

Dynamics of multiple symbiont density regulation during host development: tsetse fly and its microbial flora

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Symbiotic associations often enhance hosts' physiological capabilities, allowing them to expand into restricted terrains, thus leading to biological diversification. Stable maintenance of partners is essential for the overall biological system to succeed. The viviparous tsetse fly (Diptera: Glossinidae) offers an exceptional system to examine factors that influence the maintenance of multiple symbiotic organisms within a single eukaryotic host. This insect harbours three different symbionts representing diverse associations, coevolutionary histories and transmission modes. The enterics, obligate mutualist *Wigglesworthia* and beneficial *Sodalis*, are maternally transmitted to the intrauterine larvae, while parasitic *Wolbachia* infects the developing oocyte. In this study, the population dynamics of these three symbionts were examined through host development and during potentially disruptive events, including host immune challenge, the presence of third parties (such as African trypanosomes) and environmental perturbations (such as fluctuating humidity levels). While mutualistic partners exhibited well-regulated density profiles over different host developmental stages, parasitic *Wolbachia* infections varied in individual hosts. Host immune status and the presence of trypanosome infections did not impact the steady-state density levels observed for mutualistic microbes in either sex, while these factors resulted in an increase in *Wolbachia* density in males. Interestingly, perturbation of the maternal environment resulted in the deposition of progeny harbouring greater overall symbiont loads. The regulation of symbiont density, arising from coadaptive processes, may be an important mechanism driving inter-specific relations to ensure their competitive survival and to promote specialization of beneficial associations.

Keywords: tsetse; symbiosis; *Wigglesworthia*; *Sodalis*; *Wolbachia*; coevolution

1. INTRODUCTION

Extraordinary metabolic and adaptive abilities allow bacteria to occupy a vast range of niches, including permanent habitation within other organisms (endosymbiosis). These relationships can be multifaceted, varying in their impact on host biology (spanning a wide breadth from parasitism to mutualism), prevalence and coevolutionary history. Hosts frequently exploit beneficial symbioses to augment their functional capabilities and to facilitate their expansion into novel niches. Among the eukaryotes that have succeeded in limited and often inhospitable ecological terrains are members of the Class Insecta, due in part to fitness advantages granted by symbiosis.

Obligate mutualists enable the survival of many insects on nutritionally deficient diets such as blood, plant phloem or wood (Buchner 1965). In turn, this restricted

nutritional flexibility, strict intracellular localization within host tissues, and vertical transmission mode over a long evolutionary time span have impacted the genome size and functional repertoire of these captivated microbes. One of the more dramatic testimonies of these tailoring processes is exemplified by the intracellular obligate mutualists, where metabolic capabilities no longer necessary for symbiont survival have been purged, while functions indispensable for host fitness and selective maintenance of the symbiotic interaction have been preserved (reviewed in Wernegreen (2002)). In addition to these intracellular obligates, many insects also harbour secondary symbionts which exhibit a wider range of tissue tropism. In contrast to the obligate mutualists, which have a primarily nutrient provisioning role in host biology, a broader variety of functional contributions have been attributed to secondary symbionts, including tolerance to heat stress (Montllor *et al.* 2002), resistance to parasitoid infections (Oliver *et al.* 2003), broadening of host plant range (Tsuchida *et al.* 2004) and a role in host morphogenesis (Rakoff-Nahoum *et al.* 2004). In addition, parasitic microbes, such as *Wolbachia* and *Spiroplasma* spp., increase their symbiotic prevalence by inflicting various host reproductive alterations (reviewed in Bandi *et al.* (2001)).

The tsetse fly (Diptera: Glossinidae), which relies on multiple microbial species for fitness, provides a unique model to investigate and compare the population

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dynamics of symbionts that display varying levels of integration with host biology. The obligate mutualist, *Wigglesworthia glossinidia* (Proteobacteria: Enterobacteriaceae), is localized within epithelial cells (bacteriocytes) in the adult gut and shares an ancient concordant history of over 50 million years with its tsetse host species (Chen *et al.* 1999). The *Wigglesworthia* genome, approximately 700 kb in size, displays a drastically streamlined functional capacity. Despite this reduction, *Wigglesworthia* encodes a plethora of vitamin biosynthetic products that may promote host reproduction. This function may provide a selective pressure for the retention of the symbiotic system (Akman *et al.* 2002). In fact, in the absence of *Wigglesworthia*, tsetse flies are rendered sterile, although vitamin supplementation of the blood meal diet can rescue host sterility (Nogge 1976, 1982). In contrast, *Sodalis glossinidius* (Proteobacteria: Enterobacteriaceae), does not display a concordant evolutionary history with tsetse (Aksoy 1995), suggestive of a more recent symbiotic initiation. This secondary symbiont, with intra- and extracellular localization, is primarily found in the midgut, although it can also be detected in the haemolymph and other tissues depending on the tsetse species (Cheng & Aksoy 1999). While its 4.2 Mb genome size is close to those of related free-living enterics, analysis of its proteome indicates an unusually low coding density (49.2%), and a high number of pseudogenes, both of which are indicative of an ongoing genome erosion phenomenon and adaptation to the host's restricted ecology (Toh *et al.* 2006). The functional role of *Sodalis* symbiosis in tsetse is relatively unknown, although its specific elimination has been reported to result in a reduction in host longevity (Dale & Welburn 2001). Lastly, some tsetse populations harbour a third microbe related to *Wolbachia pipientis* (Proteobacteria: Rickettsiaceae). The tissue distribution and prevalence of *Wolbachia* in tsetse species ranges from intracellular infection of only germ-line tissue to persistence in various somatic tissues (Dobson *et al.* 1999; Cheng *et al.* 2000). Facultative *Wolbachia*, identified in numerous arthropods (Werren *et al.* 1995; Jeyaprakash & Hoy 2000), are generally considered parasitic. A wide range of phenotypic effects, such as host reproductive alterations, premature death and behavioural modifications have been ascribed to *Wolbachia* infections (reviewed in O'Neill *et al.* (1998)), but its role in tsetse remains to be elucidated.

