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Mapping *Wolbachia* distributions in the adult *Drosophila* brain

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Summary

The maternally inherited bacterium *Wolbachia* infects the germline of most arthropod species. Using *Drosophila simulans* and *D. melanogaster*, we demonstrate that localization of *Wolbachia* to the fat bodies and adult brain is likely also a conserved feature of *Wolbachia* infection. Examination of three *Wolbachia* strains (W_{Mel}, W_{Riv}, W_{Pop}) revealed that the bacteria preferentially concentrate in the central brain with low titres in the optic lobes. Distribution within regions of the central brain is largely determined by the *Wolbachia* strain, while the titre is influenced by both, the host species and the bacteria strain. In neurons of the central brain and ventral nerve cord, *Wolbachia* preferentially localizes to the neuronal cell bodies but not to axons. All examined *Wolbachia* strains are present intracellularly or in extracellular clusters, with the pathogenic W_{Pop} strain exhibiting the largest and most abundant clusters. We also discovered that 16 of 40 lines from the *Drosophila* Genetic Reference Panel are *Wolbachia* infected. Direct comparison of *Wolbachia* infected and cured lines from this panel reveals that differences in physiological traits (chill coma recovery, starvation, longevity) are partially due to host line influences. In addition, a tetracycline-induced increase in *Drosophila* longevity was detected many generations after treatment.

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Introduction

An estimated 66% of all arthropods are infected with *Wolbachia* (Hilgenboecker *et al*., 2008), a Gram-negative, intracellular bacterium that is transmitted through the maternal germline (Serbus *et al*., 2008; Werren *et al*., 2008). *Wolbachia* infection often affects the host's reproduction to promote its own transmission (Werren *et al*., 2008). Therefore, much research has focused on *Wolbachia*–host interactions in the germline. However, in some species, including *Drosophila melanogaster, Wolbachia*'s self-promoting effects are weak and it is unclear how *Wolbachia* infection is maintained (Hoffmann *et al*., 1998; Yamada *et al*., 2007). Reports demonstrating *Wolbachia* localization to somatic tissues raise the possibility that *Wolbachia* may influence somatic processes to the benefit of the host. For example, *Wolbachia* has been shown to decrease *Drosophila*'s susceptibility to viral infection (Hedges *et al*., 2008; Teixeira L and Ashburner, 2008; Osborne *et al*., 2012). Other studies point towards a less general *Wolbachia* effect and highlight a host-species dependence of the *Wolbachia* influence. For example, in *D. melanogaster*, *Wolbachia* influences host size (Hoffmann *et al*., 1998) and longevity (Driver *et al*., 2004; Fry *et al*., 2004; Toivonen *et al*., 2007), but the extent of the *Wolbachia* effect and even the direction of the effects vary widely among host strains and species. In some *D. melanogaster* lines, altered behaviour has also been associated with *Wolbachia* infection, such as olfactory-cued locomotion (Peng *et al*., 2008), mating rate (de Crespigny *et al*., 2006; Gazla and Carracedo, 2009) and fertility (Gazla and Carracedo, 2009), but these results also vary with host and *Wolbachia* strains. In laboratory *Drosophila* lines that have been evolved separately towards tolerance for various toxins over a 30-year time period, *Wolbachia* has been shown to contribute significantly to mating discrimination between these populations (Koukou *et al*., 2006). However, no *Wolbachia* effect has been detected within populations, indicating that *Wolbachia* can enhance a mating bias that has evolved independently in these populations (Koukou *et al*., 2006).

Previous studies demonstrated the presence of *Wolbachia* in the brains of *Drosophila* (Min and Benzer, 1997; Albertson *et al*., 2009) and *Collembola* (springtails) (Czarnetzki and Tebbe, 2004), and deduced a *Wolbachia* infection by qPCR in *Eurema hecabe* (Butterfly) (Narita *et al*., 2007) and *Drosophila* (Dobson *et al*., 1999;

McGraw *et al*., 2002). However, it remains unclear whether this is a sporadic event or a conserved feature of *Wolbachia* infection. Here we directly address this issue by taking advantage of *Drosophila simulans* and *D. melanogaster* lines established from wild populations, including lines established in 2003 from a farmer's market collection in North Carolina (Edwards *et al*., 2009). Known as the '*Drosophila* Genetic Reference Panel', these lines have been inbred for at least 20 generations and are widely used for behaviour studies and expression profiles (Ayroles *et al*., 2009; Edwards *et al*., 2009; Morozova *et al*., 2009; Mackay, 2010). The lines were found to have a range of differences in starvation resistance, lifespan, chill coma recovery time, copulation latency and other traits (Ayroles *et al*., 2009). Of the 192 RAL (Raleigh) *D. melanogaster* lines deposited at the Bloomington Stock centre, we used the core group of 40 lines to analyse them with regard to *Wolbachia* infection and related effects on physiological parameters. Significantly, 16 of the lines are infected with *Wolbachia* and were analysed for a bacterial presence in the brain as well as behavioural and physiological responses.

Wolbachia has been shown to localize to the *Drosophila* brain during larval and adult stages (Albertson *et al*., 2009). The *Drosophila* brain arises from divisions of neuronal stem cells. During embryogenesis, neuroblasts continuously divide asymmetrically to produce a self-renewing neuroblast and a primary neuron of the embryonic and larval central nervous system (CNS) (Doe, 2008; Egger *et al*., 2008). Neuroblast divisions continue into larval stages, producing secondary neurons that give rise to the adult central nervous system (Spindler and Hartenstein, 2010). In dividing neuroblasts of infected embryos and larvae, *Wolbachia* has been found to preferentially localize to the self-renewing neuroblast rather than to the primary neuron (Albertson *et al*., 2009). This asymmetric distribution may influence the final *Wolbachia* distribution in the adult *Drosophila* brain.

The adult *Drosophila* brain is composed of approximately 100 000 neurons. The soma of neurons coalesce in certain regions and project their neurites into densely interwoven neuropils, which form distinct lobes (Spindler and Hartenstein, 2010; Yu *et al*., 2010). Functional studies of neuropil have defined the major brain centres (Vosshall and Stocker, 2007; Olsen and Wilson, 2008; Tanaka *et al*., 2012). These include (i) the protocerebrum (several distinct interlinked neuropils), (ii) mushroom body (learning and memory), (iii) antennal lobes (olfactory chemosensory pathways), (iv) the subesophageal ganglion (gustatory neurons and taste behaviour), (v) antennal nerves (convergence of olfactory receptor neurons), (vi) the ventrolateral protocerebrum (visual projection neurons connecting the central brain and optic lobe) and (vii) the optic lobes (comprising the compound eye) (Hanesch *et al*., 1989; Pereanu *et al*., 2010). As described below, we have generated detailed *Wolbachia* distribution maps of the bacterial infection in the brain of four host/*Wolbachia* combinations: W_{Riv} in *D. simulans*, W_{Mel} in *D. simulans*, W_{Mel} in *D. melanogaster* and W_{Pop} in *D. melanogaster*. These studies demonstrate that bacteria localization to the adult brain is a conserved feature of *Wolbachia* infection, yet the specific distribution within the brain differs among different *Drosophila* species and *Wolbachia* strains.

Results

Localization to adult brains is a conserved feature of Wolbachia *infections*

This study examines whether brain infection is always present in *Wolbachia*-infected *D. melanogaster* and *D. simulans* lines. To survey *D. melanogaster*, we assayed the 'core' group of 40 lines from the '*Drosophila* Genetic Reference Panel' (Ayroles *et al*., 2009) for the presence of *Wolbachia*. Infection was determined by PCR using entire flies and by cytology of the ovarioles (Fig. S2). In all 40 lines examined, the PCR and cytological analysis were in accord: 24 lines were uninfected and 16 lines were stably *Wolbachia* infected (Table 1). Sequencing the *wsp* gene from four of the infected lines (304, 360, 712, 820) produced sequences identical to the published *wsp* sequence from w_{Mel} (Tigr, cmr.jcvi.org). *wsp* is one of the fastest evolving *Wolbachia* genes (Baldo *et al*., 2010) and identical *wsp* sequences from flies in a limited geographical may indicate that the lines are infected with the same bacterial strain, although examples of divergent *Drosophila* lines with identical *wsp* sequences exist (Riegler *et al*., 2005). Adult brains from each of the infected *D. melanogaster* lines were dissected from at least two male and female flies and stained with Syto-11, a DNA dye that preferentially stains *Wolbachia* (Casper-Lindley *et al*., 2011). *Wolbachia* was clearly present in all *D. melanogaster* brains examined (Fig. S1). *Wolbachia* infections were also determined by PCR for a field population of *D. melanogaster*, captured in Albion, MI. Six infected lines were stained with Syto-11 and showed *Wolbachia* in the adult brain (data not shown).

To survey *D. simulans*, isofemale lines were established from *D. simulans* flies captured near Davis, California, and tested for *Wolbachia* infection by PCR (Michael Turelli, UC Davis). Fourteen infected lines were analysed for bacteria distribution in somatic and germline tissues. The adult ovaries and brains of four adult females per line were dissected and stained; both tissues showed robust *Wolbachia* titre with a 100% infection frequency ($n = 56$, Fig. S1). *Wolbachia* infections were also determined by PCR for a field population of *D. simulans* captured in the

Table 1. Infection status of flies from the '*Drosophila* Reference Panel'.

Bloomington strain number	RAL number	Infection status
25174	208	–
25175	301	–
25176	303	–
25179	307	–
25180	313	–
25181	315	–
25182	324	–
25184	357	–
25185	358	–
25187	362	–
25188	375	–
25189	379	–
25192	391	–
25192	399	–
25193	427	–
25194	437	–
25196	514	–
25197	517	–
25744	705	–
25745	714	–
25203	732	–
25204	765	–
25205	774	–
25207	799	–
25177	304	+
25178	306	+
25183	335	+
25186	360	+
25445	365	+
25190	380	+
25195	486	+
25198	555	+
25199	639	+
25200	707	+
25201	712	+
25202	730	+
25206	786	+
25208	820	+
25209	852	+
25210	859	+

Flies were analysed by PCR and oocyte cytology to determine the *Wolbachia* infection status of the inbred lines.

Landels-Hill Big Creek Reserve, CA. Several infected lines were stained with Syto-11 and showed *Wolbachia* brain localization similar to the field populations described above (data not shown). Taken together, these data indicate that localization of *Wolbachia* to the *Drosophila* adult brain is likely a conserved feature of *Wolbachia* infection.

Wolbachia *exhibit distinct intra and extracellular distributions in the adult* Drosophila *brain*

Wolbachia localization patterns were further examined at the cellular level within the adult central brain of the *D. melanogaster* laboratory strain that was infected either with the native W_{Mel} *Wolbachia* or with the pathogenic W_{Pop} variant (Min and Benzer, 1997), and within brains of the *D. simulans* laboratory strain infected with W_{Riv} or W_{Mel}. To quantify *Wolbachia*, dissected brain tissue was fixed and stained with propidium iodide (PI), anti-CG9850 antibody, and fluorophore-conjugated phalloidin (Fig. 1). Propidium Iodide stains both host and bacterial DNA, anti-CG9850 fortuitously cross-reacts with *Wolbachia* (Cho, 2004), and phalloidin labels the actin-rich host cell cortex. In uninfected flies, low-intensity anti-CG9850 staining is observed, with occasional small background puncta (Fig. 1). In contrast, infected flies stained with anti-CG9850 show intense puncta that tightly colocalize with propidium iodide puncta. All *Wolbachia* strains that are analysed in this study show this colocalization. In quantitative analyses, *Wolbachia* were scored as both CG9850- and PI-positive staining puncta unless otherwise noted. The stainings also reveal that *Wolbachia* does not reside in axons (Fig. 2A–C), but in or next to the host cell bodies of neurons in the central brain. All examined *Wolbachia* strains either occurred as few, individually discernible dots within the host cell bodies (Fig. 2D–F), or as larger clusters (Fig. 2G–I). *Wolbachia* clusters were especially large and abundant in the W_{Pop} strain and many clusters appeared to be extracellular. Figure 2J shows Z-slices through an area with a large W_{Pop} cluster in *D. melanogaster*. The actin stain (green in the merged image and the top row of individual channel images) shows that the cluster is not surrounded by host cortical actin, indicating that it is extracellular. Furthermore, the identical stain of PI (DNA) and the anti-CG9850 antibody (purple in the merged image and the second and third row of individual channel images) indicates that there is no host DNA in this *Wolbachia* cluster.

