Title: Bacteria contribute to mosquito development via folate biosynthesis and energy storage

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Abstract

The mosquito microbiota impacts the physiology of its host and is essential for normal larval development, thereby influencing transmission of vector-borne pathogens. Germ-free mosquitoes generated with current methods show larval stunting and developmental deficits. Therefore, functional studies of the mosquito microbiota have so far mostly been limited to antibiotic treatments of emerging adults. In this study, we developed a novel approach to produce germ-free *Aedes aegypti*. It is based on reversible colonisation with bacteria genetically modified to allow complete decolonisation at the end of larval development. We show that, unlike germ-free mosquitoes produced using sterile diets, reversibly-colonised mosquitoes showed no developmental retardation and reached the same size as control mosquitoes. This allowed us to study the role of bacteria during larval development by inducing decolonisation at the beginning of the third larval instar. Based on a transcriptome analysis and diet supplementation experiments, we propose that bacteria support larval development by contributing to folate biosynthesis and by enhancing energy storage. Overall, this study provides a new tool to study the microbiota in insects and deepens our knowledge on the metabolic contribution of bacteria to mosquito development.

Introduction

Vector-borne diseases are estimated to account for 17 % of all cases of infectious diseases worldwide and cause over 700 000 deaths per year (WHO, 2017). The mosquito *Aedes aegypti* is the main vector for several arboviruses and it is widely distributed across tropical and neotropical regions. Due to the spread of insecticide resistance, the factors shaping mosquito fitness and vector competence (*i.e.* its ability to acquire and transmit viruses) are under study in order to identify alternative strategies for transmission blockade. Among these, the microbial

community colonising the mosquito is known to directly impact vector competence and in a broader way to alter vectorial capacity, which represents the potential of a mosquito population to transmit disease, hence it includes wider aspects of mosquito physiology. Several pieces of evidence indicate that the microbiota influences not only viral replication and dissemination in the mosquito body, but also mosquito development, lifespan, mating choice, fecundity and fertility (Coon et al., 2014; de Gaio et al., 2011; Gendrin et al., 2015; Pike et al., 2017; Wu et al., 2019; Xi et al.). In particular, live bacteria are essential for normal larval development, hindering possibilities for the production of germ-free controls for microbiota studies. Therefore, most studies investigating the mosquito microbiota were based on conventional rearing and use of antibiotics at adult emergence, which may have off-target effects and does not fully sterilize mosquitoes. In the past, several studies proposed rearing protocols of germ-free larvae, which could not be reproduced in recent experiments (Correa et al., 2018; Golberg et al., 1945; Lang et al., 1972). Lately, mosquito larvae have been shown to develop in the absence of bacteria when provided an enriched diet delivered as bits rather than mashed food, but development time is tripled and germ-free mosquitoes are smaller than their conventional controls (Correa et al., 2018).

The exact contribution of the microbiota to larval development is still a conundrum. It was proposed that the bacteria growing in the mosquito gut consume oxygen and that the resulting hypoxia is a developmental signal for moulting from one stage to the next (Coon et al., 2017). However, successful larval development in fully germ-free conditions challenged this model and metabolic contribution of bacteria to larval development was alternatively proposed (Correa et al., 2018).

We developed a transient bacterial colonisation method to support larval development while producing germ-free adults. We showed that this approach allowed development to occur within a standard timeline and did not affect adult size, suggesting that these germ-free mosquitoes represent an ideal tool for microbiota studies. We further used transient colonisation during early larval development to characterise the role of bacteria in larval development using a transcriptomic approach and diet complementation. Our data indicate that bacteria contribute to folate biosynthesis and energy storage and that folate supports larval development.

Results

Transient colonisation with auxotrophic bacteria allows the production of germ-free adult mosquitoes

After microbiological sterilisation of mosquito eggs, hatchlings provided with autoclaved usual rearing food are able to survive for weeks but remain first-instar larvae; their development can be rescued by bacterial colonisation, for instance with *Escherichia coli* (Coon et al., 2014; Correa et al., 2018). Therefore, we hypothesised that transient bacterial colonisation would be an efficient way to support larval development and produce germ-free adults. To test this, we used HA 416, an *E. coli* HS-derived strain that is auxotrophic for two amino-acids required for peptidoglycan synthesis (*meso*-diaminopimelic acid, *m*-DAP, and D-alanine, D-Ala) and that was previously used to transiently colonise the mouse gut (Cuenca et al., 2016). We

supplemented the food of gnotobiotic larvae with these amino-acids to support the proliferation of auxotrophic *E. coli* during larval development only (Figure 1A). These supplements were toxic to first instar larvae when provided at the concentrations previously used in mice, but 4-fold lower concentrations supported growth of auxotrophic *E. coli* (Figure S1) without causing any toxicity to larvae. The auxotrophic *E. coli* strain rescued the development of axenic larvae with the same efficiency as control wild-type *E. coli* ($p = 0.29$, Figure 1B). The proportion of larvae reaching adulthood was higher among gnotobiotic larvae than among untreated larvae, carrying their conventional microbiota ($F_{2,16} = 7.9$, $p < 0.001$, Figure 1B).
To monitor colonisation efficiency, we quantified bacterial loads at different time points of larval and pupal development in auxotrophic *E. coli*-carrying larvae, using wild-type *E. coli* gnotobiotic larvae as controls (Figure 1C). After surface sterilisation, larval and pupal homogenates were plated on LB supplemented with *m*-DAP and D-Ala. Comparable bacterial amounts were found in third instar larvae harbouring each *E. coli* strain ($F_{1,8^{\wedge}6} = 0.14$, $p = 0.71$). Bacterial counts gradually decreased in auxotrophic *E. coli* gnotobiotic hosts from the fourth instar and were 1.3 and 8-fold lower than wild-type *E. coli*-carrying larvae at the first and second day of the fourth instar, respectively (day 1: $F_{1,1.6e12} = 4.5$, $p = 0.033$; day 2: $F_{1,3.4e12} = 44$, $p < 0.001$). During the pupal stage, bacteria were undetected in most auxotroph-carrying individuals (92.1 ± 7.7 %) while bacterial load also decreased in the controls, as previously documented (Moll et al., 2001) ($F_{1,65} = 66$, $p < 0.001$). The lower bacterial loads found in fourth-instar larvae harbouring auxotrophic *E. coli* might be explained by the exhaustion of *m*-DAP and D-Ala by auxotrophic bacteria and their resulting inability to grow. Since before metamorphosis larvae stop to feed and empty their gut, the lower bacterial loads found in late fourth instar larvae and in pupae might be further explained by the resulting intestinal inaccessibility of *m*-DAP and D-Ala. The presence of bacterial DNA was still detectable via qPCR in 22 % of 1-day old adult mosquitoes originating from auxotrophic *E. coli*-carrying larvae and in 40 % of mosquitoes originating from wild-type *E. coli* gnotobiotic larvae ($F_{1,52} = 0.34$, $p = 0.56$, Figure S2), but not in 7-week old adults ($p < 0.001$, Figure S2). Adult mosquitoes originating from auxotrophic *E. coli*-carrying larvae were confirmed by culture to be completely sterile during the first three days following emergence, while bacteria were culturable from 50% of the wild-type *E. coli* gnotobiotic mosquitoes at the same time-points (Figure 1D). The absence of culturable bacteria in some adult mosquitoes immediately after emergence has been previously observed in different mosquito species, including *Ae. aegypti* (Moll et al., 2001). To further quantify the loss of bacteria, we measured the bacterial loads in adult mosquitoes originating from auxotroph-carrying gnotobiotic larvae on a larger sample size. No culturable bacteria was isolated from 97.6 ± 2.1 % (34/34; 118/122; 311/321) of 1-to-2-day old adults (Figure S3).

Effect of bacterial colonisation on mosquito development

We investigated whether larvae harbouring auxotrophic *E. coli* presented any developmental deficits. Firstly, we quantified the duration of each developmental stage to assess if the colonising bacterial strains had an impact on development kinetics. The gnotobiotic treatments slightly shortened the beginning of larval development, while total development time was unaffected (Figure 1E, L1: $F_{2,550} = 18$, $p < 0.001$; L2: $F_{2,526} = 3.1$, $p = 0.044$; L3: $F_{2,503} = 6.9$,

p = 0.001; L4: $F_{2,475}$ = 2.9, p = 0.057; P: $F_{2,467}$ = 1.0, p = 0.37, see the figure for single comparisons).

Secondly, we evaluated whether auxotrophic *E. coli* supported the development of both sexes. We observed no significant differences in sex ratio between mosquitoes carrying a conventional microbiota or reared on wild-type and auxotroph bacteria (Figure S4, F_2 = 4.2, p = 0.50). A slightly lower adult female:male ratio was observed among conventionally-reared individuals compared to wild-type *E. coli*-carrying mosquitoes.