Tsetse reproductive biology is unusual—an adult female produces a single egg at a time that hatches and develops *in utero* (viviparity). After a period of maturation and sequential molting within the mother, a fully mature third instar larva is deposited and pupates shortly thereafter in the soil. As one of the few insect k-strategists, each tsetse female can deposit five to six offspring during her two- to three-month lifespan (Leak 1999). During intrauterine development, the enteric symbionts *Sodalis* and *Wigglesworthia* are transmitted through the mother's milk gland secretions to the larval progeny, where they establish new infections (Ma & Denlinger 1974; Cheng & Aksoy 1999). *Wolbachia*, however, infects germ-line cells and utilizes a transovarial transmission route (O'Neill *et al.* 1993). Given the unique reproductive biology of tsetse, all three symbionts, however, are in essence maternally acquired by the progeny. In addition to the multiple bacterial symbionts, tsetse flies can also acquire the

protozoan parasite African trypanosomes from the mammalian hosts they feed on. Trypanosomes replicate and can establish chronic infections in the fly midgut in close proximity to the gut symbionts, and be transmitted to tsetse's vertebrate hosts through an infected bite.

We utilized the *Glossina morsitans morsitans* system to examine the population dynamics of multiple microbial symbionts through development in the same host individuals. The three symbionts analysed have different associative properties, transmission modes and coevolutionary histories with their tsetse host. The evolutionary success of enteric symbionts requires a highly faithful maternal transmission route to counteract the bottleneck process confronted at each generation. We investigated the influence of extrinsic factors that could impact symbiotic homeostasis, such as alterations in host immune status, presence of competitive parties, and humidity fluctuations in the maternal environment. We predicted that beneficial symbiotic associations would exhibit population dynamics profiles in concert with host physiological processes through development and critical life events. We also expected the perturbations in the maternal environment to have adverse effects on the transmission and maintenance of symbiosis in subsequent generations, leading to fitness loss of the biological system.

2. MATERIAL AND METHODS

(a) *Tsetse and trypanosome cultures*

The *Glossina morsitans morsitans* colony maintained in the insectary at Yale University was established from puparia collected in Zimbabwe approximately twenty years ago. Flies were maintained at 24 ± 1 °C with 50–55% relative humidity and received defibrinated bovine blood every other day by using an artificial membrane system (Moloo 1971). Females were mated at days 3–5 post emergence, males were removed after 48 h, and approximately 30 fertile females were maintained in each breeding cage, unless otherwise specified. Procytic stage *Trypanosoma brucei rhodesiense* (strain YTAT 1.1) were maintained at 28 °C in SDM-79 medium supplemented with 10% heat-inactivated fetal bovine serum (Brun *et al.* 1979).

(b) *Sample preparation and DNA isolation*

Total DNA was extracted, using the Holmes–Bonner method (Holmes & Bonner 1973), from larvae, teneral (newly enclosed; unfed) two-week-old virgin and four-week-old virgin adults. DNA was similarly extracted from early (24–48 h post deposition) and late (28–30 days post deposition) pupae and 2–8-week-old mated females. Intrauterine larva were surgically removed from mothers and identified as L1, L2 and L3 based on morphological characteristics (Leak 1999). Flies were processed for DNA isolation 48 h following their last blood meal.

(c) *Measuring symbiont density*

Real-time quantitative PCR (QT-PCR) was performed with an icycler iQ real-time PCR detection system (Bio-Rad, Hercules, CA). Symbiont specific thiamine biosynthesis protein (*thiC*) (GenBank Accession no. AB063522), exochitinase (*chi*) (GenBank Accession no. BSPY11391) and outer surface protein (*zusp*) (GenBank Accession no. AF020079) were used for *Wigglesworthia*, *Sodalis* and *Wolbachia* quantification, respectively. While duplications of *Wolbachia zusp*

have been noted (Wu *et al.* 2004), a single amplicon was obtained using WolF¹ and WolR¹ primers (R. V. M. Rio 2003, unpublished data). The tsetse *chitinase* gene (*Gmchi*, GenBank Accession no. AF337908), which is also single copy, was used to normalize for DNA extraction efficiency (Yan *et al.* 2002). Specific fluorescent probes designed to the central portion of the PCR product for the *chi* and *Gmchi* amplicons were utilized for quantification of *Sodalis*'s genome and host cell copy numbers, respectively. The fluorometric intensity of SYBR Green I Dye (a fluorescently labelled double-stranded DNA binding dye) was used for quantification of the *thiC* and *wsp* genes.

Symbiont density is defined as the number of symbiont genomes relative to host cell number. DNA from each experimental sample was analysed to quantify the density of the three symbionts. Internal standard curves were generated for each primer combination using the four above-mentioned amplicons, each cloned into pGEM-T vector (Promega). Density estimates were obtained by comparison to a standard curve using Bio-Rad icycler iQ Multi-Color Real Time PCR data analysis software. All assays were carried out in duplicate, and replicates were averaged for each sample. Negative controls were included in all amplification reactions. The PCR-amplification parameters, reagents, primer design criteria and sequences used for quantification, are summarized in the electronic supplementary material.

