Stability of the effect of silencing fibronectin type III domain-protein 1 (FN3D1) gene on Anopheles arabiensis reared under different breeding site conditions

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Abstract

Background: Malaria vector mosquitoes acquire midgut microbiota primarily from their habitat. The homeostasis of these microbial communities plays an essential role in the mosquito longevity, the most essential factor in the mosquito vectorial capacity. Our recent study revealed that silencing genes involved in regulation of the midgut homeostasis including FN3D1, FN3D3 and GPRGr9 reduced the survival of female adult Anopheles arabiensis mosquitoes. In the present study, we investigate the stability of the gene silencing efficiency of mosquitoes reared in three different breeding conditions representing distinct larval habitat types: town brick pits in Jimma, flood pools in the rural land of Asendabo and roadside pools in Wolkite.

Methods: First-instar larvae of An. arabiensis mosquitoes were reared separately using water collected from the three breeding sites. The resulting adult females were micro-injected with dsRNA targeting the FN3D1 gene (AARA003032) and their survival was monitored. Control mosquitoes were injected with dsRNA LacZ. In addition, the load of midgut microbiota of these mosquitoes was determined using flow cytometry.

Results: Survival of naïve adult female mosquitoes differed between the three sites. Mosquitoes reared using water collected from brick pits and flood pools survived longer than mosquitoes reared using water collected from roadside. However, the FN3D1 gene silencing effect on survival did not differ between the three sites.

Conclusions: The present study revealed that the efficacy of FN3D1 gene silencing is not affected by variation in the larval habitat. Thus, silencing this gene has potential for application throughout sub-Saharan Africa.

Keywords: Anopheles arabiensis, Larval breeding sites, Gene silencing stability, Survival, FN3D1 gene

Background

Malaria still thrives in sub-Saharan Africa. The region holds over 90% of the 219 million malaria cases with an estimated 435,000 malaria deaths in 2017 [1]. This disproportionate share is due to the principal malaria vectors in the region that exhibit a higher vectorial capacity, i.e. Anopheles gambiae (s.s), Anopheles coluzzii, Anopheles arabiensis and Anopheles funestus. Key parameters for vectorial capacity include preference to feed on humans, susceptibility to Plasmodium infection and longevity of the mosquito. It can take two weeks and beyond for the Plasmodium parasites to complete the mosquito stage development after which they can be transmitted to another human host. On the other hand, in tropical regions, the average lifespan of Anopheles mosquitoes...
mosquito is poorly understood. However, there is speculation that exposure of larvae to toxins dissolved in the water might contribute to the rising prevalence of vector resistance to pyrethroids in the cities across the region [20, 21]. Anopheles larvae have shown a high resilience to the high toxicity of some of these wastes in the urban and suburban sites, but the impact of such pollution on the life traits of the resulting adult mosquito is poorly understood. However, there is speculation that exposure of larvae to toxins dissolved in the water might contribute to the rising prevalence of vector resistance to pyrethroids in the cities across the region [20, 21]. It was also suggested that larvae developing in polluted water can lead to a significant fitness cost [22].

Based on the above facts, we assessed the survival of adult female An. arabiensis mosquitoes reared in water collected from three different breeding sites. In our previous study we observed that silencing the midgut gene fibronectine type III domain-protein 1 (FN3D1) involved in the midgut homeostasis reduced the longevity of An. arabiensis mosquitoes [23]. Therefore, in the present study we further assessed the stability of the FN3D1 gene silencing effect on An. arabiensis mosquitoes reared in water collected from three different breeding sites.

Methods

Study sites and sample collection

Water samples for mosquito rearing were collected from three malaria endemic sites including Wolkite, Jimma and Asendabo, south-west Ethiopia (70°40′0.01″N, 36°49′59.99″E) (Fig. 1). From each site, a single pool confirmed to support An. arabiensis larvae was selected to collect rearing water using a clean jug and plastic container/jerry cans. Simultaneously water samples for bacterial counts were collected using sterile, screw cup bottles following standard operational procedures [24]. The water collected for rearing purposes was filtered using a fine mesh linen cloth in order to remove debris and mosquito eggs and larvae. The filtered water was then transferred directly to clean larvae rearing pan or boiled for 10 min and cooled before transferring to the rearing pans.