Finally, we compared the larval body length and the wing length of conventionally-reared and gnotobiotic individuals to assess whether the different microbiota compositions impacted the mosquito size, which is a readout of the insect nutritional and metabolic status. At the fifth day after egg hatching, gnotobiotic fourth-instar larvae for both *E. coli* strains reached the same length as conventionally-reared larvae (Figure 1F, $F_{2,481}$ = 2.5, p = 0.083) and wing size was comparable among the three rearing conditions in both males and females (Figure 1G, males: $F_{2,199}$ = 2.0, p = 0.14; females: $F_{2,176}$ = 1.7, p = 0.19).

Transcriptomic analysis after terminating bacterial colonisation in larvae

We tested whether reversible colonisation could also be interrupted during larval development by placing the auxotroph-harbouring larvae in a rearing medium lacking *m*-DAP and D-Ala (Figure 2A). We rinsed third-instar larvae and placed them in sterile water containing only sterile fish food and monitored the bacterial loads in single larvae. As soon as 2h after *m*-DAP and D-Ala depletion, we observed a 1000-fold reduction in bacterial loads, which became a 10^4-fold reduction after 5 h. Larvae were germ-free 12h after amino-acid depletion (Figure S5). We processed wild-type *E. coli*-carrying larvae in the same way to test whether this reduction in the microbiota load was specific to the bacterial auxotrophy or if it was simply due to the transfer of larvae to a new sterile rearing environment and the active expulsion of bacteria from the larval gut. Two hours after the transfer, we observed a 500-fold lower bacterial load in the gut of transferred larvae with respect to non-transferred larvae, suggesting that the change of medium has a significant effect on the microbiota load ($F_{1,33}$ = 38, p < 0.001). However, 5h after the transfer, the bacterial load of transferred larvae started to increase and after 12h reached values similar to non-transferred larvae, although still significantly lower (Figure S5, 5h: $F_{1,32}$ = 36, p < 0.001; 12h: $F_{1,3702}$ = 23, p < 0.001; 20h: $F_{1,2344}$ = 14, p < 0.001). This indicates that the colonisation with auxotrophic bacteria can efficiently be switched off during larval development. As larvae develop at a similar speed with wild-type or auxotrophic bacteria, we reasoned that this switch would allow a well-controlled investigation of the bacterial contribution to larval development. We decided to address this question via transcriptomic analysis, focusing on third instar larvae because their size allowed precise dissections and because this developmental stage is intermediate between two larval stages, and therefore more representative of larval development than fourth instar larvae which will moult into pupae.

We analysed the transcriptome of third instar larvae in individuals having lost auxotrophic *E. coli* and in individuals carrying wild-type *E. coli*. We synchronised larvae by selecting individuals starting their third instar within a 5h timeframe, after which we transferred auxotroph-carrying larvae for *m*-DAP and D-Ala depletion (Figure 2A). We sampled guts and whole larvae from both germ-free and wild-type *E. coli* gnotobiotic conditions 12h and 20h later, starting as early as 12h after amino-acid depletion to monitor direct changes caused by

the absence of bacteria rather than secondary impacts linked to any potential developmental delay in germ-free larvae. After extraction of total RNA, we confirmed the loss of bacteria in reversibly colonised mosquitoes by qPCR (Figure S6, gut 12h: $F_{1,2} = 39$, $p = 0.023$; gut 20h: $F_{1,3} = 81$, $p = 0.001$; whole larvae 12h: $F_{1,4} = 45$, $p = 0.004$, whole larvae 20h: $F_{1,2} = 68$, $p = 0.015$). cDNA libraries were produced and sequenced on an Illumina NextSeq 500. A total of 2735 transcripts were significantly differentially regulated (adjusted $p < 0.001$) at least in one sample type at one time point. The sample distance heatmap showed that for each tissue type and time-point, the three experimental replicates clustered together (Figure S7). The principal component analysis (PCA) on gut samples identified a first component explaining 45% of the variance and discriminating sampling time-points and a second component representing 26% of the variance and separating samples by the presence/absence of bacteria (Figure 2B). PCA on whole larvae data revealed a more important separation of samples by time-point (77% of the variance) and only 13% of the variance explained by the microbiota composition. The 12h time point was early enough to detect early transcriptional modifications in the gut before any body-scale transcriptional change, which was detectable 20h post-amino-acid depletion (Figure 2B).

Among the differentially regulated genes, we selected those transcripts with a $\log_2$ fold value < -1.5 or > 1.5, obtaining a total of 573 up-regulated and 293 down-regulated genes in germ-free larvae compared to colonised larvae (Table S1). The Gene Ontology enrichment analysis of transcripts up-regulated in germ-free larvae included phosphatase/alkaline phosphatase/phosphoric ester hydrolase activity, oxidoreductase activity, heme binding, iron ion binding, monooxygenase activity, cofactor binding, and catalytic activity (Figure 2C and Table S2). Several alkaline phosphatases were found amongst the most highly upregulated genes (Figure 2D and Table S3). The Kyoto encyclopaedia of gene and genomes (KEGG) enrichment analysis revealed the folate (Vitamin B9) biosynthesis and thiamine (Vitamin B1) metabolism up-regulated in germ-free larvae in both gut and whole larva samples (Figure 2E). As many genes coding for proteins involved in these pathways are not present in the *Ae. aegypti* genome, this suggests that, as reported in other insects, the mosquito microbiota participates in vitamin B biosynthesis. Focusing on the downregulated genes, we detected 6 hexamerin 2 beta genes as significantly downregulated, including 4 in the 10 most downregulated genes (Figure 2D and Table S4). Hexamerins are juvenile hormone binding proteins which are reported to participate in amino-acid storage during the end of larval development and during metamorphosis. Interestingly, we also identified several down-regulated genes encoding proteins involved in lipid storage and transport, such as *Vitellogenin*, *Vitellogenin*-related gene (*AAEL008598*) and two lipophorin genes (*AAEL009955* and *AAEL019755*) (Figure 2D and Table S4). Together, our transcriptomic analysis suggests that the microbiota participates in vitamin B biosynthesis and in macromolecule storage during larval development.

To determine whether hypoxia-induced signalling was affected in third-instar germ-free larvae, we specifically checked the expression level of hypoxia-induced factors (HIF)-alpha and -beta (AAEL019499 and AAEL010343) and of prolyl hydroxylase (PHD)-1 and -2 genes (AAEL024908 and AAEL028025) in our samples, as HIF were reported to be induced in the presence of a microbiota in first instar larvae (Valzania et al., 2018) and are targets of PHDs. Only *phd-2* was slightly down-regulated at 20h (gut: $\log_2$ fold = -1.39, *p*adj = 0,0015; whole larvae: $\log_2$ fold = -0.6, *p*adj = 0,015). This is expected to happen in hypoxic conditions, since

PHD hydrolyses HIFs in normoxic but not in hypoxic conditions and lower PHD levels would result in higher HIF transcripts. However, none of the HIF genes was found to be differentially regulated in our samples. We also measured the oxygen levels in larval guts 12h post-transfer, which should correspond to the lowest level of oxygen concentration in the presence of bacteria (Coon et al., 2017). No difference in oxygen levels was detected between larvae carrying the wild-type *E. coli* (left in their medium or transferred in new sterile medium) and the germ-free larvae (Figure S8, $F_{2,82} = 0.52$, $p = 0.60$).

Folate supports late stages of development

The absence of a significant correlation between the loss of the microbiota and the hypoxia-induced signalling, and the regulation of many genes involved in vitamin biosynthesis and macromolecule storage after microbiota loss support the hypothesis that the microbiota contributes metabolically to mosquito development. To further investigate this theory, we tested whether, as already known for first-instar larvae, late larval development was affected by the loss of bacteria. When third instar larvae were deprived of *m*-DAP and D-Ala, they all moulted into the fourth instar (99 ± 0.9 %, mean ± SEM), but only 45 ± 2.7 % moulted to pupa and only 30 ± 5.4 % fully developed into adults (Figure 3A, L4: $F_{2,4} = 1.0$, $p = 0.44$; Pupae: $F_{2,4} = 34$, $p = 0.003$; Adults: $F_{2,4} = 37$, $p = 0.002$). The third and fourth instars of germ-free larvae were prolonged by an average of 0.25 and 2.5 days respectively (Figure 3B, L3: $F_{2,404} = 19$, $p < 0.001$; L4: $F_{2,253} = 128$, $p < 0.001$), while metamorphosis duration was not affected ($F_{2,233} = 2.9$, $p = 0.054$). These observations suggest that bacteria also participate in late larval development, while some individuals make it to the adult stage, hypothetically using energy stored during their early development.

A similar reduction in development success was observed among wild-type *E. coli*-carrying larvae that were transferred into sterile water (Figure 3A), indicating that the presence of a sufficient microbiota load throughout larval development is critical. The duration of the fourth instar was also affected by this transient decrease in bacterial load (Figure 3B).