(d) Genome copy number determination per *Wigglesworthia* cell

Bacteriomes were dissected, gently homogenized and DNA was isolated from a known number of *Wigglesworthia* cells (quantified by haemocytometer measurements). The copy number of *thiC* was determined by QT-PCR in serially diluted samples, and numbers of genomes per cell were extrapolated in three independent experiments (see electronic supplementary material).

(e) Impact of environmental stress on offspring symbiont density

Newly mated *G. m. morsitans* females were reared in single cages under fluctuating humidity levels (39–63%) for two weeks, then returned to normal conditions (50–55%). Offspring were collected in the order of deposition (i.e. 1st, 2nd, 3rd and 4th). Similarly, the offspring of females maintained under normal rearing conditions were sequentially collected. Following pupation, symbiont densities in the progeny were determined in the early (24–48 h post deposition) and late (28–30 days post deposition) pupal periods.

(f) Impact of host immune induction and third party competition on symbiont density

(i) Host immune induction

Teneral flies received a single blood meal containing *Escherichia coli* K12 (10^6 cells ml⁻¹) and were subsequently maintained on a normal diet as previously described (Hao *et al.* 2001). Symbiont genome densities were analysed 48 h and two weeks following bacterial challenge.

(ii) Third party competition

Teneral flies received a single blood meal containing mammalian stage trypanosomes (10^5 cells ml⁻¹) and were subsequently maintained on a normal diet (Hao *et al.* 2001). Two weeks post acquisition of the infective blood meal, a

PCR amplification assay was used to distinguish flies with trypanosome midgut infections (infected) from those that had successfully cleared their parasites (recovered). For this assay, primers (TBR1 and TBR2) specific for the *T. brucei* subspecies were used (Moser *et al.* 1989). Two weeks, which is sufficient time to determine trypanosome infection status in flies (Gibson & Bailey 2003), was used as the time point for examining symbiont densities as a function of third party competition.

(g) Statistical analysis

Data were analysed using SAS system v. 8.02 for Windows (Littell *et al.* 1996). Student's *t*-tests, one-way analysis of variance (ANOVA) and *post hoc* pairwise comparison of the mean were performed to determine whether densities significantly differed between time points. Where densities appeared skewed, the data were log transformed to satisfy normality. *F*-tests were applied to assess homogeneity of variances. When necessary, final ANOVA models were modified to account for unequal variances across groups.

To examine the impact of environmental stress on timing of offspring deposition, a general linear model for repeated measures was developed. This model employed an unstructured covariance matrix that allowed the duration between offspring depositions to be correlated across pupae (first through fourth). Likewise, as for the offspring symbiont density analyses, interaction between group (control versus experimental) and pupal stage was also investigated and included in the final model as necessary.

3. RESULTS

(a) Symbiont genome density through host development

We used a real-time quantitative PCR assay to capture and compare genome densities of the three different symbionts harboured within the same individual host throughout development (i.e. spanning from first instar larva (L1) to 30 days into adulthood). Symbiont prevalence among the individuals analysed was 100%, in that every fly examined was infected with *Wigglesworthia*, *Sodalis* and *Wolbachia*.

Multiple genome copies per bacterium have previously been described for obligate insect symbionts with the abundance varying among different host developmental stages (Komaki & Ishikawa 2000). We determined the genome copy number per *Wigglesworthia*, and our results also indicate the presence of multiple genomes per bacterial cell, varying from 3 to 23 copies in the two-week-old female bacteriome (see electronic supplementary material). Thus, the symbiont density data presented here reflect symbiont genome copy number relative to tsetse host cell.

In general, *Sodalis* was maintained at a density three orders of magnitude lower than *Wigglesworthia* and *Wolbachia* through development (figure 1a). Although *Sodalis* shares the same transmission route into host progeny as *Wigglesworthia* (through maternal milk gland secretions), it colonizes the intrauterine larva during L1 instar, while *Wigglesworthia* does so in the L2 instar stage (figure 1a,b). During larval development, the enteric symbionts proliferated in parallel to host cell replication. However, significant differences in both symbiont densities were observed between early and late pupal stages, resulting in a threefold increase in *Sodalis* (figure 1a) and

