Wolbachia inhibits ovarian formation and increases blood avidity in female *Aedes aegypti* – a novel time-specific reproductive alteration

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Abstract

Wolbachia, a gram-negative endosymbiotic bacterium widespread in arthropods, is well-known for changing the reproduction of its host in ways that increase its rate of spread, but there are also costs to hosts that can reduce this. Here we investigated a novel reproductive alteration of *Wolbachia w*AlbB on its host *Aedes aegypti*, based on studies of mosquito life history traits, ovarian dissections and gene expression assays. We found that an extended period of the larval stage as well as the egg stage as previously shown can increase the proportion of *Wolbachia*-infected females that become infertile; the impact of *Wolbachia* on infertility therefore accumulated before pupation. We found that ovarian formation was blocked in infertile females, even though infected females had relatively lower *Wolbachia* densities than fertile infected females. Infertile females also showed a higher frequency of blood feeding following a prior blood meal, indicating that they do not enter a complete gonotrophic cycle. Treatments leading to infertility decreased expression of genes related to reproduction. Our results demonstrate effects related to the causes and consequences of infertile *w*AlbB-infected *Ae. aegypti* females with implications for *Wolbachia* releases and have evolutionary implications for *Wolbachia* infections in novel hosts.

Keywords: *Wolbachia*; reproductive alteration; *Aedes aegypti*; ovarian formation

Introduction

Wolbachia, a Rickettsia-like maternally inherited endosymbiotic bacterium widespread in insects [1], can alter insect reproduction in multiple ways to enhance its transmission [2]. Specific alterations include parthenogenesis – infected females can produce female offspring

without mating; feminization – infected genetic males are transformed into functional females and produce female offspring; male killing – infected immature males die while females survive, and cytoplasmic incompatibility (CI) – females infected with the predominant *Wolbachia* strain obtain a fertility advantage in reproduction because males infected with *Wolbachia* can cause female embryonic lethality when they mate with uninfected females or females infected with a different *Wolbachia* strain [3, 4]. Aside from these alterations that enhance the spread of *Wolbachia*, there can also be costs to the infection that slow its spread [5-7]. These effects of *Wolbachia* are normally based on investigations of natural *Wolbachia* strains that have existed in their hosts for many years, but are also frequently found in artificial infections [8].

In the last decade, artificially introduced *Wolbachia* have been used in reducing the transmission of arboviral diseases, with typical examples being the use of *Wolbachia w*Mel [9] and *w*AlbB [10] in *Aedes aegypti* mosquitoes. Studies on native hosts tend to indicate only a weak fitness cost of *Wolbachia* [6], but *Wolbachia* can have diverse fitness costs on its new introduced host *Ae. aegypti*, such as life-shortening, heat sensitivity, quiescent egg viability and reduced blood feeding success [11-14]. The effects of *Wolbachia* in native and introduced hosts points to the possibility of a mutualistic relationship between *Wolbachia* developing over time as a consequence of co-evolution and adaptation. In fact, it is acknowledged that sometimes *Wolbachia* can act as both parasite and mutualist [5, 15, 16]. As a result, *Wolbachia* field release and establishment in new populations can be useful for studying evolutionary changes in *Wolbachia* given that the invasion history of the population is known [17-19].

In our previous study of the effects of *Wolbachia w*Mel and *w*AlbB on *Ae. aegypti* eggs, we discovered a novel reproductive effect induced by *w*AlbB, in which infected females that hatched from quiescent eggs lost their fertility [20]. Several other reproductive fitness costs of *Wolbachia*, including those on the number and viability of eggs, can be attributed to the consequence of nutritional competition between *Wolbachia* and its host [21] and represent quantitative effects. However, in our study *Wolbachia*-infected females lost their fertility entirely, indicating a qualitative effect difficult to attribute to nutritional competition [20], so we suspect the involvement of other mechanisms.

In this paper, we investigate the causes and consequences of *Ae. aegypti* female infertility induced by *Wolbachia w*AlbB infection after egg quiescence. Firstly, we tested if the effects of *Wolbachia* on female infertility are permanent and dissected female ovaries. Secondly, we performed a larval starvation experiment combined with real-time PCR assays to understand the influence of *Wolbachia* on female development during different stages that could lead to infertility effects. Finally, we tested the blood avidity of fertile and infertile mosquitoes to see the impacts of the loss of ovaries on the rate of female blood feeding. Our study highlights the novel nature of reproductive effects altered by *Wolbachia w*AlbB in its new host *Ae. aegypti*, with implications for invasion of *Wolbachia* into uninfected populations and the evolution of mutualism more generally.

