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Intercommunity effects on microbiome and GpSGHV density regulation in tsetse flies

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Abstract

Tsetse flies have a highly regulated and defined microbial fauna made of 3 bacterial symbionts (obligate *Wigglesworthia glossinidia*, commensal *Sodalis glossinidius* and parasitic *Wolbachia pipientis)* in addition to a DNA virus (*Glossina pallidipes* Salivary gland Hypertrophy Virus, GpSGHV). It has been possible to rear flies in the absence of either *Wigglesworthia* or in totally aposymbiotic state by dietary supplementation of tsetse's bloodmeal. In the absence of *Wigglesworthia*, tsetse females are sterile, and adult progeny are immune compromised. The functional contributions for *Sodalist* are less known, while *Wolbachia* cause reproductive manupulations known as Cytoplasmic Incompatibility (CI). High GpSGHV virus titers result in reduced fecundity and lifespan, and have compromised efforts to colonize flies in the insectary for large rearing purposes. Here we investigated the within community effects on the density regulation of the individual microbiome partners in tsetse lines with different symbiotic compositions. We show that absence of *Wigglesworthia* results in loss of *Sodalis* in subsequent generations possibly due to nutritional dependancies between the symbiotic partners. While an initial decrease in *Wolbachia* and GpSGHV levels are also noted in the absence of *Wigglesworthia*, these infections eventually reach homeostatic levels indicating adaptations to the new host immune environment or nutritional ecology. Absence of all bacterial symbionts also results in an initial reduction of viral titers, which recover in the second generation. Our findings suggest that in addition to the host immune system, interdependencies between symbiotic partners result in a highly tuned density regulation for tsetse's microbiome.

Keywords

symbiont; virus; intercommunity; tsetse

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1. Introduction

Tsetse flies are the sole vectors responsible for cyclical transmission of protozoan trypanosomes that cause human African trypanomiasis (HAT or sleeping sickness) and African animal trypanosomiasis (AAT or nagana). HAT causes devastating effect on humans, while AAT impacts agricultural development and nutritional resources in about 37 countries in sub-Saharan Africa (Welburn et al., 2009). There are no effective vaccines for disease control and drugs available for treatment are expensive and difficult to administer (Aksoy, 2011). Control of the tsetse populations however has had considerable success for disease control. However, disease elimination requires vector control methods that are cheap and effective for sustainability. In addition to transmitting trypanosomes, tsetse harbors several symbiotic microbes. Knowledge on tsetse symbiosis, which is essential for nutrition, fecundity and immunity can lead to novel approaches for vector control (Aksoy, 2000; Aksoy et al., 2008a; Aksoy et al., 2008b; Rio et al., 2004). Tsetse females have an unusual viviparous reproductive biology. Females develop a single oocyte per gonotrophic cycle. The oocyte is ovulated, fertilized and undergoes embryonic development in-utero. The resulting larva hatches and is carried in the intrauterine environment through three larval instars before being deposited. During its intrauterine life, the larva receives all of its nutrients in the form of milk secreted by the female accessory glands, milk glands. Tsetse feed exclusively on vertebrate blood, which is limited in nutrients. To supplement its diet with metabolites missing from its diet, tsetse has established symbiosis with *Wigglesworthia glossinidia* (called Primary endosymbiont) (Aksoy, 1995). In addition tsetse can carry the facultative commensal *Sodalis glossinidius* (called Secondary endosymbiont) and parasitic *Wolbachia pipientis* (Dale and Maudlin, 1999; O'Neill, 1993). While *Wolbachi*a is vertically transmitted trans-ovum, *Sodalis* and *Wigglesworthia* are maternally transmitted via mother's milk (Attardo et al., 2008; Cheng and Aksoy, 1999). *Wolbachia* symbionts are wide spread intracellular bacteria that have been estimated to infect over 60% of insects (Hilgenboecker et al., 2008). *Wolbachia* infections have been shown to cause a number of reproductive modifications in their hosts, the most common being cytoplasmic incompatibility (CI) (Saridaki and Bourtzis, 2010; Werren, 1997). CI occurs when a *Wolbachia* infected male mates with an uninfected female or with a female infected with a different strain of *Wolbachia* (Serbus et al., 2008; Werren et al., 2008). In tsetse, *Wolbachia* is localized exclusively within germ-line tissue and induces strong CI (Alam et al., 2011; Cheng et al., 2000). In addition to CI, some *Wolbachia* infections can benefit host fitness, including nutrition provision, influencing lifespan, and conferring resistance to pathogens (Aleksandrov et al., 2007; Glaser and Meola, 2010; Hosokawa et al., 2010; Kambris et al., 2010; Moreira et al., 2009; Walker et al., 2011). Furthermore, presence of certain *Wolbachia* strain infections in mosquitoes have been associated with resistance to other pathogen infections, including dengue and plasmodium (Brelsfoard and Dobson, 2011a; Kambris et al., 2010; Walker et al., 2011). The facultative commensal endosymbiont *Sodalis* has established intra and extra-cellular infections in various diverse tissues in tsetse, including midgut, hemolymph and milk gland (Attardo et al., 2008; Cheng and Aksoy, 1999). The genome sequence of *Sodalis* has shown that it has reduced coding capacity (around 51%), and contains a large number of fragmented CDSs and pseudogenes, which are apparently non functional in the restricted nutritional ecology of its host. Thus, *Sodalis* represents an evolutionary intermediate transitioning from a free-living to a mutualistic lifestyle (Toh et al., 2006). The function of *Sodalis* in tsetse is unclear, but it has been suggested to play a role in vector competence by favoring trypanosome establishment (Dale and Welburn, 2001; Farikou et al., 2010). While all individuals in laboratory lines harbor *Sodalis*, infection prevalence in natural populations vary from 0% to 85% in the different species analyzed (Lindh and Lehane, 2011; Maudlin et al., 1990). Furthermore presence of multiple *Sodalis* genotypes have also been described in natural population (Geiger et al., 2005).

