Quality control of long-term mass-reared *Aedes albopictus* for population suppression

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Abstract

The endosymbiotic bacterium *Wolbachia* is being developed as a tool to suppress mosquito populations and their transmitted pathogens, with successful field trials in multiple countries having resulted in efforts to scale up the capacity to mass-produce mosquitoes for release. However, major challenges exist to achieving this goal, including concerns that mass-reared mosquitoes will adapt to laboratory conditions during long-term maintenance and experience inbreeding depression, resulting in poor performance of the released mosquitoes in the field. Here, we assessed the performance of the *Aedes albopictus* HC line infected with a triple-strain *Wolbachia* after mass-rearing at scaled-up densities of up to 15 million mosquitoes per week for over 50 generations. In comparison with the wild-type GUA line, the HC mosquitoes had desirable characteristics for mass-rearing and release, including robust male mating competitiveness, high female reproductive capacity, reduced vector competence for dengue virus, and increased *Wolbachia* density. Although the larval survival rate of the HC and GUA lines was similar, the HC larvae developed significantly faster, possibly because of up-regulation of the molting hormone 20-hydroxyecdysone-related gene *E75* in the HC larvae. Our results indicate that over many generations mass-reared mosquito lines can retain their quality if large effective population sizes with sufficient genetic heterogeneity are maintained under optimized rearing conditions and demonstrate the long-term feasibility of deploying *Wolbachia*-based approaches for area-wide management of mosquito vectors for disease control.

Keywords *Wolbachia* · Mosquito · Mass-rearing · Dengue · Vector control

Key message

- Successful field trials have been accomplished to develop *Wolbachia* for mosquito suppression.

- *Aedes albopictus* HC line carrying a novel *Wolbachia* has been mass-reared for > 50 generations.
- Long-term mass-reared HC males maintain strong mating competitiveness and flight ability.
- Long-term mass-reared HC females maintain resistance to dengue virus, with life-span reduced.
- Long-term mass-reared HC larvae develop faster than their wild-type counterparts.

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Introduction

Pathogens transmitted by mosquitoes, including malaria parasites, and dengue and Zika viruses, cause severe mortality and morbidity globally, but traditional vector control approaches are insufficient to protect humans from infection. Successful implementation of the sterile insect technique (SIT) for the control of several insect pests of agricultural and veterinary importance (Dyck et al. 2005) has encouraged significant efforts to develop analogous techniques for

mosquito control (Harris et al. 2011; Mains et al. 2016). One of them is the incompatible insect technique (IIT), in which males infected with the maternally inherited endosymbiotic bacteria *Wolbachia* are released to induce incompatible matings with target females who are either uninfected or infected with different *Wolbachia* strains (Xi and Joshi 2016). IIT field trials have resulted in both historical success in eradicating *Culex quinquefasciatus* (Laven 1967) and recent success in effectively suppressing *Aedes aegypti* and *Aedes albopictus* (Baton et al. 2021; Crawford et al. 2020; Zheng et al. 2019).

Area-wide application of either IIT or SIT for mosquito vector control requires the capacity to scale up the production of incompatible/sterile males with robust mating competitiveness relative to the wild-type males. Despite significant efforts, mosquito mass-production has not yet attained the industrial scale reached by the operational SIT programs for screwworm and medfly control, two successful examples of the application of this technology (Dyck et al. 2005; Enkerlin et al. 2017). In contrast to the breeding and release of insects for screwworm and medfly control programs, there is a potential risk of arbovirus transmission among those involved in—or associated with—the mass-rearing or mass-release of mosquitoes, either through escape of adult females within the rearing facility itself, or immediately following male release, if any contaminant females remain as a consequence of imperfect sex separation. It is neither ethical nor responsible to mass-produce and release mosquitoes with vector competence—the ability of a mosquito to acquire and ultimately transmit a pathogen—higher than wild-type mosquitoes. IIT has provided a solution to minimize this risk due to the ability of some *Wolbachia* strains to confer mosquito resistance to a broad spectrum of human pathogens (Bian et al. 2013, 2010; Kambris et al. 2009; Moreira et al. 2009).