Based on our transcriptomic results, we investigated whether the addition of folate to the rearing water of late germ-free larvae would support further larval development and metamorphosis. When providing folate upon *m*-DAP and D-Ala depletion, we observed that development success to adulthood was doubled without impacting development time (Figure 3C, L4: $F_{2,2} = 1.0$, $p = 0.41$; Pupae: $F_{1,2} = 38$, $p = 0.025$; Adults: $F_{1,2} = 17$, $p = 0.032$; Figure 3D, L3: $F_{1,248} = 0.39$, $p = 0.53$; L4: $F_{1,145} = 0.018$, $p = 0.89$; pupal stage: $F_{1,100} = 2.1$, $p = 0.15$).

We then investigated the consequences of the second transcriptomic signal observed in germ-free larvae, namely the downregulation of genes involved in amino-acid and lipid storage. To this aim, we quantified by fluorescence the lipid levels in the gut and in the pelt of germ-free larvae and in larvae carrying wild-type *E. coli*, transferred or not transferred to sterile rearing water. We found significantly higher lipid signals in the gut of germ-free larvae compared to larvae carrying a microbiota (Figure 4A, B, $F_{2,124} = 7.7$, $p < 0.001$), while the amount of lipids in pelts, which are composed for the majority of the cuticle and the fat body, was lower in larvae that lost their microbiota (Figure 4C, $F_{2,97} = 18$, $p < 0.001$). The lower lipid levels detected in the pelt of germ-free larvae correlated with, and possibly led to, a slower growth of

these larvae, as reflected by the lower DNA signal and the smaller larval size (Figure 4D, $F_{2,97} = 16$, $p < 0.001$; Figure 4E, $F_{2,96} = 19$, $p < 0.001$).

Discussion

The mosquito microbiota impacts its hosts' physiology at the larval and adult stage via largely uncharacterised mechanisms. The current study allowed the development of a new approach to produce fully germ-free adult mosquitoes and sheds light on the metabolic contribution of the microbiota to larval development.

The production of a relevant negative control has long been a technical issue for the study of the microbiota in adult mosquitoes, which was either solved by allowing normal development and treating emerging mosquitoes with antibiotics or by producing stunted germ-free mosquitoes (Correa et al., 2018; de Gaio et al., 2011). However, antibiotic treatments are known to reduce bacterial load and modify microbiota composition rather than produce germ-free mosquitoes, and off-target effects cannot be ruled out. Moreover, several pieces of evidence underline the requirement for standardised conditions during development due to carry over effects of the larval environment from larvae to adults. For instance, larval microbiota composition and larval diet affect host lifespan and vector competence, while crowding and starvation affect metabolic profiling in the adult fat body (Dickson et al.; Linenberg et al., 2016; Price et al., 2015). Here we provide a tool which allows the development of germ-free and colonised adults in comparable conditions, allowing further functional studies of the bacterial microbiota in adults.

Interestingly, we found that development success was slightly higher in gnotobiotic than conventionally-reared mosquitoes (Figure 1B). This may be due to the presence of bacteria with a low larvicidal activity in our colony, which could also explain the slight variation in sex ratio (Figure S3) as females develop slightly slower than males and therefore may have a prolonged contact with such pathogens. Alternatively, some individual larvae may not carry enough bacteria for successful larval development, which correlates with development success deficits after the transfer of wild-type *E. coli* harbouring third-instar larvae (Figure 3A). We were intrigued that as much as 99.8 % of the bacteria were lost within 2h after transferring larvae to sterile water, and that bacterial load was recovered within around 10h. Further work is required to assess whether this is due to quantification of cuticular bacteria, if the quick surface sterilisation has limited efficacy, or whether it reflects an extremely fast shedding of gut bacteria during development.

Our transcriptomic data clearly suggest the involvement of the folate biosynthesis pathway during larval development. These results are in line with an older study on the nutrition of *Ae. aegypti*, where folic acid was shown to promote pupation and with a recent report of B vitamins as a key contribution of the microbiota to tick development (Duron et al., 2018; Golberg et al., 1945). Folate biosynthesis also appeared among the regulated pathways in adult *Anopheles* mosquitoes deprived of their microbiota (Rodgers et al., 2017). The genome of *Ae. aegypti* only encompasses some genes of this biosynthesis pathway, while the *E. coli* genome codes a full pathway except the gamma-glutamyl-hydrolase, which we found up-regulated in germ-free larvae (Kanehisa et al., 2017). This suggests that the host and its microbiota cooperate

towards the production of this vitamin. When comparing our transcriptomic data to a previous transcriptome analysis in first instar larvae (Vogel et al., 2017), we can identify a few shared features including the importance of metabolic differences between gnotobiotic and axenic larvae and the low representation of immune genes. However, the identified pathways of interest are not shared, which may be due to differences between early and late larval development or linked to the fact that the previous study focused on same age larvae while our attention was driven to synchronicity.

In conclusion, our study provides a new tool for microbiota analysis in mosquitoes or other insects requiring bacteria for their development. It also allowed us to build a model for the metabolic contribution of the microbiota to larval development. Our model suggests that bacteria are required for folate biosynthesis, which supports the later stages of development, and for energy storage in the form of proteins and lipids, which promotes larval growth.

Materials and Methods

Bacteria and mosquitoes

E. coli HS (wild-type) and *E. coli* HA 416 (auxotroph; Cuenca et al., 2016) were grown in lysogeny broth (LB). *E. coli* HA 416 cultures were supplemented with 50 µg/mL kanamycin, 50 µg/mL *meso*-diaminopimelic acid (*m*-DAP) and 200 µg/mL D-alanine (D-Ala). For all experiments, *E. coli* cultures were inoculated from single fresh colonies in liquid LB with appropriate supplementation and incubated at 30 °C, shaking at 200 rpm for 16 h. Growth curves of auxotrophic *E. coli* with optimal amino-acid concentrations (50 µg/mL *m*-DAP and 200 µg/mL D-Ala) and concentrations compatible with first-instar larvae (12.5 µg/mL *m*-DAP and 50 µg/mL D-Ala) were obtained by inoculating 2 mL of LB supplemented with the two amino-acid concentrations with the same diluted bacterial culture in a 24-well plate. The optical density at 600 nm (OD_{600}) was monitored every 15 minutes using a FLUOstar plate reader (BMG Labtech). The experiment was performed two times.

The *Ae. aegypti* New Orleans colony was maintained under standard insectary conditions at 28-30 °C on a 12:12 h light/dark cycle. Gnotobiotic mosquitoes were maintained in a climatic chamber at 80% relative humidity on a 12:12 h light/dark 30°C/25°C cycle.

Generation of germ-free (axenic) larvae

Inside a microbiological safety cabinet, eggs were scraped off the paper they were laid on and transferred onto a disposable vacuum filtration system. Eggs were surface sterilised via incubation in 70% ethanol for 5 min, then in 1% bleach for 5 min and finally in 70% ethanol for 5 min. After each incubation step, a vacuum was applied to the filtration unit for fast replacement of the sterilising liquid. After rinsing three times with sterile water, the eggs were resuspended in ~20 mL of sterile water and transferred to a sterile 25-mL cell-culture flask with a vented cap. Approximately 20-30 eggs were inoculated in 3 mL of liquid LB and incubated overnight at 30 °C shaking at 200 rpm to confirm sterility. Flasks were kept standing in a climatic chamber at 80% relative humidity on a 12:12 h light/dark 30°C/25°C cycle for eggs to hatch overnight.

Generation of gnotobiotic larvae and axenic adults via reversible colonisation

Depending on the experiment type, two rearing methods were used: for experiments aiming to analyse adult mosquitoes, a batch rearing in cell-culture flasks (10-15 larvae per flask) was used; for experiments requiring the follow-up of single larvae, larvae were placed in single wells of sterile 24 well-plates (Romoli and Gendrin, in press).

A 16 h culture of each *E. coli* strain was pelleted and cells were resuspended in 5 times the initial culture volume of sterile deionised water to reach a concentration of 2-5*10^8 CFU/mL. For the generation of larvae gnotobiotic for the auxotrophic *E. coli*, *m*-DAP (12.5 µg/mL) and D-Ala (50 µg/mL) were also added to the bacterial suspension. For batch rearing, 10 mL of bacterial suspension and 750 µL of autoclaved suspension of TetraMin Baby fish food (50 mg/mL in water) were added to each flask. For rearing of individualised larvae, 2 mL of bacteria and 50 µL of fish food suspension were added to each well. Larvae were kept in the climatic chamber until pupation.

Pupae were rinsed once in sterile water and then transferred to 15 mL tubes placed in autoclaved polypropylene cups for emergence. A smaller tube containing a rod of sterile cotton dipped into a 10 % sterile sucrose solution was placed in each cup. Tubes containing pupae and sugar were held in place using specifically designed 3D printed components.

Duration of development

All measurements for the investigation of the developmental dynamics were performed on wild-type and auxotrophic *E. coli*-carrying gnotobiotic individuals and from conventionally-reared individuals obtained from non-sterilised eggs and reared in non-sterile conditions (using tap water as conventionally used for our mosquito colony). Three independent mosquito batches were examined for each type of analysis.