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All tsetse individuals harbor the obligate mutualist *Wigglesworthia,* which has coevolved with the tsetse host over 50 million years (Chen et al., 1999). *Wigglesworthia* resides intracellularly in the midgut bacteriome organ, and extracellularly in mother's milk secretions (Attardo et al., 2008; Ma and Denlinger, 1974; Pais et al., 2008). *Wigglesworthia* genome has been drastically reduced to about 700 kb in size and has retained functions presumably necessary for the hosts such as the vitamin coding genes, which are thought to supplement tsetse's nutritionally restricted blood diet with vitamins (Akman et al., 2002; Rita V.M. Rio, 2012). Furthermore, *Wigglesworthia* has retained functional flagella and motility coding genes, which are expressed preferentially in the milk gland and early larval developmental indicating that they may be responsible for the transmission of *Wigglesworthia* from mother to progeny and for the colonization in early intrauterine larva (Rita V.M. Rio, 2012). It has been possible to generate *Wigglesworthia* free flies by maintaining fertile females on ampicillin supplemented blood diets (Pais et al., 2008). The ampicillin eliminates the extracellular *Wigglesworthia* population residing in the milk gland, but does not impact the intracellular *Wigglesworthia* within bacteriocytes. Thus, females continue to reproduce but give rise to progeny, which lack *Wigglesworthia* and hence which are reproductively sterile. It has also been possible to maintain flies fertile in the absence of all of their endosymbionts by supplementing their antibiotic containing diets with 1% (w/v) yeast extract (Alam et al., 2011). Thus, by supplementing the diet of fertile *G. morsitans morsitans* (*Gmm*) females by either ampicillin or tetracycline, lines have been developed that either lack only *Wigglesworthia* while retaining *Sodalis* and *Wolbachia* (Gmm^{Wig-}) or that lack all symbionts (aposymbiotic, Gmm^{Apo}). Absence of *Wigglesworthia* during larval progeny development has been associated with compromised immunity in emerging adults, indicating that *Wigglesworthia* is essential for immune maturation during development in addition to its nutrient supplementation role in adults (Pais et al., 2008; Weiss et al., 2011).

Finally, in addition to the three bacterial symbionts, laboratory flies and a number of natural populations carry a nuclear rod-shaped enveloped DNA virus (*Glossina pallidipes* Salivary gland Hypertrophy Virus, GpSGHV), that was first identified by Jaenson as the causative agent of salivary gland hypertrophy (Jaenson, 1978). GpSGHV can be maternally transmitted either through trans ovum or infected milk glands. In laboratory maintained colony, it can also be horizontally transmitted during blood feeding on an *in vitro* membrane (Abd-Alla et al., 2011). High virus titers result in reduced fecundity and lifespan, and have compromised efforts to colonize flies in the insectary for large rearing purposes (Abd-Alla et al., 2011; Sang et al., 1999).

Environmental bacteria other than the symbiotic partners have been described from *G. fuscipes fuscipes* in Kenya and *G. palpalis palpalis* in Angola (Geiger et al., 2009; Lindh and Lehane, 2011), but their relative densities, transmission and prevalence in natural populations remains to be seen. While each symbiont exhibits different levels of integration with host biology and can impact different aspects of host physiology, there may also be within community dynamics that impacts their density regulation. For example, based on genome comparative analysis, it appears that *Sodalis* and *Wigglesworthia* may show metabolic complementarity (Belda et al., 2010; Snyder et al., 2010). It appears that *Sodalis* is unable to complete thiamine biosynthesis pathway but encodes a thiamine transporter. In our earlier studies, we investigated symbiont density dynamics thru development (Rio et al., 2006). These results showed lack of proliferation during juvenile development, followed by a narrow window of opportunity for proliferation in young adults after hatching, but regulated growth in adulthood. We reported highly variable densities for *Wolbachia*, but in light of the recently discovered chromosomal insertions for the *wsp* gene used for analysis in *G. m. morsitans,* these results do not indicate the true cytoplasmic *Wolbachia* density measurements (Doudoumis, 2011).

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In this paper, we examined the microbiome density regulation through development in tsetse. In particular, we measured cytoplasmic *Wolbachia* density regulation through different host developmental stages and sex. We also used the host lines we developed, Gmm^{Wig-} that lacks *Wigglesworthia* but retains *Sodalis*, *Wolbachia* and GpSGHV, and Gmm^{Apo} that lacks all symbionts but retains GpSGHV, to understand the intercommunity dynamics on symbiotic density regulation outcomes.

2. Materials and Methods

2.1 Insects and trypanosomes

Ampicillin and tetracycline treated *Glossina morsitans morsitans* females were maintained as described (Alam et al., 2011; Pais et al., 2008). Briefly, females were fed on blood meal supplemented with ampicillin (50 μg/ml) or with tetracycline (20 μg/ml) and yeast extract (1 mg/ml) (BD, Franklin Lakes, NJ). Pupal progeny from the second and third gonotrophic cycles of ampicillin treated mothers were collected and reared to adulthood (denoted as Gmm^{Wig-}-*F1*). Gmm^{Wig-}-*F1. F1* progeny were mated and their progeny (denoted as Gmm^{Wig}-*F2*) were obtained. Similarly the progeny of Gmm^{Wig-}-*F2* were collected (denoted as Gmm^{Wig-}-*F3)*. Two generations of progeny of tetracycline treated mothers, denoted as Gmm^{Apo}-*F1 and* Gmm^{Apo}-*F2* were collected similarly. Schematic protocol for rearing methods is shown in Fig. 1. All progeny received bloodmeals supplemented with yeast extract (10 mg/ml) to keep them fertile in the absence of the obligate *Wigglesworthia*. Normal teneral flies were fed on same yeast supplemented blood until 40 days old and were used as controls.

2.2 Wolbachia density in tsetse

For *Wolbachia* quantifications, we used primers specific for the heat-shock protein 60, *groel*, which we have confirmed to be absent from *G. m. morsitans* chromosomal DNA (Doudoumis, 2011). For measurements, we used same tissue and developmental DNA samples as described (Rio et al., 2006). Briefly, mothers and their four sequential offsprings (1^{st}, 2^{nd} 3^{rd} and 4^{th} deposition) were collected and DNA was extracted using Holmes-bonner method (Holmes and Bonner, 1973). DNA from larva (three stages), early pupae (24–48 hr post deposition) and late pupae (28–30 day post deposition), 1 week, 2 week and 4 week old females and males were similarly extracted.

2.3 Quantification of symbiont and SGHV densities in Gmm^{Wig-} and Gmm^{Apo} flies

At least four females of Gmm^{Wig-} (F1, F2 and F3) and Gmm^{Apo} (F1 and F2) were collected when adults were 40 days old and surface sterilized by washing twice in 1% bleach and twice in 75% ethanol, respectively. Total DNA was extracted using the Holmes-bonner method (Holmes and Bonner, 1973). Symbionts and GpSGHV were quantified by qPCR using the primers listed in Table 1. All data were normalized to host β-*tubulin*. All data were expressed as fold change compared to the same samples collected from normal controls (Gmm^{WT}). Values are presented as means. Proportional data was Arcsinsqrt transformed prior to statistical analysis and significance was determined using a one-way ANOVA test.