We have recently been able to mass-rear more than 15 million mosquitoes per week enabling the weekly production and mass-release of ~5 million males of the transinfected *Ae. albopictus* HC line which has an artificial triple-strain *Wolbachia* infection (Zheng et al. 2019). As an essential component of any vector control program, quality control of the mass-reared HC mosquitoes has been extensively performed to ensure the robust fitness of the released mosquitoes relative to wild-type mosquitoes at the beginning of mass-rearing for the field trial (Zhang et al. 2015, 2016; Zheng et al. 2019). However, more than 50 generations have passed during the 4-year period since the HC line was first developed for mass-rearing, raising concerns that this mosquito line could have adapted to the artificial laboratory conditions, and/or have experienced rapid genetic changes and inbreeding depression, and, therefore, reduced performance in the field. Previous studies have found that laboratory-adapted *Aedes* mosquitoes show faster development and changes in body size (Ross et al. 2019; Yeap et al. 2013),

increased sensitive to stress (Hoffmann and Ross 2018; Jong et al. 2017), and reduced male fecundity relative to their field counterparts (Ponlawat and Harrington 2007; Richardson and Williams 2013). Under laboratory settings, inbreeding was previously shown to be extremely costly to *Ae. aegypti*, resulting in reduced survivorship, increased development time, and lowered energy reserves for flight, dispersal, and basic metabolic functions (Koenraadt et al. 2010; Ross et al. 2019). Inbreeding depression could occur through mating between closely related individuals, resulting in an increase in homozygosity and the expression of deleterious traits (Reed and Frankham 2003; Wright et al. 2008). Loss of fitness in *Ae. aegypti* due to inbreeding correlated strongly with decreased effective population size, but, if population sizes were sufficiently high, colonized mosquitoes were able to maintain their fitness (Ross et al. 2019). Furthermore, interactions between *Wolbachia* and mosquito hosts might evolve under mass-rearing, resulting in reduction in both *Wolbachia* density and its mediated viral blocking effect, as evidenced by the previous observations that *Wolbachia* density was subject to environmental conditions of the mosquito larvae (Ross et al. 2017; Wiwatanaratnabutr and Kitayapong 2009). Thus, the early detection of reductions in fitness and other quality parameters of mass-reared mosquitoes resulting from laboratory adaptation and/or inbreeding depression are essential for the success of vector control programs involving mass-release of artificially bred insects.

In the present study, we evaluated key biological traits of HC mosquitoes, and compared them to the wild-type GUA strain, in order to examine the possibility that the HC mosquitoes are experiencing fitness loss as a result of long-term mass-rearing. Except for a minor reduction in female longevity, we found that HC mosquitoes otherwise retain key biological traits upon prolonged mass-rearing, including male mating competitiveness, *Wolbachia*-mediated virus blocking, and other life-history traits. These findings support the long-term feasibility of deploying IIT for area-wide management of mosquito vectors for disease control.

Materials and methods

Mosquito colonies

The *Ae. albopictus* HC line, infected with *Wolbachia* strains wAlbA, wAbB, and wPip, was produced at the mass-rearing factory run by Guangzhou Wolbaki Biotech Co., Ltd. (Wolbaki), according to a standard mass-rearing protocol (Zhang et al. 2017, 2018; Zheng et al. 2019), and is referred to as the “mHC” (i.e., mass-reared HC) line in this study. mHC had been mass-reared for over 50 generations at the time of the experiments reported here. The wild-type

mosquito line GUA was recently established by collecting larvae from eight different field sites in Guangzhou (Supplementary Table 1). Approximately 200 larvae were collected from each location, then pooled together, and reared to adults (G_0) under standard low-density laboratory conditions to establish the colony, which was used for experiments within five generations. Both mHC and GUA were reared under standard conditions during the experiment for measurement of biological traits. Larvae and adults were maintained in a standard mosquito rearing room at $27 \pm 2^\circ\text{C}$ and $75 \pm 5\%$ relative humidity with a 12:12-h light:dark photoperiod. Adults were provided with fresh 10% sucrose solution, blood-fed on mice, and allowed to lay eggs, according to a protocol approved by the Ethics Committee on Laboratory Animal Care of the Zhongshan School of Medicine (approval number 2017-041).

Male mating competitiveness

Three replicate cages (30 cm $\times$ 30 cm $\times$ 30 cm) were each filled with 20 GUA and 20 mHC virgin adult males 1–2 days post-eclosion. After 24 h, 20 adult virgin GUA females of the same age as the males were also introduced into each replicate cage. Cages for an additional two groups, a fertile control ($\text{GUA}_{\text{♀}} \times \text{GUA}_{\text{♂}}$) and a sterile control ($\text{GUA}_{\text{♀}} \times \text{mHC}_{\text{♂}}$), were set up in parallel. Mosquitoes were kept in the cages for 3 days and then blood-fed on mice once. Three days later, oviposition cups were provided to the females for 2 days. After the eggs were collected and allowed to mature for an additional 5 days, they were hatched to determine the egg hatch rate, which was used to calculate Fried's Competitiveness Index (Fried 1971). Briefly, after the mean egg hatch rates of fertile control cages (H_n), sterile control cages (H_s), and treatment cages (H_o) were measured, the male mating competitiveness index (CI) was calculated using the equation: $\text{CI} = [(H_n - H_o)/(H_o - H_s)] * (N/S)$, where N and S were the numbers of GUA and mHC males.

