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***Wolbachia* enhance *Drosophila* stem cell proliferation and target the germline stem cell niche**

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Abstract

Wolbachia are widespread maternally-transmitted intracellular bacteria that infect most insect species and are able to alter the reproduction of innumerable hosts. The cellular bases of these alterations remain largely unknown. Here we report that *Drosophila mauritiana* infected with a native *Wolbachia* wMau strain produces about four times more eggs than the non-infected counterpart. *Wolbachia* infection leads to an increase in the mitotic activity of germline stem cells (GSCs) as well as a decrease in programmed cell death in the germarium. Our results suggest that upregulation of GSCs division is mediated by a tropism of *Wolbachia* for the germline stem cell niche (GSCN), the cellular microenvironment that supports GSCs.

Wolbachia are maternally transmitted intracellular bacteria infecting a large number of invertebrates such as insects and parasitic worms (1). Many invertebrates that harbor these bacteria are either the vectors (e.g. mosquitoes) or the causative agent (e.g. filarial nematodes) of devastating human infectious diseases. By understanding the biology at the interface between *Wolbachia* and their hosts, advances in the treatment of filarial diseases and the control of disease vectors are made possible (2–7). Furthermore, *Wolbachia* can dramatically alter host reproduction, affecting the evolutionary history of numerous invertebrates (1). Therefore, understanding how *Wolbachia* affect their hosts is an important ecological, evolutionary and human health question.

To investigate the influence of *Wolbachia* on their hosts at the cellular level, we used the *Drosophila* gonad, a powerful experimental system. We have previously shown that in *Drosophila melanogaster*, *Wolbachia* target the somatic stem cell niche (SSCN, Fig. 1A) – the microenvironment that supports the somatic stem cells – in the female ovary (8). Further work shows that *Wolbachia* also target the somatic stem cell niche in the ovary of other insects (9, 10). Here we report two additional stem cell niches preferentially colonized (i.e., cell tropism) by *Wolbachia*: the female germline stem cell niche (GSCN, Fig. 1A), and the hub, at the apical tip of the testis (discussed below). In a *D. mauritiana* stock infected with

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Wolbachia wMau, we consistently noticed an intense accumulation of bacteria in the GSCN, the structure harboring the GSCs (infection frequency = $91\% \pm 5.7\%$, $N = 958$ germaria) (see *Wolbachia*, labeled green in Fig. 2A and B, Fig. 3A and fig. S1A). This GSCN accumulation was absent in *D. melanogaster* (GSCN infection frequency = 0% , $N = 180$ germaria, see fig. S1, B compared to A). Electron microscopy and three-dimensional reconstruction of confocal images show that the vast majority of the cytoplasmic volume of the GSCN is occupied by *Wolbachia* wMau [see Fig. 1B, the *Wolbachia* cells (a red asterisk indicates a single bacterial cell) occupy most of the GSCN (colored in green), compared to the non-infected control in fig. S1C; see also Sup. Movie]. Because GSCN function is essential for stem cell maintenance and activity (11), we hypothesized that the high levels of infection in the niche would impair its associated stem cells to a certain degree. An easy readout of GSC activity is egg production, since every egg produced originates from the division of a stem cell associated with the GSCN (Fig. 1A'). The total number of eggs laid per *Wolbachia*-infected female was 3.5 times higher than that observed in non-infected flies (herein referred to as "W-"; the genetic background of the W- flies was homogenized by successive backcrossing to infected males, as shown in fig. S2). This experiment was repeated under different temperature, humidity and age conditions (see SOM Methods and Table S1) (12). Under these different conditions, infected flies (referred to as "W+") still produced approximately fourfold more eggs than the non-infected females (see Fig. 1C and table S1).

Given these levels of egg production, we reasoned that W+ ovaries contain GSCs that are more active. To test this possibility, we measured the frequency of GSC division in W+ and W- flies using three different markers for three distinct phases of the cell cycle. The initial assessment was performed using an anti-phospho-histone H3 antibody, that labels cells in mitosis (Fig. 2, A and C, fig. S3G) (12). The labeling of GSCs in W+ flies was on average $2.7 (\pm 0.23)$ fold higher than in W- flies (Fig. 2E and table S2). This increase could indicate either a higher GSC division in infected germaria or an arrest during the mitotic phase of the cell cycle.