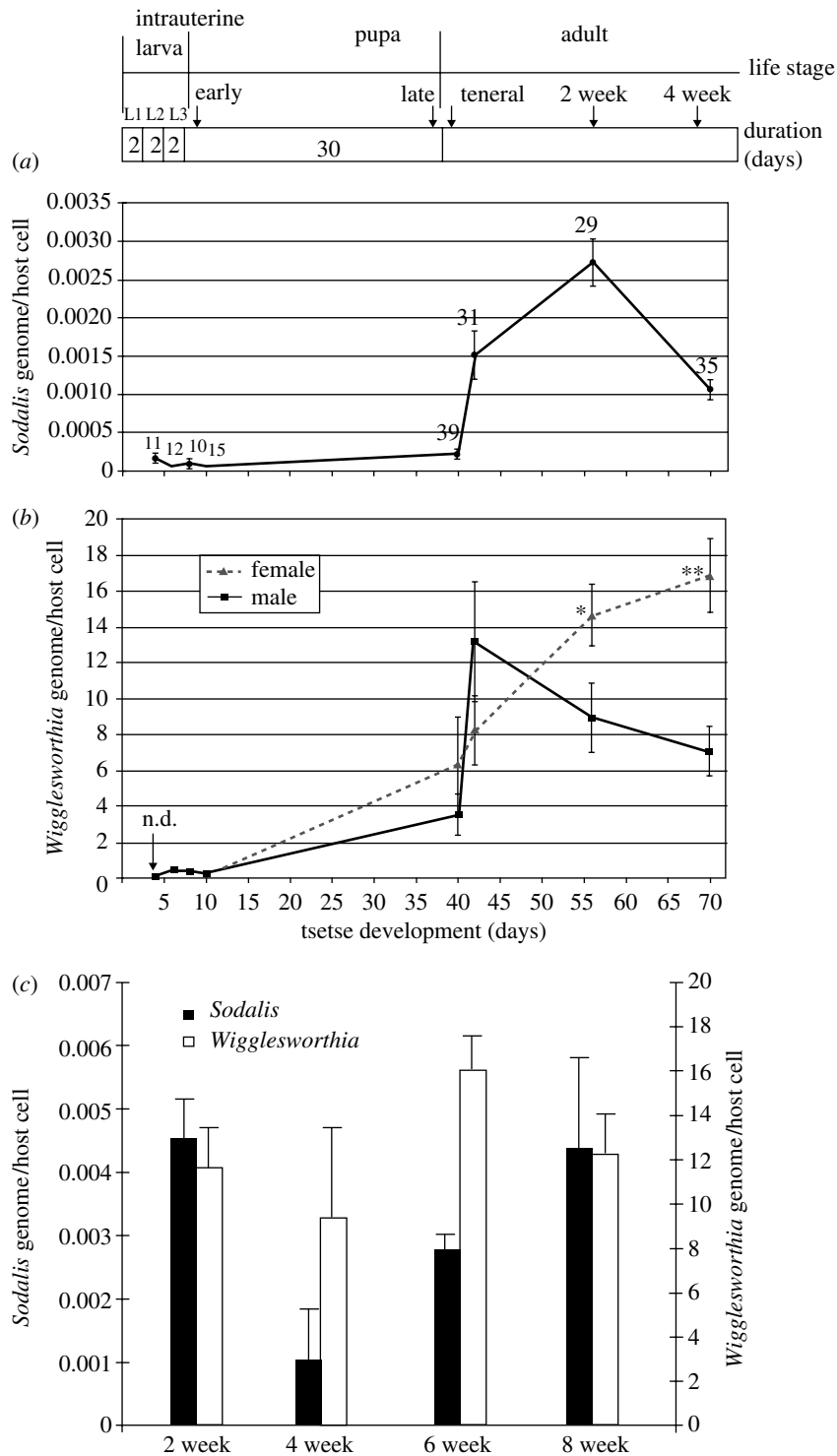


Figure 1. Mutualistic symbiont density through host development and mating status. (a) *Sodalis* density. Sample sizes are indicated adjacent to each time point. (b) *Wigglesworthia* density, *Wigglesworthia* was not detectable (n.d.) in the majority of L1 samples. Male and virgin female densities are represented with solid and hatched lines, respectively ($n \geq 5$ samples at each time point for each sex). Each time point refers to a mean, error bars represent 1 standard error of the mean (s.e.m.). *, ** indicate significant differences between corresponding time points ($p < 0.05$, 0.005 ; respectively). (c) *Sodalis* and *Wigglesworthia* densities in mated females. *Sodalis* (black bars) and *Wigglesworthia* (white bars) densities in 2, 4, 6 and 8 week old mated females. Each time point refers to a mean, error bars represent one 1 s.e.m. ($n \geq 5$ samples at each time point).

16- and 30-fold increases in *Wigglesworthia* in male and female adults, respectively (figure 1b). This proliferation apparently occurs late during pupal development, as analyses of both symbiont densities at mid-pupal development (day 15 of an approximately 30 day pupal development period) indicated minimal expansion (data not shown). Following eclosion, symbiont proliferation continued for at least 48 h (figure 1a,b). During the adult

lifespan monitored, *Sodalis* densities increased about twofold relative to teneral (figure 1a). Although host sex did not appear to influence *Sodalis*'s density, male and virgin female adult hosts exhibited different *Wigglesworthia* population dynamics (figure 1b). In males, the peak densities of *Wigglesworthia* were detected in teneral, corresponding to a fourfold increase from the late pupal stage, while in virgin *Wigglesworthia* females continued to

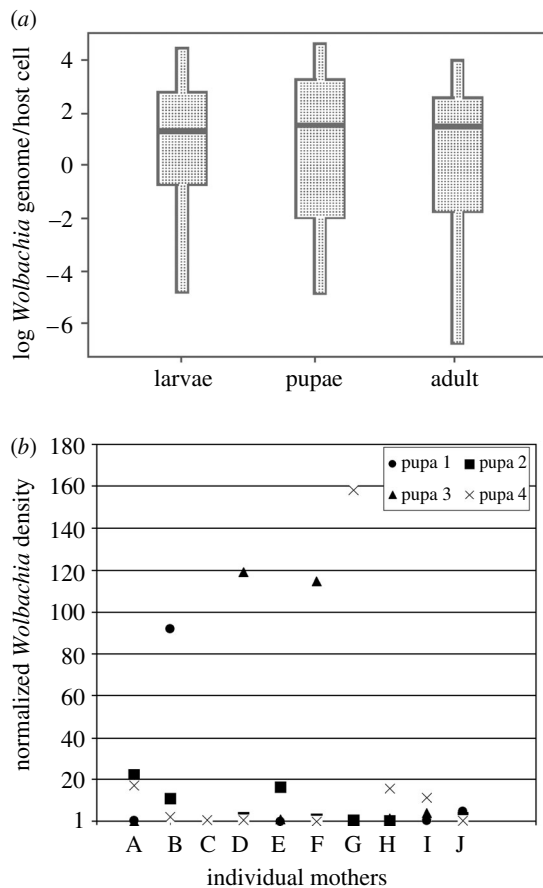


Figure 2. *Wolbachia* density through host development and in different offspring. (a) Box-and-whiskers plots of *Wolbachia* density and distribution of variance through host development. Host development was separated into three discrete categories; larvae ($n=30$), pupae ($n=109$) and adult ($n=58$). The median (heavy line) ranged from the 25th to 75th percentile (boxed) and 10th and 90th percentiles (whiskers) are shown. (b) *Wolbachia* densities from 1st, 2nd, 3rd and 4th offspring of individual mothers (A–J) measured immediately following larval deposition.