Flight ability test

Mosquito flight ability was measured using a modified flight test device (kindly provided by Jérémy Bouyer) (Fig. S1), according to a previously reported approach (Culbert et al. 2018) with minor modifications. In brief, GUA and mHC mosquitoes were reared to the pupal stage, and male pupae were collected and reared to the adult stage, with 10% sucrose solution provided once the adults had emerged. Approximately 100 males (2–3 days old) were collected using a mouth aspirator and transferred to the flight test tube. The fan above the tube was then turned on and allowed to run for 2 h. The device was then placed in a -20°C freezer to kill all the tested mosquitoes. The number of mosquitoes outside the flight tube (the escaped group) and the number

inside the tube (the non-escaped group) were recorded, and "the escape rate" was calculated as the number of escaped mosquitoes divided by the total number of tested mosquitoes (i.e., the proportion of all mosquitoes that escaped).

Mosquito vector competence for dengue virus

Mosquitoes were infected with dengue virus serotype 2 (DENV-2, New Guinea C strain) through blood-feeding as previously described (Bian et al. 2010). Fresh supernatant harvested from DENV-2-infected C6/36 cells was mixed 1:1 with human blood (final virus titer, 5×10^5 PFU/ml), and then, the mixture was injected into feeders (Wolbaki) covered with pig intestine as a membrane. Feeders were connected to a water bath circulating system (Fisher) to keep the blood at 37°C . Mosquitoes were allowed to feed on the mixture for 30–45 min. Only engorged mosquitoes were collected and maintained under standard rearing conditions. At 14 days post-blood-feeding, saliva samples were individually collected from twenty mosquitoes using the forced salivation technique, with minor modifications (Anderson et al. 2010): Mosquitoes were anesthetized with CO_2 , and their legs and wings were removed, and then the mosquito proboscis was inserted into a 10- μl pipette tip containing 6 μl of FBS and kept for 30 min at room temperature. The number of copies of DENV-2 in the saliva was quantified by RT-qPCR to reflect DENV-2 transmission potential of the mosquitoes.

DNA extraction and quantification of *Wolbachia* density

In order to minimize the influence of larval crowding on *Wolbachia* density, approximately 300 first-instar larvae were reared in a plastic tray (36 cm $\times$ 25 cm $\times$ 5 cm) with 1.5 L dH₂O. Genomic DNA was extracted from 3 to 4 adult females (5 to 6 days post-eclosion) pooled together and homogenized in 500 μl STE buffer with 20 μl proteinase K (20 mg/mL) and 70 μl SDS (10%) and then incubated at 55°C for 1 h. DNA was then purified using phenol/chloroform extraction. *wAlbA*-, *wAlbB*-, and *wPip*-specific primers were used in real-time PCR to individually quantify each *Wolbachia* strain, as normalized by the reference gene *ribosomal protein S6* (*rps6*). The total *Wolbachia* density was calculated as the sum of the individual densities of each of the three *Wolbachia* strains. Real-time PCR was performed using ChamQ SYBR qPCR Master Mix (Vazyme) on a Roche 96 machine with the following conditions: 95°C for 30 s, then 40 cycles of 90°C for 5 s, and 60°C for 30 s, followed by melting curve analysis. The specific PCR products generated by each set of primers were cloned into the pMD-18T vector (Takara) to generate standard curves. Primers for *rps6* (Zheng et al.

2019) and *wsp* of *wPip* (Liang et al. 2020), *wAlbA* (Tortosa et al. 2008), and *wAlbB* (Zheng et al. 2019) have been previously reported.

Larval development and determination of sex ratio

Eggs were immersed for ~2 h into hatching solution: 1% (w/v) yeast powder in dH₂O. The resulting newly hatched first instar larvae were transferred to the larval rearing trays (17 cm × 11.8 cm × 5.5 cm) at a density of 100 larvae/300 mL water/tray. Liver powder was weighed and suspended in 5–10 ml water and then provided to the 1st and 2nd instar larvae at 0.5 mg/larvae/day and 1.0 mg/larvae/day for the 3rd and 4th instar larvae. From day 5, pupae were collected daily and sex-sorted until all larvae developed into pupae. Male and female pupae were distinguished by microscope examination of their terminalia (Benedict 2014), with the number of each sex recorded to measure the sex ratio. The larval survival rate was calculated as the number of pupae that developed divided by the total number of 1st instar larvae and then multiplied by 100 so that it was expressed as a percentage.