We further investigated GSC proliferation using two additional markers: incorporation of the thymidine analog BrdU, an indicator of DNA synthesis during S phase (fig S3, A, D and G), and a particular fusome morphology characteristic of GSCs in G2 (fig. S3, B, E and H). The fusome is a germline-specific organelle that assumes the shape of an exclamation mark (!) during G2 (13, 14). Both markers corroborated a higher GSC proliferation rate in W+ (Fig 2E). The probability of stem cell division is significantly increased in *Wolbachia* infected flies (SOM Methods and table S2) (12). In nine independent experiments, utilizing three different methods, stem cell division in W+ flies was on average doubled (2.12 ± 0.66 , table S2). Although significant, this amount by itself does not suffice to explain the fourfold increase in egg production in infected flies. An additional cellular event that could alter egg production in a *Wolbachia*-dependent manner could be cell death in the ovary. Programmed cell death (PCD) is a known key regulator of egg production in *Drosophila melanogaster* (15). Furthermore, previous studies in wasps and in human neutrophils have shown that the presence of *Wolbachia* or *Wolbachia*-derived proteins, respectively, inhibits host apoptosis (16, 17).

We quantified the influence of *Wolbachia* infection on two developmentally-regulated PCD events that modulate egg production in *Drosophila*, the first in the germarium (Fig. 1A, left red arrow) and the second during the onset of vitellogenesis (Fig. 1A, right red arrow) (15, 18). In the parasitic wasp *Asobara tabida*, removal of *Wolbachia* causes sterility through massive cell death in mid-oogenesis, at the pre-vitellogenic stages (16). Therefore, we initially measured PCD at these stages. We found that the differences in PCD between W+ and W- previtellogenic egg chambers were highly variable and not significant regarding *Wolbachia*'s effects at this developmental point (fig. S4 and table S3) (12).

Accordingly, we measured the levels of PCD in the germarium. Using two different assays – DNA fragmentation in fixed tissue (TUNEL, Fig. 2, B and D) and visualization of dead cells via live imaging (Acridine Orange, fig. S3, C and F) – *Wolbachia* infection consistently decreased PCD in the germarium by approximately one half as compared to non-infected flies (Fig. 2F, and table S4) (12). *Wolbachia*-driven reduction of PCD in the germarium was statistically significant (Fig. 2 and table S4). Together, these results indicate that the increase in egg production in W+ *D. mauritiana* is due to both increased GSC mitosis and decreased PCD in the germarium.

Next, the mechanistic foundation for *Wolbachia*'s manipulation of GSC mitotic activity was examined. Considering that GSCN regulates stem cell physiology (19), we designed an experiment to test if levels of *Wolbachia* in the GSCN correlate with mitotic activity of the GSC (see fig. S5). During this assay we used only *Wolbachia* infected flies. Even though in these W+ flies most of the GSCNs were highly infected ($91\% \pm 6.5\%$, $N = 788$) (Fig. 3, A and C), there is a small population of niches that have either very low or no *Wolbachia* present. These distinct types of niches were termed “LN” (low infection in the niche, Fig. 3B) and their infected counterparts “HN” (high infection in the niche, Fig. 3A; see also fig. S6, A compared to B, fig. S7). Because these distinct populations of GSCs are present inside the same infected flies, all of the environmental and systemic factors are exactly the same. In four independent experiments, the mitotic activity of GSCs residing in LN niches was substantially lower or absent in comparison to HN niches (Fig. 3, C and D). There is a statistically significant association of GSC mitosis with the high density of *Wolbachia* in the niche ($P = 2.4 \times 10^{-2}$, see table S5) (12). This observation favors a mechanism in which *Wolbachia*'s infection in the niche modulates stem cell activity, although it does not rule out a contribution from systemic or stem cell-intrinsic signals (see SOM text S1, fig. S8, S9).

We found that *Wolbachia* wMau also target the hub, a group of somatic cells that form the niche supporting germline and somatic stem cells of the testis (20). In males, both the targeting of the hub (64%, $N = 77$, Fig. 3, E and F) as well as the upregulation of GSC division did not occur to the same degree as in females (fig. S10, table S6). It is possible that phenotypic consequences of niche tropism are diverse in males. *Wolbachia* and other maternally inherited endosymbionts can evolve drastically different germline manipulation phenotypes between sexes (21).

The vast majority of insects have symbiotic associations with bacteria that are vertically transmitted through the egg cytoplasm (22). Because of maternal transmission, these host-bacteria partnerships evolve to favor the reproductive success of infected mothers (1, 23–

25). In the *Drosophila* genus, there are several reports of *Wolbachia*-induced changes in fecundity, including cases of rapid evolution of both partners, changing from a parasitic to mutualistic association in 20 years (24, 26–29). There is little understanding of these dramatic and widespread interferences with host reproduction at the cellular and molecular level (30). Here, we have identified two cellular events that are manipulated by *Wolbachia*. The combination of *Wolbachia*-induced alterations of both PCD in the germarium and GSC mitosis results in higher egg production, which further promotes *Wolbachia* spreading through maternal transmission. These findings provide the cellular mechanisms for *Wolbachia*'s effects on host fecundity observed in this infected *D. mauritiana* strain over its non-infected counterpart (see SOM text S2).

