only a few generations in the laboratory before being assayed, and with densities in colonized and field-collected *Cx. pipiens.* In all cases, the density of *Wolbachia* in somatic tissues of Ben95 *Cx. quinquefasciatus* was significantly higher than densities in the other mosquitoes tested, and furthermore, the lower densities of somatic *Wolbachia* in the other mosquito populations do not appear to inhibit WNV infection and reduce vector competence.

Materials and Methods

Mosquito Colonies. Adult mosquitoes were maintained on 10% sucrose ad libitum at 26°C and 50% relative humidity (RH) and fed chicken blood for egg laying. Larvae were reared at 250 larvae per liter with a water depth of 1.5 cm and fed standardized volumes of ground koi pellets (Kaytee Products, Chilton, WI). The Ben95 colony of *Cx. quinquefasciatus* was established from mosquitoes obtained from Benzon Research, Inc. (Carlisle, PA). The provenance of the Ben95 mosquitoes is unknown, but they have been continuously maintained in the laboratory for at least

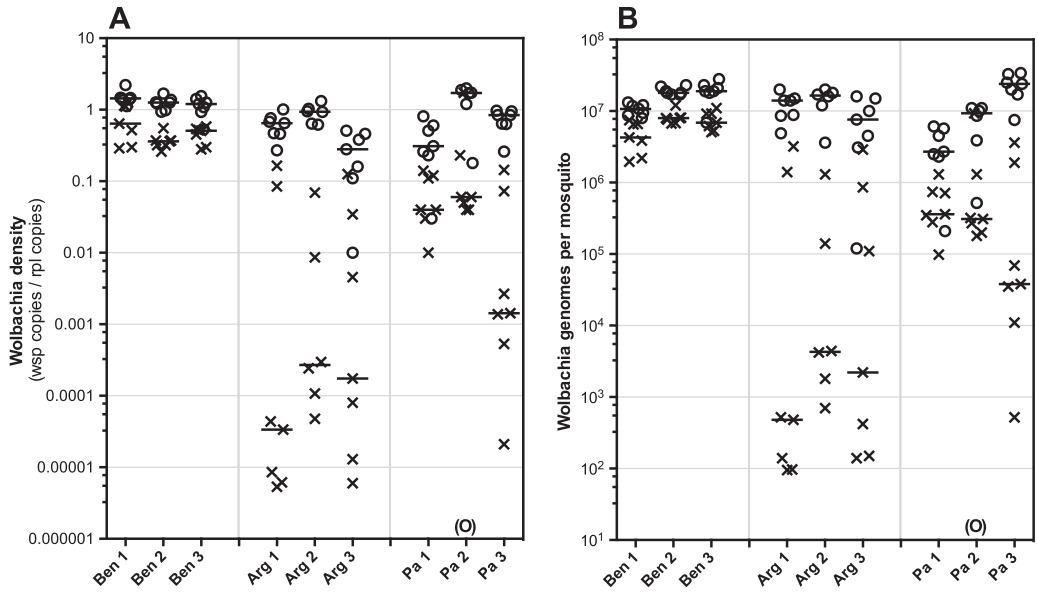

Fig. 1. Levels of *Wolbachia* in the Ben95 (Ben) and Arg12 (Arg) colonies of *Cx. quinquefasciatus*, and the Pa04 (Pa) colony of *Cx. pipiens*. The number of *Wolbachia wsp* gene sequences was measured by PCR and (A) shown normalized to the number of host nuclear ribosomal *RpL32* gene sequences or (B) shown as the absolute number of *Wolbachia* genomes per mosquito. DNA was extracted from sibling progeny from each of three separate females (families numbered 1–3 for each mosquito strain). DNA was extracted from whole females (O) or from ovariectomized females (X). Median values are indicated by a separate horizontal line for each group of whole female and ovariectomized female samples for each family. Samples for which no *Wolbachia wsp* gene sequences were detected are indicated in parentheses at the bottom of the graph.

40 yr (G. Benzon and A. Yousten, personal communications). The Arg12 strain of *Cx. quinquefasciatus* was established at the Wadsworth Center in 2012 from egg rafts collected in Buenos Aires province, Argentina (Micieli et al. 2013). Three egg rafts from the F3–4 generation were used for the analysis of *Wolbachia* density illustrated in Fig. 1. The Pa04 colony of *Cx. pipiens* was established at the Wadsworth Center in 2004 from egg rafts collected in Pennsylvania (Ciota et al. 2007).

Field Collections. Egg rafts of *Cx. pipiens* were collected during a single field season from mid-June through mid-September and from oviposition traps located in urban, suburban, and rural locations separated from each other by ≈10 km and located in and around the city of Albany, NY (42° 39′ N, 73° 45′ W). A rabbit chow infusion spiked with 3-methylindole was used as the attractant in the oviposition traps (Beehler et al. 1994). Each egg raft was hatched, and DNA extracted from first-instar larvae was used in a polymerase chain reaction (PCR) reaction to identify egg rafts that were from *Cx. pipiens* vs. *Culex restuans* Theobald or *Culex salinarius* Coquillett, species that are also found in the region. The primers used were CP16, PQ10, and R6, as described previously (Crabtree et al. 1995). Species identification was confirmed by examining the morphology of fourth-instar larvae. Larvae from each *Cx. pipiens* egg raft were reared to adulthood under standardized laboratory conditions, after which *Wolbachia* levels were measured in adult females 3–5 d old.