Assays for fecundity, egg hatch rate, and wing size

After GUA and mHC pupae enclosed to adults, males were allowed to copulate with females for 5–7 days. Then, female mosquitoes were blood-fed with mice, and 62 engorged mosquitoes for each line were individually reared in a 50-ml plastic tube supplied with sugar and wet filter paper for laying eggs. Six days later, the filter papers were removed from the tubes and kept wet for 3 more days to allow the eggs to mature. Fecundity was measured by counting the number of eggs from each mosquito. Thirty randomly selected egg papers were individually immersed in hatching solution for 24 h, and then egg hatch rate per individual female was calculated as the number of decapped eggs divided by the total number of eggs.

Wing length was used to estimate adult body size according to the approach reported previously, with a slight modification (Styer et al. 2007). In brief, wings were dissected from 5- to 7-day-old females and males (15 individuals of each sex), laid on glass slides, and then photographed together with a scale plate using a Cannon EOS 800D camera. The length (mm) from the humeral cross vein to the wing tip was measured with Adobe Photoshop software, version CS6.

Measurement of the relative expression of the 20-hydroxyecdysone (20E) primary response gene *E75* in mosquito larvae

One hour after eggs were immersed in hatching solution, 500 each of newly hatched mHC and GUA larvae were transferred to rearing trays (36 cm × 25 cm × 5 cm, filled with about 2 L of water), with food provided as described above. At 48, 72, 96, and 128 h after egg hatching, five samples, each containing 10 to 20 larvae, were collected to measure the relative expression of *E75* in the two lines using the $2^{-\Delta\Delta C_t}$ method (Schmittgen and Livak 2008).

RNA extraction, reverse transcription, and qRT-PCR

Total RNA from either mosquito saliva or larvae was extracted using RNAiso (Takara) according to the manufacturer's protocol. Extracted RNA was dissolved in RNase-free water, treated with gDNA wiper (Vazyme) at 42 °C for 2 min, and then immediately reverse-transcribed using the HiScript Q RT SuperMix for qPCR (Vazyme). The resulting cDNA was stored at -20 °C until used. Real-time PCR was performed using ChamQ SYBR qPCR Master Mix (Vazyme) on a Roche LightCycler 96 machine with the following conditions: 95 °C for 30 s, then 40 cycles of 90 °C for 5 s and 60 °C for 30 s, followed by melting curve analysis. In order to calculate the number of DENV-2 virus copies, the DENV-2 *NS5* gene was cloned into the pMD-18T vector (Takara) as described previously (Zhang et al. 2013). A serial dilution of the recombinant plasmid was used to generate a standard curve. DENV-2 *NS5* primers were newly designed: forward 5'-AACCATGGAAGCTGTACGCA-3', reverse 5'-AGCTTCATCTCACCTTGCGC-3'. The mosquito *E75* expression level was normalized with *rps7*. Primers for *rps7* have been previously reported (Zhang et al. 2013), and those for *E75* were newly designed: forward 5'-CGCCTCCACTGTACGATACC-3', reverse 5'-CCGCTCCCATACTGCTACTG-3'.

Adult longevity assays

Thirty newly emerged virgin male or female adults were transferred to small cages (20 cm × 20 cm × 20 cm) and kept under standard rearing conditions as described above. This setup was replicated three times for each sex of each mosquito line. Fresh 10% sucrose solution was provided to the mosquitoes. Every 2 days, the mosquitoes were checked, and dead mosquitoes were counted and removed until all mosquitoes had died.

Statistical analysis

All data were statistically analyzed by SPSS 13.0 and Graph-Pad Prism 6.0 software. The normality of the datasets was checked using D'Agostino and Pearson omnibus normality test, and then, Student's *t* test was used for data with normal distributions and Mann–Whitney *U* test for data with non-normal distributions. Larval development time was compared between mHC and GUA using a two-tailed Student's *t* test. The relative expression level of *E75*, male mating competitiveness, larval survival rate, sex ratio, adult size, fecundity, fertility, escape rate, and the number of DENV-2 *NS5* copies in saliva samples were compared between mHC and GUA using two-tailed Mann–Whitney *U* tests. The log-rank (Mantel–Cox) test was used to compare longevity within the two sexes between the two mosquito lines. DENV-2 prevalence in mosquito saliva samples was compared between mHC and GUA using Fisher's exact test.

Results

Impact of long-term mass-rearing on the quality of adult HC males, in terms of mating competitiveness and flight ability

In order to test whether long-term mass-rearing reduces the ability of HC males to mate with wild-type females, we measured the mating competitiveness of mass-reared HC males (mHC) using the GUA line recently established from the field. The results showed that the average mating competitiveness index of mHC males was 1.07 ± 0.20 , and not significantly different from 1, indicating that mHC males and GUA males showed equal mating competitiveness under laboratory conditions (Table 1).

