Effect of larval density and additional anchoring surface on the life-history traits of a laboratory colonised *Anopheles funestus* strain.

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Abstract

Optimal rearing conditions, inclusive of larval rearing density, are critical for sustained mosquito productivity. There is limited information on favourable conditions for the larval rearing of *Anopheles funestus*, the dominant malaria vector in east and southern Africa. This work investigated the effects of larval rearing densities and additional anchoring surface on *An. funestus* development using a life table approach. Larval cohorts were reared at four different larval densities using the same rearing surface area, larval food concentrations and temperature conditions. Rearing larvae at high densities extended larval developmental time and reduced adult productivity. Adding an extra larval anchoring surface when rearing larvae at high density resulted in extended larval developmental time, increased larval survivorship and produced bigger adults. These findings improve our understanding of the relationship between larval density and developmental traits in *An. funestus* and provides baseline information for *An. funestus* rearing under laboratory conditions.

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Introduction

Anopheles funestus (Diptera: Culicidae) is an important primary malaria vector throughout sub-Saharan Africa (Gillies and De Meillon 1968; Gillies and Coetzee 1987; Coetzee and Koekemoer, 2013; Dia *et al.,* 2013; Djamouko-Djonkam *et al*., 2020). Despite the significance of this species as a vector, many aspects of its larval biology, including optimal larval rearing density, are not understood. This is attributed to difficulties in the colonisation of colonising this species (Coetzee and Koekemoer, 2013; Ngowo *et al*., 2021). Laboratory colonisation of *An. funestus* remains a challenge due to its tendency to avoid mating in confined places, making its proliferation in laboratory cages challenging (Gillies and De Meillon, 1968). Understanding various aspects of *An. funestus* rearing biology, including impact of larval density, is critical in obtaining baseline information that can be used to develop standard laboratory rearing procedures for colonising this species.

Mosquito larvae are omnivorous, opportunistic aquatic feeders that feed on aquatic microbes (detritus, algae and microorganisms) to acquire nourishment for growth and accumulation of excess nutrients in the body for utilisation in later developmental stages (Gillies and De Meillon, 1968; Clements, 1992; Bond *et al.*, 2005). In nature, *An. funestus* larvae tend to favour inhabiting more permanent waters such as lakes and swamps (Gillies and de Meillon 1968; Gillies and Coetzee, 1987; Nambunga *et al*., 2020; Debrah *et al.,* 2021) and may also develop in locations along sluggish streams and rivers where there is vegetation. They can also be found in artificial habitats such as rice fields, wells and domestic water containers (Evans, 1938; Gillies and De Meillon 1968; Dia *et al.*, 2013).

The key limiting factors to *An. funestus* larval development includes salinity and extreme temperatures (Gillies and De Meillon 1968; Koekemoer *et al.*, 2014; Dia *et al.,* 2013). Other

critical larval rearing conditions include rearing density and diet. If all these parameters are optimal, it promotes simultaneous larval development, adequate adult size and a sustained production cycle (Benedict *et al.*, 2009; Khan, 2010; Hood-Nowotny *et al.*, 2012). Of these factors, the effect of larval density on the mosquito developmental cycle is not clearly understood. For instance, Lyimo *et al.* (1992) showed that high *An. gambiae* larval densities decreased the developmental time in *An. gambiae*. Contrary to this, other researchers showed an extended developmental time in *An. stephensi*, *An. coluzzii*, *An. gambiae* and *An. arabiensis* (Muriu *et al.*, 2013; Yadav *et al.*, 2017; Epopa *et al.*, 2018; Mamai *et al.*, 2018).

Another parameter affected by rearing larvae at high density is early instar survivorship. Several studies showed that over-crowding larvae increase early instar mortality (Roberts and Kokkinn, 2010; Epopa *et al.*, 2018). Premature instar mortality can be due to intra-species cannibalism due to limited food sources. In some instances, rearing larvae at high-density results in the build-up of toxic chemicals in the rearing water from larval excreta resulting in retardant growth (Moore and Fisher, 1969; Roberts, 1998). Other studies have linked larval overcrowding with physical effects whereby moving larvae continually disturb each other and sometimes collide, creating waves of turbulence that affects their ability to feed properly (Roberts and Kokkinn, 2010). All these effects have been shown to have a downstream effect on subsequent developmental stages. Of note is the impact on adults. Various studies revealed that adult body size and survival are negatively affected by high larval density rearing (Fisher *et al.*, 1990; Ng'habi *et al.*, 2005; Muriu *et al.*, 2013; Epopa *et al.*, 2018).