Materials and Methods

Mosquito populations

We used uninfected and *Wolbachia w*AlbB-infected *Aedes aegypti*. The uninfected population was derived from eggs collected in 2019 from regions in Cairns, Queensland, Australia where *Ae*. *aegypti* were not infected with *Wolbachia*. The *w*AlbB-infected population was generated by microinjection [22] and infected females were crossed to males from the uninfected population regularly [23, 24] to maintain a similar genetic background between populations. In following experiments, uninfected females were hatched from eggs stored for less than one month at 26 $\pm$ 1 °C as long-term storage does not influence the female fertility of uninfected populations [20]. However for consistency, uninfected females had the same rearing and feeding conditions as *w*AlbB-infected fertile and infertile females to which they were compared. *w*AlbB-infected females from non-stored eggs were hatched from freshly-laid eggs that had been dried and preserved for one week at 26 $\pm$ 1 °C. The process of mosquito rearing and “isolation and identification” is referred to Supplementary material 1.

Infertility across gonotrophic cycles

We hatched a mixture (approximately 1 : 1) of *w*AlbB-infected eggs that had been stored for two or four months, and did an “isolation and identification” for 200 females, fertile and infertile females were then provided with a second blood meal and 20 from each group were randomly selected for oviposition in 70 mL cups individually for another week to test if their fertility status was maintained.

Infertile female dissection and *Wolbachia* density

To examine ovarian development, fertile and infertile females after "isolation and identification" were dissected in saline (0.9% sodium chloride) and their ovaries were observed under a compound light microscope (Motic B1 series, Australian Instrument Services Pty. Ltd., Australia). The development of ovaries was further examined by dissecting females 4 ± 1 days post-emergence (before their first blood meal). Then we used an NIS Elements BR imaging microscope (Nikon Instruments, Japan) for photography and measurement.

We also measured *Wolbachia* density of fertile and infertile females after "isolation and identification", for 16 individuals per group. Screening based on real-time PCR assays followed methods described previously [25]. For each replicate, all samples were set up in a 384-well plate with white wells and measured by primers *aeg* and *wMwA* [26], two consistent replicates ($\Delta Ct < 1$) were obtained and their values were averaged for the density analysis.

Impact of larval stage extension on mosquito infertility

Based on results obtained from experiments described in previous sections, we suspected that mechanical changes causing female infertility occurred before metamorphosis. To further investigate whether the impact of *Wolbachia* infection on female infertility was only mediated through the egg stage or whether the prepupal stage also had an impact, we deprived 2nd instar *w*AlbB-infected larvae of food for two weeks before feeding them again until pupation then followed with an "isolation and identification". In non-starved controls, *w*AlbB-infected larvae were provided with food *ad libitum*. Each starvation treatment was performed with both stored eggs (stored at 26 ± 1□ for 10 weeks) and non-stored eggs (stored at 26 ± 1□ for 1 week) for a total of four treatments. For the group where infected females were neither stored nor starved, we had two replicates, while for the other three experimental groups we had three replicates, with each replicate containing 30 individual females. Before the blood meal, 15 individual females from each treatment 4 ± 1 days post-emergence were screened for *Wolbachia* infection and *Wolbachia* density, using screening methods for fertile and infertile females identical to those described in the previous section. After "isolation and identification", females were also dissected to confirm their fertility status by checking for the presence of ovaries. We also performed all four treatments with uninfected *Ae. aegypti* (with the same egg storage and larval starvation conditions) and

dissected 15 individuals 4 ± 1 days post-emergence from each replicate to check for existence of ovaries.

Gene expression assays

We selected three genes related to mosquito reproduction and tested their expression levels in females at different developmental stages: gene *vitellogenin receptor* (*vgr*), *ecdysone receptor* (*ecr*) and *eggshell organizing factor* (*eof*) (Supplementary material 2). Gene expression level was quantified relative to a control gene, *RPS17*, which encodes ribosomal protein S17 [27]. We tested gene expression in female pupae 1 ± 0.5 days post-pupation and in female adults 1 ± 0.5 days post-emergence in *w*AlbB-infected females that been stored as eggs for 14 weeks as well as both *w*AlbB-infected and uninfected females that had not been stored. For the *w*AlbB-infected females that had been stored as eggs, we also tested gene expression levels in fertile and infertile females after "isolation and identification". Mosquito samples were given a second blood meal and stored in RNA*later*® (Sigma Aldrich Cat No. R0901-100ML) on the third day after, followed with the reverse transcription and real-time PCA assays (Supplementary material 2)