Gnotobiotic or conventionally-reared larvae were placed in single wells of 24-well plates to monitor the duration of the different developmental stages in single individuals and to determine the developmental success of each rearing condition, measured as the percentage of larvae reaching the adult stage. Plates were inspected three times per day and the moment at which larvae moulted was noted. The sex of the adult mosquito or the time of death were also recorded for each individual. The experiment was stopped after 15 days, generally 5 days after the last emergence. Individuals stacked at the larval stage were marked as non-developed. For each replicate, at least 48 individuals per condition were analysed.

Reversible colonisation dynamics

To validate reversible colonisation, adult mosquitoes emerged from auxotrophic *E. coli*-gnotobiotic larvae were collected 1-3 days after emergence, surface sterilised for 3-5 min in 70% ethanol, rinsed three times in sterile PBS and homogenised individually in 100 µL of sterile LB. To quantify the number of bacterial CFUs present in each individual, serial dilutions of the homogenates were plated on LB agar plates supplemented with 50 µg/mL *m*-DAP and 200 µg/mL D-Ala. Figure S3 show data from three independent replicates based on 34, 123 and 321 individuals. To further investigate the colonisation dynamics of the auxotrophic *E. coli* strain, the bacterial loads in third and fourth instar larvae and pupae were investigated and compared to those of individuals carrying the wild-type strain. Larvae and pupae were surface sterilised for 15-30 s in 70% ethanol, rinsed in sterile water and homogenised individually in

100 µL of sterile LB. Serial dilutions of the homogenates were plated on LB agar plates supplemented with 50 µg/mL *m*-DAP and 200 µg/mL D-Ala and the number of bacterial cells present in each individual was determined. Three replicates of at least 12 individuals per replicate were analysed at each time point.
To detect the presence of bacterial DNA on adult mosquitoes, DNA was extracted from single 1-day old whole mosquitoes produced with the two *E. coli* strains. Mosquitoes were placed individually in 2 mL screw-cap tubes containing 0.5 mm glass beads and homogenised using a bead beater (Precellys Evolution, Bertin) for 2x1 min at 9,000 rpm in 190 µL of Lysis Buffer (20 mM Tris-HCl, 2 mM EDTA, 1.2% Triton X, 20 mg/mL lysozyme). Two blank samples were extracted in parallel and used as negative controls. After 2h incubation at 37 °C, Proteinase K (1 mg/mL final concentration) was added and samples were incubated 4h at 55 °C. DNA was purified using the ZymoBIOMICS DNA 96 MagBead kit (Zymo Research). The presence of bacterial DNA was detected and quantified via amplification of the *16S* rRNA bacterial gene (FOR: 5'- TCCTACGGGAGGCAGCAGT-3'; REV: 5'-GGACTACCAGGGTATCTAATCCTGTT-3', (Nadkarni et al., 2002) and the ribosomal mosquito gene *S7* (FOR: 5'-CCTTTCACTGTACCGAACGC-3'; REV: 5'-TTTGACAGAACAGCCGACCA-3') via qPCR using the SYBR qPCR Premix Ex Taq (Takara) in a Light Cycler 480 (Roche). Mosquitoes were considered germ-free when their *16S* Ct were higher than those of negative controls. Three replicates of 9 individuals per replicate were analysed.

Larval and wing length measurements
For larval length measurements, larvae were collected at the fifth day from hatching. Larvae were fixed in PBS with 4% paraformaldehyde for 20 min and placed on a microscope slide. For each larva, the distance between the anterior border of the head and the posterior border of the last abdominal segment excluding the siphon (Romoli and Gendrin, in press) was measured using a dissection microscope equipped with a camera. Four independent mosquito batches were examined and, for each replicate, at least 28 individuals per condition were analysed.
For wing length measurements, adult mosquitoes were collected 2 days post emergence and kept at -20°C until analysis. The measurements were conducted on the left wing of both male and female mosquitoes. Wings from several individuals were dissected and placed on a microscope slide. The distance between the alular notch and the radius 3 vein was measured for each wing using a dissection microscope equipped with a camera (Romoli and Gendrin, in press). Four independent mosquito batches were examined and, for each replicate, 8 to 20 individuals per condition were analysed.

Experimental setup and sample collection for transcriptomics on colonised vs germ-free larvae
Gnotobiotic larvae for both bacterial strains were obtained as previously described, reared individually in 24-well plates and kept in a climatic chamber at 80% RH with 12:12 h light/dark 30°C/25°C cycle. Larvae gnotobiotic for the auxotrophic strain were supplemented with *m*-DAP (12.5 µg/mL) and D-Ala (50 µg/mL). To ensure synchronicity, larvae moulting to third instar between 48h and T_0=53h after addition of bacteria were selected. At T_0, larvae carrying the auxotrophic *E. coli* were washed in sterile water and individually transferred to new 24-well plates containing 2 mL of sterile water and 50 µL of sterile fish food. Twelve hours later

(T_{12}), 120 larvae per condition were collected for sampling and another 120 larvae reversibly colonised by the auxotrophic *E. coli* were transferred to new 24-well plates containing 2 mL of sterile water and 50 µL of sterile fish food to further reduce bacterial colonisation. Another 8h later (T_{20}), 120 larvae per condition were collected for sampling.
At both T_{12} and T_{20}, for each condition, 60 larval guts (dissected on ice) and 60 whole larvae were snap frozen in prechilled 2 mL screw-cap tubes containing 0.5 mm glass beads and kept in a cooling block below -40 °C. A gut sample included gastric caeca, anterior and posterior midgut, while hindgut and Malpighian tubules were excluded. Three replicates were performed using independent mosquito batches.

RNA extraction, library preparation, RNA sequencing and data analysis
After collection, 1 mL of TRIzol (Thermo Fisher Scientific) was added to all samples. Samples were homogenised using a bead beater (Precellys Evolution, Bertin) for 2x1 min at 9,000 rpm and stored at -80 °C until RNA extraction. Total RNA was extracted using TRIzol according to the manufacturer's instructions. After RNA extraction, samples were treated for 30 min at 37 °C with 10 U of DNase I (Thermo Fisher Scientific) and purified using the MagJET RNA Kit (Thermo Fisher Scientific) in a KingFisher Duo Prime system (Thermo Fisher Scientific). Samples were mixed with RNA stable (Biomatrica), vacuum dried using a SpeedVac and shipped for library preparation and sequencing.
RNA quality evaluation, library preparation and sequencing were performed by Biofidal (Vaulx-en-Velin, France - http://www.biofidal-lab.com). RNA integrity was evaluated on a BioAnalyzer (Agilent Technologies) using an RNA 6000 Pico RNA chip. Single-end libraries (75 bp) with poly(A) selection were constructed using the Universal Plus mRNA-Seq Library Preparation with NuQuant kit (Nugen Tecan Genomics) from 1 µg of total RNA. After quality check, libraries were pooled in equimolar concentrations and sequenced on a NextSeq 500 high-output flow cell (Illumina). The resulting raw reads were de-multiplexed and adaptor sequences were trimmed by the sequencing facility. FASTQ sequences were trimmed for their quality using Trimmomatic (minimum quality score: 15, minimal length: 40) and their quality was evaluated using FASTQC. After quality trimming, an average of 15 million reads (min 8.4 million, max 25 million) were obtained per sample. Reads were mapped on the *Ae. aegypti* genome (assembly AeaegL5, geneset AeaegL5.2) using HISAT2 with a mean alignment rate of 94%. Read counts were obtained with FeatureCounts and differential expression analysis was conducted in R using the DESeq2 package. Only genes with an adjusted p-value < 0.001 and a $\log_2$ FoldChange < -1.5 or > 1.5 were subjected to the Gene Ontology analysis with g:Profiler.
To quantify the bacteria loss on samples subjected to RNA sequencing, RNA was retro-transcribed using the PrimeScript RT-PCR Kit (Takara) and analysed via qPCR using the SYBR qPCR Premix Ex Taq (Takara) in a Ligth Cycler 480 (Roche). The bacterial load was quantified via amplification of the *16S* rRNA bacterial gene (primers previously described) and normalised to the ribosomal mosquito gene *S17* (FOR: 5'- AAGAAGTGGCCATCATTCCA-3'; REV: 5'- GGTCTCCGGGTCGACTTC-3'; Dzaki et al., 2017).

Analysis of bacterial loss dynamics

Third-instar synchronous gnotobiotic larvae were obtained as previously described. At T_0, larvae carrying the wild-type or the auxotrophic *E. coli* were washed in sterile water and individually transferred to new 24-well plates containing 2 mL of sterile water and 50 µL of sterile fish food. Non-transferred larvae were reared in parallel. After 2h, 5h, 12h and 20h from the transfer, at least 6 larvae per condition were surface sterilized for 15-30 s in 70 % EtOH, rinsed in sterile water and homogenised individually in 100 µL of sterile LB. Serial dilutions of the homogenates were plated on LB agar plates supplemented with 50 µg/mL *m*-DAP and 200 µg/mL D-Ala and the number of bacterial cells present in each individual was determined. Two replicates were analysed at each time point.