3. Results

3.1 Maternal transmission of Wolbachia

To investigate the transmission density dynamics of the three symbionts from mother to offspring, mothers and female offspring of their four depositions were collected and measured. Our prior data had indicated that there was no significant variation in the number of symbionts (*Sodalis* and *Wigglesworthia*) acquired from mother to her sequential progeny (Rio et al., 2006). Here we analyzed the same samples for cytoplasmic *Wolbachia* density

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variations using primers that do not amplify from the chromosomal insertions. Our findings show that each of the sequential progeny of the mothers acquired *Wolbachia* from the mother and there were no significant variations in the number of symbionts acquired between the different sequential larval progeny analyzed immediately upon deposition (Fig. 2). Significant differences in comparisons of the *Wolbachia* density of mothers and her offspring was observed (ANOVA; $F_{1,14} = 5.0$, P =0.01) (Fig. 2). The mothers were shown to have a significantly greater *Wolbachia* density than their offspring (Fig. 2), however there was high variability in the number of *Wolbachia* present in the mothers analyzed. Our data indicate perfect maternal inheritance of *Wolbachia* infection through trans-ovum transmission, and a high variability in *Wolbachia* densities in adult females.

3.2 Wolbachia density in immature stages

We next examined the proliferation status of *Wolbachia* during the three larval stages *in utero* obtained by microscopic dissections and during early and late pupal stages obtained post parturition. We present our data both as absolute *Wolbachia* numbers and as normalized to host β-*tubulin* levels to better gain information on symbiotic proliferation. The presence of *Wolbachia* was detected in the 1st larval state and its relative density during larval growth changed significantly when comparing the larval stages through the pupa stages (ANOVA, $F_{4,16} = 5.4$, P = 0.006) when normalized by the tsetse β-*tubulin* gene (Fig. 3A). *Wolbachia* density did not increase during pupal growth either (the early and late pupal stages) (Fig. 3A). However, no difference in the absolute copy number of *Wolbachia* was observed through development suggesting that *Wolbachia* does not proliferate significantly during juvenile development in tsetse (ANOVA, $F_{4,16} = 0.16$, P =0.95) (Fig 3B). This proliferation is different from the other two symbionts *Sodalis* and *Wigglesworthia,* which proliferate in parallel with host cell growth during immature stages (Rio et al., 2006).

3.3 Wolbachia density in adults

We next measured the *Wolbachia* density from adult male and female flies at 1, 2 and 4 weeks post eclosion. *Wolbachia* infection density was significantly higher in males than in females and increased with age in females (ANOVA, $F_{2,17} = 3.80$, P = 0.04) but not in males (ANOVA, $F_{2,19} = 1.35$, P = 0.28) (Fig. 4A and B, respectively). However, in older females (4 weeks old) and in males (2 and 4 weeks old), the relative *Wolbachia* density varied significantly (Fig. 4B), suggesting that in some individuals *Wolbachia* infections may proliferate without tight host regulation in the adulthood, unlike what we observed during juvenile development. It appears that particularly males carry higher loads of *Wolbachia* and are subject to varying proliferations in the adulthood.

3.4 Dynamics of symbionts density in Gmm^{Wig-} flies

Development of *Wigglesworthia* free flies (Gmm^{Wig-}) allowed us to study the intercommunity dynamics for symbiont density regulation in the host. We investigated density regulation of *Sodalis* and *Wolbachia* in Gmm^{Wig-} from the three generations of 40 day old Gmm^{Wig-}. When normalized against the levels detected in the corresponding control Gmm^{WT} flies that retained *Wigglesworthia*, *Sodalis* numbers in the first generation (Gmm^{Wig-}-F1) increased initially, returned to similar levels as the Gmm^{WT} controls by the second generation (Gmm^{Wig-}-F2), but significantly declined in the third generation (Gmm^{Wig-}-F3) (Fig. 5A). It is likely that there is a complementary relationship between *Sodalis* and *Wigglesworthia* given the restricted nutritional ecology they share in the tsetse midgut. At least one product, thiamine has been shown to be limiting for *Sodalis* growth as only the thiamine transporter is present in its genome (Toh et al., 2006). Given that *Wigglesworthia* can synthesize thiamine, it is possible that *Sodalis* may benefit and exploit *Wigglesworthia* metabolites for its growth *alone* (Belda et al., 2010; Snyder et al., 2010). Without *Wigglesworthia, Sodalis* levels may decline in parallel with the decreasing level of

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nutrition. Interestingly, *Wolbachia* density analyzed from the same samples showed a decrease in the first two generations (F1 and F2), but reached a stable level in the F3 revealing that mutual beneficial relationships may also exist between *Wolbachia* and *Wigglesworthia* (Fig. 5B).

3.5 Dynamics of GpSGHV in Gmm^{Wig-} and Gmm^{Apo} flies

We found that the prevalence for GpSGHV infection in our laboratory tsetse colony is 100% based on PCR assay. We do not however observe any salivary gland hypertrophy phenotype in the colony flies (data not shown). To further investigate the interaction of this virus with the symbiotic fauna of the host, GpSGHV levels were examined in Gmm^{Wig-} and Gmm^{Apo} flies. GpSGHV load decreased significantly in the absence of *Wigglesworthia* by over 100 fold in the first two generations (Gmm^{Wig-}-F1 and Gmm^{Wig-}-F2), but reached normal density levels in the third generation flies (Gmm^{Wig-}-F3) (Fig. 6). In symbiont-free Gmm^{Apo} flies, we saw the same trend, GpSGHV level was reduced in the first generation (Gmm^{Apo}-F1) by over 500 fold, but increased significantly in the second (Gmm^{Apo}-F2) reaching normal levels. These data suggest that tsetse's microbiome can influence GpSGHV densities either directly or indirectly and that in the absence of the microbiome, proliferation of the virus also suffers. It remains to be seen if the higher levels of GpSGHV we noted in the aposymbiotic flies results in atrophy of salivary glands or in sterility in the males.