We also compared the flight ability of mHC and GUA males by measuring, as an indirect indicator of male competitiveness, the capacity of mosquitoes to escape flight tubes (Culbert et al. 2018). There was no significant difference in the escape rates between mHC (0.86 ± 0.03) and GUA males

Fig. 1 Flight ability of mHC and GUA males. Flight ability was measured using a flight test device (as shown in Supplementary Fig. 1) and calculated as the “escape rate” (i.e., the proportion of all males that escaped their flight test tube). Each circle represents the value for a single replicate, and the bars shown are the means of the three replicates $\pm$ SEM for each mosquito line. Mann–Whitney *U* test, $P=0.827$

(0.86 ± 0.04) (Fig. 1), further supporting the inference that the mating competitiveness of HC males is not negatively affected by long-term mass-rearing.

Impact of long-term mass-rearing on the quality of adult HC females, in terms of reproduction and vector competence

Female reproductive quality, a key factor that determines the cost of mass production, can be reduced during long-term mass-rearing under factory conditions. Thus, we compared both the fecundity (i.e., the number of eggs produced by each female) and fertility (i.e., the proportion of eggs hatching) of mHC and GUA females. Again, we saw no significant difference in either fecundity (mHC 77.95 ± 3.6 versus GUA 74.6 ± 2.6 eggs per female) or fertility (mHC 0.84 ± 0.02 vs GUA 0.89 ± 0.02 hatch rate) between the two lines (Fig. 2a, b), indicating that there was no evidence of any impact of our mass-rearing conditions on the reproductive quality of HC females.

One advantage of using the HC line for IIT is that its artificial infection with the wPip *Wolbachia* strain increases

Table 1 Mating competitiveness index of mass-reared *Ae. albopictus* HC (mHC) males

Mating groups	Number of replicates	Egg hatch rate ($\pm$ SEM)	Competitiveness index (CI)	<i>P</i>
GUA♀ $\times$ (GUA♂: mHC♂)	3	0.44 ± 0.04 (H_o)	1.07 ± 0.20	0.487
GUA♀ $\times$ GUA♂	3	0.90 ± 0.02 (H_n)	–	
GUA♀ $\times$ mHC♂	3	0.00 ± 0.00 (H_s)	–	

H_n mean egg hatch rate of fertile control cages; H_s mean egg hatch rate of sterile control cages; H_o mean egg hatch rate of treatment cages; CI male mating competitiveness index, calculated as: $CI = [(H_n - H_o) / (H_o - H_s)] * (N/S)$, where *N* and *S* were the numbers of GUA and mHC males. The calculated CI value was compared with the theoretical value of 1 (i.e., no difference in mating competitiveness) using a Mann–Whitney *U* test

Fig. 2 Reproductive capacity of mHC and GUA females. **a** The number of eggs laid by each individual female ($n=62$ for each mosquito line). **b** The hatch rate of a subset of 30 individuals for each mosquito line randomly selected from those shown in **a**. Horizontal bars represent the mean $\pm$ SEM. Mann–Whitney U test: eggs per female, $P=0.31$; egg hatch rate, $P=0.0682$

mosquito resistance to arbovirus infection (including that with both dengue and Zika viruses), so that any residual females resulting from imperfect sex separation, which are mixed with the males destined for field release, are less likely to be competent vectors (Zheng et al. 2019). The strength of mosquito resistance to arboviral infection is believed to be related to the density of *Wolbachia* in the somatic tissues of mosquitoes, which may be subject to changes during long-term mass-rearing. Therefore, we compared between mHC and GUA mosquitoes vector competence for dengue virus serotype 2 (DENV-2), which was previously used to assay the vector competence of the HC line when its mass-rearing

was first initiated (Zheng et al. 2019). Fourteen days after the mosquitoes were orally infected with DENV-2 virus, we collected their saliva and measured the copy numbers of the viral genomes. All of the mHC saliva samples were negative, whereas 35% of the GUA saliva samples were positive (Fig. 3a, Fisher's exact test, $P=0.008$). The density of *Wolbachia* in the whole bodies of 5-to-6-day-old adult females was also compared between the two mosquito mHC and GUA lines. The total *Wolbachia* density in the mHC females was about twofold higher than in the GUA females (Fig. 3b, Mann–Whitney U test, $P=0.001$). While *wAlbB* was dominant strain (81%) in GUA, *wPip* counted for 88% of total

Fig. 3 Mosquito vector competence for dengue virus and *Wolbachia* density in mHC and GUA mosquitoes. **a** mHC and GUA mosquitoes were orally infected with DENV-2. At 14 days post-infection, individual saliva samples ($n=20$ for each mosquito line) were collected, and DENV-2 genome copies were measured by RT-qPCR. **b**. Five-to-six-day-old mHC and GUA females were collected ($n=8$ pools of