Analysis of development and folate supplementation

Third-instar larvae reared with wild-type *E. coli* and transferred to new rearing medium and germ-free larvae were obtained as previously described. Non-transferred larvae were reared in parallel. To investigate the effect of folate on development success, 250 µg/ml folate (Sigma Aldrich) was added to germ-free larvae. Plates were inspected daily and the developmental stage of each individual was noted. Three replicates were performed using independent mosquito batches. For each replicate, 24 to 48 larvae were analysed.

Gut hypoxia measurements

Third-instar larvae reared with wild-type *E. coli* and transferred to new rearing medium and germ-free larvae were obtained as previously described. Non-transferred larvae were reared in parallel. At least 9 larvae per condition were collected at the 12h time-point, washed in sterile water and incubated at dark for 30 min in sterile PBS supplemented with 5 µM Image-iT Red Hypoxia Reagent (Thermo Fisher Scientific) and fish food. In parallel, two larvae per condition were processed without the dye as a negative control. Larvae were frozen 3 min at -80 °C and placed in a single slide. Images were acquired with an EVOS FL Auto system (Thermo Fisher Scientific) equipped with a Texas Red filter using a 10X objective. The multiple images were stitched automatically using the EVOS FL Auto Software to reconstitute a single image including all samples. Fluorescence intensities and area surfaces of single guts were measured using FIJI (Schindelin et al., 2012). Three replicates were performed using independent mosquito batches.

Lipid content and length measurements

Third-instar larvae reared with wild-type *E. coli* and transferred to new rearing medium and germ-free larvae were obtained as previously described. Non-transferred larvae were reared in parallel. Larvae were sampled 44h after the transfer, when wild-type *E. coli* carrying larvae were at the second day of the fourth instar. Guts and pelts (composed by the cuticle and the fat body) were dissected, placed in PBS with 4% paraformaldehyde and fixed overnight at 4 °C. After three 5 min washes in PBS, guts were stained at dark for 20 min with DAPI (Thermo Fisher Scientific, final concentration 0.1 µg/mL) and for 20 min with BODIPY 505/515 (Thermo Fisher Scientific, final concentration 1 µM), rinsed three times in PBS for 5 min and imaged. All guts from the same experiment were placed in the same slide and images were acquired with an EVOS FL Auto system (Thermo Fisher Scientific) equipped with DAPI and GFP filters using a 10X objective. The multiple images were stitched automatically using the

EVOS FL Auto Software to reconstitute a single image including all samples. Fluorescence intensities and area surfaces of single guts were measured using FIJI (Schindelin et al., 2012). A different protocol was used to quantify lipids in the pelts to avoid the loss of fat body parts during staining and rinsing steps. After fixation, pelts were washed three times in PBS for 5 min and placed in individual tubes with 75 µL of DAPI (0.1 µg/mL) and 75 µL of BODIPY 505/515 (1 µM). Pelts were homogenized, incubated at the dark for 20 min and transferred to a 96-well plate. Fluorescence intensities were measured using a FLUOstar plate reader equipped with DAPI and GFP filters, using a solution of the two dyes as blank. At least 12 larvae per condition were analysed in three independent replicates.
Larval lengths were measured as previously described on larvae obtained with the same protocol and collected at the 44h time-point. Two independent replicates were performed using 9 and 24 individuals per condition.

Statistical analyses
Statistical analyses were performed using generalized linear mixed models (GLMM) using the lme4 package in R (version 3.6.0) and setting the rearing condition (bacterium) as fixed effects and the replicate as a random effect. For categorical data (development success, sex ratio) an ANOVA was performed on a logistic regression (glmer). For quantitative data (CFU counts, duration of development, larval and wing length measurements, qPCR results, hypoxia, lipid and DNA quantifications) an ANOVA was performed on a linear regression (lmer). Data shown in Figure S2B were analysed in Prism (version 8.3.0) using a Welsh's t test.

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References

Coon, K.L., Vogel, K.J., Brown, M.R., and Strand, M.R. (2014). Mosquitoes rely on their gut microbiota for development. Molecular Ecology *23*, 2727–2739.

Coon, K.L., Valzania, L., McKinney, D.A., Vogel, K.J., Brown, M.R., and Strand, M.R. (2017). Bacteria-mediated hypoxia functions as a signal for mosquito development. Proceedings of the National Academy of Sciences of the United States of America *114*, E5362–E5369.

Correa, M.A., Matusovsky, B., Brackney, D.E., and Steven, B. (2018). Generation of axenic Aedes aegypti demonstrate live bacteria are not required for mosquito development. Nature Communications *9*, 4464.

Cuenca, M., Pfister, S.P., Buschor, S., Bayramova, F., Hernandez, S.B., Cava, F., Kuru, E., Nieuwenhze, M.S., Brun, Y.V., Coelho, F.M., et al. (2016). D-Alanine-Controlled Transient Intestinal Mono-Colonization with Non-Laboratory-Adapted Commensal E. coli Strain HS. PLOS ONE *11*, e0151872.

Dickson, L.B., Jiolle, D., Minard, G., Moltini-Conclois, I., Volant, S., Ghozlane, A., Bouchier, C., Ayala, D., Paupy, C., Moro, C., et al. Carryover effects of larval exposure to different environmental bacteria drive adult trait variation in a mosquito vector. Science Advances *3*, e1700585.

Duron, O., Morel, O., Noël, V., Buysse, M., Binetruy, F., Lancelot, R., Loire, E., Ménard, C., Bouchez, O., Vavre, F., et al. (2018). Tick-Bacteria Mutualism Depends on B Vitamin Synthesis Pathways. Current Biology.

de Gaio, A.O., Gusmão, D.S., Santos, A.V., Berbert-Molina, M.A., Pimenta, P.F., and Lemos, F.J. (2011). Contribution of midgut bacteria to blood digestion and egg production in aedes aegypti (diptera: culicidae) (L.). Parasites & Vectors *4*, 105.

Gendrin, M., Rodgers, F.H., Yerbanga, R.S., Ouédraogo, J.-B., Basáñez, M.-G., Cohuet, A., and Christophides, G.K. (2015). Antibiotics in ingested human blood affect the mosquito microbiota and capacity to transmit malaria. Nature Communications *6*.

Golberg, L., Meillon, B. de, and Lavoipierre, M. (1945). The Nutrition of the Larva of Aedes Aegypti L: II. Essential Water-Soluble Factors from Yeast. J Exp Biol *21*, 90–96.

Kanehisa, M., Furumichi, M., Tanabe, M., Sato, Y., and Morishima, K. (2017). KEGG: new perspectives on genomes, pathways, diseases and drugs. Nucleic Acids Research *45*, D353–D361.

Lang, C., Basch, K., and Storey, R. (1972). Growth, composition and longevity of the axenic mosquito. The Journal of Nutrition *102*, 1057–1066.

Linenberg, I., Christophides, G.K., and Gendrin, M. (2016). Larval diet affects mosquito development and permissiveness to Plasmodium infection. Scientific Reports *6*, 38230.

Moll, R., Romoser, W., Modrzakowski, M., Moncayo, A., and Lerdthusnee, K. (2001). Meconial peritrophic membranes and the fate of midgut bacteria during mosquito (Diptera: Culicidae) metamorphosis. Journal of Medical Entomology *38*, 29–32.

Nadkarni, M.A., Martin, F., Jacques, N.A., and Hunter, N. (2002). Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. Microbiology (Reading, England) *148*, 257–266.

Pike, A., Dong, Y., Dizaji, N., Gacita, A., Mongodin, E.F., and Dimopoulos, G. (2017). Changes in the microbiota cause genetically modified Anopheles to spread in a population. Science *357*, 1396–1399.

Price, D.P., Schilkey, F.D., Ulanov, A., and Hansen, I.A. (2015). Small mosquitoes, large implications: crowding and starvation affects gene expression and nutrient accumulation in Aedes aegypti. Parasites & Vectors *8*, 252.

Rodgers, F.H., Gendrin, M., Wyer, C.A., and Christophides, G.K. (2017). Microbiota-induced peritrophic matrix regulates midgut homeostasis and prevents systemic infection of malaria vector mosquitoes. PLoS Pathogens *13*, e1006391.

Romoli, O., and Gendrin, M. Manipulating the mosquito microbiota to study its function. In Immunity in Insects, (Springer), *in press*

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., et al. (2012). Fiji: an open-source platform for biological-image analysis. Nature Methods *9*, 676–682.

Valzania, L., Coon, K.L., Vogel, K.J., Brown, M.R., and Strand, M.R. (2018). Hypoxia-induced transcription factor signaling is essential for larval growth of the mosquito Aedes aegypti. Proceedings of the National Academy of Sciences of the United States of America *115*, 457–465.

Vogel, K.J., Valzania, L., Coon, K.L., Brown, M.R., and Strand, M.R. (2017). Transcriptome Sequencing Reveals Large-Scale Changes in Axenic Aedes aegypti Larvae. PLoS Neglected Tropical Diseases *11*, e0005273.

WHO (2017). Global vector control response 2017–2030 (World Health Organization).