Blood avidity of female mosquitoes

Normal fully engorged fertile females are reluctant to feed within a gonotrophic cycle. In this experiment, we test the blood avidity of uninfected, *w*AlbB-infected fertile and infertile females. After "isolation and identification", we measured blood avidity of female mosquitoes by providing fertile and infertile females a third blood meal three days after they had become engorged in the second blood feeding, when blood was almost digested. The same volunteer who provided previous blood meals placed their forearm into a BugDorm cage and left it there for 15 minutes. Fully engorged females were considered as having successfully fed after this feeding attempt. Replicates were processed through different batches of eggs and three replicates were completed with 20–30 individual females from each replicate. We also randomly weighed 20 or to the maximum of females that had fully fed and 20 that had not been provided with a blood meal from all three replicates to estimate their blood meal weight.

Statistics

We used R v. 3.6.0 with R studio v. 1.1.453 to conduct data analyses and visualizations [28]. After real-time PCR measurement, we used the $2^{-\Delta\Delta Ct}$ method to compare gene expression

levels [29], using either uninfected females from pupae or young adult stage as controls for the same stage, or fertile females as the control group of infertile females after blood feeding. The values of $2^{-\Delta\Delta Ct}$ were log-transformed for ANOVA analysis. For *Wolbachia* density we used the $2^{\Delta Ct}$ method [30] to calculate relatively density before log transformation for ANOVA analysis. We also use Tukey's honest significant difference (HSD) tests [31] for further pairwise comparisons. For the proportion data from larval starvation and blood avidity experiments we analysed data with binomial logistic regression models [32]. In the blood avidity test, student's t-tests were used to test for changes of mosquito weight after blood feeding.

Results

Consistency of infertility across gonotrophic cycles and ovarian dissection

We did an "isolation and identification" for *w*AlbB-infected females and provided a second blood meal to fertile and infertile females. We confirmed that all females we scored as fertile or infertile after the first gonotrophic cycle maintained their phenotype in the second gonotrophic cycle. We then dissected and compared the ovarian appearance of fertile and infertile females and discovered that ovaries cannot be observed in infertile females under a microscope (Figure 1). Only in very rare cases (around one in 20) can immature ovarian structure be seen, but with a narrow width similar to that of a Malpighian tube (Supplementary material 3).

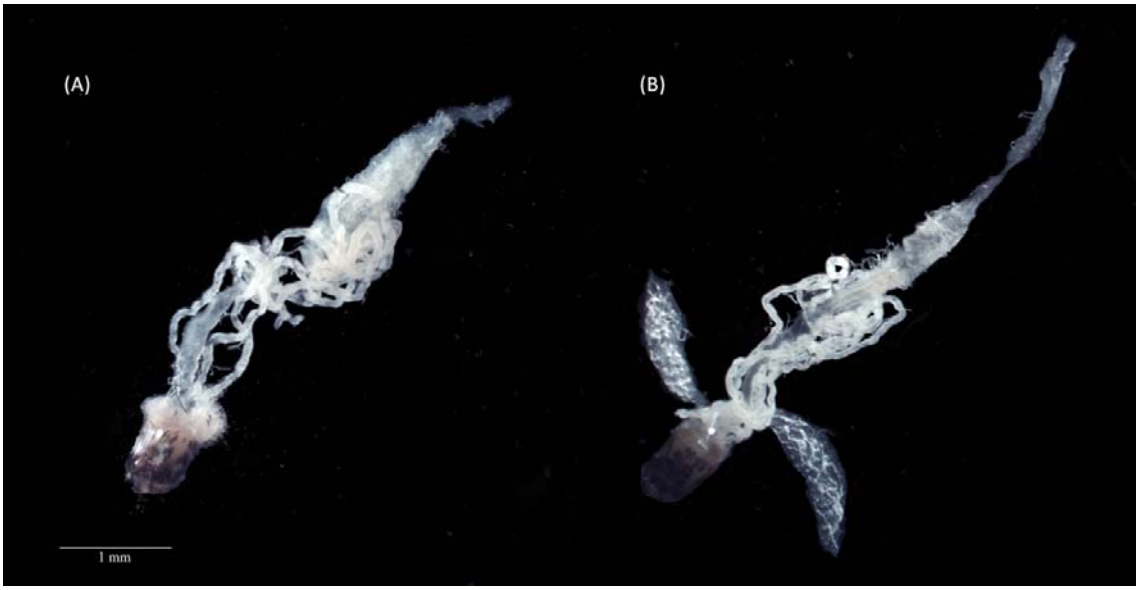

Figure 1. Appearance of internal organs in *w*AlbB-infected *Aedes aegypti* mosquitoes. (a) Infertile females lack observable ovaries, while ovaries are present in (b) fertile females. The background of the photographs was removed for clarity, with the original photographs presented in the Supplementary material 3.