proliferate during the whole age spectrum analysed (figure 1b). No significant differences in *Wolbachia* densities were observed during larval, pupal and adult development in both sexes or among individuals of the same age class. In this case, the variability was too wide to allow for mean determination (figure 2a). Although sample sizes for *Wolbachia* density estimates were increased due to the high variability observed, this did not result in conformity. Hence, the median, rather than the mean, was used as a representative measure of *Wolbachia* density through host development. The median *Wolbachia* density (approximately 10 genome copies per host cell) was similar among tsetse larval, pupal and adult life stages. Furthermore, by examining *Wolbachia* density of the offspring from individual mothers, we also determined that this variability does not arise due to a given mother depositing offspring harbouring consistently high or low symbiont loads. Instead, the *Wolbachia* densities in the multiple progeny analysed from each mother fluctuated randomly independent of their order of deposition (figure 2b).

(b) The impact of host mating on symbiont density

Symbiont densities from two-, four-, six- and eight-week-old mated females were examined to determine whether

host reproductive status influences symbiont dynamics. *Wigglesworthia* density in mated females was similar to virgin levels (figure 1b,c), while *Sodalis* density varied. The two-week-old fertile flies showed a twofold increase of *Sodalis* relative to similarly aged virgins (figure 1a,c), while the four-week-old group declined, only to return to higher levels by eight weeks. No significant differences were observed in *Wolbachia* densities as a function of age and mating status (data not shown). Hence, mating did not appear to be an influential physiological factor in the regulation of symbiont genome densities, although *Wigglesworthia* was more stable than *Sodalis*. Since *Wigglesworthia* products are thought to be indispensable for host fecundity, it remains to be seen whether they are subject to transcriptional upregulation in mated female flies.

(c) Impact of host immunity challenge and third party competition on symbiont density

Because the host immune system plays a pivotal role in controlling foreign invaders, we evaluated how stimulating tsetse immune responses affected its resident microbial flora. Symbiont densities were examined following host challenge with *E. coli* and trypanosomes. Both of these organisms have been shown to increase the expression of several tsetse immunity genes, resulting in the synthesis of antimicrobial peptides as well as reactive oxygen and nitrogen intermediates (Hao *et al.* 2001, 2003; Boulanger *et al.* 2002). Furthermore, a proportion of the tsetse flies challenged with trypanosomes had established persistent midgut parasite infections and served to explain the effects of additional trophic complexity on symbiont populations.

Sodalis density measured from two-week-old adults with midgut trypanosome infections did not differ from flies that had successfully cleared their parasite infections (figure 3a, TI versus TR). Similar non-disruptive results on *Sodalis* were observed 48 h (data not shown) and two weeks following *per os* *E. coli* challenge (figure 3a). *Wigglesworthia* densities in these same individuals also did not differ in comparison to unchallenged controls (figure 3b). These results suggest that both symbionts were unaffected by either the antimicrobial specific host immune response or the potential competitive interactions with the parasitic trypanosomes replicating in the midgut. Although *Wolbachia* densities measured from the same individuals challenged with *E. coli* were comparable to the controls, rampant *Wolbachia* replication was observed in males exposed to trypanosomes regardless of their infection status (ANOVA, $p < 0.0001$, figure 3c). In addition, both infected and recovered 6-week-old males still harboured significantly higher *Wolbachia* densities (ANOVA, $p = 0.0015$, figure 3d), suggesting either a sex-specific opportunist role for this symbiont, or loss of host control in males upon specific challenge. In contrast, *Wolbachia* densities in females were not affected by either trypanosome or *E. coli* challenge.

(d) Impact of environmental stress on offspring symbiont density

The delays between each larval deposition cycle and the offspring symbiont loads, were determined from mothers exposed to varying humidity levels (environmentally stressed) during their first gestational period. Delayed life history effects were expected solely in offspring from