Advancing our understanding of how endosymbionts subvert the cellular processes of insects will also be relevant to the growing efforts towards controlling human infectious diseases through symbiotic bacteria (3–7, 31).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. *Wolbachia* target the GSCN and infection increases egg production

(A) *Drosophila* ovariole with the germline shown in light blue and the somatic follicle cells in white. Egg chambers are formed in the germarium (left) and mature into the egg. The upward-pointing green arrow indicates germline stem cell (GSC, dark blue) division, which positively affects egg production [see inset A': GSC divides asymmetrically and one daughter cell exits the germline stem cell niche (GSCN, green) and forms the egg's germline (light blue)]. The downward-pointing red arrows indicate developmental points where the onset of programmed cell death (PCD) reduces egg production, either in the germarium or in previtellogenic egg chambers. (Lower left) A magnified view of the germarium shows both the somatic stem cell niche (SSCN, green arrowhead) and the GSCN (yellow bracket), formed by the terminal filament (light green) and the cap cells (dark green), which contact the GSCs (blue arrowhead). (B) Electron micrographs of a GSCN (green) and the GSC (blue) in infected *D. mauritiana*. Most of the cytoplasm of the cap cells (GSCN) is occupied by *Wolbachia* wMau (red asterisk, see also Sup. Movie). Scale bar = 1 μ m. The inset shows a magnified view of the GSCN, red asterisk highlighting a single *Wolbachia* wMau. (C) Fold change of total amount of eggs laid per infected female (W+, green) under different conditions normalized to non-infected (W-, yellow). Relative egg production was measured in triplicate for each condition: room temperature (RT, 20 days and 46 days, light green) or at 25°C (20 days, dark green). *Wolbachia* significantly induced fecundity gains at all

conditions (Student's t -test, $P_{RT\ 20\ days} = 6.5 \times 10^{-4}$, $P_{RT\ 46\ days} = 3.9 \times 10^{-4}$ and $P_{25^{\circ}C\ 20\ days} = 1.7 \times 10^{-2}$). See table S1 and (12).

Fig. 2. *Wolbachia* infection increases GSC mitotic activity and suppresses PCD in the germarium Representative confocal images of *D. mauritiana* germaria infected [W+, *Wolbachia* shown in green (A and B)] and non-infected [W-, (C and D)]. Arrowheads indicate the presence (red arrowhead) or absence (blue arrowhead) of GSC division [pH3 (phospho-Histone H3), red in A and C] or PCD (TUNEL, red in B and D). Germline is labeled with anti-Vasa (blue), Scale bar = 10µm. (E and F). Average fold difference for each marker indicated below graphs, normalized to W- (mean of triplicates, 15 independent experiments total). Infection significantly affects GSC mitosis (E) and PCD (F) for all markers (Logistic regression, $P_{pH3} = 5.4 \times 10^{-3}$, $N = 621$; $P_{BrdU} = 2.0 \times 10^{-2}$, $N = 1061$; $P_{Fusome} = 4.3 \times 10^{-3}$, $N = 695$; $P_{TUNEL} = 8.0 \times 10^{-3}$, $N = 802$; $P_{Acridine\ Orange} = 1.2 \times 10^{-7}$, $N = 754$, N = number of germaria.). See also tables S2, S4 and (12).

Fig. 3. High levels of *Wolbachia* at the GSCN upregulate GSC mitosis

(A – B) Niches (yellow brackets) from infected flies are classified as highly infected (HN, A) and with low infection (LN, B). Fusome staining (red) shows GSC in the HN dividing (“!” morphology in A). Scale bar = 5 μ m. (C) Frequency of HN (solid green) and LN (hatched green) in four independent experiments. The numbers in each category and the total number of germaria analyzed are indicated for each experiment. (D) For each germarium counted in graph D, the frequency of GSC division was determined by either fusome morphology (Exp. 1 and 2) or BrdU incorporation (Exp. 3 and 4). HN significantly increases GSC mitosis (logistic regression, $P = 2.4 \times 10^{-2}$). (E and F) In infected testes of *D. mauritiana*, *Wolbachia* also target the stem cell niche (aka hubs, yellow arrowhead) at high (HN, E) and low levels (LN, F). (F) pH3 staining (white) labels a dividing testis stem cell adjacent to an HN niche. Scale bar = 5 μ m.