After "isolation and identification", we tested 16 fertile and infertile females and found that fertile females had higher densities of *Wolbachia* (Figure 2: ANOVA: $F_{1,30} = 8.632$, $P = 0.006$).

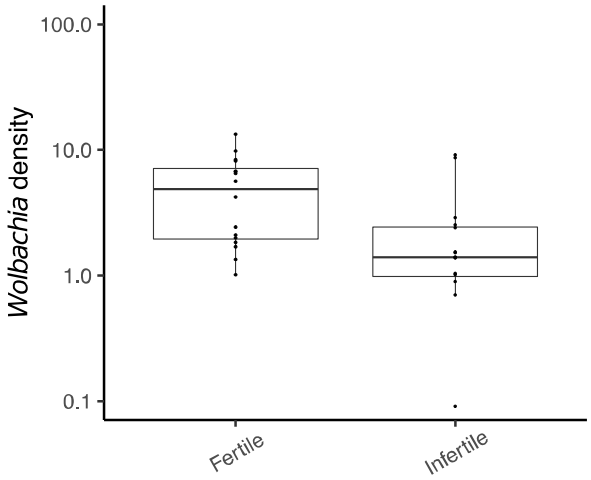

Figure 2. Box plots of relative *Wolbachia* density of fertile and infertile *w*AlbB-infected females after isolation and identification (one week after blood meal).

Impact of larval stage extension on mosquito infertility

To test if there is any impact of larval stage extension on infertility in *w*AlbB-infected females, we starved mosquito larvae to increase the duration of the larval stage from approximately one week to three weeks for mosquitoes that had or had not been stored as eggs. We found significant effects of both storage and starvation treatment, with an interaction between these terms (Figure 3A: storage: $F_{1,7} = 256.304$, $P < 0.001$; starvation: $F_{1,7} = 32.412$, $P < 0.001$; interaction: $F_{1,7} = 21.515$, $P = 0.002$). Infertility rates associated with *w*AlbB infection increased further with extension of the larval stage over and above that seen with stored eggs by 15%, and the extension of the larval stage by starvation also induced infertility of around 10% by itself. For uninfected *Ae. aegypti* treated in the same manner, all females that were dissected contained ovaries, indicating the essential role of *Wolbachia* *w*AlbB in inducing female infertility during larval starvation and egg storage.