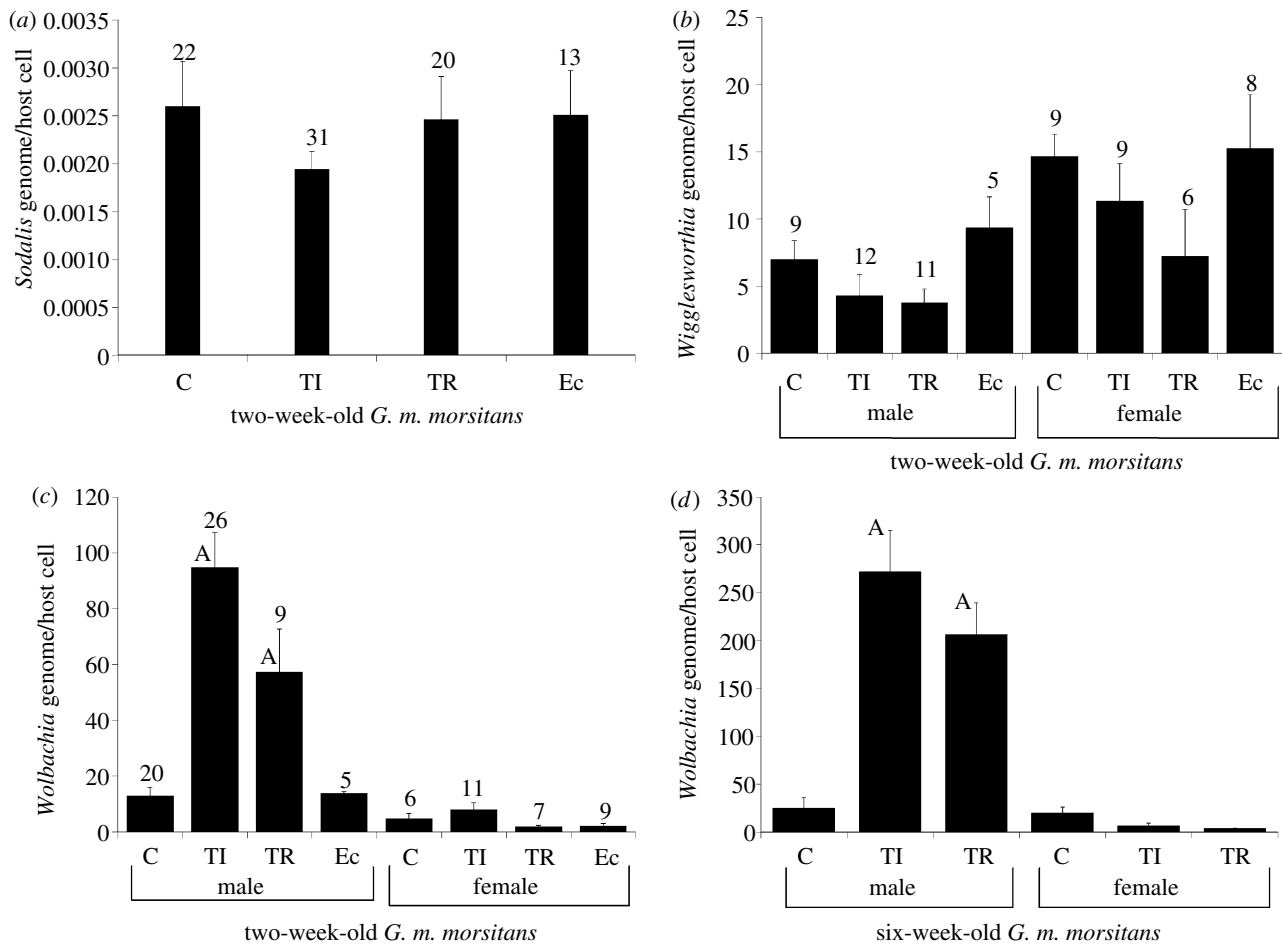


Figure 3. Impact of host immunity and third party competition on symbiont densities. (a) *Sodalis* density measured from two-week-old adults (males and females combined). (b) *Wigglesworthia* density measured from two-week-old males and females. (c) *Wolbachia* density in two-week-old males and females from groups; C (control), TI (trypanosome infected), TR (trypanosome recovered, tsetse which were challenged but able to clear the infection) and Ec (*per os E. coli* challenge). Sample sizes are indicated. (d) Mean *Wolbachia* density in six-week-old males and females corresponding to; C (control), TI (trypanosome infected) and TR (trypanosome recovered, tsetse which were challenged but able to clear the infection). $n \geq 5$ samples for each category. A designates treatments that are significantly different from others ($p < 0.005$). Means are represented and error bars signify 1 s.e.m.

the first gonotrophic cycle because mothers were directly exposed to humidity fluctuations only during this period. A general linear model for repeated measures did not support differences in the duration between larval deposition cycles among control and stressed mothers. The difference (in days) in the duration between larval deposition cycles between the two groups was found to be 0.65 days (95% confidence interval: 0.109–3.866, $p = 0.682$). However, the impact of maternal stress extended beyond the time of environmental stress. Fluctuations in humidity resulted in an increase in the density of all three symbionts in subsequent progeny (figure 4). *Sodalis* and *Wigglesworthia* density significantly differed between the first and second offspring deposited by environmentally stressed and control females, and similar differences in *Wolbachia* density were also observed in the first and third offspring. These environmental effects subsided with time, as symbiont densities in the fourth offspring were comparable to controls. It is worth noting that in the fourth offspring from the environmentally stressed mothers, deposited about one month following humidity fluctuations, *Wolbachia* densities still remained high (approximately seven times greater than those from control group), but the association is not statistically

significant (table 1). The restoration of homeostasis also occurred with progression of progeny age, as older individuals analysed from experimental and control groups as late pupae contained similar symbiont densities (data not shown).

4. DISCUSSION

Our results illustrate that host colonization by symbiotic flora is an active and continuous process, with the composition of these communities varying spatially and temporally throughout development. Our findings demonstrate plasticity of symbiont density in response to a multitude of host (i.e. development, sex, immunity) and environmental factors (i.e. humidity, third parties). Symbiont responses to these influential factors exhibited distinct dynamics probably reflective of the age of the association, the functional repertoires of the symbionts and varying levels of integration with host biology.