Wolbachia *distribution in the brain depends on both host line and* Wolbachia *strain*

The bacteria distribution within the central brain varied among the two *Drosophila* species and the different *Wolbachia* strains. Intracellular as well as cluster infection were quantified in eight brain regions. From a dorsal view of the brain (Fig. 3A and A′), the quantified regions include: (1) the soma between the superior medial protocerebra, (2) the soma posterior to mushroom body and anterior to the antennal lobe, (3) soma posterior to antennal lobes and anterior to suboesophageal ganglion, (4) soma anterior to the superior protocerebrum lobe, (5) soma lateral to antennal lobes and medial to the ventrolateral protocerebrum lobe, (6) soma posterior antennal nerve neuropil and the ventrolateral protocerebrum lobe, (7) soma lateral to the lateral superior protocerebrum, and (8) soma surrounding the optic lobe. Brain cells were quantified as having 0 bacteria, 1 bacterium, 2–5 bacteria, or > 5 bacteria (Table S1). In *D. simulans*, W_{Riv} had a very similar titre in regions 1 through 4, and in regions 5

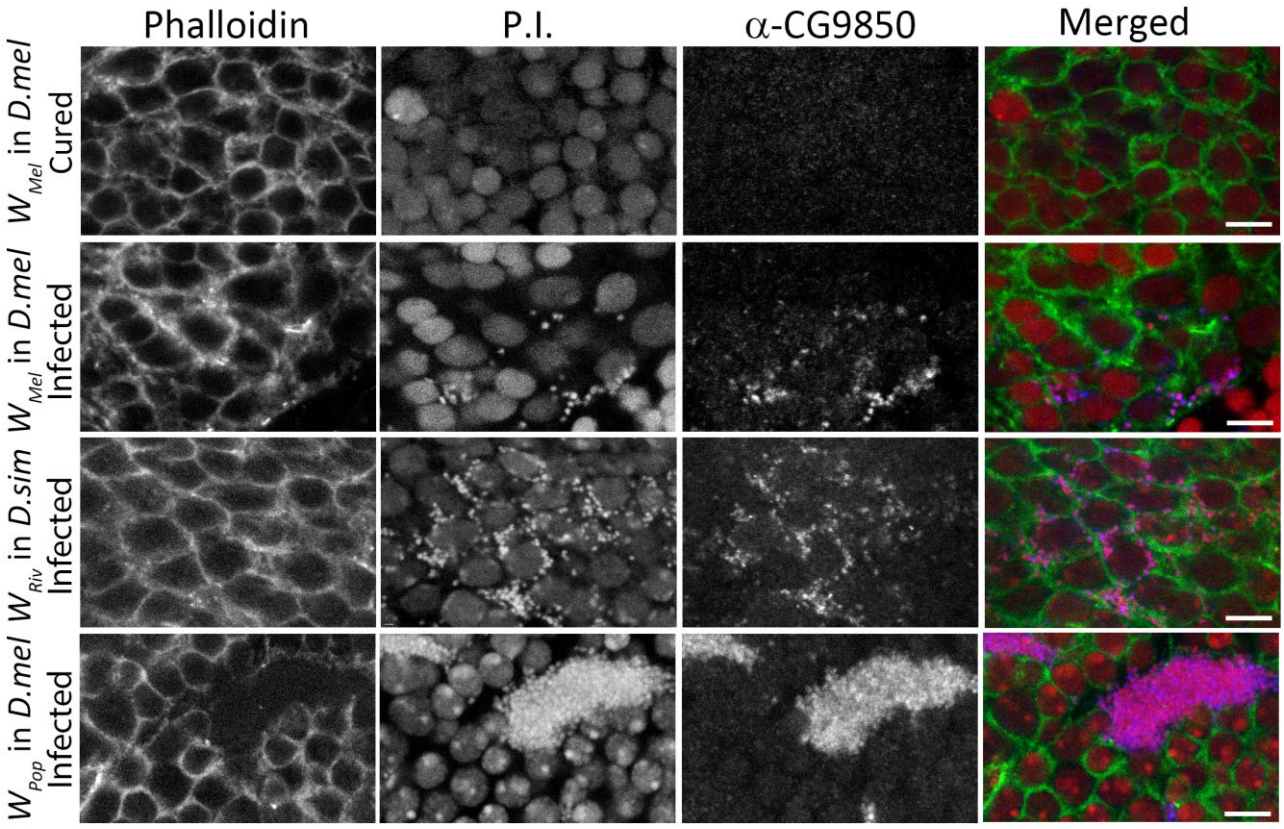
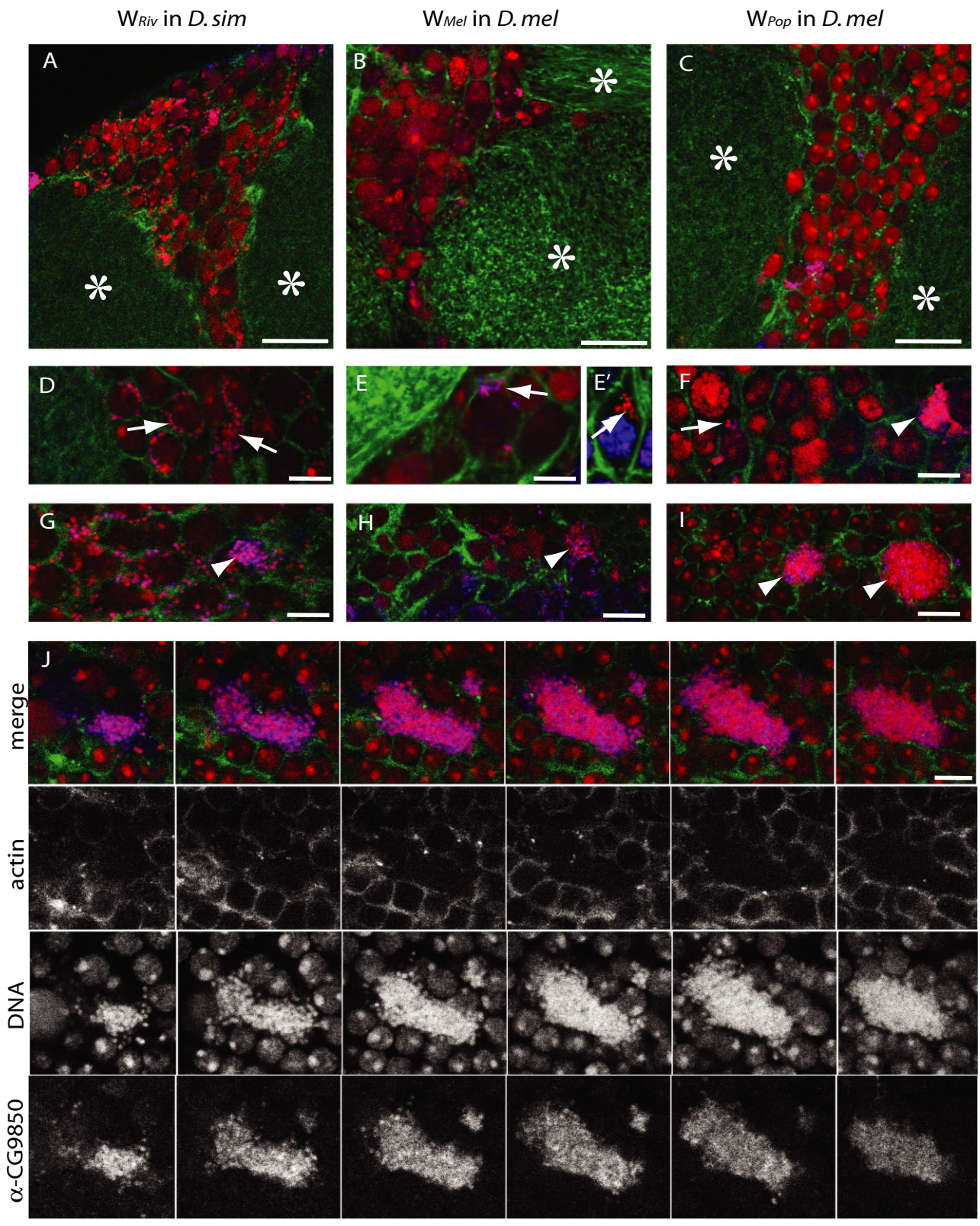


Fig. 1. Propidium Iodide and anti-CG9850 stain *Wolbachia* in the adult brain. The DNA stain propidium iodide (PI) highlights host nuclei. In infected fly lines, DNA staining of *Wolbachia* stands out as small, brighter puncta in the cytoplasm. The anti-CG9850 antibody also stains *Wolbachia* in the infected fly lines and the staining is tightly overlapping (right panels). Phalloidin marks the actin-rich cell cortex. The top row shows tetracycline-cured *D. melanogaster* and the other rows show infected fly lines as indicated. Scale bars, 5 μM.

through 7 (Table S1). Therefore, we averaged these regions in two groups: regions 1 to 4 and regions 5 to 7 (Fig. 3K). Representative images of these regions and of region 8 are shown in Fig. 3B–D. In contrast, the *Wolbachia* distribution in *D. melanogaster* was similar in all regions from 1 to 7, for either W_{Mel} or W_{Pop} (Table S1) (Fig. 3G and I). The average infection quantities of regions 1 to 4 and regions 5 to 7 for all strains are shown in Fig. 3K. Because titre level and distribution differed between W_{Riv} in *D. simulans* and W_{Mel} in *D. melanogaster*, we investigated whether the microbe or host control these differences by examining W_{Mel} in *D. simulans* hosts. W_{Mel} *Wolbachia* in *D. simulans* hosts were scored in three groups (area 1 through 4, area 5 through 7, and area 8), with the first two groups having the same titre distribution, similar to the W_{Mel} infection in *D. melanogaster* (Table S1 and Fig. 3K). However, in regions 1 through 7, W_{Mel} in *D. simulans* showed an intermediate titre between, W_{Riv} in *D.simulans* and W_{Mel} in *D. melanogaster*, as described below.

Overall, W_{Riv} in *D. simulans* had the highest number of infected cells: more than half of the cells were infected in regions 1 through 4, and over 70% were infected in regions 5 through 7 (Fig. 3K). In contrast, only about 40%

Fig. 2. In the adult central brain *Wolbachia* do not reside in axons, but are either in the cell body or as aggregates between host cells.
A–C. Overviews of the central brain infected with WRiv, WMel or WPop respectively. *Wolbachia* does not reside in the axon-rich neuropil (asterisks).
D–F. *Wolbachia* bacteria localize to the soma of neurons either as small aggregates of single bacteria (arrows) or as larger clusters (arrowheads).
G–I. Large bacteria clusters can occur with or without host cell cortical actin.
J. Images of a Z-series through a *Wolbachia* (WPop) cluster in WMel show that it is not encased in an actin-rich host membrane and lacks a host nucleus. Cortical actin (top row of the individual channels and green in the merged image) does not envelop this bacterial cluster and no host nuclei are visible within the cluster or in the nearby area.
Scale bars, 15 μM (A–C) or 5 μM (D–J). A–J (except E′) staining: cell cortex is green (phalloidin-488), host nuclei are red (PI) and *Wolbachia* are purple (PI and anti-CG9850). E′ staining: host nuclei are marked blue (anti-Histone H1).