4. Discussion

All multicellular eukaryotes live in symbiotic associations with microorganisms, which can form complex communities referred to as the microbiome. While many studies have looked at the role of the individual microorganism and the one host-one microbe dynamics, host physiology and the dynamics of the individual infections can be affected and shaped by community interdependencies. In contrast to higher eukaryotes, which are colonized by hundreds to thousands of prokaryotic phylotypes representing members of five of the six kingdoms of life, insects harbor a significantly less diverse community of microbial symbionts. Our studies here describe *Glossina* (tsetse fly) that houses a vertically transmitted microbiome composed of two enteric symbionts (genus *Sodalis* and *Wigglesworthia*) residing in the gut, one parasitic bacterium genus *Wolbachia*) present in the gonadal tissues in addition to a maternally transmitted DNA virus (GpSGHV). We used this simple symbiosis to understand symbiotic density regulation and within community effects on the individual symbiont fitness traits (density regulation and vertical transmission efficiency). Our results show that there is synergy between *Sodalis* and *Wigglesworthia* such that fly lines that lack *Wigglesworthia* eventually loose *Sodalis* infections by the third generation. This was in contrast to *Wolbachia* infections that showed wide variability in different individuals as a function of age, and that continued to maintain infections at homeostatic levels in the absence of *Wigglesworthia*. The GpSGHV levels measured without symbionts showed significant initial fluctuations only to reach again homeostatic levels by the third generation. Our results suggest interdependencies between the symbiotic partners. However, lack of extensive proliferation by any one partner in the absence of another reflects the association of a tightly regulated microbiome in tsetse.

The symbiotic fauna with the exception of *Wolbachia* are all maternal transmitted to tsetse's intrauterine larva in mother's milk secretions. *Wolbachia* is acquired by the progeny vertically trans-ovum. *Sodalis* and *Wigglesworthia* proliferate in sink with host development during immature developmental stages (larva and pupae). In young adults post eclosion however, both symbiotic densities increase for several days before being regulated by the host (Rio et al., 2006). It is interesting to note however, that in females but not in males, *Wigglesworthia* continues to proliferate for several weeks, possibly reflecting its essential role in female fecundity (Rio et al., 2006). *Wolbachia* is transmitted to offspring trans-ovum

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through germ-line infections (Alam et al., 2011). Our prior studies on *Wolbachia* dynamics had used primers that amplified *Wolbachia* fragments incorporated into tsetse's chromosomal DNA. Reanalysis of the spatial and temporal distribution of *Wolbachia* using primers specific for the cytoplasmic *Wolbachia* show that *Wolbachia* is localized in tsetse's reproductive tissues only (Doudoumis, 2011). Our data also show that there are no significant differences in the levels of *Wolbachia* acquired by the sequential progeny from their mother when analyzed in larva immediately post deposition (Fig. 2) and there is no proliferation during juvenile growth stages (Fig. 3). In the adult stages however, males have at least ten fold higher *Wolbachia* density than females and *Wolbachia* densities begin to increase around 2-weeks post eclosion (Fig. 4). Interestingly, older adults of both sexes show wide variability in *Wolbachia* levels. Results are similar to *w*AlbA *Wolbachia* strain infecting the mosquito *Aedes albopictus*, with a higher density observed in older males and wide variability in *Wolbachia* level (Tortosa et al., 2010). However, unlike tsetse a rapid decay of infection in older males only was observed (Tortosa et al., 2010). In contrast the *Wolbachia* infections in the mosquito *Culex pipiens* was observed to increase with age, and was dependent upon strain type (Duron et al., 2007).

Our data from colony flies need to be validated in field populations as nutritional ecology and environmental parameters (temperature and humidity) in the field are likely to be different than the optimal conditions maintained in the insectary. Given that *Wolbachia* densities may affect the levels of CI expression, it remains to be seen if the increase in *Wolbachia* we noted in males as a function of age may contribute significantly to CI in tsetse (Brelsfoard and Dobson, 2011b; Ikeda et al., 2003; Noda et al., 2001). However, male age has not been shown to influence CI rates in *Aedes* and *Culex* mosquitoes (Brelsfoard and Dobson, 2011b; Duron et al., 2007; Noda et al., 2001) and in two planthopper species (Noda et al., 2001). Albeit there is no clear trend on the effects of *Wolbachia* density and the age of the insect host, it seems *Wolbachia* density may be host specific and influenced by environmental, nutritional and rearing conditions (Mouton et al., 2003; Mouton et al., 2006; Mouton et al., 2007; Wiwatanaratanabutr and Kittayapong, 2009).

In addition to individual host-symbiont interactions, interactions between the community of organisms have been reported, ranging from beneficial to antagonistic in nature (Oliver et al., 2010). Metabolic interdependence between *Wigglesworthia* and *Sodalis* is predicted based on their coding capacity and an *in vitro* empirical study (Belda et al., 2010; Snyder et al., 2010). Apparently, *Sodalis* may compensate *Wigglesworthia* for folate and Coenzyme A synthesis, while *Wigglesworthia* provisions thiamine to *Sodalis*. The reduction of *Sodalis* levels in Gmm^{Wig-} flies by the third generation further confirms that *Sodalis* fitness depends on the presence of *Wigglesworthia* (Fig. 5A). The decrease in *Wolbachia* density in the absence of *Wigglesworthia* may also suggest co-adaptation or nutritional dependence. Thus, nutritional dependencies within the community can shape density regulation and transmission dynamics of the individual partners.

It is also possible that host immune responses in the absence of *Wigglesworthia* may be detrimental for commensal and parasitic microbes, including GpSGHV. Our studies have shown that to ensure optimal fecundity benefits, tsetse overexpresses an immune effector molecule peptidoglycan recognition protein (PGRP)-LB, which functions to eliminate microbe released immune elicitors such as PGN (Wang et al., 2009). PGRP-LB is also maternally transmitted to tsetse's intrauterine larva where it apparently functions again to as a negative regulator of the host immune system. In the absence of *Wigglesworthia*, there is less PGRP-LB, which in turn results in the upregulation of the host immune system. Although, studies have shown that *Sodalis* is resistant to tsetse's antimicrobial peptides, the effect of AMPs on *Wolbachia* is unknown. There may also be other effectors that may be upregulated in the absence of *Wigglesworthia* that may in turn damage the other symbionts.

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Future studies that investigate the host immune status in the absence of *Wigglesworthia* in larva and adult stages may shed some light on the role of the host immune system in community density regulation.

Tsetse GpSGHV at high densities can be pathogenic and adversely affect reproduction by suppressing vitellogenesis, causing testicular aberrations, and/or disrupting mating behavior (Lietze et al., 2011). It's interesting that despite 100% prevalence in our laboratory reared flies, no adverse effects are seen on fecundity possibly due to virus density that may be maintained below a threshold. It has been reported that lipopolysaccharide of commensals may reduce host antiviral response which in this case may help virus to transfer to offspring (Kane et al., 2011). In aphids, beneficial relationship among host, commensal microbes and virus is also observed. The aphid symbiont provides habitat for a virus, which in turn synthesizes a toxin against wasps' larva to protect the aphid host (Roossinck, 2011). Declining pattern of GpSGHV levels in Gmm^{Wig-} -F1, –F2 and Gmm^{Apo} –F1 may also suggest a beneficial community dynamics for virus maintenance. Recovery of virus titers to normal level in Gmm^{Wig-}-F3 and Gmm^{Apo}-F2 however suggests either adaption of the virus in the maternal transmission process so that higher densities are transferred to progeny, or modifications in the host immune system leading to relaxed density regulation, or viral adaptations leading to evasion of the host immune regulation. While direct effects of antibiotics on viral replication have not been noted, GpSGHV levels may be adversely affected by unknown epigenetic effects antibiotic treatment may have had on host physiology. It remains to be seen whether viral pathogenesis would be observed in subsequent generations.