3 to 4 adults for each mosquito line), and the *Wolbachia* density was measured individually for each strain using qPCR. Mean $\pm$ SEM and significance were determined using Mann–Whitney U tests (for both DENV-2 copies and *Wolbachia* density) or Fisher's exact test (for the prevalence of DENV-2). ** $P<0.01$

Wolbachia density, with *wAlbB* suppressed to a minimum in mHC (Fig. 3b). Overall, these results indicate that the *Wolbachia*-mediated virus-blocking effect, as well as high *Wolbachia* densities, was maintained during the long-term mass-rearing of the HC line.

Impact of long-term mass-rearing on the quality of HC larvae, in terms of development and survivorship

Long-term mass-rearing may impose selection on the development of mosquito larvae because the larvae are repeatedly reared under identical artificial conditions, with discrete generations produced according to a fixed schedule (e.g., pupae were always collected after the same amount of time had elapsed). We compared the larval development time and survivorship of the mHC and GUA lines and observed that the mHC larvae developed to pupae significantly more quickly than the GUA larvae. The average larval development time for both male (5.13 ± 0.04 days) and female (5.73 ± 0.02 days) mHC was significantly shorter than the analogous times for GUA males (5.54 ± 0.08 days) and females (6.20 ± 0.09 days), respectively (Students' *t* test, males: $t = 7.93$, d.f. = 4, $P = 0.001$; females: $t = 7.05$, d.f. = 4, $P = 0.002$, Fig. 4a). However, there was no significant difference in the larval survival rate between the two lines (Mann–Whitney *U* test, $P = 0.7$, Fig. 4b).

Insect larval development is controlled by a critical steroid hormone, 20-hydroxyecdysone (20E), with the level of this hormone being positively correlated with the mRNA expression of the 20E primary response gene *E75* (Li et al. 2019; Moeller et al. 2013). Therefore, we measured by qRT-PCR the level of *E75* expression in larvae at 48, 72, 96, and 120 h after egg hatching to determine whether *E75* played

any role in promoting mHC larval development when compared to that of GUA larvae. We found that the expression of *E75* was significantly higher in mHC larvae than in GUA larvae at 96 or 120 h after egg hatching, but no significant difference was observed between the two lines at 48 or 76 h after egg hatching (Fig. 4c). These results suggest a potential correlation between rapid development to pupae and increased expression of *E75* during the latter developmental stages in mHC larvae.

Impact of long-term mass-rearing on other HC adult life-history traits: sex ratio, body size, and longevity

In order to further measure the impact of long-term mass-rearing on other mosquito life-history traits that might affect the efficacy of IIT-based suppression of target field populations, we also compared the sex ratio, body size, and longevity of mHC and GUA mosquitoes. We found no significant difference in either the sex ratio or adult wing (i.e., body) size between the mHC and GUA lines (Fig. 5a, b). However, mHC females (median longevity: 44 days) had significantly shorter life span than did GUA females (median longevity: 48 days) (Fig. 5c, log-rank test, $P = 0.0007$), but both mHC and GUA males had similar life spans (Fig. 5d, log-rank test, $P > 0.05$). Thus, long-term mass-rearing seems to exert a negative impact on HC longevity in a female-specific manner.

Discussion

The HC mosquito line passed rigorous quality control during the initial phase of its mass-rearing (Zhang et al. 2015, 2016; Zheng et al. 2019). In this work, we have here further

Fig. 4 Larval development and survival of mHC and GUA mosquitoes. **a** mHC larvae developed significantly faster than did GUA larvae ($n = 3$ replicates). **b** Survival rate of mHC and GUA larvae ($n = 3$ replicates). **c** *E75* expression level in mosquito larvae at different

times after egg hatching. Each time point shows data for five biological replicates of 10–20 larvae. Mean $\pm$ SEM and significance were determined by using either a Student's *t* test (A and B) or a Mann–Whitney *U* test (C). n.s. not significant, * $P < 0.05$; ** $P < 0.01$

Fig. 5 Sex ratio, adult body size, and longevity of mHC and GUA mosquitoes. **a** Sex ratios; $n=3$ replicates. Mann–Whitney U test, $P=0.7$. **b** Wing size; $n=15$ individuals. Mann–Whitney U test, males: $P=0.305$; females: $P=0.187$. **c** Female longevity.