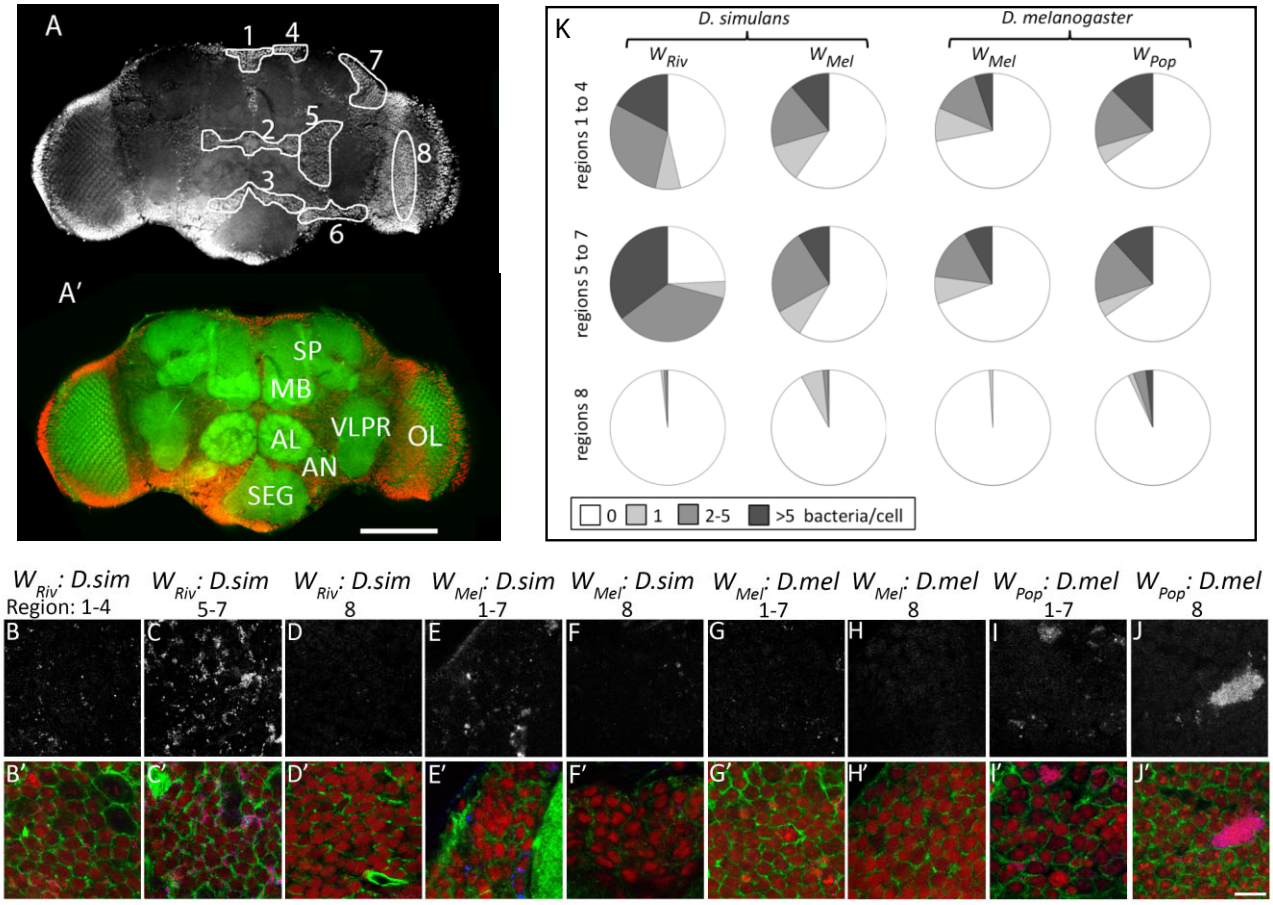


Fig. 3. *Wolbachia* distribution in the adult brain.
A and A′. Overview over the adult brain: panel (A) shows PI staining highlighting host cell bodies, and indicates the regions of *Wolbachia* quantification. (A′) is a merged image of the DNA staining (PI-red) and actin staining (green), highlighting axons and the major brain lobe centres. The regions are indicated as: MB, mushroom bodies; AL, antennal lobes, SEG, subesophageal ganglion, AN, antennal nerve; VLPR, ventrolateral protocerebrum; OL, optic lobes.
B–D. Representative images of WRiv in *D. simulans* as they are found in regions 1–4, 5–7 and 8 respectively.
E and F. Representative images of WMel in *D. simulans* in areas of regions 1–7 and 8 respectively.
G and H. Representative images of WMel in *D. melanogaster* in areas of regions 1–7 and 8 respectively.
I and J. Representative images of WPop in *D. melanogaster* in areas of regions 1–7 and 8 respectively.
Panels (B)–(J) show only the anti-CG9850 channel, (B′)–(J′) show merged images of the PI (red), the anti-CG9850 (blue) and the phalloidin (green) channels. Scale bars, 100 μM (A, A′) and 10 μM (B–J).
K. Quantification of *Wolbachia*-containing cells in the respective strains for each region average. Pie charts in shades of grey indicate the per cent of cells without *Wolbachia* (white), with one *Wolbachia* (light grey), with two to five *Wolbachia* (medium grey), or more than five *Wolbachia* (dark grey). Data for each individual region are shown in supplementary Table S1.

of all brain cells were infected in regions 1 through 7 in *D. simulans* with W_{Mel}, and less than 40% of cells where infected in *D. melanogaster* with either W_{Mel} or W_{Pop} (regions 1 through 7). Among the cells that contained bacteria, *D. simulans* with W_{Riv} exhibited the most cells with more than 5 *Wolbachia* per cell. These cells comprised nearly 20% in region 1 to 4, and about 35% in regions 5 to 7. In contrast, W_{Mel} in either *D. simulans* or *D. melanogaster* had fewer than 12% (region 1 to 4) or 14% (region 5 to 7) of cells with more than five bacteria. Similarly, only 12% of W_{Pop}-infected cells showed more than 5 bacteria. Region 8, the optical lobe, showed very little infection in all lines with 98% and 92% host cells without bacteria in *D. simulans* (W_{Riv} and W_{Mel}) and 99%, and 93% cells uninfected in *D. melanogaster* (W_{Mel} and W_{Pop}) (Fig. 3K).

Wolbachia cluster size and frequency were also quantified (Fig. 4). Extracellular *Wolbachia* clusters were defined as aggregates of bacteria with no host nuclei and an area greater than 11 μm^2 (slightly larger than an average neuronal cell body). The average cluster size is largest in W_{Pop} in *D. melanogaster* (54 μM) and significantly smaller in W_{Mel} in *D. melanogaster* and in W_{Riv} in *D. simulans* (21 and 22 μM) (Fig. 4A). W_{Mel} in

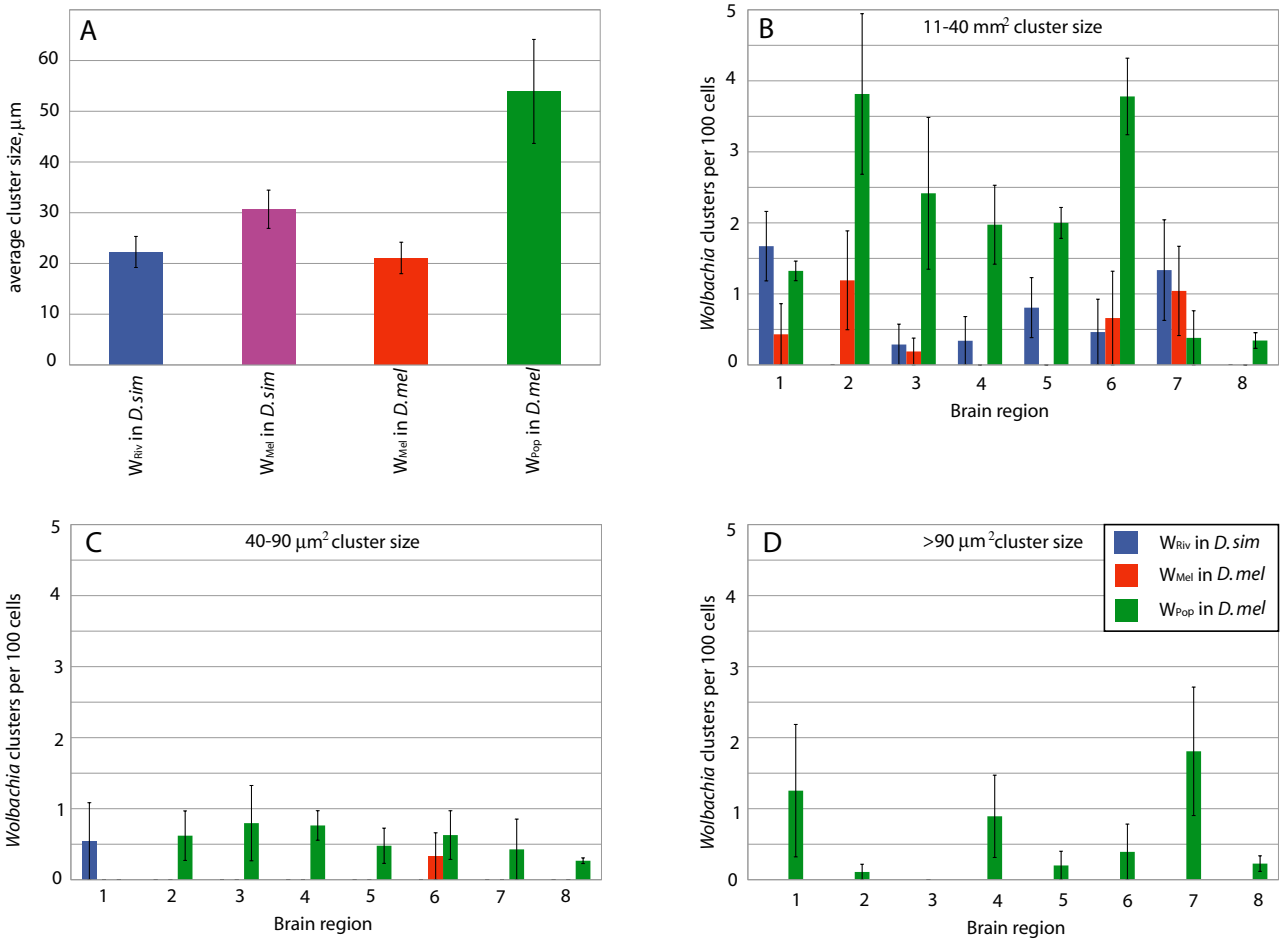


Fig. 4. *Wolbachia* cluster size and distribution in the adult brain.
A. Average cluster size in the indicated strains.
B–D. Cluster frequency per 100 cells according to cluster size (11–40 µm² cluster size: B; 40–90 µm² cluster size: C; over 90 µm² cluster size: D). Cluster frequency was counted for WRiv in *D. sim* (blue), WMel in *D. mel* (red) and WPop in *D. mel* (green). WPop forms more numerous and larger clusters.