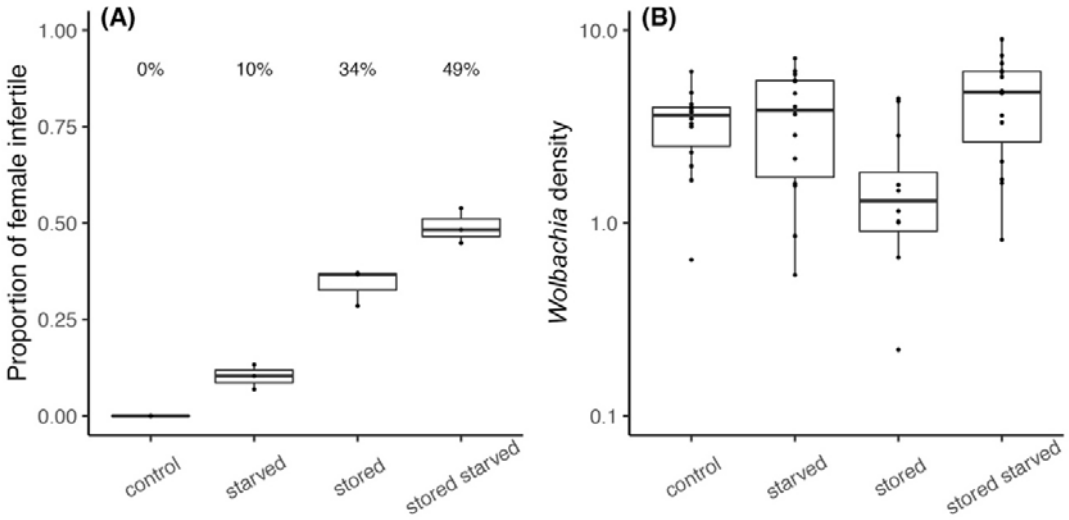

Figure 3. (A) Boxplots showing the proportion of female infertility for *w*AlbB-infected mosquitoes when stored or not stored as eggs and when larvae had been starved or not starved. Values above boxplots represent corresponding averaged percentages. (B) Boxplots of relative *Wolbachia* density in 4 ± 1 days post-emergence females.

We also screened for *Wolbachia* in all the treatments to confirm *Wolbachia* infection status. We failed to detect an infection in five out of 60 individuals and these were excluded from the density analysis (Supplementary material 4). *Wolbachia* density was significantly influenced by larval starvation but not egg quiescence, with a significant interaction (Figure 3B: ANOVA: starvation: $F_{1,51} = 7.145$, $P = 0.010$; egg quiescence: $F_{1,51} = 2.475$, $P = 0.122$, interaction: $F_{1,51} = 8.396$, $P = 0.006$). Egg quiescence decreased *Wolbachia* density, but density increased with larval starvation, potentially cancelling out this effect [33].

Gene expression assays

We selected three genes related to reproductive development and tested their expression level at different developmental stages. For comparisons before females took a blood meal (pupae and early adult stage), we considered the uninfected mosquitoes as a control. For the pupal stage, we found significant differences among the three groups of mosquitoes, but no interaction with the genes (Figure 4A, ANOVA: mosquito group: $F_{2,81} = 26.717$, $p < 0.001$; genes: $F_{2,81} = 1.044$, $p = 0.357$; interaction: $F_{4,81} = 0.423$, $p = 0.792$). Specifically, in Tukey's HSD test, *w*AlbB-infected females that been stored as eggs had lower expression levels of all of the three genes than the expression levels in uninfected females but only not gene *ecr* had lower expression level in *w*AlbB-infected females that had not been stored.

For young adults 1± 0.5 days post-emergence, significant differences of gene expression levels were found among mosquito groups, but there was no interaction with the genes (Figure 4B, mosquito group: $F_{2,81} = 15.641$, $p < 0.001$; genes: $F_{2,81} = 0.383$, $p = 0.683$; interaction: $F_{4,81} = 7.100$, $p = 0.792$). However, while differences among mosquito groups were similar across the three genes, only differences for *vgr* expression were significant between *w*AlbB-infected females that been stored and the other two mosquito groups, with some individuals from stored eggs often having very low expression levels and these females are probably infertile. The *w*AlbB infection may have increased expression of this gene, indicating that there may also have some impacts of *Wolbachia* on the reproduction of non-stored females (Figure 4B).

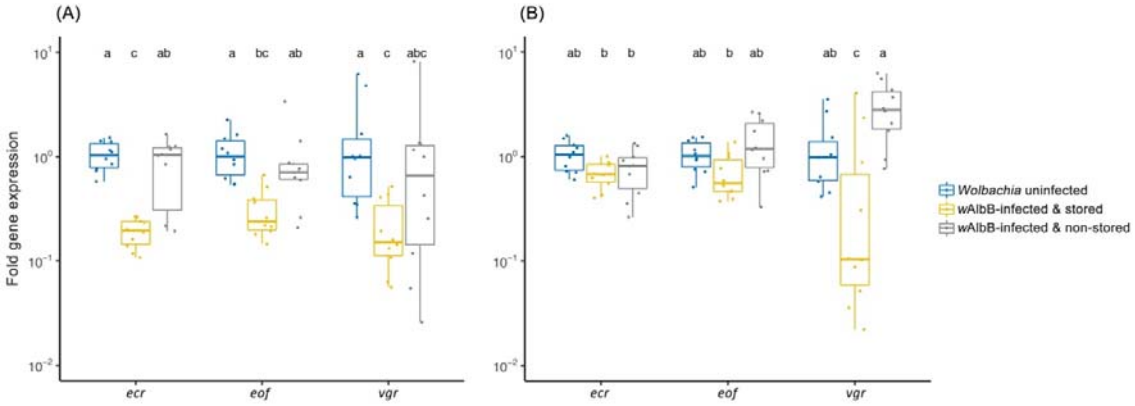

Figure 4. Boxplots of relative expression of reproduction-related genes *ecdysone receptor* (*ecr*), *eggshell organizing factor* (*eof*) and *vitellogenin receptor* (*vgr*) in female (A) pupae and (B) young adults (1 ± 0.5 days post-emergence). Values with the same letter are not significantly different based on Tukey's HSD tests.