Wu, P., Sun, P., Nie, K., Zhu, Y., Shi, M., Xiao, C., Liu, H., Liu, Q., Zhao, T., Chen, X., et al. (2019). A Gut Commensal Bacterium Promotes Mosquito Permissiveness to Arboviruses. Cell Host & Microbe *25*, 101-112.e5.

Xi, Z., Ramirez, J., and Dimopoulos, G. The Aedes aegypti Toll Pathway Controls Dengue Virus Infection. PLoS Pathogens *4*, e1000098.

Figure legends

Figure 1. Reversible colonisation allows production of germ-free mosquitoes without any delay or deficit in development. (**A**) Schematic representation of the experimental set up. Eggs are surface sterilized and give rise to axenic larvae. A bacterial culture of the *E. coli* wild-type (gnotobiotic control) or auxotrophic strain is added to axenic larvae to obtain gnotobiotic larvae. To support the growth of auxotrophic bacteria, *m*-DAP and D-Ala are provided throughout the whole larval developmental stage. After transferring pupae carrying auxotrophic *E. coli* to a new medium lacking *m*-DAP and D-Ala, germ-free mosquitoes are obtained. (**B**) Proportion of larvae developing to adulthood (striped bars) and of larvae dead or stalled in the development (full bars) after conventional rearing (CR, black), rearing with wild-type *E. coli* (WT, orange) and auxotrophic *E. coli* (AUX, blue). CR larvae developed in tap water on autoclaved fish food. (**C**) Colony Forming Units (CFU) quantification of bacterial loads (upper chart) and prevalence (lower chart) in larvae at third instar (L3), early fourth instar (L4-1), late fourth instar (L4-2), and in pupae (P). (**D**) CFU quantification of bacterial loads (upper chart) and prevalence (lower chart) in 1-3-day old mosquitoes. (**E**) Duration of the larval instars (L1-L4) and of the pupal stage (P). (**F**) Larval length measured on the fifth day after hatching. (**G**) Wing length of two-day old adult mosquitoes. In **B**-**G**, data show mean ± SEM of three independent replicates where 48 (**B**, **E**), 12 (**C**, **D**), 28 (**F**) and 8-20 (**G**) individuals were analysed per condition. NS, Non-significant; *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$. In **E**, different letters represent significantly different data for each larval instar.

Figure 2. Transcriptomic analysis after terminating bacterial colonisation in larvae. (**A**) Schematic representation of the experimental set up. To produce third-instar germ-free larvae, larvae gnotobiotic for auxotrophic *E. coli* were transferred to a rearing medium lacking m-DAP and d-Ala. Gut (n = 60) and whole larvae (n = 60) were sampled for RNA extraction 12h and 20h after transfer, using wild-type *E. coli* gnotobiotic larvae as a control. Three independent replicates were performed. (**B**) Principal Component Analysis (PCA) of guts and whole larvae transcriptomic data of wild-type *E. coli* carrying (WT, orange) and germ-free larvae (GF, white). (**C**) Venn diagram of Gene Ontology terms enriched for genes up-regulated in germ-free conditions. (**D**) Log_2 fold values and function of the ten highest up-regulated (red) and down-regulated (blue) genes in germ-free larvae, ordered by decreasing (up-regulated) or increasing (down-regulated) mean value in the four sample types. Different colour-codes indicate genes with a shared function (orange: phosphatase activity; green: hydrolytic reaction; yellow: chitin binding; purple: transferase activity; light blue: amino-acid storage; dark blue: lipid transport/storage). (**E**) Representation of the mosquito gene products that participate in the folate biosynthesis pathway and that were up-regulated in germ-free larvae. The up-regulation colour-code is the same as in panel **F** and reflects the mean of log_2 fold in the four sample types.

Figure 3. Late larval development is folate dependent. (**A**) Proportion of larvae reaching the fourth instar (L4), the pupal stage and adulthood when reared continuously with wild-type *E.*

coli (WT, orange), when transferred in new rearing medium after being reared with wild-type *E. coli* (WT transferred, light orange) and after becoming germ-free (GF, white). (**B**) Duration of the third instar (L3), fourth instar (L4) and pupal stage in the experiments shown in **A**. (**C**) Proportion of larvae reaching the fourth instar (L4), the pupal stage and adulthood in germ-free conditions (GF, white bars) and in germ-free conditions with 250 µg/mL folate supplementation (GF + folate, striped bars). (**D**) Duration of the third instar (L3), fourth instar (L4) and pupal stage in the experiments shown in **C**. Data show the mean ± SEM of three independent replicates where at least 48 (**A**, **B**) and 24 (**C**, **D**) individuals were analysed per condition.

Figure 4. Consequences of the absence of a microbiota on lipid storage and larval growth. (**A**) Exemplifying image of three guts belonging to fourth-instar larvae gnotobiotic for *E. coli* wild-type before (WT) and after the transferring to new rearing medium (WT transf) and germ-free (GF) stained with BODIPY 505/515. (**B**) Quantification of BODIPY 505/515 fluorescence intensity in the gut of fourth instar larvae (normalized to the gut area) gnotobiotic for *E. coli* wild-type before (WT) and after the transferring to new rearing medium (WT transf) and germ-free (GF). (**C**) Quantification of BODIPY 505/515 fluorescence intensity (Arbitrary Units, AU) in the pelt of fourth instar larvae gnotobiotic for *E. coli* wild-type before (WT) and after the transferring to new rearing medium (WT transf) and germ-free (GF). (**D**) Quantification of DAPI fluorescence intensity (Arbitrary Units, AU) in the pelt of fourth instar larvae gnotobiotic for *E. coli* wild-type before (WT) and after the transferring to new rearing medium (WT transf) and germ-free (GF). (**E**) Larval length measured at the second day of the fourth instar in larvae gnotobiotic for *E. coli* wild-type before (WT) and after transfer to a new rearing medium (WT transf) and germ-free (GF). Data represent the mean ± SEM of three independent replicates where at least 12 individuals were analysed per condition (**B-D**) and of two independent replicates where 9 and 24 individuals were analysed per condition (**E**).

Figure 1

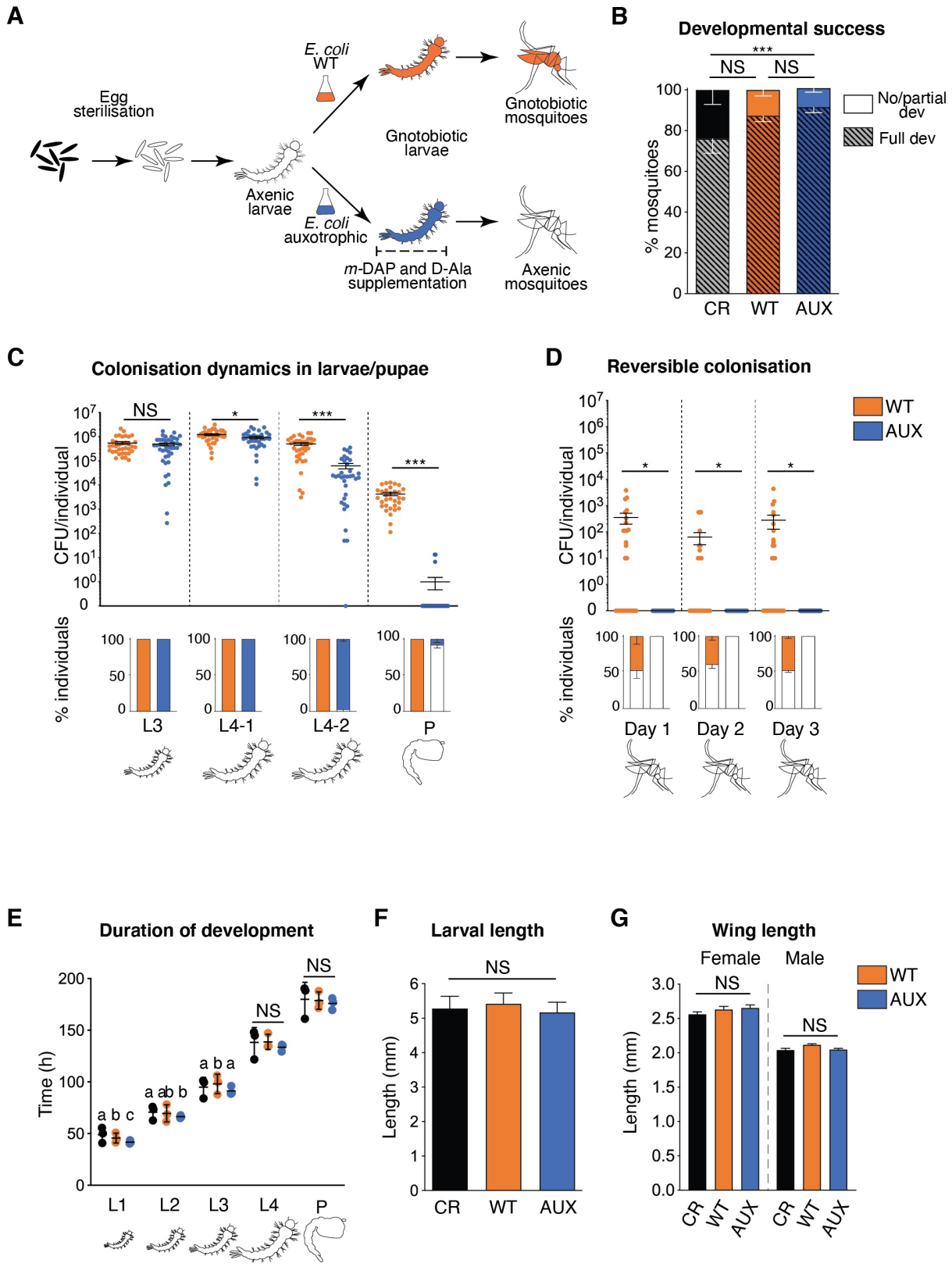
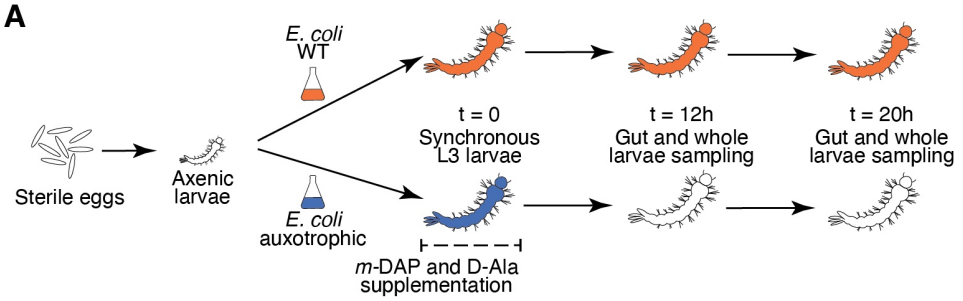