D. simulans forms intermediate size clusters (32 µM). Cluster size frequency was quantified by counting clusters of three size categories (11–40 µm², 40–90 µm², and greater than 90 µm²). Figure 4B–D shows the frequency of bacterial clusters for each size category per 100 host cell bodies. Clusters of W_{Mel} in *D. melanogaster* and of W_{Riv} in *D. simulans* occur mostly in the smallest category and were not observed at all in the optical lobe (area 8, Fig. 4B). W_{Pop} in *D. melanogaster* showed dramatically higher frequency of the small cluster size, especially in areas 2 through 6, and also showed clusters in region 8, the optical lobe. The larger cluster sizes (Fig. 4C and D) were very rare in W_{Mel} in *D. melanogaster* and W_{Riv} in *D. simulans*, yet were commonly formed by the pathogenic W_{Pop} strain, which is known to cause premature death of its *D. melanogaster* host. Also, the largest observed cluster size developed by W_{Pop} is dramatically larger (858 µm) than those of W_{Mel} in *D. melanogaster* (43 µm), in *D. simulans* (87 µm), or of W_{Riv} in *D. simulans* (71 µm).

In the ventral nerve cord, Wolbachia *preferentially localize to the neuronal cell bodies but not to axons*

The ventral nerve cord (VNC) is an integral component of the adult central nervous system and functions as a major neural circuit centre for motor activities such as walking (Burrows *et al.*, 1988; Laurent and Burrows, 1988; Yellman *et al.*, 1997) and flying (Burrows, 1975; Peters *et al.*, 1985; Reye and Pearson, 1987). An overview of the *D. simulans* VNC is shown in Fig. 5A. Intracellular *Wolbachia* are visualized in a larger magnification (Fig. 5B). *Wolbachia* localize to the VNC in both *Drosophila* species and all three examined *Wolbachia* lines (Fig. 5C–F). Similar to the central brain, we observed a higher cellular infection frequency and titre in *D. simulans* compared with

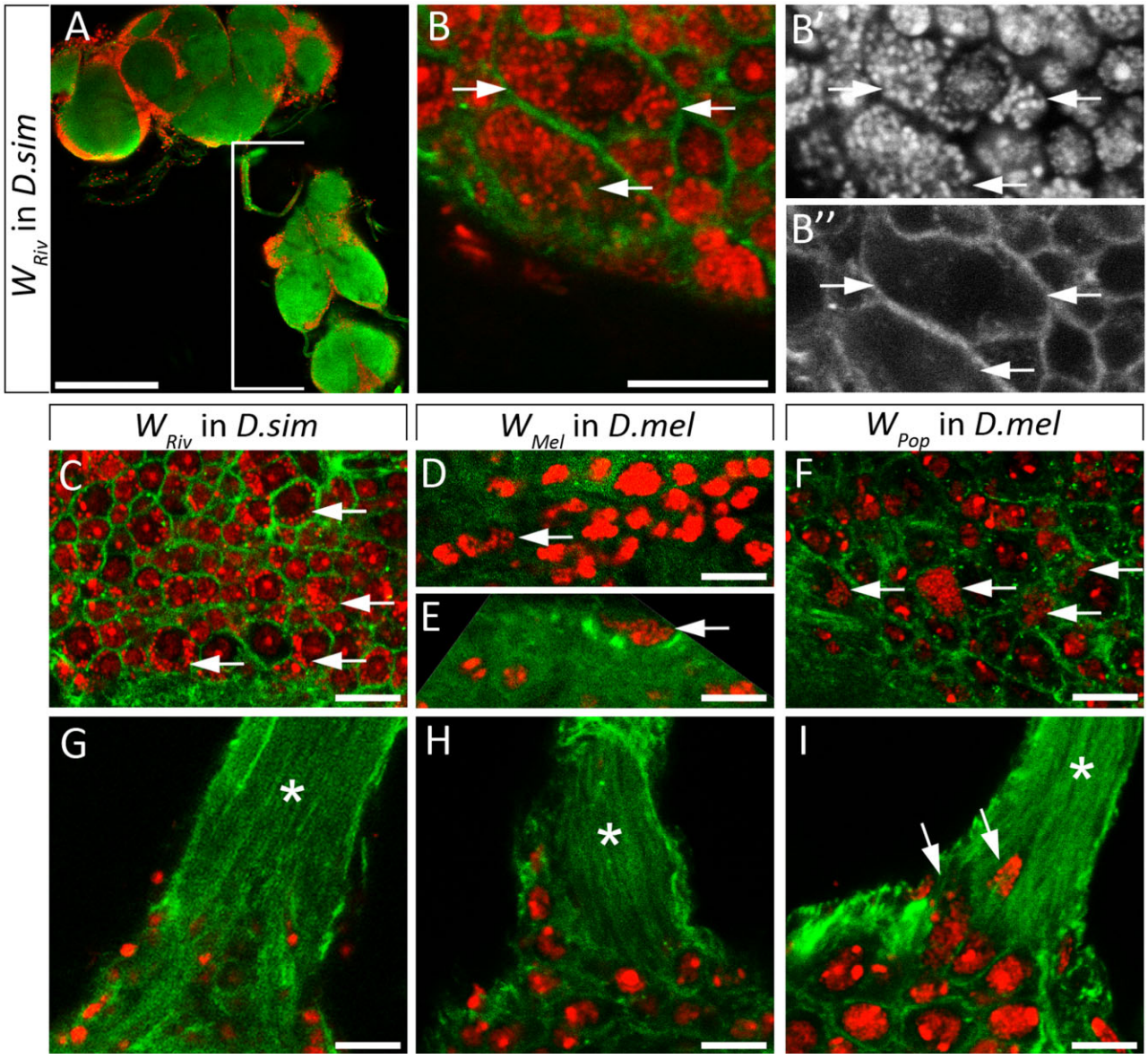


Fig. 5. *Wolbachia* localization in the ventral nerve chord (VNC).
A. Overview of the central brain and VNC (bracket) from *D. simulans* infected with WRiv.
B–B″. Higher magnification of (A), showing that *Wolbachia* is contained within the cortical actin cortex of host cell bodies in the VNC (arrows). (B) Merged image of PI (red) and actin (green), (B′) PI staining DNA, (B″) Phalloidin-488 staining cortical actin.
C–F. Bacteria in host cells: WRiv in *D. simulans*, WMel in *D. melanogaster* and WPop in *D. melanogaster* respectively.
G–I. WRiv in *D. simulans* and WMel in *D. melanogaster* do not occur in regions of axonal bundles (G, H asterisks), but WPop clusters are detected in regions of *D. melanogaster* axonal bundles (I, arrows). Scale bars, 100 μM (A) or 10 μM (B, C–I).

D. melanogaster with either *Wolbachia* line (Fig. 5C–D and data not shown). *Wolbachia* clusters are found in the VNC of W_{Pop}-infected hosts (Fig. 5F).

The central brain and the VNC are connected through a dense network of axonal tracks (Fig. 5A, upper part of the bracket). Similar to the axon-dense central brain neuropil, *Wolbachia* is generally not apparent within the axon tracks (Fig. 5G–I, asterisks) except for occasional W_{Pop} clusters in *D. melanogaster* (Fig. 5I, arrows).

Localization to the fat bodies is likely a conserved feature of Wolbachia *infections*

The *Drosophila* fat body senses the nutritional status of the flies and consequently regulates global growth (Colombani *et al.*, 2003). In addition, the fat body plays a role in mating behaviour (Lazareva *et al.*, 2007). *Wolbachia* has been observed in larval fat bodies in of *D. melanogaster* (Clark *et al.*, 2005). To analyse if this

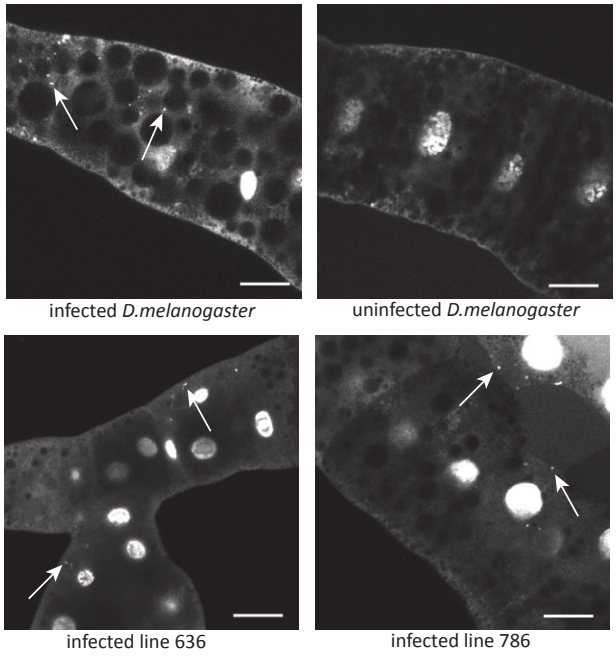


Fig. 6. *Wolbachia* in larval *D. melanogaster* fat bodies. Syto-11 staining shows that *Wolbachia* reside in fat bodies of the infected laboratory line and lines 639 and 786 (arrows). No puncta are seen in the uninfected laboratory line.

Wolbachia localization in fat body tissue is conserved, we tested all infected lines form the '*Drosophila* Genetic Reference Panel'. For fat body analysis, third instar larvae were dissected and stained for *Wolbachia* visualization with Syto-11. All infected strains showed bacteria in the fat bodies and examples are shown in Fig. 6.

Wolbachia *influence on physiology and behaviour*

It is intriguing that *Wolbachia* localization to brain and fat bodies is a conserved feature of *Wolbachia* infections in *Drosophila*. The behavioural manipulation hypothesis proposes that a microbial endosymbiont can alter host behaviour specifically to increase its own transmission (Thomas *et al*., 2005). To analyse if *Wolbachia* influences behaviour and physiology, previously published assays on strains of the *Drosophila* Genetic Reference Panel (Ayroles *et al*., 2009; Edwards *et al*., 2009; Harbison *et al*., 2009; Morozova *et al*., 2009) were reanalysed with respect to the lines' infection status. ANOVA analysis showed that infection status of a line is correlated with differences in chill coma recovery, sleep time during the day, and some of the ethanol sensitivity, locomotor startle response and olfaction responses (Table 2 column W, asterisks). There was no significant correlation between *Wolbachia* infection and quantification of the other examined traits, which include aggressive behaviour (Edwards *et al*., 2009), competitive fitness, copulation latency (Ayroles *et al*., 2009), starvation resistance and longevity (Morozova *et al*., 2009), sleep time (night) and sleep bout number (day and night) (Harbison *et al*., 2009).

To test if *Wolbachia*-induced effects existed within individual lines, we repeated the assays after curing the lines of *Wolbachia*. Nine of the 16 infected lines were used to evaluate the physiological effects of *Wolbachia* and four of the 24 uninfected lines were also treated and used to control for general tetracycline-induced effects. The assays described below directly compare cured and uncured isolines from the *Drosophila* Genetic Reference Panel.

Chill coma. Flies were kept on ice for 30 min and the time of their first movement after shifting to 25°C was recorded. Of the nine infected lines tested, females in one line and males in three lines had a significant response to tetracycline, and the response was either a longer or a shorter recovery time, depending on the line (Fig. 7A). A slower chill coma recovery was observed in uninfected, treated lines 765 (males and females) and 379 (females). Using a nested ANOVA analysis, line identity was the only significant factor in explaining the response variation (Table 3). This result indicates that neither *Wolbachia* infection nor tetracycline treatment have a systematic effect. The average chill coma recovery times for the individual lines were similar to the ones published by Ayroles (Ayroles *et al*., 2009). However, the ANOVA analysis of those untreated lines had indicated a trend for slower recovery times in infected fly lines (Table 2). We have used fewer uninfected fly strains in the second analysis, which may explain why a slower recovery time was not observed. It is noteworthy that none of the four uninfected lines had a faster recovery time after tetracycline treatment.