***Wolbachia* Quantitation.** *Wolbachia* density was measured by quantitative real-time PCR. DNA was extracted from individual females using the DNeasy Blood and Tissue Kit (Qiagen, Venlo, Netherlands). For ovariectomized females, ovaries were removed by dissection in Ephrussi-Beadle Ringer solution, being careful to ensure that all ovary tissue was removed. Mock dissection controls, in which the ovaries were removed but combined with the carcass for DNA extraction, gave the same *Wolbachia* densities as whole mosquitoes (R.L.G., unpublished data), demonstrating that the lower *Wolbachia* densities seen in ovariectomized mosquitoes were due solely to removing the ovaries and not loss during dissection of *Wolbachia* located in the hemolymph or hemocyes. *Wolbachia* density was determined by measuring the number of *Wolbachia wsp* gene sequences relative to the number of *ribosomal protein L32* (*RpL32*) gene sequences in DNA from each mosquito. General *Wolbachia wsp* gene primers 81 F and 691 R were used as described previously (Zhou et al. 1998). Insect *RpL32* gene sequences were measured using primers RpL32-F2 (5′-AAG CCG AAA GGT ATC GAC AA-3′) and RpL32-R2 (5′-CAG TAG ACG CGG TTC TGC AT-3′), which bind regions of the *RpL32* gene conserved between *Cx. quinquefasciatus* and *D. melanogaster* and give a 190-bp product. PCR reactions were done using USB Hot Start-IT SYBR Green quantitative PCR (qPCR) Master Mix buffer (Affymetrix, Santa Clara, CA), 0.2 μM of each primer, and 0.5 μl of template DNA. The same amplification profile was used for both

wsp and *RpL32*: 95°C 2 min, followed by 40 cycles of 95°C 15 s, 60°C 30 s, 72°C 1 min, followed by a standard melt curve to confirm the specificity of each reaction. Duplicate reactions were run for each gene. The Ct values for duplicate reactions, which always differed by <5%, were averaged for each calculation. After averaging, Ct values for each gene were converted to absolute copy numbers using standard curves that were run on each 96-well plate of samples. The standard curves were log dilution series of 10^6 to 10^2 copies of each gene sequence generated using cloned PCR products for the *wsp* and *RpL32* genes from *Cx. pipiens*. *Wolbachia* density was calculated as the copy number of *wsp* gene sequences divided by the copy number of *RpL32* gene sequences in each sample.

The Mann-Whitney test was used for all pair-wise comparisons to determine significance between median *Wolbachia* densities. ANOVA was used for all group-wise comparisons to determine significance of average *Wolbachia* densities among families. For statistical analyses that included negative *wsp* results, Ct values at the limit of detection for the assay were used instead of zero values or dropping the data from the analysis. This had minimal effect on the statistical results and did not affect any conclusions drawn from the analyses. Pearson's *r* correlation test was used to compare the median *Wolbachia* densities in the whole-body vs. somatic tissue samples within each family of the field-collected *Cx. pipiens.*

Vector Competence. Vector competence assays were performed essentially as described previously (Aitken 1977). Briefly, 3- to 5-d-old females were fed a bloodmeal containing WNV at a final titer of 10^8 pfu/ml. The WNV stock was 03–1956V1C1, a WNV02 genotype virus isolated from the kidney of an American crow collected in New York State in 2003. Pre-blood-fed mosquitoes were collected from the same group of females before blood feeding. Fully engorged mosquitoes were sorted into cups and held at 27°C, 55% relative humidity (RH), and a photoperiod of 16:8 (L:D) h before being assayed. At 7 and 14 d post-bloodmeal, mosquitoes were anesthetized, their legs removed and homogenized, and a sample of their saliva was collected using the capillary tube technique. After saliva collection, the ovaries were removed, and the ovariectomized bodies stored at −70°C until DNA was isolated from the bodies and somatic *Wolbachia* levels quantitated as described (in Wolbachia Quantitation). Vero cell plaque assays were used to quantitate WNV in the leg homogenates and saliva, essentially as described previously (Payne et al. 2006). Mosquitoes with no WNV in the leg homogenate or saliva were categorized as nondisseminated infections. Mosquitoes with WNV only in the leg homogenate were categorized as disseminated infections, and mosquitoes with WNV in both the leg homogenate and saliva were categorized as transmitting infections. WNV titers were measured in the whole bodies of 15 mosquitoes collected at 7 d postbloodmeal to determine the overall frequency of infection, which was 93%. All experiments involving infectious WNV were done in the Wadsworth Center's ACL-3 laboratories.