After "isolation and identification", female mosquitoes were provided a second blood meal and were screened for gene expression levels three days later. Significant differences were found between fertile and infertile females, and also between genes selected and their interaction (Figure 5, mosquito group: $F_{1,54} = 235.696$, $p < 0.001$; genes: $F_{2,54} = 68.553$, $p < 0.001$; interaction: $F_{2,54} = 68.553$, $p < 0.001$). Specifically, all three genes showed the same trend and there were significant differences between fertile and infertile females for expression of *eof* and *vgr*, and especially for *vgr* whose expression was negligible in the infertile mosquitoes.

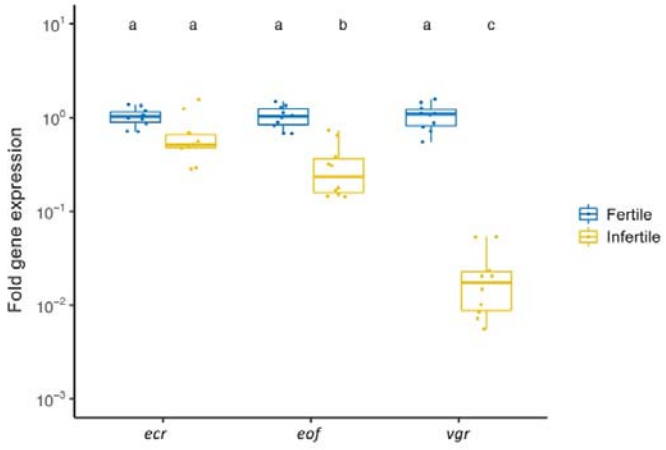

Figure 5. Boxplots of relative expression of reproduction-related genes *ecdysone receptor* (*ecr*), *eggshell organizing factor* (*eof*) and *vitellogenin receptor* (*vgr*) in fertile and infertile females one week after their second blood meal. Values with the same letter are not significantly different according to Tukey's HSD tests.

Blood avidity

After "isolation and identification", we provided female mosquitoes a second blood meal following a successively blood feeding attempt on the third day after to test their blood avidity. We found that infertile females had a higher proportion feeding compared to fertile and uninfected females (Figure 6A, $F_{2,6} = 61.037$, $p < 0.001$). We also compared the weight of fully fed and unfed females and noted significant differences between mosquito groups, feeding status and their interaction (Figure 6B, ANOVA: mosquito group: $F_{1,73} = 29.06$, $p < 0.001$; feeding: $F_{1,73} = 322.89$, $p < 0.001$; interaction: $F_{2,73} = 15.26$, $p < 0.001$). Specifically, significant weight increases were recorded for both *w*AlbB-infected fertile females ($t_{35} = 9.627$, $p < 0.001$) and *w*AlbB-infected infertile females ($t_{38} = 15.658$, $p < 0.001$), but the weight of fully fed fertile females was significantly lower compared to infertile females ($t_{35} = -4.645$, $p < 0.001$), while there was no significant difference between unfed fertile and infertile females ($t_{38} = -1.096$, $p = 0.280$), suggesting that infertile females took in a larger amount of blood during successive feeding.