Results from the tsetse system presents a well-tuned intercommunity dynamics for microbes that display varying levels of integration in host biology. Perturbations to this microbial ecology result in adverse effects on the density dynamics in the different partners of the microbiome. The eventual recovery of *Wolbachia* and GpSGHV densities in the absence of *Wigglesworthia* is in line with their anticipated roles in host biology, parasitic and pathogenic respectively. This is in contrast to *Sodalis*'s commensal relationship with its tsetse host. The extensive genomic reduction noted in *Sodalis* coding capacity already indicates interdependencies on tsetse's nutritional ecology, which may suffer in the absence of *Wigglesworthia*. Interestingly perturbations to the microbiome did not result in long-term unregulated proliferation of its individual members, implying strong density regulation effects likely imposed by the host immune system or by community interdependencies resulting from the highly restricted nutritional ecology of the tsetse host.

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Research Highlight

Three symbionts and a DNA virus reside in different tsetse tissues.

Availability of *Gmm*$^{Wig-}$ and *Gmm*^{Apo} enables analysis of intercommunity dependencies.

The symbiont densities are tightly regulated through host development.

There is cooperative interaction between *Wigglesworthia* and *Sodalis*

DNA virus is commensal without displaying pathogenesis in the absence of symbionts.

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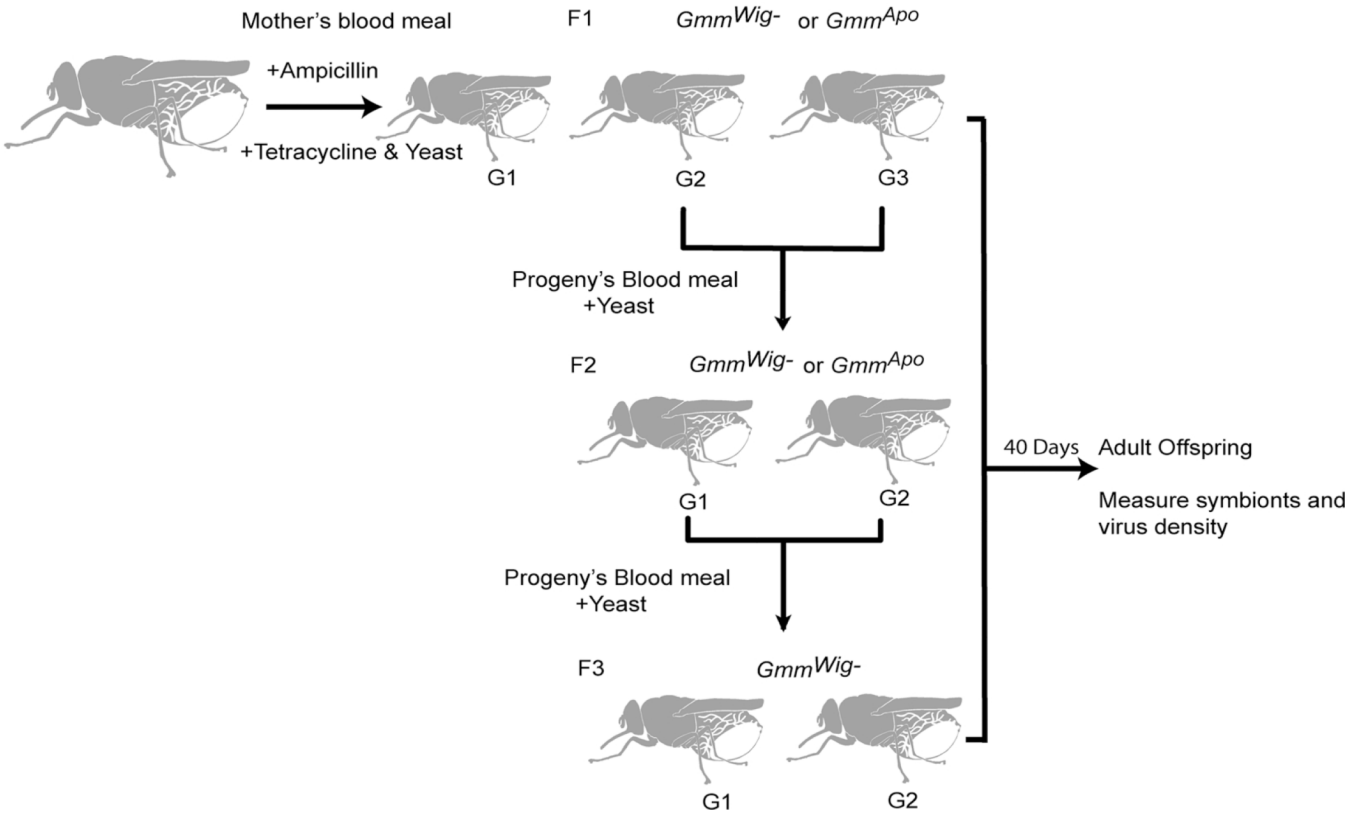

Fig. 1. Experimental scheme of sample collections

Pregnant females were maintained on bloodmeals containing either ampicillin (50 μg/ml) or tetracycline (20 μg/ml) with all supplemented with yeast extract (10 mg/ml). Second and third gonotrophic cycles (G2 and G3) were collected and maintained on bloodmeal supplemented with yeast for 40 days (called F1). Two gonotrophic cycles from F1 were collected and maintained similarly (called F2). Two gonotrophic cycles from F2 were collected and maintained similarly (called F3). All flies were analyzed at 40 days post eclosion.