Results

Cx. quinquefasciatus. *Wolbachia* levels were first measured in mosquitoes from the Ben95 colony of *Cx. quinquefasciatus* in which *Wolbachia*-induced resistance to WNV infection has been demonstrated (Glaser and Meola 2010). *Wolbachia* densities were measured as the number of *Wolbachia wsp* gene sequences normalized to the number of host nuclear ribosomal *RpL32* gene sequences in DNA extracted from individual sibling females from each of three families (Fig. 1A). *Wolbachia* densities in the whole-body samples for all the Ben95 siblings both within and between families were similar, with a median *wsp*-to-*rpl* ratio for all the samples of 1.27 (Fig. 1A, O's). *Wolbachia* densities specifically in somatic tissues were consistently two- to threefold lower than densities measured in whole bodies from siblings from the same family with a median *wsp*-to-*rpl* ratio of 0.45 (Fig. 1A, X's). Although we did not measure *Wolbachia* density directly in isolated ovaries owing to technical limitations, this result indicates that there are at least as many *Wolbachia* in the ovaries as the rest of the somatic tissues of the body combined and that the density of *Wolbachia* in ovaries must be much higher than in somatic tissues given that the ovaries in nongravid females contribute extremely little mass to each whole-body sample, yet increase *Wolbachia* density in the whole-body samples by two- to threefold.

Estimates of the absolute number of *Wolbachia* genomes in each Ben95 mosquito is shown in Fig. 1B. Note that the absolute *Wolbachia* levels are only estimates, because the measurements are not normalized for variation in total DNA content in each sample, and the efficiency of DNA extraction may not be 100%, although the total amount of DNA extracted from each mosquito was about what would be expected. There was generally good concordance in sample-to-sample variation between the measurements of *Wolbachia* density and of *Wolbachia* genome copy number (compare Fig. 1A and B), suggesting that the values for *Wolbachia* genome copy number per mosquito are probably reasonable estimates. There were ≈18 million *Wolbachia* in each Ben95 mosquito with ≈11 million in the ovaries and 7 million in the somatic tissues of the body (Fig. 1B). Based on our earlier studies, this means that the infection of somatic tissues of Ben95 mosquitoes with ≈7 million *Wolbachia* is sufficient to increase resistance to WNV infection and reduce vector competence (Glaser and Meola 2010).

We next considered how representative, with respect to *Wolbachia* levels, the colonized Ben95 mosquitoes are of *Cx. quinquefasciatus* in general, especially given that the Ben95 colony has been maintained in the laboratory for at least 40 yr, so is likely to be more genetically homogenous than mosquitoes in outbred field populations. To address this question, *Wolbachia* levels were measured in *Cx. quinquefasciatus* that were newly collected from the field

and maintained only three to four generations in the laboratory before being assayed (strain Arg12). The Arg12 *Cx. quinquefasciatus* colony was started from >50 females from at least 10 field-collected egg rafts, and hundreds of progeny were used to propagate the colony in each subsequent generation without significant rearing bottlenecks. Such large-scale inbreeding for only three to four generations would have minimal impact on genetic diversity within the population and would be unlikely to provide sufficient time for phenotypic selection or genetic drift to impact *Wolbachia* levels. Therefore, although not collected directly from the field, we reasoned that the Arg12 *Cx. quinquefasciatus* would have *Wolbachia* levels more representative of levels found in outbred field mosquitoes than in mosquitoes from a long-term highly inbred laboratory colony like Ben95.

In general, *Wolbachia* densities in the Arg12 *Cx. quinquefasciatus* were significantly lower and much more variable than in the Ben95 mosquitoes (Fig. 1A). Specifically, median *Wolbachia* densities in the whole-body samples of Arg12 were about twofold lower than in the Ben95 mosquitoes with a median *wsp*-to-*rpl* ratio of 0.57 ($P < 0.0001$), whereas *Wolbachia* densities in somatic tissues of the Arg12 mosquitoes were dramatically lower. A few individual Arg12 mosquitoes had somatic *Wolbachia* densities that approached those measured in Ben95 mosquitoes, but most had densities that were three to five orders of magnitude lower with a median *wsp*-to-*rpl* ratio of only $\approx 10^{-4}$, or 3,200-fold lower than in Ben95 mosquitoes ($P < 0.0001$). Furthermore, somatic *Wolbachia* densities varied >20,000-fold between individual Arg12 mosquitoes compared with only fivefold variation between individual Ben95 mosquitoes. The same relative differences in *Wolbachia* levels between the Ben95 and Arg12 mosquitoes and between the whole-body and ovariectomized samples were also reflected in the estimates of the absolute number of *Wolbachia* per mosquito (Fig. 1B). The median number of *Wolbachia* in whole bodies for all the mosquitoes tested was slightly lower in the Arg12 than in the Ben95 mosquitoes (13 vs. 18 million; $P < 0.05$), whereas the median number of somatic *Wolbachia* was dramatically lower (2,000 vs. 7 million; $P < 0.0001$). In fact, nearly half the Arg12 mosquitoes had less than an estimated 1,000 somatic *Wolbachia*, with some having *Wolbachia* numbers at the limit of detection for the qPCR assay.

