Nutritional sex-specificity during larval development in mosquitoes

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Abstract

Mosquitoes rely on their microbiota for B vitamin provision. We previously found that *Aedes aegypti* larvae cleared of their microbiota at the beginning of the third instar were impaired in their development and that folic acid supplementation increased the proportion of larvae reaching the adult stage. In this study, we investigated the effects of other B vitamins on the development of germ-free mosquito larvae. We found that diet supplementation using a cocktail of seven B vitamins did not improve mosquito developmental success, but rather had a significant impact on the sex-ratio of the resulting adults, with an enrichment of female mosquitoes emerging from B vitamin-treated larvae. A transcriptomic analysis of B vitamin treated male and female larvae identified few genes that may be involved in specific vitamin-induced mechanisms. The treatment of germ-free larvae with high doses of individual B vitamin solutions identified a specific toxic effect related to biotin exposure at high concentrations. The supplementation of germ-free larvae with varying biotin doses revealed divergent biotin requirements and toxic effects for male and female *Ae. aegypti* larvae. These findings shed new light on sex-specific nutritional requirements and toxicity thresholds during the development of insect larvae, and on their consequences on the sex ratio of adults.

Introduction

Aedes aegypti mosquitoes are vectors of pathogens responsible for various human diseases, notably including yellow fever or dengue fever. They are predominantly concentrated in tropical and neotropical regions, poising a significant threat to approximately 3.9 billion people who could potentially contract dengue (World Health Organization, 2020). Mosquitoes are holometabolous insects, undergoing a complete metamorphic transformation from aquatic larvae to terrestrial adults. Consequently, several adult physiological traits, such as size and lifespan, heavily hinge on the quality of the insect larval development, specifically influenced by the nutritional status during this developmental stage.

Mosquitoes heavily depend on their microbiota for vital nutrients crucial for their larval development. In their natural habitat, mosquito larvae primarily feed on microorganisms, particulate organic matter, and detritus present in their breeding sites (Clements 1992). In controlled laboratory rearing environments, the main food sources for larvae typically comprise fish food or commercial pet food designed for rodents, cats, or dogs. Mosquito larvae hatched from microbe-free eggs and maintained in sterile conditions are halted in their development when provided with a sterile conventional diet (Coon et al. 2014). However, their development can be rescued when a live microbiota is introduced or when provided nutrient-rich diet and kept in the dark, strongly implying that the microbiota plays a fundamental role in furnishing essential nutrients to its mosquito host by metabolically transforming components already present in the diet (Coon et al. 2014; Romoli et al. 2021; Correa et al. 2018; Wang et al. 2021). These essential nutrients encompass critical elements such as essential amino acids, nucleosides, and B vitamins, which are beyond the synthetic capabilities of most insects (Wang et al. 2021). The investigation of mosquito nutritional requirements and how the microbiota affects larval development could potentially unveil crucial metabolic insights that might serve as the foundation for innovative vector control strategies.

To unravel the mechanisms behind the intricate interactions between mosquitoes and their microbiota, we previously developed a method that allows to produce germ-free mosquitoes at virtually any stage of their development. Our approach consists in the colonisation of *Aedes aegypti* mosquitoes with an *Escherichia coli* strain auxotrophic for two amino acids that are crucial for

bacterial cell wall synthesis. When bacteria are supplied alongside these amino acids to germ-free larvae, mosquito development is rescued. As soon as the larval rearing medium is changed to sterile water deprived of bacteria and the two amino acids, bacterial growth is arrested and germ-free larvae are obtained. Using this method, we have been able to pinpoint folic acid as one of the critical metabolites furnished by the microbiota and required during mosquito larval development (Romoli et al. 2021).

Vitamins of the B group are cofactors in numerous cellular processes, encompassing essential functions in the electron transport chain (riboflavin, nicotinic acid), amino acid metabolism (pyridoxine, folic acid), lipid metabolism (biotin, riboflavin, nicotinic acid), and nucleic acid metabolism (folic acid, nicotinic acid). They are required in very low amounts, as they serve as coenzymes and are not consumed by the reactions to which they participate (Douglas 2017). Insects depend on their food and microbiota for B vitamin provision, as they lack the complete metabolic pathways to synthesize them (Dadd 1973). The insect model *Drosophila melanogaster* requires all B vitamins for optimal larval development (Piper 2017) and similar necessities have been hypothesized for mosquitoes. However, physiological differences such as the mosquito blood feeding behaviour might make up for specificities for mosquitoes. Over the past century, research efforts have yielded the definition of synthetic diets designed for rearing mosquito larvae in the absence of a microbiota, paving the way for a deeper understanding of mosquito nutritional requirements (Trager 1935; Lea, Dimond, and Delong 1956; Singh and Brown 1957). These diets have identified the necessity of certain elements, including sterols, amino acids, nucleosides, and B vitamins, in supporting optimal mosquito development. A more recent study has highlighted the critical roles played by amino acids and B vitamins in mosquito larval growth. Notably, riboflavin has been identified as needed throughout the entire larval development, while thiamine, pyridoxine, and folic acid are specifically required by *Ae. aegypti* larvae to initiate pupation (Wang et al. 2021).

In our previous study, we found that folic acid provision partially rescued the development success of mosquito larvae deprived of their microbiota during late instars (Romoli et al. 2021). We thus tested the effect of supplying different B vitamin doses on *Ae. aegypti* larval development. Larvae cleared of their microbiota during their third instar and supplemented with increasing amounts of B vitamins did not show any improvement in their developmental success when compared to germ-free non-supplemented counterparts. However, we observed an impact on the sex-ratio of the resulting adults, with a significant enrichment of female mosquitoes emerging from B vitamin-treated larvae. We further investigate potential mechanisms involved in this sex-specific effect of B vitamins via transcriptomics and diet supplementation.

Results

Vitamin supplementation affects mosquito sex-ratio in axenic conditions

We previously reported that *Ae. aegypti* larvae cleared of their microbiota at the third instar showed lower developmental success compared to conventionally reared larvae, with only 10–20 % of individuals successfully completing the metamorphosis stage. The proportion of individuals reaching the adult stage increased to ~50–60 % if folic acid (vitamin B9) was provided to these larvae (0.25–1.25 mg/mL), suggesting an important role of the microbiota in supplementing this B vitamin (Romoli et al. 2021). We thus wondered if other B vitamins potentially produced by the microbiota were involved in mosquito larval development and could further increase the proportion of larvae developing into adults. We produced germ-free third-instar larvae, following our transient colonization protocol (Romoli et al. 2021): after egg sterilization, we mono-colonized first instar