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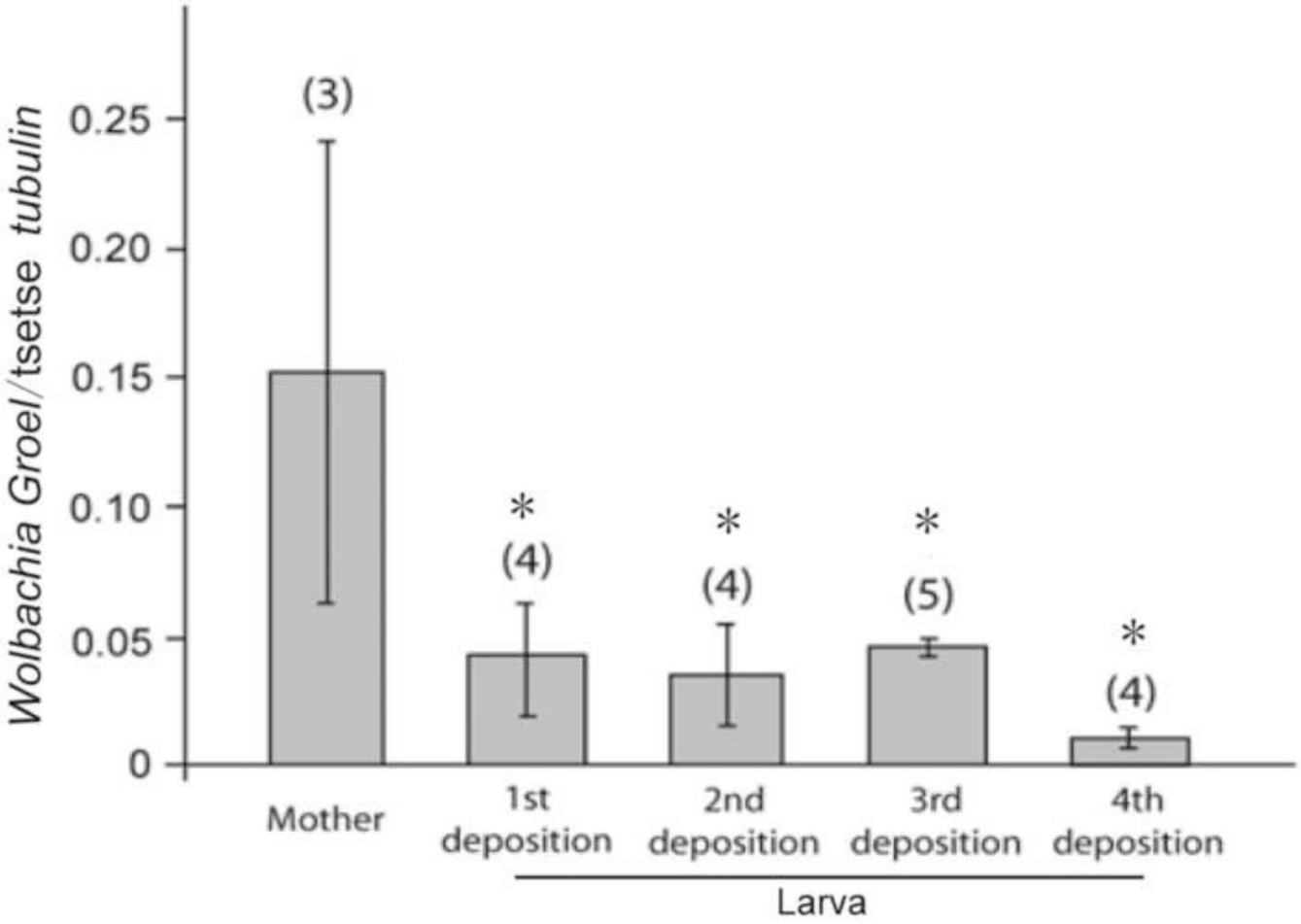

Fig. 2. *Wolbachia* density from mothers and their 1st, 2nd, 3rd and 4th larval offsprings

Error bars represent the standard error of the mean. Numbers in parentheses represent the sample size (n). Asterisks represent statistically significant data for the larva in comparison to the mothers according to Tukey-Kramer post-hoc comparisons ($P<0.05$).

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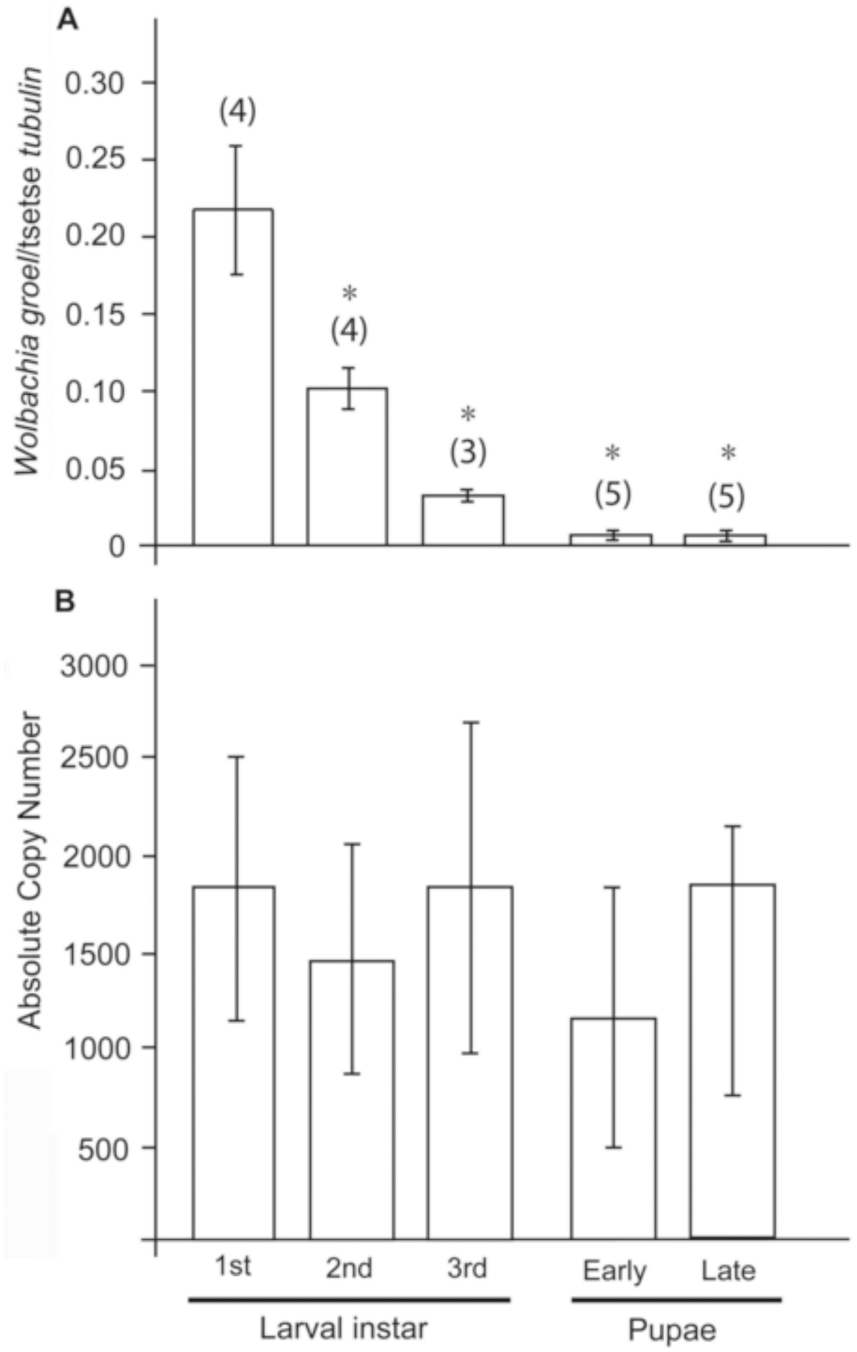