Starvation

The response to starvation was significantly influenced by tetracycline treatment in six of the eight infected lines (Fig. 7B). However, the direction of the response differed according the line and/or sex. Of the three uninfected lines, two (and males of the third) were significantly affected by tetracycline, also in differing directions. Similar to the outcome of the chill coma experiment, variation in starvation survival was dependent on the line identity and there was no general significant influence of either tetracycline or *Wolbachia* infection. However, in agreement with earlier studies (Table 2) (Ayroles *et al*., 2009; Goenaga *et al*., 2010), the duration of starvation survival was significantly dependent on sex, with females surviving for a longer duration than males.

Table 2. ANOVA analysis of the relationship between infection status and published physiological and behavioural traits.

Phenotype	W	S	W*S
Aggressive behaviour (males only)	0.9412		
Chill coma recovery time	0.0014*	0.5563	0.8274
Competitive Fitness	0.3076		
Copulation Latency	0.7874		
Ethanol sensitivity 1 (alcohol medium)	0.3673	0.3725	0.5527
Ethanol sensitivity 1 (standard medium)	0.0112*	0.5401	0.6135
Ethanol sensitivity 2 (alcohol medium)	0.1505	0.1218	0.7537
Ethanol sensitivity 2 (standard medium)	0.11	0.512	0.4851
Locomotion (activity per waking minute)	0.2915	0.0006*	0.8963
Locomotion (distance moved in 12 h)	0.0127*	0.0127*	0.9064
Locomotion (time spent moving in 12 h)	0.0181*	0.0171*	0.827
Locomotor startle response (2006 data, standard medium)	0.0606	0.9706	0.9737
Locomotor startle response (2007/8 data, dopamine medium)	0.0485*	0.4968	0.5821
Locomotor startle response (2007/8 data, ethanol medium)	0.0384*	0.7543	0.445
Locomotor startle response (2007/8 data, serotonin medium)	0.0222*	0.7433	0.665
Locomotor startle response (2007/8 data, standard medium)	0.1172	0.837	0.8344
Longevity	0.6513	0.2837	0.6216
Olfaction – 0.1% benzaldehyde (ethanol medium)	0.4285	0.5063	0.9754
Olfaction – 0.1% benzaldehyde (standard medium)	0.0429*	0.6172	0.6003
Olfaction – 0.1% benzaldehyde (tomato medium)	0.5513	0.6543	0.846
Olfaction – 0.3% benzaldehyde (ethanol medium)	0.0235*	0.4624	0.3814
Olfaction – 0.3% benzaldehyde (standard medium)	0.3934	0.3249	0.2553
Olfaction – 0.3% benzaldehyde (tomato medium)	0.0528	0.6389	0.6352
Olfaction – Acetephenone	0.6016	0.4797	0.7252
Olfaction – hexanol	0.7239	0.4946	0.8374
Sensory bristle number (abdominal bristles)	0.5655	0.0841	0.937
Sensory bristle number (sternopleural bristles)	0.1774	0.1198	0.9497
Sleep bout number (day)	0.1621	0.1924	0.2559
Sleep bout number (night)	0.1038	0.8342	0.978
Sleep time (day)	0.013*	0.0001*	0.5113
Sleep time (night)	0.9811	0.0231*	0.9529
Starvation stress resistance	0.8951	< 0.0001*	0.7204

Column W = *Wolbachia*-related effect, column S = strain identity-related effect, W*S = interaction between infection status and strain identity. Asterisks indicate a significant *Wolbachia*-related effect ($P < 0.05$).

Longevity

Of the nine infected lines tested, male longevity was extended after tetracycline treatment in all lines except for 639 (Fig. 7C). In females, four of nine lines had increased longevity after tetracycline treatment. Of the four uninfected lines, two also had significantly increased longevity after tetracycline treatment in both sexes. Data analysis using nested ANOVA indicates that tetracycline has a significant effect on longevity (Table 3), independent of *Wolbachia* infection. Line identity was also a significant factor in determining longevity.

Discussion

Localization to the adult brain and fat bodies is likely a conserved feature of Wolbachia *infection*

The success of *Wolbachia* dispersal has been attributed to its efficient localization to the male and female germ lines and successful transmission through the latter (Serbus *et al.*, 2008). However, there are a number of reports documenting the presence of *Wolbachia* in somatic tissues of larval and adult insects (Min and Benzer, 1997; Dobson *et al.*, 1999; McGraw *et al.*, 2002; Czarnetzki and Tebbe, 2004; Frydman *et al.*, 2006; Narita *et al.*, 2007; Albertson *et al.*, 2009). To determine whether this was a sporadic occurrence or a conserved feature of *Wolbachia*-host interactions, we examined two field populations of *Wolbachia*-infected *D. melanogaster* and two field populations of *Wolbachia*-infected *D. simulans* strains for the presence of *Wolbachia* in the adult brain. In all examined strains, there was a robust presence of *Wolbachia* in the adult brain. Similar studies examining the fat bodies in 14 *Wolbachia*-infected *D. melanogaster* strains also revealed the presence of *Wolbachia* in all cases. Thus, while *Wolbachia* has been traditionally viewed as a germline endosymbiont, these studies, together with the previous work described above, demonstrate that *Wolbachia* has a rich somatic life as well.

The presence of *Wolbachia* in these somatic tissues raises intriguing issues regarding their route and mechanism of localization. In the developing *Drosophila* brain, *Wolbachia* exhibits a microtubule dependent, asymmetric segregation pattern during neuroblast divisions, indicating that they rely on intracellular mitotic cues for their ultimate somatic localization (Albertson *et al.*, 2009). Alternatively,

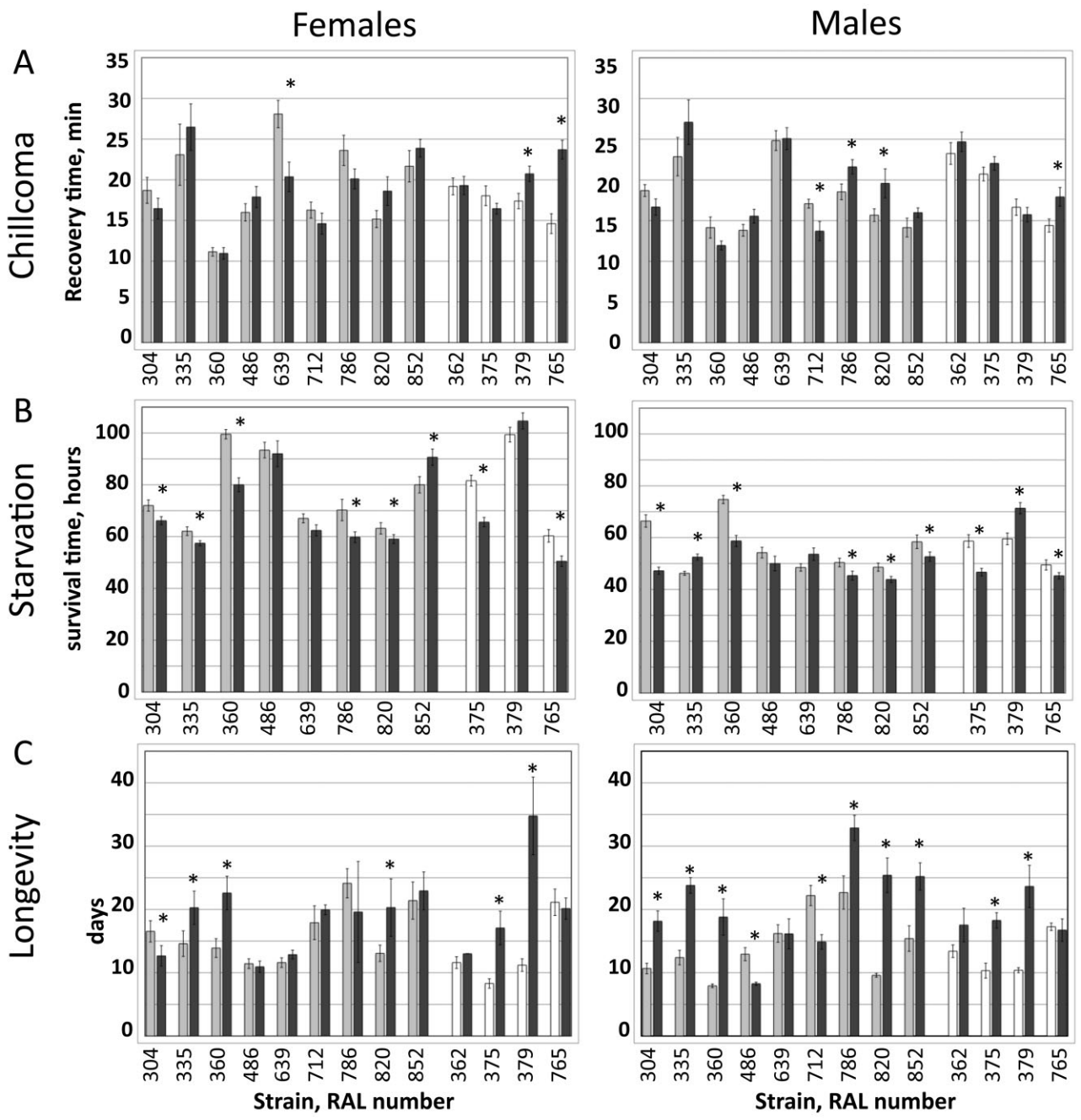


Fig. 7. Physiological traits of infected and uninfected *D. melanogaster* lines.
A. Recovery time of different fly lines after chill-induced coma.
B. Survival time without food supply.
C. Fly longevity on regular food supply.
Results of female flies are in the left graphs and results from male flies are in the right graphs. Infected lines are represented by light grey bars, uninfected lines by white bars and tetracycline-treated flies by dark grey bars. Significant differences between lines before and after *Wolbachia*-curing with tetracycline are marked by asterisks (*T*-test, $P > 0.05$). (n for each experiment are listed in supplementary Table S1).

other work demonstrates that *Wolbachia* injected into the adult abdomen is capable of an extraordinary migration to the specific somatic niche cells of the female germline (Frydman *et al*., 2006; Fast *et al*., 2011). Thus, *Wolbachia* possesses the ability for both, intracellular mitotic-based cell-to-cell transmission, and extracellular migration. This conclusion is in accord with observations of *Wolbachia* in the nematode *Brugia malayi*, where it has been

Table 3. Nested ANOVA analysis of the effect of sex, infection status, and fly line identity and tetracycline treatment on chill coma recovery, starvation survival time and longevity.

Chill coma recovery		
Source of variation	*F*	Significance
Sex	0.344	0.563
Infection status	0.061	0.810
Line	7.431	0.001*
Tetracycline treatment	1.873	0.198
Sex*tet treatment	0.179	0.676
Infection*tet treatment	1.623	0.229
Tet treatment*line	1.075	0.419
Starvation survival time		
Source of variation	*F*	Significance
Sex	68.787	0.000*
Infection status	0.337	0.575
Line	7.211	0.002*
Tetracycline treatment	3.074	0.113
Sex*tet treatment	0.053	0.820
Infection*tet treatment	0.038	0.849
Tet treatment*line	1.001	0.471
Longevity		
Source of variation	*F*	Significance
Sex	0.633	0.432
Infection status	1.737	0.203
Line	6.528	0.017*
Tetracycline treatment	5.349	0.027*
Sex*tet treatment	–	–
Infection*tet treatment	1.992	0.186
Tet treatment*line	0.293	0.982

Asterisks indicate a significant *Wolbachia*-related effect ($P < 0.05$).

demonstrated that *Wolbachia* relies on both cell-to-cell transmission and internal mitotic mechanisms for their germline localization (Landmann *et al*., 2012).