larvae with the *E. coli* HA416 strain, auxotrophic for D-Ala and *m*-DAP, in the presence of these amino acids, and starved larvae from D-Ala and *m*-DAP shortly after reaching the third instar to turn them germ-free. We supplemented these larvae with a solution containing six B vitamins (biotin, folic acid, nicotinic acid, pyridoxine, riboflavin, and thiamine) and choline (not classified as B vitamin but recognized as essential nutrient), at concentrations previously reported to support *Ae. aegypti* larval development in germ-free conditions (VIT1x (Singh and Brown 1957)). However, we did not observe any significant increase in the percentage of adult mosquitoes (Supplementary Figure 1, glmer with random effect on the proportion of adults: $F = 0.35$, $p = 0.55$, not significant (ns); see Supplementary Table 1 for details on statistics). Since the folic acid concentration supplemented in our previous study was 5–25 times more concentrated that the concentration used in Singh & Brown, 1957, we decided to test the effect of higher B vitamin concentrations on larval development, namely a four and an eight times more concentrated solution (VIT4x and VIT8x). Again, we did not observe an increase in the proportion of adult mosquitoes, rather a marginally significant decrease with VIT8x (Figure 1A, glmer with random effect on the proportion of adults: $F = 24$, $p < 0.0001$; AUX vs GF, VIT4x, or VIT8x: $p < 0.0001$; GF vs VIT4x: $p = 0.73$; GF vs VIT8x: $p = 0.058$). However, while in germ-free conditions the small number of mosquitoes completing their development was due to a similar proportion of larvae dying and being stalled at the larval stage (~30 %), the vitamin treatment significantly doubled mortality rates to ~60 % (Figure 1A, glmer with random effect on the proportion of dead larvae: $F = 22$, $p < 0.0001$; AUX vs GF, VIT4x, or VIT8x: $p < 0.0001$; GF vs VIT4x: $p = 0.010$; GF vs VIT8x: $p < 0.0001$). Interestingly, we saw a significant shift in the sex ratio of the fully developed mosquitoes, with more than 80 % of adult mosquitoes being females when larvae were treated with VIT4x and VIT8x solutions (Figure 1B, glmer with random effect: $F = 9.0$, $p < 0.0001$; AUX or GF vs VIT4x: $p = 0.0010$; AUX vs VIT8x: $p = 0.0037$; GF vs VIT8x: $p = 0.0019$). This suggested a sex-specific effect of B vitamins on mosquito development.

Transcriptomic analysis of germ-free larvae treated with B vitamins

We hypothesized that the observed impact of B vitamins on sex ratio without affecting overall development success can either be attributed to a concomitant increase and decrease in the fitness of female and male larvae respectively upon vitamin supplementation, due to differential nutritional needs and to toxic effects of these compounds at the tested concentrations, or to an influence on male trait maturation during larval development through some feminization mechanism. To unravel this question, we conducted a transcriptomic study on larvae cleared of their microbiota at the beginning of the third instar and kept in germ-free conditions (GF), supplemented with VIT1x or VIT8x solutions. If the presence of B vitamins had led to an upsurge in detoxification- or stress-related genes in males, it would lend credence to the toxicity hypothesis. Conversely, any de-regulation of genes associated with sex-determination or development would indicate the potential feminizing impact of B vitamins.

When transferring third instar larvae in a medium without mDAP and dAla to derive them germ-free, we supplemented their diet with 0x, 1x or 8x concentrations of B vitamins (Figure 2A). As the mortality was generally observed at the end of the fourth instar or during metamorphosis, we decided to sample individuals earlier, 3-6 hours after moulting to the fourth instar. We extracted RNA and DNA from individual larvae (1200 larvae in total) and sexed them by individual PCR on the male-specific *Nix* gene. The RNA of at least 48 females and males per replicate was pooled in equimolar amount. The large number of samples imposed experimental set up adjustments which resulted in more than one freeze-thaw cycle for each sample, hence the resulting RNA quality did not allow to proceed for poly-A enrichment for RNA sequencing. Instead, libraries were prepared by depleting ribosomal RNA, which allowed us to sequence non-coding RNAs together with protein-coding transcripts. After quality trimming, reads were aligned on the *Ae. aegypti* genome. An

average of 45 million reads was obtained per sample (min 22 million, max 70 million), with an average alignment rate of 80 % (min 6 %, max 90 %). Ribosomal RNA reads composed less than the 0.4 % of all reads, indicating a successful depletion of these RNA species.

As a quality control for the sex assignment of our samples, we quantified the amount of reads mapping on the *Nix* gene in each sample (AAEL0922912, Supplementary Figure 2). Read counts were always lower in females than in males, except for the third replicate of the germ-free sample. A second check on our PCR results spotted several mistakes in this replicate, thus all samples belonging to the third replicate were removed from the analysis (Figure 2A). Moreover, the sample of males treated with VIT1x collected during the first replicate showed very low alignment rates (5.6 %) and only one read aligning on the *Nix* gene, making any analyses impossible on the VIT1x condition. In summary, we performed the differential expression analysis only on germ-free and VIT8x treatments using the first and second replicates. The general low RNA quality and the suboptimal number of replicates resulted in an overall poor clustering of samples based on sex or treatment (Supplementary Figure 3A). It resulted in a low number of regulated genes, which did not allow us to perform any Gene Ontology enrichment analysis.

To verify the accuracy of sample sex assignment, we first compared the transcriptomes of male and female larvae. Principal component analysis (PCA) discriminated male and female samples in germ-free treated-larvae but not in VIT8x conditions (Supplementary Figure 3B, C). A total number of 69 and 20 genes were differentially expressed between males and females in germ-free or VIT8x conditions, respectively (Figure 2B, Supplementary Tables 2 and 3). When comparing which differentially regulated genes were commonly identified in both germ-free and VIT8x conditions, five and three genes were up- and down-regulated in males compared to females. Among these, the male determiner gene *Nix* (Hall et al. 2015) was significantly up-regulated in males in both GF and VIT8x samples, and the male-specific gene *myo-sex* (Aryan et al. 2020) was among the up-regulated genes in males kept in GF conditions, corroborating the sex-specificity of the genes identified as such in our data.

While PCA did not clearly separate samples for the vitamin treatment (Supplementary Figure 3D, E), a small number of genes was identified as differentially regulated by the vitamin treatment, with 13 and 16 genes significantly regulated in males and females, respectively (Figure 2C). The *AAEL020376* gene, paralog to the *chloride channel protein 2* gene, was commonly down-regulated with vitamins in both sexes, suggesting its involvement in a generalist vitamin-regulated mechanism (Figure 2D, E). Amongst the genes induced in males upon treatment, heat shock protein 70 encoding gene was highly up-regulated, indicating a possible activation of a stress-response in vitamin-treated male larvae (Figure 2F, *padj* < 0.001). Most of these genes were not annotated in the *Ae. aegypti* genome, but some had known orthologs. *AAEL004564* is an ortholog of *CG8420* in *Drosophila*, which encodes a male accessory gland protein, putatively component of the seminal fluid (Ram and Wolfner 2007). *AAEL013606* (Figure 2G, *padj* = 0.004) is a sex-determining region Y protein (SRY)-like ortholog, a DNA-binding transcription factor that initiates male sex-determination in most mammals. Others appear to have less clearly male-specific functions, such as *AAEL002537*, ortholog to the *Drosophila* gene *dusky*, which is involved in cell morphogenesis and imaginal disc-derived wing morphogenesis. *AAEL022708* is annotated as *Peptidyl-prolyl cis-trans isomerase*, a chaperone protein that regulates protein conformational changes by catalysing the isomerization between the *cis* and *trans* forms of peptide bonds. Its *Drosophila* ortholog (*CG11858*) is highly expressed in the larval central nervous system and is predicted to bind DNA and process rRNA.