ity. Mean $\pm$ SEM; $n=3$ replicates. Log-rank (Mantel–Cox) test, $P=0.0007$. **d** Male longevity; $n=3$ replicates. Log-rank (Mantel–Cox) test, $P>0.05$. All figures show the mean $\pm$ SEM

demonstrated that HC mosquitoes continue to show high performance, based on biological traits that are essential for the success of IIT-based population suppression, after mass-rearing in our factory facility for more than 50 generations. No differences were observed between mHC mosquitoes and their wild-type GUA counterparts in the following traits: the mating competitiveness and flight ability of males, fecundity and fertility of females, survivorship of larvae, and the sex ratio and body size of adults. As observed previously (Zheng et al. 2019), mHC females exhibit a lower vector competence for dengue virus than GUA. In addition, consistent with an elevated expression of *E75* at 96 and 120 h after egg hatching, the development of HC larvae to the pupal stage occurs significantly faster than GUA larvae. Despite the lack of any difference in the life span between HC and GUA adult males, adult GUA females live significantly longer than mHC females.

We have paid close attention to monitoring the quality of HC mosquitoes under mass-rearing conditions because previous studies have shown that long-term (from dozens of generations to more than 10 years) artificial maintenance in laboratory and/or factory settings can strongly reduce the fitness and/or heterozygosity of mosquitoes (i.e., inbreeding depression and/or adaptation to artificial environments) (Azrag et al. 2016; Jong et al. 2017; Koenraadt et al. 2010; Lainhart et al. 2015; Powell and Evans 2017; Ross et al.

2019), with the potential to cause a reduction in the quality of artificially bred mosquitoes relative to wild-type mosquitoes in target field populations (Hoffmann and Ross 2018; Reed and Frankham 2003; Ross et al. 2019). These negative impacts have not been observed in HC mosquitoes reared under our mass-rearing protocol for more than 50 generations. This may be explained by the three reasons. First, before being used in mass-rearing, the HC mosquitoes had been repeatedly outcrossed for seven generations with the wild-type GUA line, established from the field by pooling collected samples from 20 different locations in Guangzhou (Zhang et al. 2015; Zheng et al. 2019). This outcrossing enabled us to introduce considerable genetic heterogeneity into the HC mosquitoes and improved their fitness, as observed in *Ae. aegypti* (Ross et al. 2019; Yeap et al. 2011). Second, after outcrossing, the HC mosquitoes were maintained at a large population size (millions of mosquitoes in each generation), thus greatly reducing the possibility of any inbreeding depression that might occur during mass-rearing (Ross et al. 2019). Third, each step of our mass-rearing protocol has been optimized—including the density of larvae in the rearing trays, the sex ratio and density of adults in each cage, and the egg storage conditions—in order to minimize the stress to the mosquitoes as much as possible (Zhang et al. 2017, 2018). Although it remains speculative at this stage, the *Wolbachia* themselves may provide certain benefits to HC

mosquitoes under mass-rearing conditions. We have consistently observed that HC larvae develop faster than GUA larvae (Zhang et al. 2015), as supported by the increased expression of molting hormone regulating *E75* gene in the HC larvae. Overexpression of *E75* gene is positively correlated with 20E (ecdysteroid) and related to synthesis of chitin and metamorphosis (Li et al. 2016; Liu et al. 2018). *E75* has its highest peak of activity during the last stages of larval development prior to pupae formation (Margam et al. 2006), which is consistent with observations of this study. It would be interesting to explore if the overexpression of *E75* is induced by the triple-strain *Wolbachia* infection of mHC (*wAlbA*, *wAbB*, and *wPip*).

The lack of evident fitness reduction under laboratory conditions in our mass-reared HC mosquitoes suggests that, with rearing conditions optimized, long-term mass-rearing does not necessarily result in reduced quality of insects produced for mass-release in vector control programs. It was reported that a 40-year-old colonized line of the tsetse fly *Glossina palpalis gambiensis* was still competitive in a field trial in Burkina-Faso (Sow et al. 2012), and this line out-competed a recently colonized strain from the target area in Senegal (Bassene et al. 2017), indicating that long-term, large-scale breeding of insects does not necessarily have adverse effects on male mating competitiveness.

It is also possible that the 50-generation (4-year) mass-rearing is not of sufficiently long duration for detrimental effects to accumulate to the extent that can be detected by our experimental approaches. If this is the case, continuous monitoring of the essential traits of mass-reared HC mosquitoes will be necessary for quality control. Theoretically, inbreeding-related colony depression can be reversed by outcrossing with wild-type mosquitoes (Ekechukwu et al. 2015). If inbreeding depression of the HC line does occur in the future, we can outcross this line with field-collected *Ae. albopictus* males to quickly restore the level of fitness, since the triply *Wolbachia*-infected HC line causes unidirectional cytoplasmic incompatibility when these mosquitoes mate with wild-type mosquitoes (Zheng et al. 2019). This is one advantage of HC mosquitoes, as opposed to other transinfected lines that induce bi-directional cytoplasmic incompatibility with wild-type mosquitoes, and for whom removing native *Wolbachia* from field mosquitoes is required before outcrossing.