A
Egg sterilisation
Axenic larvae
E. coli WT
Gnotobiotic larvae
Gnotobiotic mosquitoes
E. coli auxotrophic
m-DAP and D-Ala supplementation
Axenic mosquitoes
B
Developmental success
***
NS
NS
% mosquitoes
No/partial dev
Full dev
CR
WT
AUX
C
Colonisation dynamics in larvae/pupae
CFU/individual
% individuals
NS
*
***
***
L3
L4-1
L4-2
P
D
Reversible colonisation
WT
AUX
CFU/individual
% individuals
Day 1
Day 2
Day 3
E
Duration of development
Time (h)
NS
NS
a b c
a ab b
a b a
L1
L2
L3
L4
P
F
Larval length
NS
Length (mm)
CR
WT
AUX
G
Wing length
Female
Male
NS
NS
Length (mm)
WT
AUX
CR
WT
AUX
CR
WT
AUX

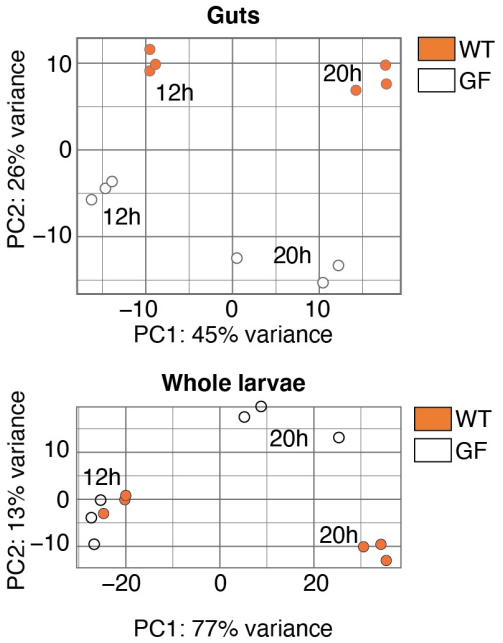
Figure 2

A



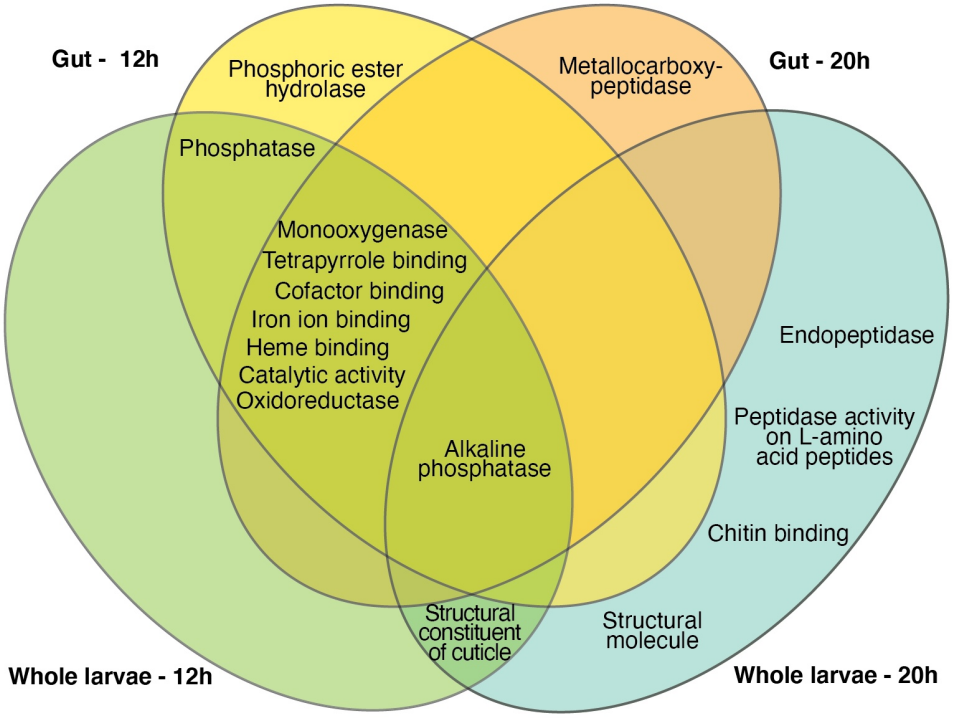
B

Principal Component Analysis



C

Gene Ontology of up-regulated genes in germ-free larvae



D

AAEL	Description					Function	Gut 12h	Gut 20h	WI 12h	WI 20h
000931	Alkaline phosphatase					Phosphatase activity	9.2	7.2	5.5	5.6
003317	Alkaline phosphatase					Phosphatase activity	7.1	6.1	7.6	6.0
003297	Alkaline phosphatase					Phosphatase activity	5.8	3.2	5.4	3.2
002555	Sodium/solute symporter					Transmembrane transport	5.7	4.5	3.6	3.1
002138	Triacylglycerol lipase					Hydrolytic reaction	4.4	3.9	4.0	4.0
003289	Alkaline phosphatase					Phosphatase activity	5.2	3.0	4.7	3.1
000323	Cysteine-rich venom protein					Trypsine inhibitor activity	4.0	3.9	3.7	3.6
029062	Unknown (Chitin binding)					Chitin binding	5.0	3.0	4.5	2.7
024870	Nose resistant to fluoxetine protein 6-like					Transferase activity	3.3	3.1	3.8	3.1
003905	Alkaline phosphatase					Phosphatase activity	4.5	2.5	3.1	3.1

AAEL	Description				Function	Gut 12h	Gut 20h	WI 12h	WI 20h
008045	Hexamerin 2 beta				Amino acid storage	0.0	-6.7	-5.3	-7.0
011169	Hexamerin 2 beta				Amino acid storage	-3.6	-4.2	-2.5	-4.2
011520	Sucrose transport protein				Sucrose transport	-4.5	-3.8	-3.1	-2.9
008817	Hexamerin 2 beta				Amino acid storage	0.0	-4.0	-3.1	-6.0
018240	Unknown (Fuseless region)				Pre-synaptic calcium channel regulation	-3.3	-3.4	-3.3	-3.0
008313	Unknown					0.0	-3.1	-4.5	-4.5
013757	Hexamerin 2 beta				Amino acid storage	0.0	-5.4	-2.2	-4.5
011661	Unknown (Cilia structure/activity)				Cilia structure/activity	-3.9	-4.1	0.0	-3.2
023132	Unknown (Lipase activity)				Hydrolytic reaction	-2.7	-4.0	0.0	-4.5
008598	Unknown (Vitellogenin domain)				Lipid transport/storage	0.0	-2.7	-2.0	-4.7