Cx. pipiens. We next considered whether the difference in *Wolbachia* levels, particularly in somatic tissues, between long-term colonized vs. newly collected *Cx. quinquefasciatus* would also be true for colonized vs. field-collected *Cx. pipiens*, another *Culex* species naturally infected by *Wolbachia*. *Wolbachia* levels were first measured in *Cx. pipiens* from a colony that has been maintained in the laboratory for 9 yr (Pa04). Median *Wolbachia* densities in whole bodies of Pa04 mosquitoes were comparable with densities measured in Ben95 and Arg12 *Cx. quinquefasciatus*, with *wsp*-to-*rpl* ratios around 1, although the Pa04 mosquitoes demonstrated more pronounced differences between families (Fig. 1A). In contrast, the median *Wolbachia* density in somatic tissues for all the Pa04 mosquitoes was 0.04 *wsp*-to-*rpl* ratio, 11-fold lower than in Ben95 mosquitoes ($P < 0.0001$) but 284-fold higher than in Arg12 mosquitoes ($P < 0.01$; Fig. 1A). There also were pronounced differences in the densities of somatic *Wolbachia* among the Pa04 families tested, with families 1 and 2 having higher and more consistent densities, similar to what was observed in the Ben95 mosquitoes, and family 3 having lower more variable densities, more similar to what was observed in the Arg12 mosquitoes. Therefore, overall, somatic *Wolbachia* densities in the Pa04 *Cx. pipiens* were intermediate between what was observed for the Ben95 and Arg12 *Cx. quinquefasciatus*. Estimates of the absolute number of *Wolbachia* in the Pa04 *Cx. pipiens* paralleled the measurements of *Wolbachia* density. The median number of *Wolbachia* in whole bodies was 8 million, which is lower than observed for the Ben95 (18 million) and Arg12 (13 million) mosquitoes, although the range among individual Pa04 mosquitoes encompassed the same range observed for the *Cx. quinquefasciatus* strains. The median number of somatic *Wolbachia* in the Pa04 mosquitoes was 310,000 per mosquito, which is intermediate between what was observed for Ben95 (7 million) and Arg12 (2,000).

Next, *Wolbachia* levels were measured in field-collected *Cx. pipiens*. *Wolbachia* densities as a function of collection week and landscape type are illustrated in Fig. 2. The median *Wolbachia* density in whole bodies for all the field-collected *Cx. pipiens* was not significantly different from the median density measured in the Pa04 colony ($P = 0.169$). In contrast, the median *Wolbachia* density in somatic tissues for the field-collected *Cx. pipiens* was 0.0024 *wsp*-to-*rpl* ratio, 17-fold lower than in the Pa04 colony ($P < 0.01$). Estimates of the absolute number of *Wolbachia* in the field-collected *Cx. pipiens* paralleled the measurements of *Wolbachia* density. The median number of *Wolbachia* in whole bodies was ≈3.4 million and in somatic tissues was ≈15,000 per mosquito. For convenience, a scale of the approximate number of *Wolbachia* genomes per mosquito in the field-collected *Cx. pipiens* is shown on the right y-axis of Fig. 2, although the data shown are the *wsp*-to-*rpl* ratio measurements of density. There were no significant differences in *Wolbachia* levels as a function of collection site or collection week other than mosquitoes from urban sites, as a group, having a slightly higher median *Wolbachia* density measured in whole bodies compared with mosquitoes from suburban (twofold; $P < 0.0001$) or rural sites (1.5-fold; $P < 0.01$). The same differences, however, were not detected for *Wolbachia* in somatic tissues, which was not surprising given the large variation in somatic densities between individual mosquitoes.

In summary, *Cx. pipiens* showed a similar trend as *Cx. quinquefasciatus* of higher more consistent densities of somatic *Wolbachia* in a long-term laboratory colony vs. lower more variable densities of somatic *Wolbachia* in field-collected (or recently colonized) mosquitoes (Figs. 1 and 2). The magnitude of the