Female larvae treated with vitamins showed a higher number of down-regulated genes, including a lipase 1 precursor encoding gene (Figure 2H, *padj* = 0.04) and two genes involved in ion transport across membranes (*AAEL010140* and *AAEL011109*). Among the up-regulated genes, three were orthologs to genes encoding for chitin binding proteins expressed during development

(*AAEL024503*, *AAEL006686* and *AAEL017212*, Figure 2I, *padj* = 0.02). Even though these results do not allow to draw strong conclusions, the up-regulation in structural genes and down regulation in lipase may reflect a positive impact on developmental processes, while the downregulation of ion transport may be a response to stress due to increasing concentrations of vitamins.

Effect of individual B vitamins on larval development and adult sex ratio

The observed mortality upon VIT8x treatment, the upregulation of a heat shock protein and downregulation of a transporter pointed towards a sex-specific threshold of toxicity of vitamins on mosquito larvae. To narrow down which B vitamin was causing this sex-specific effect, we treated decolonised *Ae. aegypti* larvae with single vitamins at high dose (50x compared to the reference concentration, (Singh and Brown 1957)). We added a 16x dose of folic acid to all tested conditions to obtain enough adult mosquitoes in the germ-free control group and have a more reliable comparisons on adult mosquito sex-ratio. We observed a significant decrease in larval development success in folic acid and biotin treatments (Figure 3A, glmer with random effect on the proportion of adults: $F = 12$, $p < 0.0001$; GF vs folic acid: $p = 0.015$; GF vs biotin: $p < 0.0001$; all other comparisons $p > 0.05$, ns). In particular, while ~90 % larvae developed in adults in the germ-free control, only ~60% and ~5 % mosquitoes reached the adult stage in the folic acid and biotin treatments, respectively. While the addition of folic acid induced a non-significant increase in the proportion of mosquitoes that were blocked in their larval development (from ~2 % in the control to ~ 20 %, Figure 3A, glmer with random effect: $F = 4.0$, $p = 0.0002$; GF vs folic acid: $p = 0.062$, ns), biotin induced a strong mortality on larvae (from ~10 % in the control to ~70 %, Figure 3A, glmer with random effect: $F = 13$, $p < 0.0001$; GF vs biotin: $p < 0.0001$). The sex-ratio of the resulting adults was not significantly affected by any vitamin supplementation, although no viable male mosquito developed from biotin-treated larvae (Figure 3B, glmer with random effect: $F = 0.63$, $p = 0.73$, ns). This low statistical effect was probably due to the small number of biotin-treated larvae that could complete their development (adult mosquitoes per replicate (sex): 1/24 (undetermined); 1/24 (female); 3/24 (females)), suggesting that the biotin 50x dose was generally toxic to both sexes. However, we could not determine if the absence of fully developed males after biotin supplementation was due to a male-killing effect or to the feminization of phenotypic characters in male mosquitoes.

Biotin requirements and toxicity are sex-specific

The experimental set up used in previous experiments was not optimal to understand whether biotin was having a male-toxic effect or if it was inducing feminization of male mosquitoes. In fact, the sole visual analysis of fully developed mosquitoes allowed us to conclude that adult mosquitoes were showing female-specific phenotypic characters but did not give us information on the genotype of those mosquitoes. We took advantage of a recently established *Ae. aegypti* genetic sexing strain (Aaeg-M) characterized by the insertion of the *eGFP* transgene in the male-specific M locus (Lutrat et al. 2023). This allowed us to sort first instar larvae by sex right after egg sterilization and perform the full experiment on male and female larvae in parallel. The effect of four increasing biotin concentrations (1x, 4x, 8x and 20x) was tested on decolonized third instar larvae. As done previously, we added 16x folic acid to all tested solutions to increase the number of adult mosquitoes.

When analysing the global results independently from the mosquito sex, we observed that the sole addition of folic acid did not significantly increase the proportion of larvae completing their development to adulthood in the Aaeg-M strain, with ~20 % of fully developed adults in both germ-free controls, with or without folic acid (Figure 3C, glmer with random effect: $F = 38.3$, $p < 0.0001$; GF vs GF+folic acid: $p = 1$, ns). The proportion of fully developed mosquitoes at 1x biotin concentration increased to ~30 %, while it decreased to ~20 % and ~10 % in 8x and 20x biotin

treatments, respectively (Figure 3C, see Supplementary Table 1 for individual comparisons). In all treatments, the variation in the proportion of adult mosquitoes was due to an equivalent change in the percentage of dead mosquitoes rather than of stunted larvae.

Data sorted by sex indicated a differential biotin requirement for male and female mosquitoes: while the biotin 1x concentration was the best one to support the development of males (~ 30 %, Figure 3D, glmer with random effect: $F = 28.3$, $p < 0.0001$, see Supplementary Table 1 for individual comparisons), the 4x concentration resulted in the highest development success in females (~ 50%, Figure 3E, glmer with random effect: $F = 16.8$, $p < 0.0001$, see Supplementary Table 1 for individual comparisons). In females, the effect of the sole addition of biotin 4X was not statistically different ($p = 0.25$, ns), but the addition of biotin 4x and folic acid was ($p = 0.0001$). The sole addition of folic acid did not significantly improve the development of germ-free larvae in these conditions ($p = 1$, ns).

When comparing male and female mosquitoes in their developmental rates to pupa (i.e., the proportion of larvae starting metamorphosis by day), we observed similar proportions of mosquitoes starting metamorphosis in all conditions, with a higher percentage of males going through pupation in germ-free, biotin 1x and 8x treatments (Supplementary Figure 4A). However, a significant proportion of these pupae were not able to complete the developmental stage and died before emergence (Supplementary Figure 4B). Interestingly, the sole addition of 16x folic acid to germ-free larvae differentially affected the pupation rates of male and female mosquitoes, suggesting that sex-dependent requirements are not exclusive for biotin (Supplementary Figure 4B). Taken together these data suggest that the clearance of the microbiota during the third instar impacts mosquito development at the metamorphosis stage, and that the addition of biotin at 1x and 4x concentrations significantly improves male and female development, respectively, while toxicity is observed at a slightly higher biotin concentration for both sexes.

For each experiment, adult mosquitoes were visually inspected to confirm that their genotype (GFP fluorescence status in L1) matched their adult phenotype. In parallel, an experiment with New Orleans mosquitoes was performed only on decolonised larvae kept germ-free or supplemented with 4x or 8x biotin. To compare mosquito phenotype and genotype, emerged mosquitoes were visually analysed to confirm their sex. Their DNA was extracted and subjected to dual PCR on *Nix* and *Actin*. PCR amplicons were analysed using a capillary electrophoresis system that allowed to automatically detect gene-specific signals. Among the analysed mosquitoes (GF: n = 70, biotin x4: n = 65, biotin x8: n = 59), none showed discordant results between genotype and phenotype, further confirming that biotin had a toxic effect but did not induce any male feminization.

Discussion

The mosquito microbiota provides essential nutrients to its host. Among these nutrients, B vitamins have been shown to be required by mosquitoes to complete their larval development (Singh and Brown 1957; Romoli et al. 2021; Wang et al. 2021). In this study, we found that the requirements of larvae in B vitamins, especially biotin, are sex-specific: males require less vitamins than females for a successful development. Combination between different requirements and low thresholds of toxicity led to a sex-ratio distortion after supplementing diet of germ-free *Ae. aegypti* larvae with increasing doses of B vitamins. Specifically, there was a notable reduction in the proportion of male mosquitoes emerging from larvae that had been subjected to vitamin supplementation.