Analysis of water from selected sites

pH, conductivity and dissolved oxygen was measured on site using a Portable Multi meter (HQ40D, HACH). Salinity was measured using a salinity meter (TRACER PocketTester) and turbidity with a turbidity meter (Wag-WT 3020, Wagtech International, Gateshead, UK). Total dissolved solids (TDS) and total suspended solids (TSS) were measured by the gravimetric method. In brief a volume of 100 ml of well-mixed water samples was filtered using a glass-fiber-filter with applied vacuum. The filtered samples were then washed with three successive 10 ml volumes of distilled water which permitted complete drainage between washings. The suction was continued for about 3 min after filtration was completed. The filtrate (with washing) was then transferred to a weighed evaporating dish and evaporated to dryness on a steam bath. If the filtrate volume exceeded dish capacity, successive portions were added to the same dish after evaporation. Finally, the samples were dried for at least 1 h in an oven at 180 ± 2 °C for TDS and 103–105 °C for TSS, allowed to cool in a desiccator to balance temperature, and weighed [24]. All the parameters were measured based on the supplier’s guidelines and the instruments were calibrated before the samples analysed. A total of two replicates was taken for each site.

The total bacterial count was assessed for the water samples using membrane filter techniques. For each sample tenfold serial dilution was prepared with a total volume of 100 ml and allowed to pass through a funnel covered with a membrane filter (with a size of 47mm diameter by 0.45 μm porosity) in order to trap the bacteria available in the water. Then the membrane with the trapped bacteria was placed in a Petri dish containing a pad saturated with sterilized M-lauryl sulfate broth (Sigma-Aldrich, Bangalore, India). The M-lauryl sulfate broth media was prepared according to the manufacturer’s guidelines. The passage of nutrients through the filter during incubation facilitates the growth of organisms in the form of colonies on the upper surface of the membrane. Then, the inoculates were incubated at a temperature of 37 °C for 24 h where
after the total number of the colonies was counted using a magnifying lens [24].

Mosquito rearing

*Anopheles arabiensis* obtained from a laboratory strain of the Adama Malaria Research Centre, Ethiopia, was used in this study. Recently hatched first-instar larvae were reared in the water collected from the three breeding sites. Two-hundred larvae from the same batch of eggs were dispensed into the rearing trays and supplied with an equal amount of a mixture of yeast and fish food. The rearing water was changed every two days and development of larvae was monitored daily. Next, the enclosed pupae were counted and transferred into

![Map showing larval breeding sites where rearing water samples were collected](image-url)
10 ml glass beakers independently and kept in adult cages (W15 × D15 × H15 cm, BugDourm, Watkins & Doncaster, Leominster, UK) until the emergence of the last pupae. The emerged adults were maintained on 10% sugar at 27 °C, 70% humidity with 12:12 h light:dark photoperiod [25]. The experiment was done in triplicate.

Adult wing size was measured to evaluate differences in the adult female body size. For this experiment, 10 individual mosquitoes were removed from each adult cage to measure their wing size as follows: a single wing was clipped from each mosquito using scalpel blade and mounted onto a glass microscope slide. Then the wing size was measured using a micrometer eyepiece with stereomicroscope. Measurements were taken from the tip of the wing (excluding fringe) to the distal end of the alula [26].

### Gene silencing and survival assay

From each breeding site, 15–24 adult female mosquitoes were CO₂ anesthetized and treated with 69 µl of the FN3D1 dsRNA gene and another group with non-mosquito dsLacZ gene. Double stranded RNA (DsRNA) for FN3D1 and the control LacZ gene was prepared from complementary DNA (cDNA) as described in our previous study [23]. The cDNA was synthesized from 1 µg of the tRNA using Prime-Script™ 1st-strand cDNA Synthesis Kit (TaKaRa, Saint-Germain-en-Laye, France) which was extracted from 10 whole female An. arabiensis mosquitoes using TRIzol reagent (Invitrogen, Carlsbad, USA). The following primer sequences tailed with the LacZ target 5′ as: forward: AGA ATC CGA CGG GTT GTT ACT; reverse: TGG ATC GTC CTC ATC ACT GT) and FN3D1 (forward: GAT GGA CGT GGA TCA GCC; reverse: GAC CAC GCT CAT CGA TAA TTT).