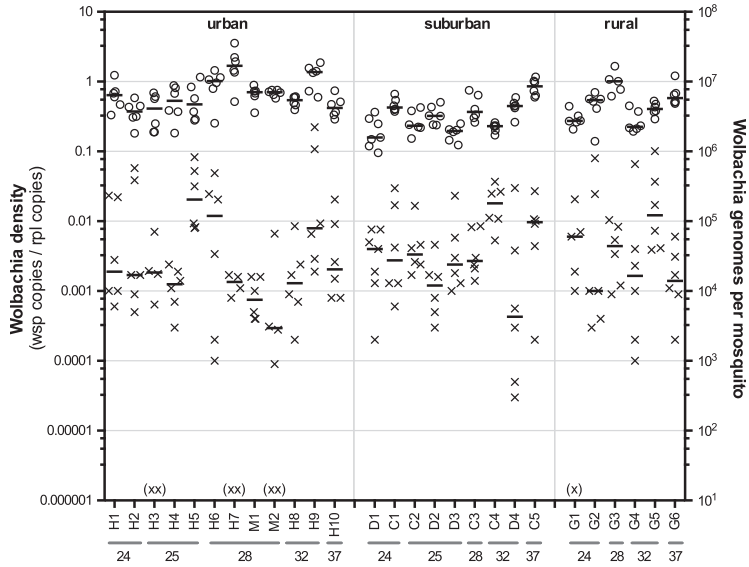

Fig. 2. Levels of *Wolbachia* in field-collected *Cx. pipiens*. Individual egg rafts were collected, larvae hatched and reared to adulthood, and *Wolbachia* densities measured in nongravid sibling females 3- to 5-d-old from each raft. DNA was extracted from whole (O) or ovariectomized females (X). Families are grouped by landscape type. Each column presents the results for a single family, which are named consecutively by collection site. The week of collection is indicated below a gray line that identifies those families collected for that week. The data shown are the number of *Wolbachia wsp* gene sequences normalized to the number of host nuclear ribosomal *RpL32* gene sequences, as indicated on the left y-axis. Median values are indicated by a separate horizontal line for each group of whole female and ovariectomized female samples for each family. The right y-axis illustrates the approximate number of *Wolbachia* genomes per mosquito for each sample. Samples for which no *Wolbachia wsp* gene sequences were detected are indicated in parentheses at the bottom of the graph.

difference, however, was much smaller for *Cx. pipiens* (17-fold) than for *Cx. quinquefasciatus* (3,000-fold).

Because the adults obtained from each individual field-collected *Cx. pipiens* egg raft were analyzed separately, familial differences in *Wolbachia* density could be assessed. There were significant differences in *Wolbachia* density among families in both whole mosquitoes ($P < 0.0001$), which primarily represents *Wolbachia* in ovary, and for *Wolbachia* in somatic tissues ($P < 0.05$). In whole mosquitoes, median *Wolbachia* density differed up to 11-fold among families, with no overlap in the distribution of *Wolbachia* densities between some families (e.g., compare families C4 and C5 in Fig. 2). The same was true in somatic tissues, with median *Wolbachia* density differing up to 71-fold among families, and no overlap in densities between some families (e.g., compare families H4 and H5 in Fig. 2). Familial differences in *Wolbachia* densities did not fall into discernible subgroupings. In fact, rank ordering the median *Wolbachia* densities for all the families illustrates that *Wolbachia* density in this field population of *Cx. pipiens* is a quantitative trait (Fig. 3). Furthermore, familial *Wolbachia* densities were not correlated between the whole-body samples and somatic tissue samples within each family (Pearson's $r = 0.016$; compare upper and lower panels in Fig. 3). Finally, significant familial differences in *Wolbachia* density also were observed in the whole-body samples for the Pa04 *Cx. pipiens* ($P < 0.01$) and Arg12 *Cx. quinquefasciatus* ($P < 0.001$), but not for the Ben95 *Cx. quinquefasciatus* ($P = 0.1191$; Fig. 1).

Vector Competence. The relatively high and consistent densities of somatic *Wolbachia* in Ben95 *Cx. quinquefasciatus* (Fig. 1A) were high enough to increase the mosquito's resistance to WNV infection and reduce their vector competence (Glaser and Meola 2010). By comparison, the densities of somatic *Wolbachia* in the Arg12 *Cx. quinquefasciatus* and the Pa04 and field-collected *Cx. pipiens* were all significantly lower (Figs. 1 and 2), raising the possibility that the levels of *Wolbachia* in these mosquitoes were too low to inhibit WNV infection. In contrast to the Ben95 *Cx. quinquefasciatus*, however, the densities of somatic *Wolbachia* in individual mosquitoes in the Arg12 *Cx. quinquefasciatus* and the Pa04 and field-collected *Cx. pipiens* varied widely, from high densities approaching those in the Ben95 mosquitoes to very low densities, estimated to be only hundreds of bacteria per mosquito, almost certainly too low to inhibit WNV infection and reduce vector competence (Figs. 1 and 2). This variability, however, raises the possibility that differences in somatic *Wolbachia* density between individual mosquitoes could contribute to individual differences in vector competence, such that mosquitoes with high densities of somatic *Wolbachia* might be more resistant to WNV infection, and therefore be less competent vectors, than mosquitoes with low densities of *Wolbachia* within the same population.