Male and female mosquitoes have different developmental dynamics at the larval stage that suggest differential nutritional requirements. Mosquito larvae must reach a critical mass to

successfully commence metamorphosis, and this mass is higher for females than males (Chambers and Klowden n.d.). Diet composition at the larval stage strongly affects the sex ratio of the adult mosquito population, with higher proportions of males emerging in starvation or suboptimal feeding conditions (Souza et al. 2019). Larval development is generally longer for females than for males and female adult body sizes are impacted more significantly than males by the composition of the larval diets (van Schoor et al. 2020). This is probably due to the fact that females need more energy storage than males for egg production, which is reflected by significantly higher body sizes in adults.

This male-killing effect appears to be a specific outcome resulting from exposure to one or a combination of B vitamins. It is worth noting that the B vitamin doses we used in our study were four and eight times higher than what has been previously reported as sufficient to sustain mosquito larval development in germ-free conditions (Singh and Brown 1957) and between two and 160 times higher than those used more recently to formulate a chemically defined medium for germ-free *Ae. aegypti* larvae (Wang et al. 2021). Both these studies identified osmotic pressure as a critical limiting factor for artificial diets, describing a rapid larval mortality when the rearing medium contained either 11.6 g/L (Singh and Brown 1957) or 113.7 g/L (Wang et al. 2021) of amino acids. The osmotic pressure of these solutions is ~25 or ~256 times higher than that of the 8x vitamin B solution we tested. Therefore, it is unlikely that the elevated mortality observed in our experiments could be due to high solute concentration. However, the up-regulation of a chloride channel encoding gene in both male and female larvae treated with the 8x vitamin solution may reflect an increase in solute concentrations.

To investigate the potential mechanisms behind the male-specific toxic effect of B vitamins, we performed a transcriptomic study on germ-free male and female larvae exposed to either a 1x or an 8x vitamin solution. We encountered several problems in the process of sample sex assignment via PCR. Misidentifications of certain samples and RNA degradation led us to exclude one replicate and the entire vitamin 1x condition. This resulted in a relatively low number of genes that exhibited sex-specific regulation when compared to a prior transcriptomic study. Notably, Matthews *et al*'s study utilized the same sex assignment technique and reported approximately 3400 genes that were differentially regulated between male and female fourth instar larvae (Matthews et al. 2018). Between 35 % and 40 % of the genes identified in our transcriptomic study overlapped with those identified by Matthews *et al*, suggesting a degree of consistency in our data. Most significantly, *Nix* was specifically enriched in male transcriptomes. These observations suggest that our dataset may exhibit relatively lower sensitivity but a high degree of specificity, meaning that while we likely missed many regulated genes, those identified in our study are true positives.

The analysis of genes differentially regulated in vitamin 8x conditions identified different sets of genes in male and female larvae. Notably, the up-regulation of genes linked to stress response, DNA binding, development or sexual reproduction was specific to male larvae. This observation suggests that the administration of high doses of vitamins interferes with critical cellular processes, as indicated by the up-regulation of a heat-shock protein coding gene. The vitamin-induced stress response might explain the observed reduction in developmental rates among male larvae. However, it remains challenging to draw definitive conclusions regarding the precise mechanisms that underlie these effects.

When we administered individual B vitamins at elevated doses (50x), we observed that biotin, and to a lesser extent, folic acid, had lethal effects on germ-free larvae. However, only biotin was found to have an impact on sex ratio at this dose. The use of the Aaeg-M GSS strain allowed us to finely analyse the developmental success of male and female larvae independently and validate the absence of a feminizing effect caused by biotin. To maintain consistent experimental conditions compared to previous tests, we added folic acid to all tested conditions. Surprisingly, this addition

in the absence of biotin did not significantly affect the proportion of fully developed adults in germ-free conditions, in contrast to what was previously observed by us ((Romoli et al. 2021), Figure 3A). This discrepancy could potentially be attributed to differences in the mosquito strains used in the two sets of experiments (New Orleans and Aaeg-M GSS, which was created from mosquitoes sourced in Thailand). However, it is more likely that the variations in the timing of the decolonisation process between the two types of experiments were responsible for the different outcomes of folate supplementation. In fact, due to the large number of larvae and to diet autofluorescence, first instar larvae of the Aaeg-M GSS strain had to be starved for 24 h while assigning the sex via GFP fluorescence before adding bacteria and food. Hence, they were initially in a deprived metabolic status that may have carry-over effects on later development stages. We hypothesize that folic acid alone is not sufficient to complement their requirements. Furthermore, it is important to note that, in our experience, these experiments showed a noteworthy degree of variability between replicates conducted with different batches of eggs. This variability underscores the influence of various factors such as the maternal gonotrophic cycle or the age of the eggs on the outcomes of larval development. The quality of the blood on which mothers have fed and the quantity of eggs laid by each female could potentially influence the amounts of vitamins in the embryos, that might affect the larval development outcome (Goos et al. 2018; Deas, Blondel, and Extavour 2019).

The use of the Aaeg-M GSS strain allowed us to show that male and female mosquito larvae cleared of their microbiota exhibit different biotin requirements for their development (0.5 µg/mL for males and 2 µg/mL for females). Importantly, both sexes show heightened mortality rates when exposed to higher biotin concentrations. This mortality was primarily observed during metamorphosis. Although more than 50 % of the larvae successfully transitioned into pupae under all tested conditions, only a range of 10 to 40 % emerged as fully developed adults (see Supplementary Figure 4A). This observation suggests that exposure to elevated biotin levels during the third and fourth instars predominantly impacts the pupal stage of development. Biotin has been found to be essential for intestinal stem cell mitosis in *Drosophila* (Neophytou and Pitsouli 2022), and is generally involved in cell cycle progression (Velazquez-Arellano and Hernandez-Vazquez 2020), suggesting its importance during life stages with high cell proliferation rates. In particular, we observed mortality during metamorphosis, when tissue remodelling increases cell proliferation, hence the number of biotin-responsive cells may be relatively high. Elevated biotin doses may specifically de-regulate the cell cycle at this stage.

Previous studies underscored the essential role of biotin in *Ae. aegypti* larval development, but they also showed that high biotin concentrations induced mortality at the pupal stage in larvae reared in both axenic or conventional conditions (TRAGER 1948; Pillai and Madhukar 1969), confirming the toxic effects of biotin during metamorphosis. Some degree of biotin toxicity has been observed in pupae of the flour beetle *Tribolium confusum* as well (Fraenkel and Blewett 1943). Furthermore, when high biotin concentrations that still allowed *Ae. aegypti* larval development were used, they could significantly reduce adult fertility; they notably caused follicle degeneration in females after the post-ovipositional period (Pillai and Madhukar 1969). This effect of biotin on fertility seems to be conserved in other insect species such as the Mexican fruit fly *Anastrepha ludens* (Benschoter and Paniagua G. 1966), the house fly *Musca domestica* (Benschoter 1967) and the beetle *Dermestes maculatus* (Cohen and Levinson 1968). In these insects, females tended to lay fewer eggs when fed biotin-rich diets and eggs showed lower hatching rates. In *D. maculatus* the impact of biotin on embryogenesis appeared to be related to the binding of this vitamin to insoluble yolk proteins, resulting in reduced amino acid availability within the embryo (Levinson and Cohen 1973).