Information on the impact of larval density on the development is well described in other mosquito species. There is a relative lack of data on the effects of larval density during the rearing of *An. funestus* and provided the motivation for this study. Furthermore, in nature, *An. funestus* anchors

on swamps and vegetation to avoid periodic flushing by heavy rainfall (Gillies and De Meillon, 1968). This behaviour is presumed to aid the survival of *An. funestus* and can be advantageous in larval survival under high-density conditions. Under laboratory conditions, *An. funestus* larvae have adapted to this phenomenon by anchoring to surfaces of rearing containers (personal observation during routine colony rearing). It can be presumed that anchoring surfaces are more important than the surface area at high larval densities. This work hypothesised that an additional anchoring surface might reduce the adverse effects of rearing *An. funestus* larvae at high-density. This study assessed the effect of larval density on the development of a laboratory-reared *An. funestus* strain and the impact of providing an additional anchoring surface on reducing the impact of overcrowding.

Materials and methods

Biological material

An *Anopheles funestus* laboratory strain (FANG) originating from field collections from southern Angola was used during this study. This strain has been under colonisation since 2002 (Zengenene *et al*., 2021). It is housed in the Botha De Meillon Insectary at the National Institute for Communicable Diseases (NICD), Johannesburg, South Africa. It is maintained under standard insectary conditions of 25-27°C, 80% relative humidity and a 12-hour day/night cycle with a 45-minute dusk/dawn transition period, using methods described by Hunt *et al.* (2005). Before using the strain for this study, its identity was confirmed using molecular methods described by Koekemoer *et al.* (2002).

Effect of larval density on the life-history traits of a laboratory-reared *An. funestus* colony

Larvae were reared in rectangular larval rearing bowls (120mm width X 200mm length X 70mm height) at four different densities of 0.42, 0.83, 1.67 and 3.33 larvae per cm^2 (i.e. 100, 200, 400 and 800 larvae in 750ml of deionised water, respectively). Each density (treatment) had five technical replicates, which constituted a biological repeat. A total of three biological repeats were included. Life history traits as detailed below were assessed and compared as previously described in Zengenene *et al*. (2021):

Larval development time and survival

First instar larvae (L1) were added to 750ml of deionised water in larval rearing containers with an anchoring perimeter of 640mm. These were fed twice daily until pupation on a mixture of finely crushed dog biscuits (West's Beeno Traditional Crunchy Biscuit Treats, Martin and Martin, South Africa) and brewer's yeast (Vital Health Foods, South Africa) (mixed at a ratio of 3:1 respectively) at a dose rate of 0.04 – 0.40mg/larva fed twice daily as described by Zengenene *et al*. (2021). Nutritional composition for the dog biscuits is 16% protein, 10% moisture, Crude fat (2.5%), crude fibre (3%), phosphorous (1.5%), vitamin C (3 mg) and E (10iu) and organic selenium (10mcg). The daily feeding rate was adjusted according to larval mortality and or pupation, such that the quantity of food per larvae remained the same. The number of larvae pupating and day of pupation were recorded daily. The proportion of larvae surviving to pupation was calculated as the number of larvae pupating compared to the total of L1 larvae used. Time to pupation was calculated as the time to develop from L1 to pupa.

Adult emergence

Upon pupation, pupae were grouped according to the day of emergence per treatment, and adult eclosion (adult emergence) was monitored daily. The number, day of emergence and gender of adults emerging were recorded daily per replicate until the emergence of the last pupae. The sex ratio of the resultants adults was determined. Only adults that successfully emerged and could fly were recorded as emerged. Those that died on water or were unable to fly or emerge were not included in the analysis. Adult productivity was calculated as the proportion of adults emerging from pupa, while eclosion time was recorded from pupation to adult emergence.