Fig. 3. *Wolbachia* density through juvenile development

(A) *Wolbachia* density normalized to host β-*tubulin* levels, (B) absolute copy number of *Wolbachia* present in the different developmental stages analyzed. Error bars represent the standard error of the mean. Numbers in parentheses represent the sample size (n). Asterisks above bars represent statistically significant data according to Tukey-Kramer post-hoc comparisons ($P<0.05$).

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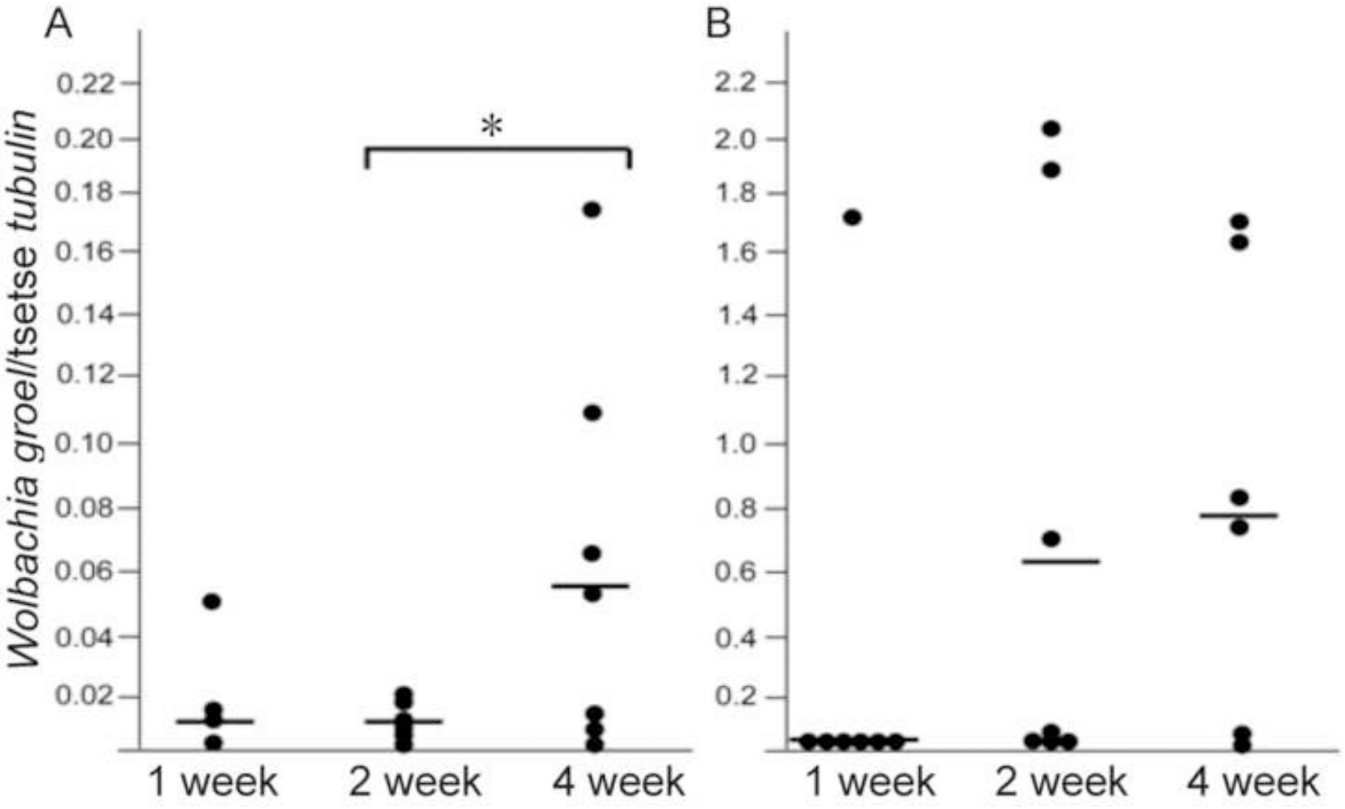

Fig. 4. Sex and age specific *Wolbachia* density

(A) females and (B) males analyzed at different adult ages post eclosion. Lines intersecting data points represent the median. *, P<0.05, Tukey-Kramer post-hoc comparisons.