Mosquitoes are incapable of synthesising B vitamins, thus relying on their diet and microbiota for the essential provision of these metabolites (Douglas 2017; Romoli et al. 2021; Wang

et al. 2021; Serrato-Salas and Gendrin 2023). Notably, mosquito larvae can develop in the absence of bacteria only if a rich diet is provided and if measures are taken to protect vitamins from light-induced degradation (Correa et al. 2018; Wang et al. 2021). Several bacterial strains have been identified as capable of rescuing mosquito development when introduced to germ-free larvae, including *E. coli* (Coon et al. 2014; Romoli et al. 2021). *E. coli* possess complete metabolic pathways for B vitamin synthesis (Serrato-Salas and Gendrin 2023), implying that this capability is indispensable to support the larval development of *Ae. aegypti*. In contrast, bacterial strains such as *Microbacterium* have been shown to be unable to support larval development when individually provided to germ-free larvae, likely due to their lack of the metabolic prerequisites for providing vitamins to the mosquito host (Coon et al. 2014; "KEGG PATHWAY: Biotin Metabolism - Microbacterium Sp. SGAir0570" n.d.). However, our findings regarding biotin toxicity and previous data indicating biotin impact on insect fertility raise the possibility that bacteria might interfere with mosquito sex ratio or, more generally, with mosquito physiology, by delivering high concentrations of some B vitamins. For instance, genomic analyses of *Wolbachia* genomes have identified a riboflavin transporter gene to be associated with cytoplasmic incompatibility (Scholz et al. 2020). Taken together, these findings underscore the critical need to explore bacterial-induced mechanisms that involve B vitamins and affect the mosquito host on various facets such as larval development, toxicity, fertility, and the alteration of the sex-ratio.

Materials and Methods

Mosquitoes and bacteria

Escherichia coli HA 416 was grown in lysogeny broth (LB) 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.

Aedes aegypti mosquitoes belonged either to the New Orleans strain or to the Aaeg-M genetic sexing strain (GSS, (Lutrat et al. 2023)). Both colonies were maintained under standard insectary conditions at 28-30 °C on a 12:12 h light/dark cycle. Mosquitoes are routinely blood-fed either on beef blood provided by the local slaughterhouse (Abattoir Territorial de Remire Montjoly) or on anesthetized mice. The protocol of blood feeding on mice has been validated by the French Direction générale de la recherche et de l'innovation, ethical board # 089, under the agreement # 973021. Gnotobiotic mosquitoes were maintained in a climatic chamber at 80 % relative humidity on a 12:12 h light/dark 28 °C/25 °C cycle.

Vitamin solutions

B vitamins (Sigma-Aldrich) were dissolved in the appropriate solvent at the concentration indicated in Table 1. Stock solutions were adjusted to pH 7.4-8.0 and filtered through a 0.22 µM membrane filter and stored at -20 °C until use.

Generation of gnotobiotic larvae

Germ-free larvae were obtained as previously described (Romoli et al. 2021). Briefly, eggs were placed on top of a filtration unit and surface sterilised by subsequent washes in 70 % ethanol for 5 min, 1 % bleach for 5 min, and 70 % ethanol for 5 min. After rinsing three times with sterile water, eggs were transferred to a sterile 25-cm^2 cell-culture flask filled with ~20 mL of sterile water. Approximately 20-30 eggs were inoculated in 3 mL of liquid LB and incubated for 48 h at 30 °C shaking at 200 rpm to confirm sterility.

The following day (day two), a 16 h culture of auxotrophic *E. coli* was centrifuged, and the bacterial pellet was resuspended in 5 times the initial culture volume of sterile water supplemented with *m*-DAP (12.5 µg/mL) and D-Ala (50 µg/mL). Larvae were individually placed in 24-well plates together with 2 mL of bacterial suspension and ~50 µL of autoclaved TetraMin Baby fish food suspension. Larvae were kept in a climatic chamber at 80 % RH with 12:12 h light/dark 28 °C/25 °C cycle.

To achieve bacterial decolonisation, larvae that moulted to the third instar in a time window of 5 h during day 4 were washed in sterile water and individually transferred in a 24-well plate filled with 1.5 mL of sterile medium and 50 µL of sterile fish food in each well. Larvae obtained with this method were shown to be largely germ-free after 12 h since transfer (Romoli et al. 2021).

The Aaeg-M GSS required a sex-sorting step prior to be transiently colonised by auxotrophic *E. coli*. This strain carries an *eGFP* transgene in the male-specific M locus, thus only male mosquitoes express GFP. Axenic first instar larvae were individually placed in 96-well plates and checked for green fluorescence under an EVOS FL Auto System (Thermo Scientific). After sex-sorting, larvae were individually placed in 24-well plates. Bacteria were added the following day (day three) therefore the experiment schedule was shifted by one day.

Vitamin treatment of axenic larvae

At the time of transfer, germ-free third instar larvae were randomly assigned to the different treatments and kept individually in 24-well plates. The larval medium consisted either of sterile water alone (germ-free condition) or sterile water supplemented with a single vitamin or with a mix

of B vitamins (see Table 1 for reference B vitamin concentration). Sterile fish food was supplied to each larva. The 24-well plates were placed in a climatic chamber at 80 % RH with 12:12 h light/dark 28 °C/25 °C cycle into plastic boxes covered with aluminium foil to prevent B vitamins from light-degradation (Wang et al. 2021).

Analysis of axenic larvae developmental success in different vitamin conditions

Larvae treated with different B vitamin conditions were observed daily to track their developmental success. For each larva, the following parameters were recorded: day of moulting to the fourth instar, day of pupation, day of adult emergence, sex; in case of failed development to adult, the day of death was recorded. Larvae were monitored for 14 days after transfer in the germ-free medium.

Experimental setup and sample collection for transcriptomics on germ-free vs vitamin treated larvae

Germ-free third instar larvae were obtained as described above. To maximise the number of larvae, at day four (day five for the Aaeg-M strain) plates were inspected every 3 h and newly moulted third instar larvae were washed and transferred in new 24-well plates filled either with sterile water (germ-free condition), or with a 1x or 8x B vitamin solution (i.e., a solution of B vitamins each at the reference concentration or at 8 times the reference concentration). The following day (day five or day six for the Aaeg-M strain), wells of fourth instar larvae were marked every 3 h and the corresponding larvae were sampled 3 h later. This experimental set up was chosen to sample "synchronized" larvae that are all in the same development timing (3 to 6 h after their moulting to fourth instar) even though they have been sampled over a 12 h period. Individual larvae were placed in individual screwcap 2 mL tubes filled with glass beads and immediately stored at -80 °C. Three independent replicates were performed. For each replicate, 48 random larvae per condition were left in the plates (i.e., not sampled) and observed for 14 days to analyse their developmental success. Up to 150 larvae per condition and replicate were collected for RNA extraction and transcriptome analysis.

DNA and RNA extraction of transcriptomic samples

DNA and RNA were simultaneously extracted from individual larvae using Trizol (Thermo Fisher Scientific). Individual larvae were homogenised in 500 µL of Trizol using a bead beater (Precellys Evolution, Bertin) for 2x1 min at 9,000 rpm. After adding chloroform and centrifuging, RNA was extracted from the upper aqueous phase following manufacturer's instructions, resuspended in 20 µL of nuclease free water and stored at -80 °C. In parallel, DNA was purified from the bottom organic phase by precipitation using 0.1 M sodium citrate and following manufacturer's instructions. DNA was resuspended in 8 mM NaOH and stored at -20 °C until use.