To test this hypothesis, we measured both *Wolbachia* density and vector competence in individual *Cx. pipiens* Pa04 mosquitoes. Pa04 mosquitoes were chosen for the analysis because they have somatic *Wolba-*

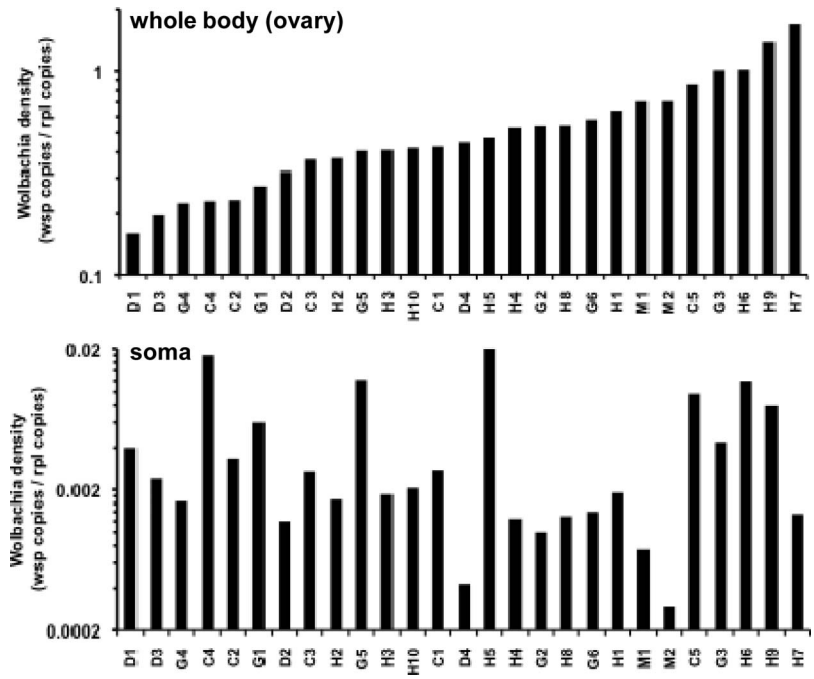

Fig. 3. Rank ordering of field-collected *Cx. pipiens* families by median *Wolbachia* density in whole females (top graph) and in ovariectomized females for the same families (bottom graph).

chia densities lower than in the Ben95 mosquitoes but higher than in either Arg12 *Cx. quinquefasciatus* or field-collected *Cx. pipiens*, yet still demonstrate significant individual variation in *Wolbachia* density across a range that includes densities observed in both the Arg12 *Cx. quinquefasciatus* and field-collected *Cx. pipiens* (Figs. 1 and 2). We reasoned that if the somatic *Wolbachia* densities that occur in the Pa04 mosquitoes were too low to reduce vector competence, then it was likely that the even lower *Wolbachia* levels in the Arg12 *Cx. quinquefasciatus* and field-collected *Cx. pipiens* would be too low to reduce vector competence in these mosquitoes as well. *Cx. pipiens* Pa04 females collected from the colony (not collected as sibling families) were given an infectious bloodmeal of WNV. Mosquitoes were collected at 7 and 14 d postbloodmeal, and legs and saliva were harvested from each mosquito and tested for WNV to identify mosquitoes that had nondisseminated, disseminated, and transmitting infections. These three categories of infection were strongly correlated with increasing WNV titers during infection (Glaser and Meola 2010). After collecting the legs and saliva, the ovaries were removed, and DNA was extracted from each ovariectomized body. The density of somatic *Wolbachia* in the body then was measured and correlated with the category of WNV infection based on the analysis of the leg and saliva samples from the same mosquito. The results of the analysis are shown in Fig. 4. The general increase in median *Wolbachia* densities seen in all the postbloodmeal samples was expected and a consequence of an age-dependent increase in *Wolbachia* density independent of virus infection, as has been reported

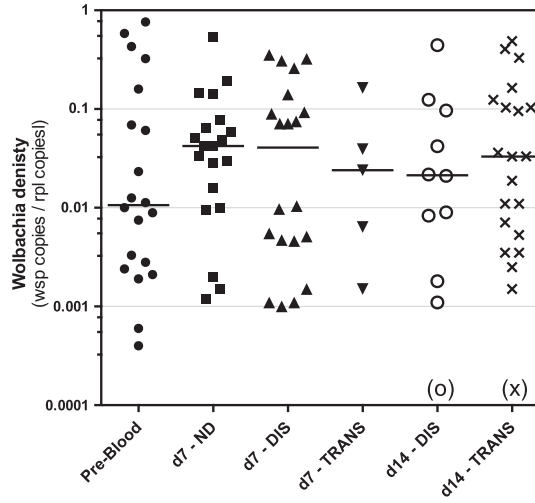

Fig. 4. *Wolbachia* density and vector competence in individual *Cx. pipiens* Pa04 mosquitoes. *Cx. pipiens* Pa04 females were given an infectious bloodmeal of WNV. At 7 and 14 d postbloodmeal (d7 and d14), WNV was measured in the legs and saliva collected from each mosquito to identify mosquitoes that had nondisseminated infections (ND), disseminated infections (DIS), and transmitting infections (TRANS). Only a couple of mosquitoes had nondisseminated infections at day 14 and were not included in the analysis. Median *Wolbachia* density is indicated by a horizontal line. Each sample for which no *Wolbachia wsp* gene sequences were detected is indicated in parentheses at the bottom of the graph.

previously for *Cx. pipiens* (Berticat et al. 2002, Duron et al. 2007). Furthermore, the overall WNV infection prevalence was 93%, based on testing for WNV in a group of whole-mosquito samples assayed from the same population of blood-fed mosquitoes used to measure vector competence. This means that all but one or two mosquitoes in the day 7, nondisseminated category did, in fact, have nondisseminated infections.