Wing size

Adult mosquitoes {50 adults (25 males and 25 females)/treatment/replicate; over three biological repeats} were randomly selected post-emergence for wing length measurement. Wing length has been shown to give a rational approximation of adult mosquito body size and is routinely used to proxy body size (Paaijmas *et al.*, 2009). To measure wing lengths, adult mosquitoes from each treatment were immobilised at 4^{o}C in a refrigerator. After immobilisation, the left-wing was removed and its length was measured from the distal edge of the alula to the end of the radius vein, excluding fringe scales (Zengenene *et al.*, 2021) at 200X magnification using an eyepiece micrometre mounted on a dissecting microscope (OLYMPUS SZX7, Olympus America Inc., Center Valley, CA, USA). The mean wing lengths were compared by gender within and between the larval density cohorts.

Assessing the impact of providing an extra anchoring surface to reduce the effects of rearing larvae at high density

An experiment was set up to evaluate the effect of providing an extra anchoring surface in reducing overcrowding at high larval density. The anchoring surface was added to the larval density that

had the most inimical overcrowding effects, in this instance, 3.33 larvae/cm^2 (800 larvae per tray). In detail, a wax paper (www.pnp.co.za) with an outer perimeter (anchoring surface) of 556mm and an inner anchoring surface of 476mm, making a total of 1,032mm additional of anchoring surface was added to each rearing container containing 800 larvae. This inevitably reduced the surface area available to the 800 larvae by 111.2cm^2 and resulted in a larval density of 3.44 larvae/cm^2 (Figure 1). The control experiment consisted of larvae reared at 3.33 larvae/ cm^2 at a standard anchoring perimeter of 640mm. The experiments contained three biological repeats, with each biological repetition constituting of five technical replicates. The impact of adding an extra anchoring surface on reducing the effects of overcrowding was assessed using the parameters described above and compared with the control. Noteworthy is the fact that the anchoring surface might also alter the food distribution in the rearing container.

Data analysis

Data were entered and managed in Microsoft Excel then analysed using IBM SPSS Statistical software (IBM Corp., Armonk, New York), version 21. Data on larval developmental time, larval survivorship, pupal survival, and adult size (wing length) was summarised as median larval developmental time (L1 to pupa), the median proportion of larvae surviving to pupation, the medianproportion of pupae surviving to the adult stage and mean wing length respectively. Time taken from larvae to pupation and pupae to adulthood was analysed using Kaplan-Meier survival analysis; for multiple comparisons, a pairwise comparison was conducted using the Log Rank-Mantel Cox test. The difference in the proportion of L1 surviving to pupation and pupae surviving to adulthood between treatments was analysed using the Chi-square test. Pearson's correlation analysis was used to analyse the relationship between larval developmental time, larval survivorship, pupal survival and the different density treatments. Wing length differences between

different larval densities were analysed using one-way ANOVA (followed by means separation by Tukey's HSD test at 5% level of significance for multiple comparisons). In contrast, those between overcrowded larvae without an additional anchoring surface (control) and overcrowded larvae with a provision of an extra anchoring surface (treatment) were analysed using the independent samples t-test. Results were interpreted at 95% confidence. Where appropriate a one-sample t-test or one-way ANOVA was used to assess if the emergence of adult males and females conformed to a one is to one ratio.

Results

Effect of larval density on the life-history traits of a laboratory-reared *An. funestus* colony

Larval development time and adult emergence

Larval developmental time to pupation and proportion of L1 developing into pupae differed between the four larval densities compared. Larval development time to pupation ranged from 16 to 21 days for all treatments (Table 1). Larvae reared at a density of 0.42 larvae/cm^2 had the fastest developmental time, while those reared at 3.33 larvae/cm^2 had the slowest development time. The difference in developmental time from L1 to pupation was statistically significant (log-rank test, χ^2 = 8,572.02, DF = 3, P = 0.00). A pairwise comparison (Log Rank-Mantel Cox) revealed that developmental time differed between all larval density treatments. Statistical analysis using Pearson's correlation analysis revealed that an increase in density significantly extended the developmental time (r(15,450) = 0.617, P = 0.00).