Determination of sex of individual larvae by PCR for transcriptomics analysis

Larvae were sexed by multiplex PCR on *Nix* (present only in males) and *actin* (reaction positive control). The primers used are listed in Supplementary Table 4. Each PCR was set-up with 1 U of FirePol DNA Polymerase (Solis Biodyne), 1x Buffer BD, 0.2 mM dNTPs, 500 nM of each primer, 1.5 mM $MgCl_2$ and 2 µL of DNA. The amplification cycle included an initial denaturation at 95 °C for 5 min, followed by 35 cycles at 95 °C for 20 sec, at 59 °C for 40 sec and 72 °C for 30 sec and a final 5 min elongation step at 72 °C. PCR products were visualised using a QIAxcel DNA screening kit in a QIAxcel instrument (Qiagen).

Pooling of RNA and sequencing

After determination of larval sex by PCR on DNA, RNA was pooled for males and females independently. Pooled samples were treated for 30 min at 37 °C with 10 U of DNase I (Thermo Fisher

Scientific) to remove any DNA trace 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.

Analysis of RNA sequencing

RNA quality evaluation, library preparation and sequencing were performed by Microsynth (Balgach, Switzerland). RNA integrity was evaluated on a BioAnalyzer (Agilent Technologies) using an RNA 6000 Pico RNA chip. Paired-end libraries (2x50 bp) were constructed using the Universal RNA-Seq with NuQuant kit (Nugen Tecan Genomics) from 250 or 45 ng of total RNA. *Ae. aegypti* ribosomal RNA was depleted using a custom AnyDeplete probe mix (Nugen Tecan Genomics). After quality check, libraries were pooled in equimolar concentrations and sequenced on a NovaSeq SP 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 (Bolger, Lohse, and Usadel 2014)(minimum quality score: 15, minimal length: 40) and their quality was evaluated using FASTQC("Babraham Bioinformatics - FastQC A Quality Control Tool for High Throughput Sequence Data" n.d.). After quality trimming, an average of 45 million reads (min 22 million, max 70 million) were obtained per sample. Reads were mapped on the *Ae. aegypti* genome (assembly AeaegL5, geneset AeaegL5.3) using HISAT2 (Kim et al. 2019). The average percentage of reads aligned concordantly one time was 49% (min 3.6%, max 58%). Read counts were obtained with FeatureCounts (Liao, Smyth, and Shi 2014)and differential expression analysis was conducted in R using the DESeq2 package (Love, Huber, and Anders 2014).

Statistical analyses

Graphs were created with GraphPad Prism (version 10.0.2). Statistical analyses on developmental success and sex-ratio of vitamin-treated larvae were performed with generalised linear mixed models (GLMM) using the lme4, lmerTest and lsmeans packages in R (version 4.3.0). The replicate was set as a random effect and an ANOVA was performed on a logistic regression (glmer). For developmental rate analyses, a Log-rank (Mantel-Cox) test was performed in GraphPad Prism testing the incremental proportion of pupae or adults per day. Supplementary Table 1 details statistical information and number of individuals analysed per replicate.

Competing Interest Statement

The authors declare no competing interests.

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Author Contributions

Conceptualization, Methodology, Validation - O.R., J.S.S., and M.G. Software, Formal analysis, Data Curation - O.R. and J.S.S. Investigation - O.R., J.S.S., C.G., P.F.I., and M.G. Writing - Original Draft, Visualization – O.R. Writing - Review & Editing - O.R., J.S.S., and M.G. Supervision, Project administration, Funding acquisition – M.G.

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Tables

Vitamin	Reference concentration (Singh and Brown 1957)	Solvent	Stock concentration
Biotin (B7)	0.5 µg/mL	NaOH 0.1 M	0.4 mg/mL
Choline	25 µg/mL	Water	50 mg/mL
Folic Acid (B9)	30 µg/mL	NaOH 0.1 M	50 mg/mL
Nicotinic Acid (B3)	10 µg/mL	NaOH 0.1 M	50 mg/mL
Pyridoxine (B6)	10 µg/mL	Water	50 mg/mL
Riboflavin (B2)	20 µg/mL	NaOH 0.1 M	10 mg/mL
Thiamine (B1)	10 µg/mL	Water	50 mg/mL

Table 1. List of the B vitamins tested with their reference and stock concentrations, and the solvent used for stock solutions.

Supplementary Table 1. Statistical analyses. For each figure, the type of analysis, response variable (dependent variable), predictor (independent variable), random effect, sample size in each replicate, test results, and results of multiple comparisons (if applicable) are shown. Glmm: generalised linear mixed model. lsmeans: least square means.

Supplementary Table 2. Genes differentially regulated between male and female larvae kept in the germ-free condition. List of genes up- (red) and down-regulated (blue) in germ-free male vs female larvae, in the absence of vitamin supplementation. For each gene, Vectorbase ID, $\log_2$ fold value, *padj*, description, and transcript type are indicated. The last column indicates whether the gene was also identified in (Matthews et al. 2018) as differentially regulated in male vs female larvae.

Supplementary Table 3. Genes differentially regulated between male and female larvae treated with the 8x vitamin solution. List of genes up- (red) and down-regulated (blue) in germ-free male vs female larvae treated with the 8x B vitamin solution. For each gene, Vectorbase ID, $\log_2$ fold value, *padj*, description, and transcript type are indicated. The last column indicates whether the gene was also identified in (Matthews et al. 2018)as differentially regulated in male vs female larvae.

Supplementary Table 4. Sequences of *Nix* and *Actin* primers used in this study. For each gene, the Vectorbase accession, 5'-3' sequence, and references are indicated.

Figures

Figure 1. Development success and sex-ratio of mosquitoes exposed to B vitamins during larval development. (**A**) Proportion of individuals completing their development (dark grey), blocked at the larval stage (white), or dying during development (light grey) when initially reared with the auxotrophic *E. coli* strain (AUX), reversibly colonized at the beginning of the third instar and kept thereafter in germ-free conditions (GF) or supplemented with a 4x (VIT4x) or 8x (VIT8x) B vitamin solution. Bar charts represent the mean ± SEM of three independent replicates of 24 to 48 mosquitoes per condition. (**B**) Proportion of male adult mosquitoes emerging from the larvae treated in (**A**). See Supplementary Table 1 for detailed statistical information.