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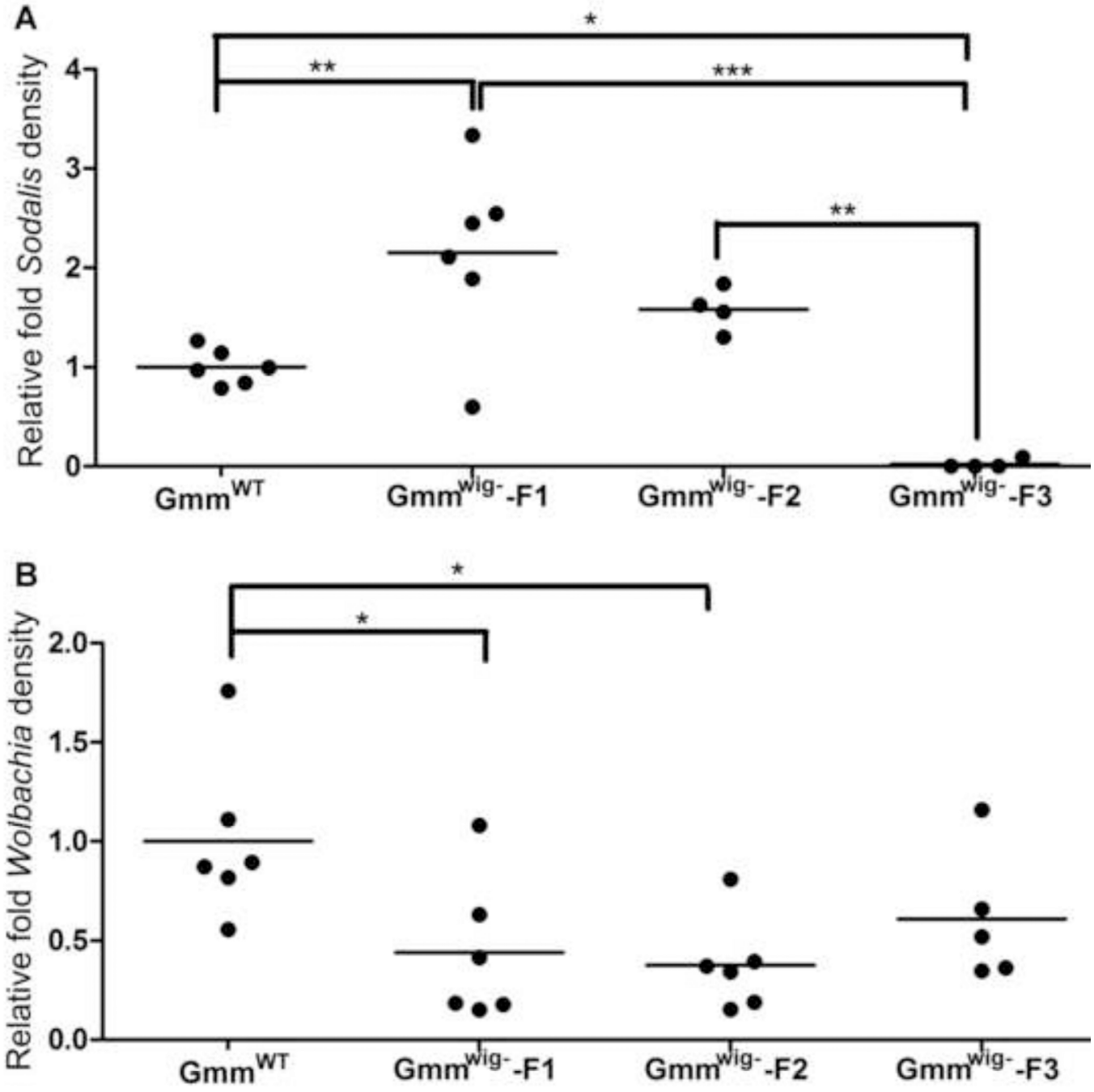

Fig. 5. Symbiont density in female *Gmm^{Wig-}*

(A) *Sodalis* and (B) Wolbachia density were measured by qPCR (n≥4). Symbiont density is expressed as relative fold difference calculated as the density measured from *Gmm^{Wig-}* state normalized to that measured from the corresponding normal state (*Gmm^{Wt}*) *, P9003C;0.05; **, P<0.001, ***, P<0.0001.

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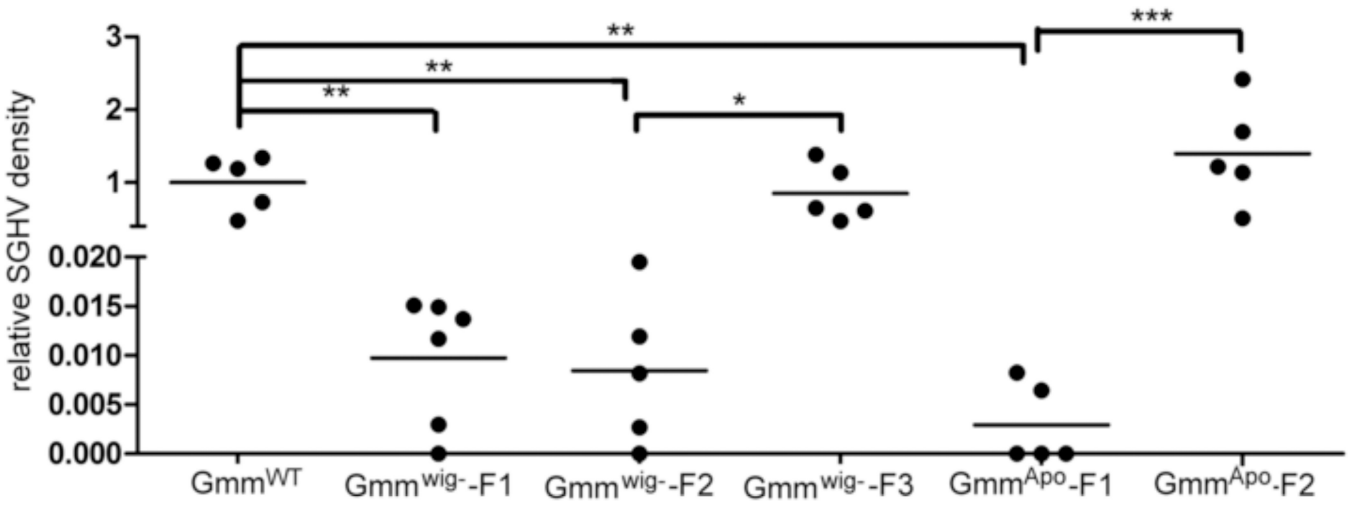

Fig. 6. GpSGHV density in *Gmm*$^{Wig-}$ and *Gmm*^{Apo} flies

GpSGHV was quantified in *Gmm*$^{Wig-}$ and *Gmm*^{Apo} and *Gmm*^{WT}. Relative virus density was determined as density measured in *Gmm*$^{wig-}$ or *Gmm*^{Apo} normalized to levels found in the corresponding control *Gmm*^{Wt} flies (n≥5). *, P<0.05; **, P<0.001, ***, P<0.0001.

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Table. 1

Primers used for qPCR reactions.

Primer name	Primer pair sequence (F: Forward, R: Reverse)
q*Gmmtub-*	F: 5’ CCATTCCCACGTCTTCACTT 3’
	R: 5’ GACCATGACGTGGATCACAG 3’
q*groel Wolbachia*	F: 5’CAGAGGATATCGAAGGTGAA 3'
	R: 5' CCTGGAGCTTTTACTGCGG 3'
q*fliC Sodalis*	F: 5' TGGGGACAGTACGATGGCAGAGC 3'
	R: 5' TCATAGGCGGTCGGGGATAATTGCG 3'
qSGHV	F: 5’ CAAATGATCCGTCGTGGTAGAA 3’
	R: 5’ AAGCCGATTATGTCATGGAAG 3’