Because we did not analyse all tissues in the adults, it is possible that *Wolbachia* are equally abundant throughout many tissues in the adult. The presence of *Wolbachia* in many host tissues has been suggested by PCR-based studies (Dobson *et al*., 1999; McGraw *et al*., 2002). Whether *Wolbachia* is specifically targeted to the brain and fat bodies, and possibly to other areas, remains unclear. However, the discovery of asymmetric *Wolbachia* segregation in the *Drosophila* neuroblasts and migration to the germline niche cells indicate that specific targeting mechanisms are involved (Frydman *et al*., 2006; Albertson *et al*., 2009; Fast *et al*., 2011). Resolving this issue will require careful cellular analysis of *Wolbachia* localization patterns in a variety of tissues. Mechanisms of targeted *Wolbachia* localization within the arthropod CNS may shed insight into strategies utilized by microbes that target specific regions in vertebrate host brains. For example, *Rickettsia*, bacteria closely related to *Wolbachia*, preferentially infect brain cells compared with endothelial cells in the mammal host (Joshi and Kovacs, 2007). *Toxoplasma gondii* is a protozoan parasite widely prevalent in animals causing a variety of neuropathologies. *T. gondii* infections target neural and glial cells in the intermediate rodent hosts, causing diverse alterations in cellular activity (Kamerkar and Davis, 2012). In the rodent, *T. gondii* preferentially localizes to limbic system of the adult brain, in particular to the medial and basolateral amygdala (Vyas *et al*., 2007). For both *Rickettsia* and *T. gondii*, little is known concerning the molecular and cellular mechanisms involved in nervous system targeting. Our finding that *Wolbachia* targets specific regions of the adult *Drosophila* central nervous system provides an excellent opportunity to apply powerful *Drosophila* genetic approaches to this issue.

Factors intrinsic to Wolbachia *influence its distribution in the brain*

Previous studies demonstrated that factors intrinsic to the *Wolbachia* strain determine its localization in the insect oocyte, while host factors played a major role in influencing titre (Veneti *et al*., 2004; Serbus and Sullivan, 2007). To determine whether *Wolbachia* or host factors influence *Wolbachia* titre and distribution in the adult brain, we examined W_{Mel} and W_{Pop} in *D. melanogaster*, W_{Riv} in *D. simulans* and W_{Mel} in *D. simulans*. All examined *Wolbachia* strains infect the optic lobes at low frequencies and predominantly reside in the central brain. Within the central brain, regional *Wolbachia* distribution differs among *Wolbachia* strains. W_{Mel} and W_{Pop} in *D. melanogaster* showed a relatively even distribution, while W_{Riv} in *D. simulans* showed significantly higher titres in specific regions (Table 4). W_{Riv} bacteria in *D. simulans* are present in much higher titres in regions 5 to 7 compared with regions 1 to 4 (regions 5 to 7 include soma medial and posterior to the ventrolateral protocerebrum lobe, and soma lateral to the lateral superior protocerebrum; regions 1 to 4 include soma between the superior medial protocerebra, soma posterior to the mushroom body, soma posterior to antennal lobes and soma anterior to the superior protocerebrum lobe). In contrast to W_{Riv}, W_{Mel} in *D. simulans* exhibited an even distribution among the regions, similar to W_{Mel} in *D. melanogaster* (Table 4). This result leads to the conclusion that – similar to *Wolbachia* localization in the oocyte – the even versus uneven pattern of *Wolbachia* localization in the brain is intrinsic to the *Wolbachia* strain rather than to the host. Also similar to the oocyte, both the host species and bacteria strain influence *Wolbachia* titre. For example, the titre in regions 1 through 4 of W_{Mel} in *D. simulans* is intermediate between that of W_{Mel} in *D. melanogaster* and W_{Riv} in *D. simulans*. In contrast, the cluster size of W_{Mel} in *D. simulans* is larger than those of either W_{Mel} in

Table 4. Summary of *Wolbachia* distribution characteristics and *Wolbachia* titre in brain and oocyte tissues.

	W_{Riv}/*D. sim*	W_{Mel}/*D. sim*	W_{Pop}/*D. mel*	W_{Mel}/*D. mel*
Region-specific distribution in CNS	Yes	No	No	No
Titre in brain (intracellular)	+++	++	+	+
Titre in brain (cluster, extracellular)	+	N/A	+++	+
Cluster size	+	++	+++	+
Posterior oocyte localization (intracellular)	No(*)	Yes(*)	Yes(**)	Yes(*)
Oocyte titre	++(*)	+++(*)	+(**)	+(*)

From (*) Serbus and Sullivan (2007) and (**) L.R. Serbus (unpubl. obs.).

D. melanogaster and that of W_{Riv} in *D. simulans* and appears to result from specific interaction between the *D. melanogaster* host with the W_{Mel} strain (Table 4). How the host impacts titre and how factors intrinsic to the *Wolbachia* strain influence tissue and cellular distribution is unclear. It may be that nutrient levels in the host cells play a key role in influencing titre while *Wolbachia* surface proteins that interact with specific host factors determine its localization. Support of this notion comes from the close association between *Wolbachia* and polarity determinants in the *Drosophila* oocyte (Serbus and Sullivan, 2007; Serbus *et al*., 2011).

Wolbachia *is present both intracellularly and extracellularly in the adult brain*

All of the examined *Wolbachia* strains formed clusters, with W_{Pop} having the largest and most numerous clusters. W_{Pop} clusters in *D. melanogaster* were first identified by EM imaging and were proposed to arise by fusion of several W_{Pop}-infected cells (Min and Benzer, 1997). However, our confocal imaging did not reveal any multinucleated cells. Furthermore, large W_{Pop} clusters lacked a host nucleus and were generally not encased within a clearly defined host membrane, indicating that bacterial clusters may also form through a different mechanism. *Wolbachia* is an obligate intracellular endosymbiont and previous work has shown that *Wolbachia* does not divide extracellularly (Rasgon *et al*., 2006). *Wolbachia* bacteria that are experimentally injected into the fly abdomen survive within the haemolymph for a few days before invading host cells, but become established only within host cells (Fast *et al*., 2011). Therefore, one possibility is that *Wolbachia* overproliferates in a small number of host cells, causing the cell to lyse. The data presented in this work raise the possibility that *Wolbachia* reside extracellularly within the adult brain. However, if these were transient aggregates that invaded nearby host cells, we would expect a high frequency of cellular infection near clusters, but the W_{Pop} cellular infection frequency was similar to W_{Mel}, indicating that bacteria originating from large W_{Pop} clusters are not invading nearby cells. It is also possible that these clusters represent the dense *Wolbachia* form that was found in EM analysis by Min and Benzer (1997) and might be a quiescent state. An alternative model to explain the presence of large bacteria clusters is aligned with the Min and Benzer cell fusion model, in that *Wolbachia* overproliferates in host cells leading to an expansion of host cell membrane. Our data revealed that some small clusters were encased by actin-rich staining (presumably remnants of a host plasma membrane). However, in this model, the host cells are severely aberrant and non-functional since they do not have an intact nucleus or host DNA. Previous studies have indicated that intracellular bacteria induce and/or block apoptosis (Gao and Abu Kwaik, 2000), as it has been shown for *Wolbachia* (Landmann *et al*., 2011; Zhukova and Kiseleva, 2012). Subcellular studies assaying factors such as organelle integrity, apoptosis and necrosis will further advance our knowledge into bacterial cluster formation and the consequences on host cell function. Surprisingly, our data indicate that, apart from the bacteria clusters, W_{Pop} has a relatively low cellular infection frequency and a low number of *Wolbachia* per cell. In this regard, the pathogenic W_{Pop} resembled the low titre W_{Mel} strain more than the high titre W_{Riv} strain.

Host line identity rather than a general effect of Wolbachia *infection determines response to chill coma, starvation and longevity*

Our observation of W_{Riv} in *D. simulans* is the first report of a microbe preferentially localizing to specific regions of the *Drosophila* central brain. At this point it is unclear if the preferential localization might translate into functional significance. Our experiments suggest that the host line identity determines how tetracycline treatment influences the flies' longevity and response to cold stress or starvation. In light of this result, it is not surprising that previous publications that analyse *Wolbachia* effects of physiological traits have come to varying conclusions. For example, removing *Wolbachia* by tetracycline decreased lifespan drastically (Toivonen *et al*., 2007), or had mixed effects (Driver *et al*., 2004; Fry *et al*., 2004). Our results confirm and extend those of Fry *et al*. who examined fitness effects (survival and fecundity) in inbred *D. melanogaster*

lines. The authors concluded that beneficial or harmful *Wolbachia* effects depend on the host genetic background and sex (Fry *et al*., 2004). We expand their observations by additional physiological parameters and also by analysing the tetracycline effect on uninfected lines. We found that tetracycline also has a line-dependent effect, confounding the analysis of *Wolbachia* infection.

Previous analyses of host behaviour in response to *Wolbachia* infection have led to results indicating that host species and *Wolbachia* strain influence the observations, for example in olfactory-cued locomotion (Peng *et al*., 2008). It would be interesting to examine whether a similar variability can be found even among lines of the same *Drosophila* species. In this study we have not examined the variation of *Wolbachia* distribution and titre in the brain within lines of one species. However, it is interesting to speculate that the line-specific variations that are observed in physiological and behaviour parameters might be linked to a different, line-specific *Wolbachia* infection pattern in the brain.

In *Drosophila*, neural circuits implicated in sexual and defensive behaviours overlap considerably, yet recent reports have identified that differential gene expression in the central brain plays a critical role in regulating behaviour. For example, the male isoform of fruitless (fruM), a key regulator of male courtship behaviour, is expressed in only 2% of neurons, which are distributed into 21 clusters in specific regions of the adult brain (Stockinger *et al*., 2005). Gene expression profiles have indicated that *Wolbachia* can induce differential gene expression in a variety of hosts, including wasp (Kremer *et al*., 2012) and silkworm (Nakamura *et al*., 2011). Specifically, W_{Mel} has been shown to alter gene expression in *D. melanogaster* (Zheng *et al*., 2011b; 2011a) while W_{Pop} and W_{Riv} have been reported to alter host gene expression in the mosquito (Hughes *et al*., 2011). To date, no genomic profiles have been performed specifically on adult brain tissue. Among the *Wolbachia* strain/host combinations examined in our studies, the data clearly indicate distinct differences in adult brains regarding *Wolbachia* distribution, cellular infection frequencies, and cluster size and frequency. Specific *Wolbachia* localization patterns in the brain may influence host-specific physiological and behavioural responses to *Wolbachia*.

Tetracycline treatment extends longevity

We observed that tetracycline increased longevity in most lines, including *Wolbachia*-free lines. In addition, increased longevity is strain dependent. This observation was surprising given that the lines were treated many generations before assaying for longevity. Because the effect was independent of a previous *Wolbachia* infection, this result could imply that the lines harbour additional bacteria that shorten their lifespan. A tetracycline effect on mitochondria has been reported for two generations after tetracycline treatment (Ballard and Melvin, 2007), but it is unlikely that mitochondria are still affected 6 months to 2 years after treatment. In contrast to our observation, a previous publication reported that a lack of bacteria in early *Drosophila* development decreases the flies' lifespan (Brummel *et al*., 2004). However, Brummel *et al*. compared flies in regular and axenic conditions, whereas our lines where grown under regular conditions that probably restored gut fauna. Min and Benzer found no tetracycline-effect on *D. melanogaster* longevity and report that tetracycline treatment immediately restores the original lifespan to flies that have been infected with the pathogenic W_{pop} bacteria strain (Min and Benzer, 1997). A tetracycline-induced life-shortening effect, attributed to *Wolbachia* loss, was observed in *indy* mutants, although an additional *Wolbachia*-infected strain was found to be unchanged by the antibiotics in that study (Toivonen *et al*., 2007). This long-lasting, line-dependent tetracycline effect will need to be taken into account in future comparisons of *Wolbachia*-infected lines and their cured control counterparts.