The distribution of somatic *Wolbachia* densities did not differ among mosquitoes that had nondisseminated, disseminated, or transmitting infections (Fig. 4). Most importantly, mosquitoes with the highest somatic *Wolbachia* densities were not selectively underrepresented among mosquitoes that were most susceptible to WNV infection. For example, mosquitoes with the highest *Wolbachia* densities occurred just as frequently among mosquitoes with transmitting infections (most WNV susceptible; highest vector competence) as mosquitoes with only disseminated infections (less WNV susceptible; lower vector competence), and even as frequently as mosquitoes with only nondisseminated infections (least WNV susceptible; lowest vector competence). This result suggests that even the highest *Wolbachia* densities that occur in the somatic tissues of *Cx. pipiens* Pa04 mosquitoes are too low to inhibit WNV infection and reduce vector competence.

Discussion

Wolbachia clearly has the capacity to increase resistance to WNV and reduce vector competence in *Cx. quinquefasciatus* mosquitoes in the Ben95 laboratory colony (Glaser and Meola 2010). However, the data presented in the current report demonstrated that somatic *Wolbachia* densities in the Ben95 colony were significantly higher than densities in a newly established colony of *Cx. quinquefasciatus* (Arg12), in a colony of *Cx. pipiens* (Pa04), and in field-collected *Cx. pipiens* (Figs. 1 and 2). Furthermore, the lower levels of somatic *Wolbachia* observed in the mosquitoes other than Ben95 did not appear to be high enough to inhibit WNV infection and reduce vector competence, at least as tested in the Pa04 *Cx. pipiens* (Fig. 4). Therefore, to the extent that the Arg12 *Cx. quinquefasciatus* and the Pa04 and field-collected *Cx. pipiens* were representative of *Cx. quinquefasciatus* and *Cx. pipiens* generally, these results suggest that the native *Wolbachia* infections in these two mosquito species may not play a significant role in modulating vector competence for WNV in many, perhaps most, field populations.

Despite the similarly low *Wolbachia* densities in the Arg12 *Cx. quinquefasciatus*, and Pa04 and field-collected *Cx. pipiens*, the possibility that some populations of *Cx. quinquefasciatus* or *Cx. pipiens* might have *Wolbachia* densities high enough to inhibit virus infection and reduce vector competence cannot be excluded. For example, different strains of *Ae. albopictus* differ in the extent to which *Wolbachia* can inhibit dengue virus infection because of differences in *Wolbachia* density (Bian et al. 2010, Mousson et al. 2012). However, unlike the relatively consistent levels of total *Wolbachia* that thus far have been reported in whole-body field-collected *Cx. pipiens* (Berticat et al. 2002, Echaubard et al. 2010, this study), *Wolbachia* levels in field-collected *Ae. albopictus* can vary dramatically between individual mosquitoes in at least some populations, perhaps contributing to more frequent differences in the ability of *Wolbachia* to inhibit viral infection in *Ae. albopictus* (Ahantarig et al. 2008).

We considered the possibility that *Wolbachia* levels might vary in field populations and potentially reach high enough levels to induce viral resistance and impact vector competence as a function of location, landscape type, or time during the season, but somatic *Wolbachia* levels in the field-collected *Cx. pipiens* did not vary significantly at least over the 10-km range, three landscape types, and single field season that were analyzed (Fig. 2). In addition to supporting the general conclusion that *Wolbachia* levels in *Cx. pipiens* were too low to impact vector competence, this observation also argues against *Wolbachia* playing a role in the significant spatiotemporal variation in vector competence that has been reported for *Cx. pipiens* (Kilpatrick et al. 2010), including some of the same general locations used in our study.