Results on larval productivity (proportion of pupae emerging into adults) are presented in Table 1. Larvae reared at 0.83 larvae/cm^2 had the highest median proportion of L1 developing through to pupa (83.00 (0)%, n = 11,360) whereas those reared at 3.33 larvae/cm^2 recorded the lowest median pupal production (60.00 (1)% , n = 7,256). Statistical analysis showed that the differences in the

192 proportion of L1 developing through to pupae between the different treatments were significant
193 (χ^2 = 864.70, DF = 6, *P* = 0.00). The proportion of first instar larvae surviving to pupation
194 significantly decreased as larval density increased (Pearson's correlation analysis: *r*(8) = -0.175,
195 *P* = 0.00). Pupal productivity decreased with increased larval density (Pearson's correlation
196 analysis: *r*(8) = -0.569, *P* = 0.00) and differences were significant (χ^2 = 167.81, DF = 6, *P* = 0.00).
197 The median time taken for pupae to emerge into adults in all the treatments was two days.
198 Statistical analysis showed no significant difference in time to adult emergence between the
199 different treatments (Log-rank test, χ^2 = 87.18, DF = 3, *P* = 0.37). The ratio between the different
200 genders of the resultant adults did not deviate statistically from the 1:1 ratio (Suplementary Table
201 1).

202 ***Wing size***

203 The highest wing lengths were recorded from adults emerging from larvae reared at 0.83
204 larvae/cm^2, whereas the lowest was observed from adults emerging from larvae reared at 3.33
205 larvae/cm^2. The adult wing lengths were statistically different between the treatments irrespective
206 of gender (one-way ANOVA, F= 61.67, DF = 3, *P* = 0.00). Pairwise comparison revealed two
207 groups of adult sizes. The first group were adults emerging from larvae reared at 0.42 and 0.83
208 larvae/cm^2, while the second group were adults reared at 1.67 and 3.33 larvae/cm^2. Generally,
209 females were larger than males in all treatments (Table 1). However, this gender difference in sizes
210 was not statistically significant in all treatments (independent samples t-test, t = 0.50, DF = 22, *P*
211 = 0.48). The largest male wing length was recorded in adults emerging from larvae reared at a
212 density of 0.83 larvae/cm^2 (2,598.93 ± 0.72 µm, n = 150). In comparison, the smallest was reported
213 from adults originating from larvae reared at 3.33 larvae/cm^2 (2,303.02 ± 0.34 µm, n = 600).
214 Statistically, there was a significant difference in male sizes between all treatments (one-way

ANOVA, F= 17.87, DF = 3, *P* = 0.001). The wing length of females followed the same trend (one-way ANOVA, F= 57.67, DF = 3, *P* = 0.00), the largest and smallest wing sizes were recorded from 0.83 and 3.33 larvae/cm^2 respectively (Table 1).

Impact of providing an extra anchoring surface on reducing overcrowding

Following assessment of the effect of larval density on the life-history traits of a colonised *An. funestus* strain, larvae reared at 3.33 larvae/cm^2, was considered most affected by overcrowding. This density resulted in longer developmental time, reduced larval survival and smaller adults. As a result, an experiment was set up where an extra anchoring surface was added to determine if this could reduce overcrowding when rearing larvae at high density.

Larval development time and adult emergence

The median larval developmental time from L1 to pupation was 16.00 ± 1.62 days (n = 6,719) for larvae reared without an extra anchoring surface (control) and 18.00 ± 1.83 days (n = 10,530) for those reared with an extra anchoring surface (treatment) (Table 2). The difference in developmental time between the two cohorts was statistically significantly (log rank test, χ^2 = 5,941.36, DF = 1, *P* = 0.00). The median proportion of larvae developing through to pupae for larvae reared without an extra anchoring surface and those reared with an extra anchoring surface was 56.00 (1)% (n = 6,719) and 88.00 (0)% (n = 10,530) respectively (Table 2). This difference in pupal productivity was statistically significant (χ^2 = 2,993.35, DF = 1, *P* = 0.00). Proportion of pupae emerging into adults was 94.00 (0)% (n = 6245) for pupae emanating from larvae reared without an extra anchoring surface and 91.00 (0)% (n = 9701) for those reared with an extra anchoring surface. Statistically, this difference was not significant (χ^2 = 55.74, DF = 1, *P* = 0.16). The median time taken by pupa to emerge into adults was two days for both pupal cohorts (Table

237 2). Sex ratio (male:female) of the resultant adults was 1: 1 for both the control (one- sample t- test,
238 t = 0.13, DF = 6,244, *P* = 0.99) and treatment (one- sample t- test, t = 0.11, DF = 9,700, *P* = 0.91).