Conclusion

Wolbachia infection of adult brains is a conserved feature in *D. simulans* and *D. melanogaster*. Bacteria distribution within different brain regions depends on the *Wolbachia* strain, whereas the titre in the brain is determined by both the host species and the bacteria strain. In addition, we found that the pathogenic W_{Pop} *Wolbachia* strain infects host cells at a similar frequency as non-pathogenic strains, but forms more numerous and larger bacteria clusters than the benign strains W_{Mel} and W_{Riv}. It appears that some of these aggregates are not contained within host cells, which may indicate that the bacteria have lysed the host cells. In spite of *Wolbachia* distribution into areas that control physiology and fly behaviour, the effect of a *Wolbachia* infection on individual *D. melanogaster* lines varies with the individual host lines.

Experimental procedures

Drosophila *stocks*

All RAL *Drosophila* lines were obtained from the Bloomington stock centre and are described by Ayroles *et al*. (2009). Infected and uninfected Oregon-R stocks used in this study are described by Ferree *et al*. (2005). *D. melanogaster* with W_{Pop}, *D. simulans* with W_{Riv} or W_{Mel} and *D. melanogaster* with W_{Mel} are labstocks (Serbus and Sullivan, 2007). The infected 'Turelli' *D. simulans* flies were captured in California by the Michael Turelli lab (University of California, Davis). The *Drosophila* lines were reared on cornmeal-molasses-yeast food at 25°C, with a 12/12 h light/dark cycle.

Tetracycline treatment

Females laid eggs on food vials containing 25 mg of tetracycline in 100 ml of regular cornmeal-molasses food. Lines were established from individual females raised from egg to adulthood in these tetracycline-spiked vials. The offspring was analysed for *Wolbachia* infection by PCR and cytology to ensure they were cured. Experiments were performed on paired infected and cured lines at least seven generations after tetracycline treatment and up to 2 years after treatment.

Assaying Wolbachia *infection status by PCR*

Wolbachia infection status of each line was analysed by PCR and cytology. PCR: flies were crushed in PCR buffer (Sambrook *et al*., 1989) containing proteinase K (0.8 mg ml^{-1}), heated to 60°C for 45 min, and to 95°C to for 10 min. The *wsp* sequence was amplified using the following primers: aacgctactccagcttctgc (reverse) and gatcctgttggtccaataagtg (forward). When indicated, the PCR products were cloned into pCR®2.1-TOPO (Invitrogen) and sent for sequencing (UC Berkeley sequencing facility).

Fixed cytology

Ovaries from adult female *Drosophila* were dissected in PBS and fixed in 3.7% Paraformaldehyde and Heptane, as described previously (Ferree *et al*., 2005). After RNase treatment, *Wolbachia* and host DNA were stained with Propidium Iodide (Ferree *et al*., 2005). Adult brain cytology: 2- to 5-day-old adult flies were briefly anaesthetized with CO_2 and placed in a watch glass containing PBS (with 0.02% Triton X-100). Dissected brains were immediately fixed in PEM (100 mM PIPES, 2 mM EGTA, 1 mM MgSO4) with 2% paraformaldehyde for 16–18 min. Primary antibodies include mouse anti-Histone H1 (1:100, Millipore), and mouse anti-FasII (1:100, DSHB). For *Wolbachia* detection, we used mouse anti-CG9850 (1:100, a generous gift from Kyungok Cho, Korea Advanced Institute of Science and Technology), which was found to specifically bind to *Wolbachia*. It is often the case that antibodies generated against an *Escherichia coli*-expressed protein highlight *Wolbachia* (Cho, 2004). We verified the specificity of this antibody with the established Propidium Iodide staining (Fig. 1). Brains were incubated in primary antibody + PBST (0.1% Triton X-100) for 4 h at room temperature (or overnight at 4°C). Secondary antibodies included anti-mouse Cy5 (1:150, Invitrogen), anti-mouse Alexa Fluor 488 (1:400, Invitrogen), and conjugated-phalloidin488 (1:100 Invitrogen). Brains were incubated in secondary antibody + PBST (0.1% Triton X-100) for 1 h at room temperature. For PI staining, fixed brains were incubated in RNase (15.4 mg ml^{-1} PBS) for 2 h in a 37°C water bath and mounted in mounting medium containing PI (10 μg ml^{-1} PI, 1× PBS, 70% glycerol in water).

Live cytology

Adult flies were dissected in PBS and ovaries, brains, or fat bodies (from male and female flies) were placed into a drop of Syto-11 (Invitrogen, 1:100 dilution of stock in PBS) on a coverslip, 20 min on ice. Samples were then overlaid with a smaller coverslip and analysed for *Wolbachia* by confocal microscopy (Leica TCS SP2) (Casper-Lindley *et al*., 2011). For brains, broken pieces of coverslips were used as spacers to avoid sample squashing.

Behavourial and physiological assays

All assays were performed as described in Ayroles *et al*. (2009).

Image capture, quantification and preparation

All images were collected with a TCS SP2 confocal system on a Leica DM IRB inverted microscope. For adult brains, *x–y–z* three-dimensional image stacks were analysed and quantified with Lecia LAF AS Lite software. The *Wolbachia* cluster areas were measured by multiplying the longest axis and the orthogonal axis of the cluster. For circular-shaped clusters, area was measured as π-r2. Images were assembled with Photoshop CS4.

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References

Albertson, R., Casper-Lindley, C., Cao, J., Tram, U., and Sullivan, W. (2009) Symmetric and asymmetric mitotic segregation patterns influence *Wolbachia* distribution in host somatic tissue. *J Cell Sci* **122:** 4570–4583.

Ayroles, J.F., Carbone, M.A., Stone, E.A., Jordan, K.W., Lyman, R.F., Magwire, M.M., *et al*. (2009) Systems genetics of complex traits in *Drosophila melanogaster*. *Nat Genet* **41:** 299–307.

Baldo, L., Desjardins, C.A., Russell, J.A., Stahlhut, J.K., and Werren, J.H. (2010) Accelerated microevolution in an outer membrane protein (OMP) of the intracellular bacteria *Wolbachia*. *BMC Evol Biol* **10:** 48.

Ballard, J.W., and Melvin, R.G. (2007) Tetracycline treatment influences mitochondrial metabolism and mtDNA density two generations after treatment in *Drosophila*. *Insect Mol Biol* **16:** 799–802.

Brummel, T., Ching, A., Seroude, L., Simon, A.F., and Benzer, S. (2004) *Drosophila* lifespan enhancement by exogenous bacteria. *Proc Natl Acad Sci USA* **101:** 12974–12979.

Burrows, M. (1975) Monosynaptic connexions between wing stretch receptors and flight motoneurons of the locust. *J Exp Biol* **62:** 189–219.

Burrows, M., Laurent, G.J., and Field, L.H. (1988) Proprioceptive inputs to nonspiking local interneurons contribute to local reflexes of a locust hindleg. *J Neurosci* **8:** 3085–3093.

Casper-Lindley, C., Kimura, S., Saxton, D.S., Essaw, Y., Simpson, I., Tan, V., and Sullivan, W. (2011) Rapid fluorescence-based screening for *Wolbachia* endosymbionts in *Drosophila* germ line and somatic tissues. *Appl Environ Microbiol* **17:** 4788–4794.

Cho, K.O. (2004) *Wolbachia* bacteria, the cause for false vesicular staining pattern in *Drosophila melanogaster*. *Gene Expr Patterns* **5:** 167–170.

Clark, M.E., Anderson, C.L., Cande, J., and Karr, T.L. (2005) Widespread prevalence of *Wolbachia* in laboratory stocks and the implications for *Drosophila* research. *Genetics* **170:** 1667–1675.

Colombani, J., Raisin, S., Pantalacci, S., Radimerski, T., Montagne, J., and Leopold, P. (2003) A nutrient sensor mechanism controls *Drosophila* growth. *Cell* **114:** 739–749.

de Crespigny, F.E., Pitt, T.D., and Wedell, N. (2006) Increased male mating rate in *Drosophila* is associated with *Wolbachia* infection. *J Evol Biol* **19:** 1964–1972.

Czarnetzki, A.B., and Tebbe, C.C. (2004) Detection and phylogenetic analysis of *Wolbachia* in Collembola. *Environ Microbiol* **6:** 35–44.

Dobson, S.L., Bourtzis, K., Braig, H.R., Jones, B.F., Zhou, W., Rousset, F., and O'Neill, S.L. (1999) *Wolbachia* infections are distributed throughout insect somatic and germ line tissues. *Insect Biochem Mol Biol* **29:** 153–160.

Doe, C.Q. (2008) Neural stem cells: balancing self-renewal with differentiation. *Development* **135:** 1575–1587.

Driver, C., Georgiou, A., and Georgiou, G. (2004) The contribution by mitochondrially induced oxidative damage to aging in *Drosophila melanogaster*. *Biogerontology* **5:** 185–192.

Edwards, A.C., Ayroles, J.F., Stone, E.A., Carbone, M.A., Lyman, R.F., and Mackay, T.F. (2009) A transcriptional network associated with natural variation in *Drosophila* aggressive behavior. *Genome Biol* **10:** R76.

Egger, B., Chell, J.M., and Brand, A.H. (2008) Insights into neural stem cell biology from flies. *Philos Trans R Soc Lond B Biol Sci* **363:** 39–56.

Fast, E.M., Toomey, M.E., Panaram, K., Desjardins, D., Kolaczyk, E.D., and Frydman, H.M. (2011) *Wolbachia* enhance *Drosophila* stem cell proliferation and target the germline stem cell niche. *Science* **334:** 990–992.

Ferree, P.M., Frydman, H.M., Li, J.M., Cao, J., Wieschaus, E., and Sullivan, W. (2005) *Wolbachia* utilizes host microtubules and Dynein for anterior localization in the *Drosophila* oocyte. *PLoS Pathog* **1:** e14.

Fry, A.J., Palmer, M.R., and Rand, D.M. (2004) Variable fitness effects of *Wolbachia* infection in *Drosophila melanogaster*. *Heredity* **93:** 379–389.

Frydman, H.M., Li, J.M., Robson, D.N., and Wieschaus, E. (2006) Somatic stem cell niche tropism in *Wolbachia*. *Nature* **441:** 509–512.

Gao, L., and Abu Kwaik, Y. (2000) Hijacking of apoptotic pathways by bacterial pathogens. *Microbes Infect* **2:** 1705–1719.

Gazla, I.N., and Carracedo, M.C. (2009) Effect of intracellular *Wolbachia* on interspecific crosses between *Drosophila melanogaster* and *Drosophila simulans*. *Genet Mol Res* **8:** 861–869.

Goenaga, J., Jose Fanara, J., and Hasson, E. (2010) A quantitative genetic study of starvation resistance at different geographic scales in natural populations of *Drosophila melanogaster*. *Genet Res* **92:** 253–259.

Hanesch, U., Fischbach, K.-F., and Heisenberg, M. (1989) Neuronal architecture of the central complex in *Drosophila melanogaster*. *Cell Tissue Res* **257:** 343–366.

Harbison, S.T., Carbone, M.A., Ayroles, J.F., Stone, E.A., Lyman, R.F., and Mackay, T.F. (2009) Co-regulated transcriptional networks contribute to natural genetic variation in *Drosophila* sleep. *Nat Genet* **41:** 371–375.