Consistent densities relative to host cell numbers observed among similarly aged tsetse individuals for *Sodalis* and *Wigglesworthia* suggest an adaptive regulation through development for these beneficial interactions that are established through maternal milk gland secretions. The teneral adult stage (24 h after eclosion and prior to

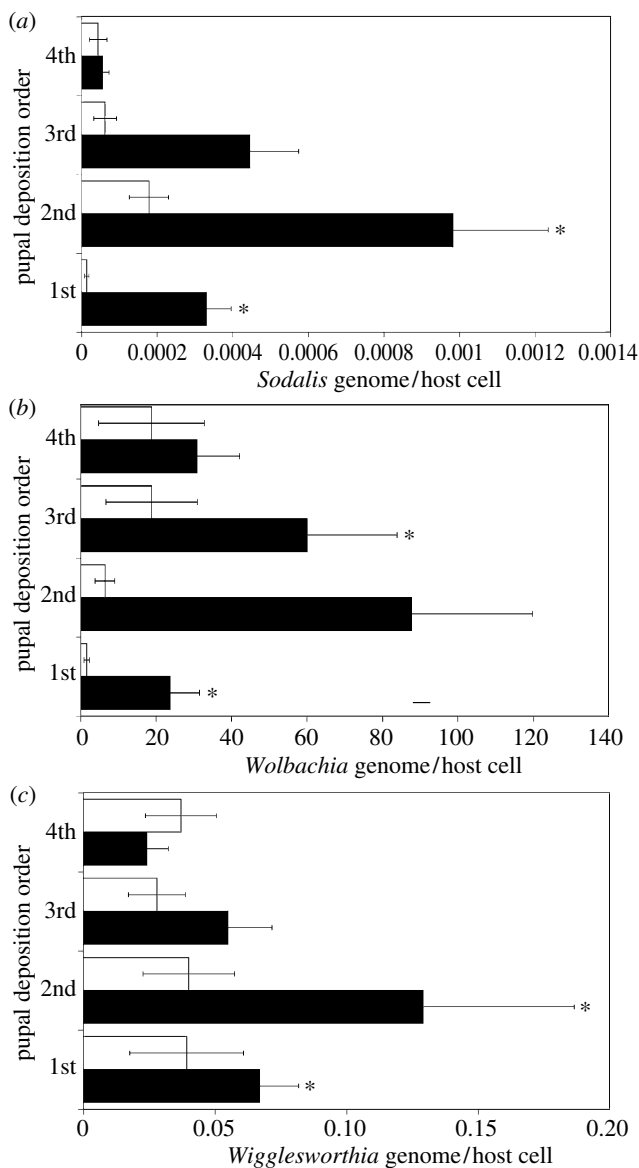


Figure 4. Symbiont density in the offspring of environmentally stressed and control mothers. (a) *Sodalis* density. (b) *Wigglesworthia* density. (c) *Wolbachia* density in the first four offspring of environmentally stressed and control mothers. Means are represented and error bars signify 1 s.e.m. ($n \geq 10$ samples for each group). * indicates significant difference between corresponding offspring ($p < 0.05$). Open bars: control, 24–48 h; filled bars: experimental, 24–48 h.

the first blood meal) provided the one exception, during which increased proliferation was documented for both symbionts. The teneral state has also been associated with greater susceptibility to trypanosome infections (Welburn & Maudlin 1992), possibly because the immature state of tsetse immunity allows for increased parasite survival. The lack of a robust immune status may also account for the increased proliferation noted during late pupal and early adult development for *Wigglesworthia* and *Sodalis*. This increase in the density of both symbionts may also be either a host or symbiont mediated response, resulting from the transition to a life stage independent of the maternal environment. More specifically, symbiont flora contributions may not be as significant during *in utero* development, as resources can be directly allocated from the mother. By maintaining lower symbiont loads, the

metabolic cost of infections during these immature stages of development may be reduced.

In contrast to the beneficial symbionts, wide variability was observed in different individuals of similar ages for *Wolbachia* density through development. The variations in *Wolbachia* densities suggest the lack of optimal host-symbiont dynamics for this organism, perhaps consequential to its recent invasion, potentially parasitic nature, or oocyte transmission mode. These findings are similar to other insect species, where high variability in *Wolbachia* loads among similarly aged individuals were also reported (McGraw *et al.* 2001; Noda *et al.* 2001; Ikeda *et al.* 2003). Potential causes of variability may include unequal vertical transmission into progeny as we detected in the sequential offspring from the same mother, or stochastic variation in *Wolbachia* replication rates (Veneti *et al.* 2005), both of which might result from the lack of sufficient co-adaptation (McGraw *et al.* 2001). Interestingly, median *Wolbachia* density, relative to host cell number, was similar throughout tsetse development suggesting global stability. Host selection for reductions in parasite densities can normally give rise to a conflict in optimal densities in parasitic associations. However, if the parasite infections confer a reproductive advantage to the host, as seen with CI expression in *Wolbachia*, hosts may be expected to exhibit a relaxed density control over these infections. It will now be interesting to elucidate whether the variations in *Wolbachia* density we observe in tsetse individuals of similar age may lead to differences in the intensity of the potential physiological modifications they may manifest, such as male killing or cytoplasmic incompatibility, as has been reported in other host systems (Hurst *et al.* 2000; McGraw *et al.* 2001; Clark & Karr 2002; Veneti *et al.* 2003).

An additional level of specificity towards the host physiological state was observed with the obligate mutualist *Wigglesworthia*, where higher densities in females suggest a role in hosts' reproductive fitness. Nogge (1976, 1982) demonstrated that the sterility observed in females in the absence of the obligate mutualist could be rescued by provisioning the fly's diet with vitamin supplements, supporting a putative nutritional role for *Wigglesworthia* in fecundity. In support of this role, more prominent bacteriomes have been described in females, in comparison to males, presumably related to the increased metabolic processes necessary for support of progeny (Nogge & Ritz 1982). Higher *Wigglesworthia* densities during the female adult stages may allow for both an increase in the production of metabolites such as vitamins required for fertility, and also greater symbiont transmission efficiency to progeny. It will be interesting to determine whether the increase in *Wigglesworthia* genome copy we noted represents an increase in cell numbers or genome copies per cell given that these bacteria have multiple chromosomes.