Figure 2. Transcriptomic analysis of male and female larvae exposed to B vitamins. (**A**) Experimental set-up: mosquito larvae reared in gnotobiotic conditions with the auxotrophic *E. coli* strain were decolonized at the beginning of the third instar (L3) to obtain germ-free larvae. Larvae were either kept in germ-free conditions or treated with 1x or 8x B vitamin solutions. After three to six hours since moulting to the fourth instar (L4), up to 150 larvae per condition were individually collected. Three independent replicates were performed. DNA was extracted from individual larvae for sex assignment using the *Nix* gene, while RNA was extracted for transcriptome sequencing on male and female larvae. A quality control step on the proportion of *Nix* reads and of overall alignment rates and a second check of initial *Nix* PCR results, allowed to select samples for data analysis. (**B**) Number of genes up- (red) and down-regulated (blue) in male vs female mosquitoes when larvae were kept in germ-free conditions or treated with the 8x B vitamin solution. The numbers in parenthesis represent the number of genes that are shared between our study and the Matthews *et al* transcriptome (Matthews et al. 2018). (**C**) Number of genes up- (red) and down-regulated (blue) in B vitamin vs germ-free conditions in male and female larvae. (**D, E**) List of genes up- (red) and down-regulated (blue) in male (**D**) and female (**E**) larvae treated with the B vitamin solution. For each gene, Vectorbase ID, $\log_2$ fold value, *padj*, description, and transcript type (coding gene: yellow; noncoding RNA (ncRNA): green; pseudogene: blue) are indicated. The last column shows paralog or ortholog genes with the name of the species and a brief function description. Asterisks (*) indicate description copied from FlyBase. (**F-I**) Reads Per Kilobase per Million mapped read (RPKM) values of representative genes differentially regulated with B vitamins in males (**F, G**) and females (**H, I**).

Figure 3. Development success and sex-ratio of mosquito larvae exposed to high doses of single B vitamins or to increasing doses of biotin. (**A**) Proportion of mosquitoes completing their development (dark grey), blocked at the larval stage (white), or dying (light grey) when reversibly colonized at the beginning of the third instar and kept in germ-free conditions (GF) or supplemented with a 50x solution of folic acid, choline, thiamine, nicotinic acid, biotin, riboflavin, or pyridoxine. A 16x folic acid solution was also added to all tested conditions. Bar charts represent the mean ± SEM of three independent replicates except for riboflavin (two replicates) of 24 mosquitoes per condition. (**B**) Proportion of male adult mosquitoes emerging from the larvae treated in (**A**). (**C-E**) Proportion of mosquitoes (Aaeg-M strain) completing their development (dark grey), blocked at the larval stage (white), or dying (light grey) when reversibly colonized at the beginning of the third instar and kept in germ-free conditions (GF) or provided with a 1x, 4x, 8x, or 20x biotin solution. A 16x folic acid solution was also added to all tested conditions. Bar charts represent the mean ± SEM of four independent replicates except for GF+folic acid (three replicates) of 16 to 190 mosquitoes per sex and condition. Data in (**C**) are not sorted by sex, while data in (**D**) and (**E**) represent results for male and female mosquitoes, respectively. See Supplementary Table 1 for detailed statistical information.

Supplementary Figure 1. Development success of mosquitoes exposed to B vitamins at 1x concentrations during larval development. Proportion of mosquitoes completing their development (dark grey), blocked at the larval stage (white), or dying (light grey) when reversibly colonized until the beginning of the third instar and kept in standard germ-free conditions (GF) or supplemented with a 1x (VIT1x) B vitamin solution. Bar charts represent the mean ± SEM of three independent replicates of 48 mosquitoes per condition.

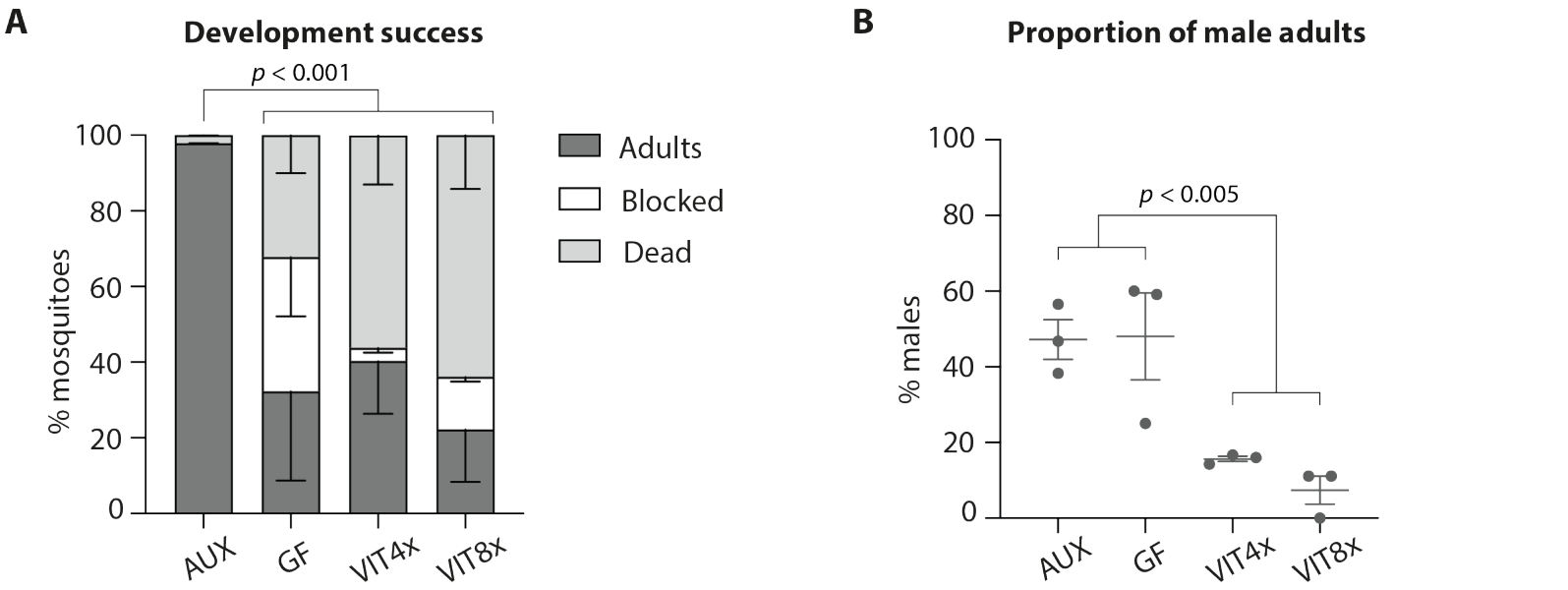
Supplementary Figure 2. Raw counts of read mapping on the *Nix* gene in the sequenced transcriptomes. Total number of reads mapping on the *Nix* gene in the transcriptomes of female

and male larvae reversibly colonized at the beginning of the third instar and kept in germ-free conditions (GF) or supplemented with a 1x (VIT1x) or 8x (VIT8x) B vitamin solution. Results from the three replicates (A, B, and C) are shown separately.

Supplementary Figure 3. Principal Component Analysis (PCA) of transcriptomes passing the quality check and used for the final analysis. (**A**) PCA of transcriptomes of female (star) and male (circle) larvae kept in germ-free conditions (white) or treated with vitamins (VIT8x, yellow). (**B, C**) PCA of transcriptomes of female (star) and male (circle) larvae kept in germ-free conditions (**B**) or treated with vitamins (**C**). (**D, E**) PCA of transcriptomes of male (**D**) and male (**E**) larvae kept in germ-free conditions (white) or treated with vitamins (yellow).

Supplementary Figure 4. Developmental rates to pupa or adult of mosquitoes exposed to increasing doses of biotin during larval development. Proportion of male (black) and female (red) larvae becoming pupae (**A**) or adult (**B**) per day when reversibly colonized at the beginning of the third instar and kept in germ-free conditions (GF) or provided with a 1x, 4x, 8x, or 20x biotin solution. A 16x folic acid solution was also added to all tested conditions. Each dot represents the mean ± SEM of four independent replicates except for GF+folic acid (three replicates) of 16 to 190 mosquitoes per sex and condition. See Supplementary Table 1 for detailed statistical information.