Our results show that the native *Wolbachia* strain *wAlbB* in the HC line is strongly suppressed to a very low level, suggesting the presence of competition between the two strains in the supergroup B (*wPip* and *wAlbB*), which infect HC. A similar result was also observed in another *Ae. albopictus* line carrying a triple-strain *Wolbachia* infection, in which *wMel* inhibited *wAlbA*, indicating competition between two strains in the supergroup A (Liang et al. 2020). It is worth noting that the HC line expresses unidirectional cytoplasmic

incompatibility when it is crossed with wild-type mosquitoes (Zheng et al. 2019), indicating that even the low level of *wAlbB* in HC eggs is sufficient to rescue sperm modification.

As it was technically challenging to mass-rear GUA at the same scale as mHC, comparisons of these two lines were performed under the standard laboratory condition in this study. Thus, our results mainly reflect the intrinsic properties of the two mosquito lines, and different results may be observed if they are compared under mass-rearing conditions. It is also possible that the approach used in the current study only reflects the level of quality of traits such as male mating competitiveness in the setting of the laboratory, and these levels may not represent the real situation in the field (Soemori et al. 1980). Previous studies showed that a transgenic line of *Ae. aegypti* with female-specific flightless phenotype performed well in laboratory experiments but was not competitive when tested in semi-field experiments (Facchinelli et al. 2013), while mating competitiveness of another transgenic line observed in the field was much lower than results from laboratory and larger semi-field mating cage studies (Carvalho et al. 2015). Conversely, another study showed that the field-derived males had decreased mating vigor when they were confined in a small cage to which mass-reared males had been adapted, demonstrating that “control” wild-type insects might also exhibit lower quality in experimental laboratory settings (Soemori et al. 1980). Future studies should be performed to compare male mating competitiveness in semi-field cages and field settings.

Unlike a previous report of no difference in life span between HC and GUA females (Zhang et al. 2015), we saw a significant reduction in the longevity of HC females when compared to GUA females when both lines were fed on sugar alone. It is unknown whether this is caused by a reduction in genetic variability of HC mosquitoes during the long-term mass-rearing. Augmented fertility and fecundity accompanied by increased longevity was previously reported for *Ae. albopictus* infected with *wAlbA* and *wAlbB* (Dobson et al. 2002). Further studies will investigate whether this difference is caused by a change in *Wolbachia* composition and abundance, and whether a different result will be obtained when a bloodmeal is provided to the females (Joshi et al. 2014). It is worth noting that reduced female longevity decreases the risk of viral transmission associated with unintentional release of residual females into the field.

Overall, our results highlight the robustness of the biological traits in HC mosquitoes under long-term mass-rearing conditions, which can affect both the mass-rearing efficacy and male mating performance in the field, thus encouraging further development and deployment of *Wolbachia*-based IIT for mosquito vector control. The effort to maintain sufficient genetic heterogeneity in a large population under optimized rearing conditions is intended to impose less stress on mosquito

colony and may contribute to avoiding the problem of inbreeding depression and reductions in insect quality. It would be interesting to further understand *Wolbachia*–mosquito interactions in relation to the maintenance of strong mating competitiveness in HC males, the high level of viral blocking in HC females, and the rapid development of HC larvae. Our future work includes developing rapid, sensitive assays that can be used in both laboratory conditions and field settings for quality control and early diagnosis of fitness loss, as well as novel treatment approaches for boosting mosquito quality when colony depression occurs. Further efforts will also be made to explore novel *Wolbachia* strains and their combinations in order to develop the next generation of transinfected lines that are best suitable for both mass production and field conditions, in terms of cost-effectiveness in operation, robustness, and stability of desired characteristics in various environments.

Author contributions

YL and ZX conceived the idea and designed the experiments. ZX, ZH, and XZ supervised the project. YL performed the majority of the experiments and analyzed the data. MZ and XW performed the remaining experiments. YL and ZX wrote the manuscript.

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Availability of data and materials All datasets presented in this study are included in the article/Supplementary Material.

Compliance with ethical standards

Conflict of interest Y. L., X.W., and Z.X. are affiliated with Guangzhou Wolbaki Biotech Co., Ltd.

Ethical approval Mosquitoes were fed on the blood of anesthetized mice according to a protocol approved by the Ethics Committee on Laboratory Animal Care of the Zhongshan School of Medicine (Approval Number 2017-041).

Informed consent Informed consent was obtained from all co-authors of the study.

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