Wigglesworthia and *Sodalis* densities remained stable during stimulation of the host immune system. These findings suggest that these organisms are either able to evade the effects of their host's immune arsenal due to their intracellular localization, or display greater acquired resistance as a result of their long coevolutionary histories (Hao *et al.* 2001, 2003; Boulanger *et al.* 2002). In support of the latter hypothesis, *Sodalis* does exhibit a greater resistance towards the bacteriocidal actions of tsetse's antimicrobial peptides Dipteracin and Attacin (Hao *et al.* 2001; Hu & Aksoy 2005), possibly reflecting cellular

Table 1. ANOVA estimation of the geometric mean density ratios of first through fourth offspring symbiont loads deposited by environmentally stressed versus control tsetse.

| offspring deposition number | <i>Sodalis</i> | | | | <i>Wigglesworthia</i> | | | | <i>Wolbachia</i> | | | |
|-----------------------------|-----------------------------------|--------|---------|---------|-----------------------------------|--------|--------|---------|-----------------------------------|--------|---------|---------|
| | geometric mean ratio ^a | 95% CI | | p-value | geometric mean ratio ^a | 95% CI | | p-value | geometric mean ratio ^a | 95% CI | | p-value |
| | | lower | upper | | | lower | upper | | | lower | upper | |
| 1 | 38.092 | 10.870 | 133.847 | <0.0001 | 4.330 | 1.559 | 12.025 | 0.0057 | 30.435 | 3.505 | 264.225 | 0.0022 |
| 2 | 4.665 | 1.425 | 15.270 | 0.0115 | 4.651 | 1.525 | 14.188 | 0.0078 | 6.505 | 0.865 | 48.901 | 0.0685 |
| 3 | 2.739 | 0.705 | 28.789 | 0.1438 | 2.135 | 0.700 | 6.513 | 0.1784 | 12.752 | 1.423 | 114.263 | 0.0233 |
| 4 | 0.763 | 0.175 | 3.329 | 0.7166 | 1.130 | 0.370 | 3.446 | 0.8275 | 7.037 | 0.624 | 79.376 | 0.1133 |

^a Ratio of geometric mean densities between stressed versus control mothers; 95% CI, 95% confidence interval.

adaptations acquired as a result of constant exposure to host physiology. In *G. m. morsitans* males, *Wolbachia* has a broader tissue distribution, being detected in the head, thorax and abdomen. In contrast, this bacterium in females is strictly restricted to germ-line tissue in this species of flies (Dobson *et al.* 1999). This lack of density regulation of *Wolbachia* in males is further accentuated following trypanosome exposure, regardless of the eventual infection status of the host. It remains to be seen whether density regulation in females is host- or symbiont-mediated. The cost of any fitness damage in females, through increases in density and extended tissue tropisms, would have greater detrimental impact on *Wolbachia* fitness than does harming males.

Environmental factors, including temperature, food quality and host rearing density, alter symbiont densities and the intensity of their effects on directly impacted individuals (Chen & Purcell 1997; Clancy & Hoffmann 1998). Furthermore, our results demonstrate that perturbations in the maternal environment have dramatic effects on the transmission and maintenance of symbiosis in subsequent generations. However, restoration of mutualist density levels following these perturbations was observed with time (i.e. symbiont densities returned to levels comparable to the controls in progeny deposited later in maternal life as well as with progeny age). Temperature fluctuations have been shown to alter *Wolbachia* and *Sodalis* abundance (Chen & Purcell 1997; Welburn & Maudlin 1991), suggesting that these symbionts are able to respond to fluctuations in the host environment. Indeed, the recently sequenced genome of *Sodalis* indicates the presence of various two-component regulatory systems that may mediate such recognition (Toh *et al.* 2006). This is in contrast to the *Wigglesworthia* proteome, which lacks any such regulatory systems. How *Wigglesworthia* density is regulated in the host remains to be determined, and possibly may involve host-specific factors.

As a k-strategist, tsetse deposit few offspring. Hence, fidelity of mutualistic symbiont transmission in tsetse is of utmost importance for the evolutionary success of the system. The bottlenecks encountered during maternal transmission of *Wigglesworthia* and *Sodalis* populations necessitate accommodations in host reproductive biology to ensure successful colonization of progeny. Given that traits involved in the specialization of symbiotic associations are likely under strong selective pressure (Thompson 1982), there may be signalling events tightly

regulating the invasion of larval gut tissues and subsequent maintenance of the infections in progeny. It is interesting that *Sodalis*'s genome has retained three putative type-III secretion systems (TTSS), reminiscent of the apparatus that related pathogenic microbes utilize to gain access to their eukaryotic host cells. At least two of these TTSSs are transcriptionally expressed during the early larval development stage in tsetse, and might be involved in the transmission and/or symbiotic establishment processes in the progeny (Toh *et al.* 2006). The annotated genome sequences of tsetse symbionts can now be used to examine the expression profiles of genes, such as those involved in invasion, quorum sensing and biofilm formation processes, which may be central for the described temporal dynamics, symbiotic acquisition, progression and maintenance processes. Our experimental results described here with controlled laboratory lines strongly suggest a correlation between the length of coevolutionary history and symbiont density dynamics, where fine-tuned regulation is consequential of adaptive processes. The coordination of density may be an influential factor for reducing conflict between partners, driving inter-specific associations and further promoting specialization of established symbioses.

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