Log2Fold GF/WT: +9, 0, -9

E

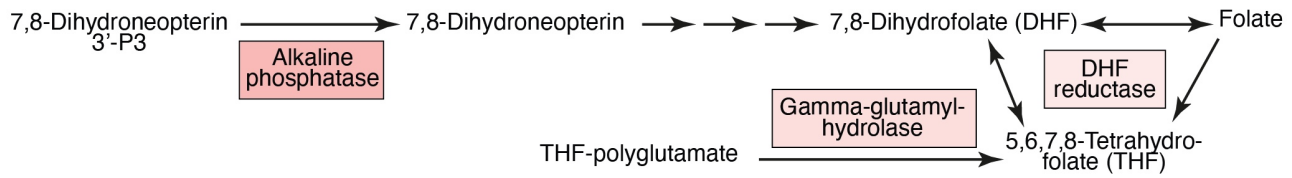


Figure 3

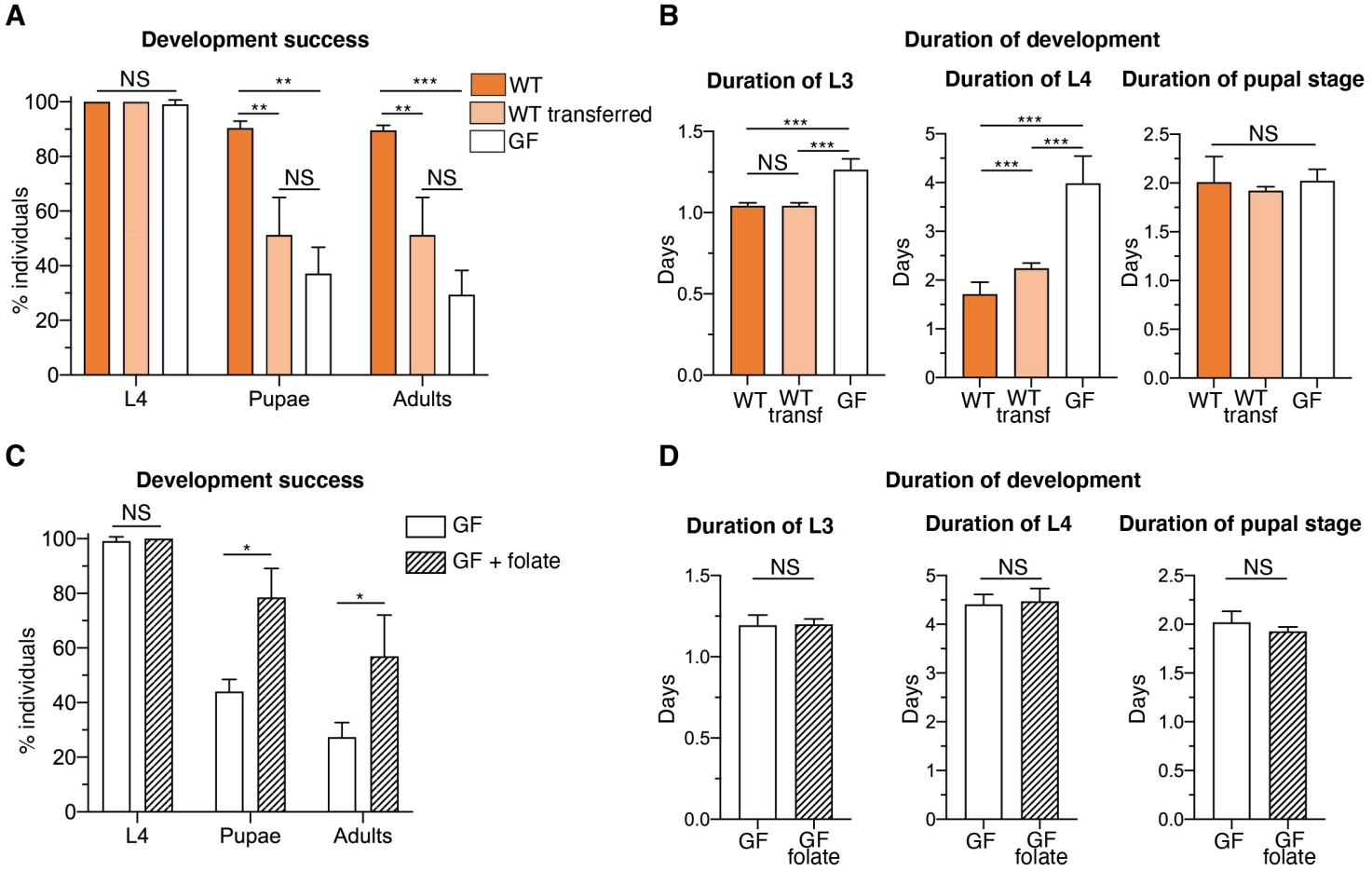
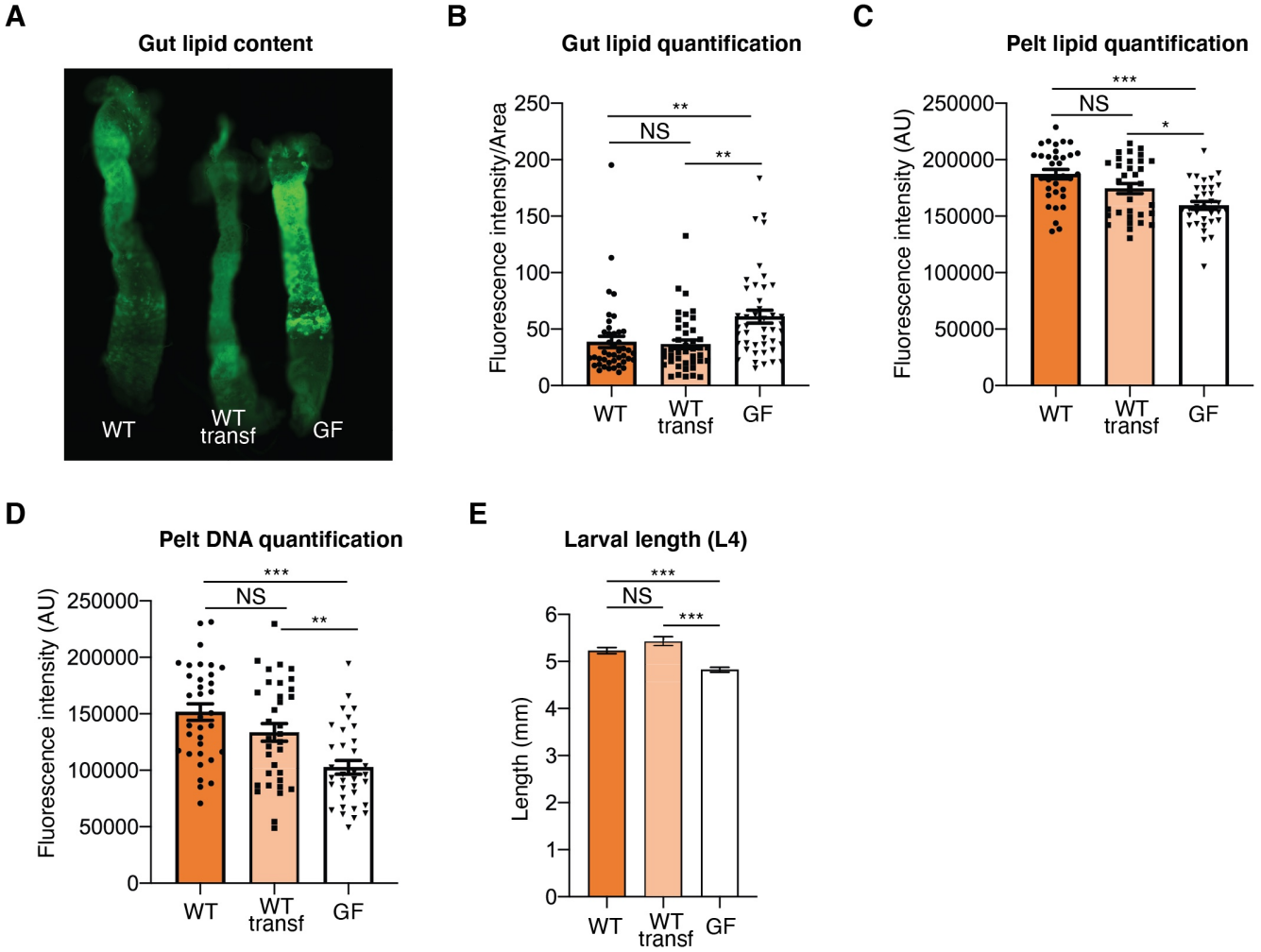

A
Development success
% individuals
NS
**
***
WT
WT transferred
GF
L4
Pupae
Adults
B
Duration of development
Duration of L3
Duration of L4
Duration of pupal stage
Days
WT
WT transf
GF
C
Development success
GF
GF + folate
D
Duration of development
Duration of L3
Duration of L4
Duration of pupal stage
GF folate

Figure 4


A
Gut lipid content
WT
WT transf
GF
B
Gut lipid quantification
Fluorescence intensity/Area
250
200
150
100
50
0
**
NS
**
WT
WT transf
GF
C
Pelt lipid quantification
Fluorescence intensity (AU)
250000
200000
150000
100000
50000
0
***
NS
*
WT
WT transf
GF
D
Pelt DNA quantification
Fluorescence intensity (AU)
250000
200000
150000
100000
50000
0
***
NS
**
WT
WT transf
GF
E
Larval length (L4)
Length (mm)
6
5
4
3
2
1
0
***
NS
***
WT
WT transf
GF