On day four, after the mosquitoes were starved overnight, they were fed on blood. At 24 h post-blood-meal, 5 mosquitoes were sampled for each breeding site independently for both unboiled and boiled water and their midguts were dissected. The midgut samples were then homogenized in 100 µl 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). The number of bacteria was then counted using flow cytometry from five pooled midgut samples per replicate [27]. A total of 3 replicates of microbiota analysis were performed.

The survival of mosquitoes was monitored starting from 24 h post-injection for 20 days. All mosquitoes were supplied with 10% sugar solution and monitored daily, and they obtained blood meal at 4-day intervals post-injection. Three replicates each consisting of 15–24 mosquitoes were performed.

### Data analysis

Data analysis was performed using the statistical software package R version 3.3.2. Survival of the FN3D1 gene- and LacZ gene-silenced mosquitoes and the naïve mosquitoes with boiled and unboiled water were depicted by Kaplan Meier survival curves as a function of site. The effect of gene silencing on survival was modelled by the Cox proportional hazards frailty model [28], with replicate as frailty term and including as covariates the gene (the FN3D1 target and LacZ control gene), the site (Jimma, Asendabo and Wolkite) and the two-way interaction. The effect of site and boiling in the naïve mosquitoes was analysed in the same way. The hazard ratio was used as a summary statistic, together with the median time to death. The bacterial counts were first log-transformed and then compared by a mixed model with replicate as random effect and the F-test was used to compare the silencing of the FN3D1 target gene and the control LacZ gene on the one hand, and the boiling on the other hand. The ratio of the bacterial counts in the control LacZ gene and the FN3D1 target gene was used as a summary statistic. The t-test was used to compare the number of emerged adults and pupae and the adult wing length between the three breeding sites. All tests were performed at a significance level of 5%.

### Results

#### Analysis of water samples

Pictures of the three breeding sites are shown in Fig. 2 and their physicochemical characteristics are provided in Table 1. The water samples from Jimma and Asendabo had close to the neutral pH, while water samples from Wolkite were alkaline with the highest salinity. Nevertheless, the pH for all the sites was within a range that was previously reported suitable for the natural breeding habitats of An. arabiensis [29]. The level of dissolved oxygen was considerably higher for Wolkite and Asendabo compared to Jimma. The Asendabo had also the highest total bacterial count compared to Jimma or Wolkite.

#### Larval development

Our results demonstrated that, generally, the larvae reared with water from Asendabo performed better when compared to the rest of the sites (Table 2). For instance, the pupation rate for Asendabo (85.8%) was significantly higher compared to Wolkite (68.5%) (t = 5.69, df = 4, P = 0.005) but not to Jimma (78%) (t = 5.69, df = 4, P = 0.062). No significant differences of percentage of pupae developed in adult mosquitoes could be found between Asendabo, Wolkite and Jimma. The mean adult wing length was significantly larger for Asendabo.
(3.2 mm) compared to Jimma (3.0 mm) \( (t = 2.58, df = 87, P = 0.012) \) and Wolkite (2.7 mm) \( (t = 8.23, df = 87, P < 0.001) \). Together, these results suggest that the above parameters may be positively impacted by the level of dissolved oxygen and bacterial count in the breeding water. Accordingly, the water from Asendabo is considered most suitable for larval development.

Survival rate of adult mosquitoes
Survival of the mosquitoes is depicted as a function of time in Fig. 3 for the different settings, i.e. different sites and water boiled or not. There was no significant interaction between the site and boiling effect, i.e. the effect of boiling the water has a similar effect for the three sites. A significant effect of boiling the water
Table 2  The rates of pupation and adult emergence (n = 3 batches of 200 eggs), and the mean adult wing size (n = 30) in mosquitoes reared in the water collected from the three sites

| Breeding site | Pupa (%) | Adult (%) | Mean wing size (mm) |
|---------------|----------|-----------|---------------------|
| Wolkite       | 68.8     | 93.7      | 2.7                 |
| Jimma         | 78.2     | 93.6      | 3.0                 |
| Asendabo      | 85.8     | 95.9      | 3.2                 |

(χ² = 17.2, df = 1, P < 0.001) and an almost significant difference between the sites (χ² = 5.64, df = 2, P = 0.059) was found. The hazard ratio of the mosquitoes grown in boiled water compared to unboiled water was 2.25 (95% CI: 1.42–3.56). The hazard ratio of Jimma compared to Wolkite was 0.53 (95% CI: 0.32–0.88) whereas the hazard ratio of Asendabo compared to Wolkite was 0.51 (95% CI: 0.31–0.84). The adult gut bacterial count ratios in mosquitoes grown in the unboiled versus the boiled water for the three sites are presented in Fig. 4.