The ability of *Wolbachia* to inhibit WNV infection is correlated with the higher densities of somatic *Wolbachia* in the Ben95 *Cx. quinquefasciatus* mosquitoes compared with lower somatic *Wolbachia* densities in the Arg12 *Cx. quinquefasciatus*, and Pa04 and field-collected *Cx. pipiens*, consistent with studies demonstrating that *Wolbachia*-induced viral resistance is density dependent (Figs. 1 and 2; Moreira et al. 2009, Frentiu et al. 2010, Lu et al. 2012, Osborne et al. 2012). The density of somatic *Wolbachia* in Ben95 mosquitoes may be higher than in the other mosquitoes analyzed, but the density is still significantly lower than densities that are unable to inhibit viral infection in *Drosophila* (Osborne et al. 2012). Osborne and colleagues reported that *Drosophila simulans* Sturtevant infected with *Wolbachia* at densities of 39–118 *Wolbachia* genomes per fly genome were resistant to viral infection (Osborne et al. 2012). Similarly, the density of somatic *Wolbachia* in the Ber1 strain of *D. melanogaster*, which is highly resistant to WNV infection (Glaser and Meola 2010), is ≈77 *Wolbachia* genomes per fly genome (unpublished data). In contrast, *D. simulans* with *Wolbachia* densities at or below 6 *Wolbachia* genomes per fly genome were not protected from infection (Osborne et al. 2012). By comparison, the average density of somatic *Wolbachia* in Ben95 mosquitoes is only 0.46 *Wolbachia* genomes per mosquito genome, or 13-fold lower than densities unable to protect *D. simulans.* It is not known why the lower densities of *Wolbachia* in the Ben95 *Cx. quinquefasciatus* are still able to inhibit infection.

It is unclear why the density of somatic *Wolbachia* in Ben95 *Cx. quinquefasciatus* is so much higher than the other mosquito populations analyzed. It is possible that the density reflects the *Wolbachia* density that was present in the field mosquitoes from which the

Ben95 colony was originally established. This possibility seems unlikely, however, unless there are larger differences in *Wolbachia* density than have thus far been reported for *Culex* mosquitoes. Alternatively, the density of *Wolbachia* in the Ben95 mosquitoes may be a consequence of long-term colonization. Perhaps during the 40 yr that the Ben95 mosquitoes have been maintained in the laboratory, phenotypic selection or genetic drift has produced high somatic *Wolbachia* densities. It is interesting to consider the possibility that exposure of colonized mosquitoes to pathogens present in the laboratory environment over an extended period of time might select for the increased resistance phenotype provided by higher *Wolbachia* densities. It is also interesting to consider the Pa04 colony of *Cx. pipiens* that have been colonized for 9 yr and have somatic *Wolbachia* densities that are intermediate between the high levels in the Ben95 *Cx. quinquefasciatus* and the lower levels in the Arg12 *Cx. quinquefasciatus* and field-collected *Cx. pipiens* (Figs. 1 and 2). Perhaps the 9 yr of colonization for the Pa04 mosquitoes, compared with 40 yr for the Ben95 mosquitoes, has produced an intermediate stage in the selection process.

Wolbachia densities in the field-collected *Cx. pipiens* demonstrated significant familial variation (Fig. 2). The most parsimonious explanation for the origin of this variation is that *Wolbachia* density is genetically determined and that there is sufficient genetic heterogeneity within the *Cx. pipiens* host, *Wolbachia* symbiont populations, or both, to produce the observed continuous distribution of median familial densities (Fig. 3). Familial variation was not a consequence of variation in environmental parameters, as egg rafts were collected within a few hours of being laid and then hatched and reared under standardized laboratory conditions. Unfortunately, the heritability of the familial differences seen in the *Cx. pipiens* could not be tested because the mosquitoes did not mate under laboratory conditions. In addition to the field mosquitoes, however, significant familial differences were also observed in the Pa04 colony of *Cx. pipiens* and Arg12 colony of *Cx. quinquefasciatus* (Fig. 1). Because the colonies were maintained under the same environmental conditions each generation, the observed familial differences, at least in the laboratory colonies, must be caused by genetic heterogeneity that exists within the colony.

Familial differences were observed in *Wolbachia* density in the field-collected *Cx. pipiens* in both the ovary (whole-body samples) as well as somatic tissues of the body (ovariectomized samples), but the familial differences were not correlated between the two tissue types within each family (Fig. 3). This observation suggest that *Wolbachia* infection of somatic tissues, at a minimum, is not simply a consequence of random mislocalization of *Wolbachia* during embryonic development of each mosquito and that the genetics determining *Wolbachia* density in somatic tissues is different from the genetics determining *Wolbachia* density in the ovary.

Herein, we provided the first characterization of variation in somatic *Wolbachia* levels in the natural *Wolbachia*–mosquito host systems of *Cx. quinquefasciatus* and *Cx. pipiens*, both important vectors of WNV. We found that the densities of *Wolbachia* in somatic tissues of Ben95 *Cx. quinquefasciatus* were significantly higher than densities in a newly established colony of *Cx. quinquefasciatus* or in colonized and field-collected *Cx. pipiens.* Furthermore, the lower densities of somatic *Wolbachia* in these mosquito populations are likely too low to inhibit WNV infection and reduce vector competence as occurs in the Ben95 *Cx. quinquefasciatus.* These results indicate that *Wolbachia*-induced resistance to WNV is not generally applicable to all populations of *Cx. quinquefasciatus* and *Cx. pipiens*, and that the impact of *Wolbachia* on vector competence in field populations, if any, is likely to be limited to specific populations of these mosquito species.

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