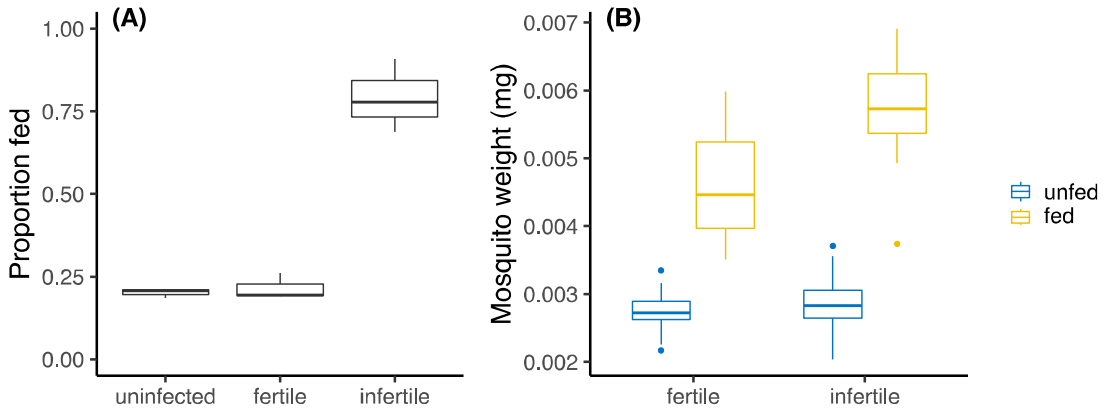

Figure 6. Boxplots of (A) female proportion that blood fed three days after they had been fully fed; (B) the weights of fed and unfed females.

Discussion

Wolbachia is a well-known bacterium that can alter the reproduction of its host to benefit its spread. In our previous study, *Wolbachia w*AlbB was found to cause *Aedes aegypti* females to become infertile following egg storage [20], inhibiting their ability to transmit vertically. In this paper, we investigated this phenomenon and obtained the following results: 1) female infertility is permanent; the development of ovaries has been interrupted by *Wolbachia*, no mature ovaries can be observed and low expression levels of reproductive genes in infertile females; 2) the effect accumulates across pre-pupal stages, in that the frequency of female infertility depends on the duration before metamorphosis (including egg and larval stages); and 3) infertile females maintain other female characteristics such as mating and blood feeding, but they do not enter a gonotrophic cycle and blood feeding occurs more frequently. We therefore confirmed that *Wolbachia w*AlbB altered the reproductive process in its new host *Ae. aegypti*.

The effect of *Wolbachia* on female infertility is different to other fitness impacts of *Wolbachia* and the four typical reproductive alterations (parthenogenesis, feminization, male killing and cytoplasmic incompatibility) in which *Wolbachia* alters host reproduction in ways that increase its vertical transmission success [4, 34]. Feminization found in lepidopteran insects that have female-heterogametic chromosomes [35, 36] can also act on the development of reproductive organs [37]. Unlike Lepidoptera, *Ae. aegypti* mosquitoes have a heterogametic sex determining region [38], which results in maleness being expressed in the

heterozygous form [39]. However, feminization converts genetic males into functional females, while the infertility effect converts functional females into non-functional females without male characteristics, suggesting different *Wolbachia*-related mechanisms [40, 41].

The development of mosquito ovaries can be divided into multiple stages [42], including previtellogenic stages when follicles remain dormant before a blood meal, and vitellogenic stages, when ovarian development can be observed within 30 minutes after females engorge [43]. The feeding process stimulates the expression of a steroid hormone, 20-hydroxyecdysone (20E), which plays a critical role in reproductive regulation by interacting with its intracellular receptor, and consists of two protein subunits: ecdysteroid receptor (EcR) and ultraspirale [44, 45]. EcR can bind to specific DNA sequences and regulates the expression of related genes such as vitellogenin (*Vg*) in the mosquito fat body, which encodes a yolk protein precursor (YPP) [46-49]. The YPP is then secreted to the haemolymph and internalized through related receptor VgR in developing oocytes [50, 51]. At a late stage of egg production, the gene *eof* is expressed, and encodes an essential protein for eggshell formation [52]. In this study, we tested the expression level of *vgr*, *ecr* and *eof* at the female pupal, young adult before blood feed and old adult after blood feed stage to compare expression between stored and non-stored/fertile and infertile females. Our data show that differences in gene expression were substantial at each stage. For pupae, large variations of expression levels were found among individuals, especially for gene *vgr*, probably indicates its expression focus on pupae of a certain age, and *w*AlbB-infected *Ae. aegypti* females that had been stored long-term as eggs had lower expression levels of the three reproductive genes compared to uninfected females, and the expression level of gene *vgr* was significantly lower than both uninfected and *w*AlbB-infected normal females at young adult stage, suggesting the formation and development of oocytes was impacted, which is also supported by the very low relative expression of *vgr* in infertile females when compared with fertile females at the third day after their blood feed, and the inability to observe mature ovaries in infertile females. What is more, we also found substantial lower expression level of genes *ecr* and *eof* in *w*AlbB-infected female pupae hatched from long-stored eggs, while this difference was not found in young adults. As *ecr* is also expressed in other organs as it encodes the receptor of a hormone [53], the down-regulated of *ecr* may indicate that egg quiescence have substantial impact on the pupation process of *w*AlbB-infected females. However, interactions between *Wolbachia* and specific reproductive signals and mechanisms are poorly understood and require further investigation such as transcriptome and pathway analysis [54, 55].