Effect of the FN3D1 gene silencing
Survival of the FN3D1- and LacZ-silenced mosquitoes is depicted as a function of time in Fig. 5 for the different sites. There was no significant interaction between the gene silencing effect and the site, i.e. the effect of silencing was the same for the three sites. Both gene silencing (χ² = 25.7, df = 1, P < 0.001) and site (χ² = 8.15, df = 2, P = 0.017) had a significant effect on survival. The hazard ratio of the FN3D1-silenced mosquitoes compared to the LacZ-silenced mosquitoes was 1.96 (95% CI: 1.58–2.43). The hazard ratio of Jimma compared to Wolkite was 0.69 (95% CI: 0.54–0.89) whereas the hazard ratio of Asendabo compared to Wolkite was 0.86 (95% CI: 0.67–1.10).

Significant effects for the microbiota were found for the gene silencing (F(1, 14) = 7.59, P = 0.016) and for the interaction between gene silencing and site (F(2, 14) = 4.23, P = 0.038). Therefore, we study the gene effect at each location separately. A significantly higher bacterial load in the FN3D1-silenced mosquitoes as compared to the LacZ-silenced mosquitoes was only found for Asendabo, with a ratio equal to 2.50 which differs significantly from 1 (F(1, 14) = 14.21, P = 0.002) (Fig. 6).
mosquitoes with the largest wing compared to Jimma and Wolkite. A similar variation in the wing size among the mosquitoes grown in different larval habitats was reported earlier for *Anopheles stephensi* [9] and *Anopheles darlingi* [8, 39]. It was concluded that such variation in the wing size is closely linked to the nutrient availability in the habitat [8, 9].

Wing size is directly correlated with survival of adult female mosquitoes, i.e. the mosquitoes with longer wing showed a higher longevity than short winged mosquitoes [9, 12, 40, 41]. Comparison of the survival of the mosquitoes reared with water from the three sites in the present work was supported by the above observation. For instance, the mosquitoes reared in the water from the Asendabo site that had the longest wing displayed the longest survival.

Previously we have demonstrated that the depletion of some midgut gene proteins including the *FN3D1*, *FN3D3* and *GPRGr9* genes markedly shorten the longevity of female *An. arabiensis* [23]. In the present study, we assessed whether the variation between breeding habitats may affect the gene silencing effect. Our survival data revealed that the effect of gene silencing was not affected by variation in the larval breeding site, i.e. for all the study sites the *FN3D1*-treated mosquitoes had a similarly reduced survival rate compared to the control LacZ group. Thus, the variation in breeding sites does not affect the gene silencing effect on reducing the longevity of *An. arabiensis* mosquitoes. Gene silencing induces mosquito mortality by disrupting the midgut homeostasis [23], which is evidenced also in the present study where a higher bacterial load was observed in the *FN3D1*-silenced mosquitoes as compared to the LacZ silenced mosquitoes.

**Conclusions**

The longevity of the *An. arabiensis* mosquitoes varied between the three larval breeding sites depending on biotic as well as abiotic characteristics of the larval breeding water. However, there was no evidence that these differences compromise the gene silencing effect of the *FN3D1* gene on the mosquito survival. Therefore, interventions based on the silencing of such genes, and thus the reduction of mosquito longevity, offer a universal strategy to block malaria transmission.

**Abbreviations**

cDNA: complementary DNA; DNA: deoxyribonucleic acid; DO: dissolved oxygen; dsRNA: double-stranded RNA; *FN3D1*: fibronectin type III domain-protein 1; *FN3D3*: fibronectin type III domain-protein 3; *GPRGr9*: G protein-coupled receptor protein 9; PBS: phosphate-buffered saline; PFA: paraformaldehyde; RNA: ribonucleic acid; TDS: total dissolved solids; TSS: total suspended solids.
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Authors' contributions
TH, LD and GKC developed the research idea. SD and TH performed the mosquito field studies. TH and SD took care of all lab analyses. LD analysed and interpreted the data. SD, TH, GKC and LD wrote the manuscript. All authors read and approved the final manuscript.

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Datasets are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate
Ethical clearance was obtained from the research and ethical review board of Agriculture and Veterinary Medicine, Jimma University, Ethiopia.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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