Romoli_Serrato-Salas_Fig1



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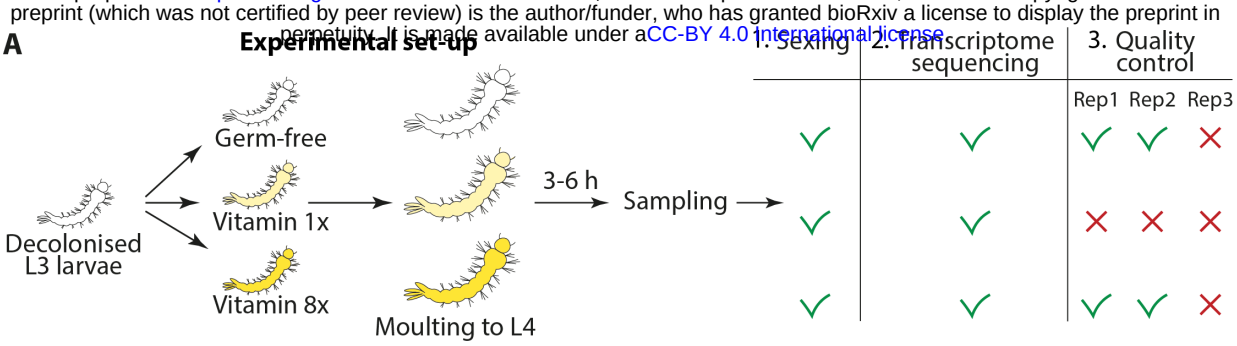

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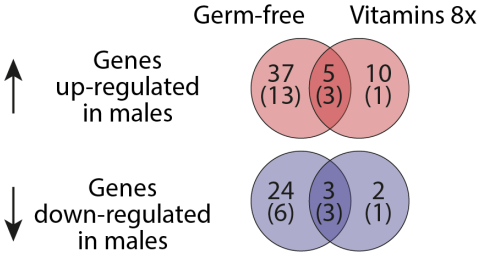
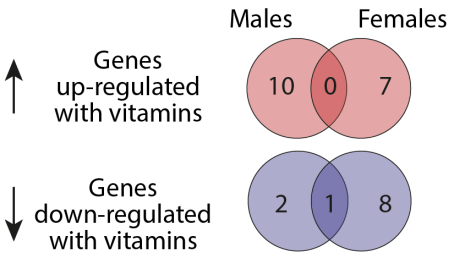


C



D

Males with vitamins 8x - Differentially regulated genes

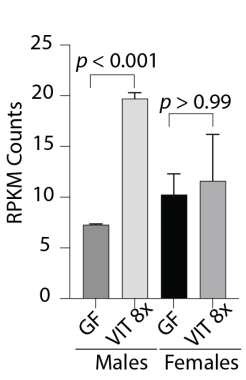
ID	log2FoldChange	padj	Transcript product	Transcript type	Paralog/Ortholog, species (description)
AAEL021701	-2.72	2.8E-03	unspecified product		cuticle protein 63-like, *Aedes albopictus*
AAEL020376	**-2.43**	**2.2E-03**	**unspecified product**		**chloride channel protein 2, *Aedes aegypti***
AAEL028740	-1.96	6.8E-08	unspecified product		
AAEL007377	1.01	1.9E-02	ammonium transporter		ammonium transporter, *Aedes aegypti*
AAEL010390	1.27	3.0E-02	glucosyl/glucuronosyl transferases		glucosyl/glucuronosyl transferase, *Aedes aegypti*
AAEL022253	1.44	1.3E-02	unspecified product		
AAEL017976	1.51	3.4E-05	heat shock protein HSP70		heat shock protein HSP70, *Aedes aegypti*
AAEL004564	2.02	5.1E-03	unspecified product		CG8420, *Drosophila melanogaster* (involved in sexual reproduction*)
AAEL013606	2.85	3.8E-03	unspecified product		sex-determining region Y (SRY) protein-like, *Aedes albopictus*
AAEL002537	2.88	9.1E-03	unspecified product		dusky, *Drosophila melanogaster* (regulates epidermal cell size/shape changes during wing morphogenesis*)
AAEL027398	3.82	5.3E-03	unspecified product		DUF4773 domain-containing protein, *Anopheles albimanus*
AAEL000484	4.71	2.8E-03	unspecified product		prespore vesicle protein-like, *Aedes albopictus*
AAEL022708	6.76	1.9E-02	Peptidyl-prolyl cis-trans isomerase		CG11858, *Drosophila melanogaster* (predicted to enable DNA binding activity, involved in rRNA processing*)

E

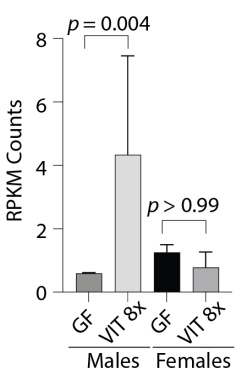
Females with vitamins 8x - Differentially regulated genes

ID	log2FoldChange	padj	Transcript product	Transcript type	Paralog/Ortholog, species (description)
AAEL012340	-8.01	3.8E-02	lipase 1 precursor		lysosomal acid lipase, putative, *Aedes aegypti*
AAEL020376	**-2.93**	**6.8E-03**	**unspecified product**		**chloride channel protein 2, *Aedes aegypti***
AAEL017678	-2.47	1.9E-02	U4 spliceosomal RNA		
AAEL010140	-1.71	3.9E-02	sodium/potassium-dependent ATPase beta-2 subunit		sodium/potassium-transporting ATPase subunit beta-1-like, *Aedes albopictus*
AAEL017646	-1.53	1.1E-03	U1 spliceosomal RNA		
AAEL017691	-1.21	3.9E-02	U1 spliceosomal RNA		
AAEL011109	-1.13	3.8E-02	unspecified product		sodium/hydrogen exchanger 9B2, *Aedes albopictus*
AAEL028652	-1.10	1.4E-02	unspecified product		
AAEL017826	-0.96	3.9E-02	U1 spliceosomal RNA		
AAEL017212	0.99	3.6E-02	unspecified product		acidic leucine-rich nuclear phosphoprotein 32 family member B-like, *Aedes albopictus*
AAEL028790	1.17	3.8E-02	unspecified product		
AAEL017067	1.34	1.6E-02	unspecified product		peritrophin-48-like, *Aedes albopictus*
AAEL006686	1.49	3.8E-02	unspecified product		CG43294, *Drosophila melanogaster* (predicted to enable chitin binding activity*)
AAEL024503	1.68	9.2E-07	unspecified product		AAEL024503, *Aedes albopictus* (contains a chitin binding Peritrophin-A domain)
AAEL028730	3.30	9.1E-24	unspecified product		
AAEL022711	6.51	1.6E-02	unspecified product		

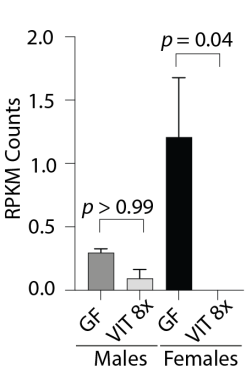
F



G



H



I