The novel reproductive alteration induced by *Wolbachia w*AlbB could slow the spread of the infection in mosquito populations. This may occur not only when the egg desiccation period is extended during dry and warm seasons [56, 57], but also when the larval period is extended due to poor provision of food [58, 59], for females are less likely to produce offspring. These effects can reduce the efficiency of *Wolbachia* invasion which aims to inhibit the transmission of arboviruses [8, 24, 60]. While *w*AlbB has established successfully in Malaysia which is warm and humid year-round precluding extended egg desiccation periods and promoting fast development of larvae [10, 61], our discovery provides guidance for future releases in other climates and highlights the importance of future monitoring in release areas [20].

Our observation that infertile females show increased blood avidity highlights a potential risk of increased nuisance biting following a *Wolbachia* release program, which may lead to community discontentment. Normal *Ae. aegypti* females ingest human blood to obtain unique nutrients for egg development [62, 63]. However, while infertile females lack ovaries, they still behave like normal females in terms of blood feeding. Previous research found that multiple feeding within a gonotrophic cycle is caused by nutritional reserve depletion or feeding interruption [64]. We tested blood feeding behaviour after the first gonotrophic cycle of “isolation and identification”, which can also reduce the influence of nutrition reserves as multiple blood feeding may rescue the effect of nutritional deprivation [65, 66]. We selected a three-day interval which is the period mosquitoes need for blood digestion to test the frequency of refeeding, and found that a large proportion of infertile females will refeed, indicating they do not follow a gonotrophic cycle. This behavioural change of *Wolbachia*-infected infertile females has significant implications for vector control population release strategies [8, 67]; in particular it would be interesting to test whether the ability of these infertile females to carry and transmit arbovirus has changed as the density of their *Wolbachia* infection may be lower than that of fertile females [68, 69]. Similar concerns have been raised about increased biting frequencies of irradiated female mosquitoes in sterile insect technique programs [70], however the prevalence of infertile females is likely to be much higher in *Wolbachia* mosquito populations compared to irradiated females that are only released accidentally.

The reproductive alterations of *Wolbachia* discovered in this study have significant evolutionary implications. So far our work relates to *Ae. aegypti*, where the *Wolbachia* *w*AlbB was artificially introduced recently from a close relative of *Ae. aegypti*, *Aedes albopictus*, in order to reduce the transmission of arboviral diseases [71]. A *Wolbachia* strain may induce similar fitness costs in a new host when compared to its native host, such in the case of life-shortening of *w*MelPop which is expressed in both *Drosophila melanogaster* and *Ae. aegypti* [11], and remains stably expressed following long-term laboratory culture [72]. We do not yet know if there are similar reproductive effects of *Wolbachia* *w*AlbB expressed in its native host, *Ae. albopictus*, while previous study indicating that low temperature adaptation [33, 73], a different effect of *w*AlbB, might be associated. On the other hand, we have also discovered a smaller loss of female infertility in *w*Mel infected *Ae. aegypti* [20], though the native host of *w*Mel, *D. melanogaster*, does not enter egg quiescence, reduced fecundity of *w*Mel-infected females was discovered under dormancy conditions [74]. Although other *Wolbachia* strains remain to be investigated, it is also possible that *Ae. aegypti* has mechanisms underlying ovarian formation that can be interrupted by endosymbionts. However, it is unclear why the frequency of infertile females increases when the density of *Wolbachia* decreases as the result of increased periods of egg storage or larval development time, and why infertile females have lower *Wolbachia* densities than fertile females in the same treatment. There may be cumulative effects of *Wolbachia* not evident from density measures at early stages of development contributing to failure of ovarian formation. One possible hypothesis is that *Wolbachia* competes for some essential but sparse nutrients with its host *Ae. aegypti* at an early life stage, which requires further investigation.

In conclusion, we further investigate a novel reproductive alteration of *Wolbachia* that we discovered previously [20]. We confirmed that *Ae. aegypti* females infected with *Wolbachia* *w*AlbB can become infertile when they are unable to form functional ovaries during metamorphosis, but these females retain other female characteristics, leading to an increased biting frequency. Our study provides significant guidance for future *Wolbachia* releases and has important evolutionary implications for understanding the reproductive alteration of *Wolbachia*, especially in novel hosts.

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