Hedges, L.M., Brownlie, J.C., O'Neill, S.L., and Johnson, K.N. (2008) *Wolbachia* and virus protection in insects. *Science* **322:** 702.

Hilgenboecker, K., Hammerstein, P., Schlattmann, P., Telschow, A., and Werren, J.H. (2008) How many species are infected with *Wolbachia*? – A statistical analysis of current data. *FEMS Microbiol Lett* **281:** 215–220.

Hoffmann, A.A., Hercus, M., and Dagher, H. (1998) Population dynamics of the *Wolbachia* infection causing cytoplasmic incompatibility in *Drosophila melanogaster*. *Genetics* **148:** 221–231.

Hughes, G.L., Ren, X., Ramirez, J.L., Sakamoto, J.M., Bailey, J.A., Jedlicka, A.E., and Rasgon, J.L. (2011) *Wolbachia* infections in Anopheles gambiae cells: transcriptomic characterization of a novel host-symbiont interaction. *PLoS Pathog* **7:** e1001296.

Joshi, S.G., and Kovacs, A.D. (2007) Rickettsia rickettsii infection causes apoptotic death of cultured cerebellar granule neurons. *J Med Microbiol* **56:** 138–141.

Kamerkar, S., and Davis, P.H. (2012) Toxoplasma on the brain: understanding host-pathogen interactions in chronic CNS infection. *J Parasitol Res* **2012:** 589295.

Koukou, K., Pavlikaki, H., Kilias, G., Werren, J.H., Bourtzis, K., and Alahiotis, S.N. (2006) Influence of antibiotic treatment and *Wolbachia* curing on sexual isolation among *Drosophila melanogaster* cage populations. *Evolution Int J Org Evolution* **60:** 87–96.

Kremer, N., Charif, D., Henri, H., Gavory, F., Wincker, P., Mavingui, P., and Vavre, F. (2012) Influence of *Wolbachia* on host gene expression in an obligatory symbiosis. *BMC Microbiol* **12** (Suppl. 1)**:** S7.

Landmann, F., Voronin, D., Sullivan, W., and Taylor, M.J. (2011) Anti-filarial activity of antibiotic therapy is due to extensive apoptosis after *Wolbachia* depletion from filarial nematodes. *PLoS Pathog* **7:** e1002351.

Landmann, F., Bain, O., Martin, C., Uni, S., Taylor, M.J., and Sullivan, W. (2012) Both asymmetric mitotic segregation and cell-to-cell invasion are required for stable germline transmission of *Wolbachia* in filarial nematodes. *Biol Open* **1:** 536–547.

Laurent, G., and Burrows, M. (1988) A population of ascending intersegmental interneurons in the locust with mechanosensory inputs from a hind leg. *J Comp Neurol* **275:** 1–12.

Lazareva, A.A., Roman, G., Mattox, W., Hardin, P.E., and Dauwalder, B. (2007) A role for the adult fat body in *Drosophila* male courtship behavior. *PLoS Genet* **3:** e16.

McGraw, E.A., Merritt, D.J., Droller, J.N., and O'Neill, S.L. (2002) *Wolbachia* density and virulence attenuation after transfer into a novel host. *Proc Natl Acad Sci USA* **99:** 2918–2923.

Mackay, T.F. (2010) Mutations and quantitative genetic variation: lessons from *Drosophila*. *Philos Trans R Soc Lond B Biol Sci* **365:** 1229–1239.

Min, K.T., and Benzer, S. (1997) *Wolbachia*, normally a symbiont of *Drosophila*, can be virulent, causing degeneration and early death. *Proc Natl Acad Sci USA* **94:** 10792–10796.

Morozova, T.V., Ayroles, J.F., Jordan, K.W., Duncan, L.H., Carbone, M.A., Lyman, R.F., *et al*. (2009) Alcohol sensitivity in *Drosophila*: translational potential of systems genetics. *Genetics* **183:** 733–745, 731SI–712SI.

Nakamura, Y., Gotoh, T., Imanishi, S., Mita, K., Kurtti, T.J., and Noda, H. (2011) Differentially expressed genes in silkworm cell cultures in response to infection by *Wolbachia* and *Cardinium* endosymbionts. *Insect Mol Biol* **20:** 279–289.

Narita, S., Nomura, M., and Kageyama, D. (2007) Naturally occurring single and double infection with *Wolbachia* strains in the butterfly Eurema hecabe: transmission efficiencies and population density dynamics of each *Wolbachia* strain. *FEMS Microbiol Ecol* **61:** 235–245.

Olsen, S.R., and Wilson, R.I. (2008) Cracking neural circuits in a tiny brain: new approaches for understanding the neural circuitry of *Drosophila*. *Trends Neurosci* **31:** 512–520.

Osborne, S.E., Iturbe-Ormaetxe, I., Brownlie, J.C., O'Neill, S.L., and Johnson, K.N. (2012) Antiviral protection and the importance of *Wolbachia* density and tissue tropism in *Drosophila simulans*. *Appl Environ Microbiol* **78:** 6922–6929.

Peng, Y., Nielsen, J.E., Cunningham, J.P., and McGraw, E.A. (2008) *Wolbachia* infection alters olfactory-cued locomotion in *Drosophila* spp. *Appl Environ Microbiol* **74:** 3943–3948.

Pereanu, W., Kumar, A., Jennett, A., Reichert, H., and Hartenstein, V. (2010) Development-based compartmentalization of the *Drosophila* central brain. *J Comp Neurol* **518:** 2996–3023.

Peters, B.H., Altman, J.S., and Tyrer, N.M. (1985) Synaptic connections between the hindwing stretch receptor and flight motor neurons in the locust revealed by double cobalt labelling for electron microscopy. *J Comp Neurol* **233:** 269–284.

Rasgon, J.L., Gamston, C.E., and Ren, X. (2006) Survival of *Wolbachia* pipientis in cell-free medium. *Appl Environ Microbiol* **72:** 6934–6937.

Reye, D.N., and Pearson, K.G. (1987) Projections of the wing stretch receptors to central flight neurons in the locust. *J Neurosci* **7:** 2476–2487.

Riegler, M., Sidhu, M., Miller, W.J., and O'Neill, S.L. (2005) Evidence for a global *Wolbachia* replacement in *Drosophila melanogaster*. *Curr Biol* **15:** 1428–1433.

Sambrook, J., Fritsch, E.F., and Manniatis, T. (1989) *Molecular Cloning – A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Serbus, L.R., and Sullivan, W. (2007) A cellular basis for *Wolbachia* recruitment to the host germline. *PLoS Pathog* **3:** e190.

Serbus, L.R., Casper-Lindley, C., Landmann, F., and Sullivan, W. (2008) The genetics and cell biology of *Wolbachia*–host interactions. *Annu Rev Genet* **42:** 683–707.

Serbus, L.R., Ferreccio, A., Zhukova, M., McMorris, C.L., Kiseleva, E., and Sullivan, W. (2011) A feedback loop between *Wolbachia* and the *Drosophila gurken* mRNP complex influences *Wolbachia* titer. *J Cell Sci* **124:** 4299–4308.

Spindler, S.R., and Hartenstein, V. (2010) The *Drosophila* neural lineages: a model system to study brain development and circuitry. *Dev Genes Evol* **220:** 1–10.

Stockinger, P., Kvitsiani, D., Rotkopf, S., Tirian, L., and Dickson, B.J. (2005) Neural circuitry that governs *Drosophila* male courtship behavior. *Cell* **121:** 795–807.

Tanaka, N.K., Endo, K., and Ito, K. (2012) Organization of antennal lobe-associated neurons in adult *Drosophila melanogaster* brain. *J Comp Neurol* **520:** 4067–4130.

Teixeira L, F.A., and Ashburner, M. (2008) The bacterial symbiont *Wolbachia* confers resistance to viruses in *Drosophila melanogaster*. In *49th Annual Drosophila Research Conference*. San Deigo, CA.

Thomas, F., Adamo, S., and Moore, J. (2005) Parasitic manipulation: where are we and where should we go? *Behav Processes* **68:** 185–199.

Toivonen, J.M., Walker, G.A., Martinez-Diaz, P., Bjedov, I., Driege, Y., Jacobs, H.T., *et al*. (2007) No influence of Indy on lifespan in *Drosophila* after correction for genetic and cytoplasmic background effects. *PLoS Genet* **3:** e95.

Veneti, Z., Clark, M.E., Karr, T.L., Savakis, C., and Bourtzis, K. (2004) Heads or tails: host-parasite interactions in the *Drosophila–Wolbachia* system. *Appl Environ Microbiol* **70:** 5366–5372.

Vosshall, L.B., and Stocker, R.F. (2007) Molecular architecture of smell and taste in *Drosophila*. *Annu Rev Neurosci* **30:** 505–533.

Vyas, A., Kim, S.K., Giacomini, N., Boothroyd, J.C., and Sapolsky, R.M. (2007) Behavioral changes induced by Toxoplasma infection of rodents are highly specific to aversion of cat odors. *Proc Natl Acad Sci USA* **104:** 6442–6447.

Werren, J.H., Baldo, L., and Clark, M.E. (2008) *Wolbachia*: master manipulators of invertebrate biology. *Nat Rev Microbiol* **6:** 741–751.

Yamada, R., Floate, K.D., Riegler, M., and O'Neill, S.L. (2007) Male development time influences the strength of *Wolbachia*-induced cytoplasmic incompatibility expression in *Drosophila melanogaster*. *Genetics* **177:** 801–808.

Yellman, C., Tao, H., He, B., and Hirsh, J. (1997) Conserved and sexually dimorphic behavioral responses to biogenic amines in decapitated *Drosophila*. *Proc Natl Acad Sci USA* **94:** 4131–4136.

Yu, H.H., Kao, C.F., He, Y., Ding, P., Kao, J.C., and Lee, T. (2010) A complete developmental sequence of a *Drosophila* neuronal lineage as revealed by twin-spot MARCM. *PLoS Biol* **8:** e1000461.

Zheng, Y., Ren, P.P., Wang, J.L., and Wang, Y.F. (2011a) *Wolbachia*-induced cytoplasmic incompatibility is associated with decreased Hira expression in male *Drosophila*. *PLoS ONE* **6:** e19512.

Zheng, Y., Wang, J.L., Liu, C., Wang, C.P., Walker, T., and Wang, Y.F. (2011b) Differentially expressed profiles in the larval testes of *Wolbachia* infected and uninfected *Drosophila*. *BMC Genomics* **12:** 595.

Zhukova, M.V., and Kiseleva, E. (2012) The virulent *Wolbachia* strain wMelPop increases the frequency of apoptosis in the female germline cells of *Drosophila melanogaster*. *BMC Microbiol* **12** (Suppl. 1)**:** S15.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Egg chambers (upper panels) and brain sections (lower panels) from wild-caught, *Wolbachia*-infected *D. simulans* lines. Host DNA and *Wolbachia* are stained with Propidium Iodide and *Wolbachia* are visible as small puncta in the egg chambers or among the host neuroblast nuclei. Scale bars, 20 μM. Similar images were obtained with all examined *D. melanogaster* lines.

Fig. S2. A. Egg chambers from infected lines (712 and 852), tetracycline-cured lines (712 and 852) and uninfected lines (375 and 517).

B. PCR from infected and cured *D. melanogaster* lines as indicated. Scale bars, 10 μM.

Table S1. Quantification of individual *Wolbachia* bacteria in different brain regions (data of the summary shown in Figs 3K and 4)

Table S2. *n* of the physiological experiments shown in Fig. 7. Status indications are: I = infected, T = infected, treated, U = uninfected, UT = uninfected, treated.