239 ***Wing length measurements***

240 The overall mean wing length regardless of gender for adults emerging from larvae reared with an
241 extra anchoring surface was 2,493.38 ± 3.37 µm, and 2,458.72 ± 6.98 µm for larvae reared without
242 an extra anchoring surface (Table 2). The difference in overall wing sizes was statistically
243 significant (independent samples t-test, t = -4.47, DF = 2,398, *P* = 0.00). Generally, females were
244 consistently bigger than males regardless of treatment. When adult sizes were split by gender,
245 females emerging from larvae reared with an extra anchoring surface had larger wing sizes
246 2,523.13 ± 4.08 µm (n = 600) compared to those from larvae reared without an extra anchoring
247 surface (2,505.58 ± 6.82 µm, n = 600), this difference was statistically significant (independent
248 samples t-test, t = -0.207, DF = 1,198, *P* = 0.03). The same result was observed in males, 2,463.62
249 ± 4.46 µm (n = 600) and 2,411.85 ± 11.25 µm (n = 600) for adults from the treatment and control
250 respectively and this difference was also statistically significant (independent samples t-test, t = -
251 4.28, DF = 1,198, *P* = 0.00).

252 **Discussion**

253 This study is the first to report the effect of larval density on various life-history traits of a colonised
254 *An. funestus* strain. The objective was to obtain the optimal and restrictive larval rearing density
255 for *An. funestus* to provide guidelines for the colonisation of this species. FANG has been under
256 colonisation for numerous years, but the suitable larval rearing density in routine colony
257 maintenance has been based on supposition and experience. In addition, the effect of larval density
258 on the development of this species has never been studied.

Substantial differences in larval developmental time between larvae reared at different densities were observed. With the daily feeding regimen used in the study, it was unlikely to account for the food that remained unconsumed and constitutes a limitation of the study. An increase in density significantly prolonged the developmental time of the *An. funestus* strain used in this study. The notion that rearing *An. funestus* larvae at high density affect their developmental time was established. Some studies have shown that larval developmental time increases with increased larval density in *An. arabiensis* (Mamai *et al.*, 2018), *An. gambiae* (Muriu *et al.*, 2013) and *An. stephensi* (Yadav *et al.*, 2017). In contrast, other authors have shown that rearing larvae at high density shorten the developmental time in *An. gambaie* (Lyimo *et al.*, 1992), however, this might be due to different strains and rearing conditions. Several factors might be attributed to the longer larval developmental time observed at higher density during this study. It could be possible that competition among larvae suppressed larval weight resulting in prolonged developmental time (Roberts and Kokkinn, 2010). The production of growth retardant chemicals (Moore and Fisher, 1969; Ikeshoji and Mulla, 1970; Roberts, 1998), physical disturbance caused by larval collision, and increased production of metabolic wastes (Roberts and Kokkinn, 2010) disrupt growth. The effects of prolonged developmental time are not restricted to the affected generation but may affect subsequent generations. This, in turn, negatively affects laboratory colonisation success, particularly in the standpoint of mass rearing. Moreover, extended time to pupation results in delayed adult eclosion (Warner and Chesson, 1985), consequently increasing the operational cost of rearing.

This study showed a reduced proportion of first instar larvae surviving to pupation as larval density increased. The same was also observed in the proportion of pupae emerging to adults. This observation in *An. funestus* supports results from other species e.g. *An. arabiensis*, *An. gambiae*,

An. coluzzii and *An. stephensi* (Giles *et al.*, 2011; Muriu *et al.*, 2013; Yadav *et al.*, 2017; Epopa *et al.*, 2018). Reduced larval and pupal survival observed in this study is most likely due to intraspecific competition caused by larval crowding resulting in exhaustion of nutrients and the production of several toxic wastes by the overcrowded larvae (Bédhomme *et al.*, 2005). Furthermore, overcrowding causes turbidity in the water surface due to larval waste and microbial growth. This can result in reduced oxygen diffusion on the water surface as well as mechanical hindrance of siphonal respiration, adversely affecting the survival of larvae and pupae (Asahina, 1964). High larval and pupal mortality rates are undesirable when rearing mosquitoes in a laboratory as these decrease the overall rate of insect production, negatively impacting laboratory colonisation potential and success.

In this work, FANG adult wing lengths, i.e. adults sizes varied depending on the larval density in which the adults originated. Generally, larvae reared at low densities produced the largest adults regardless of gender, while those reared at high densities had smaller adults emerging. This result is congruent with several other studies that showed a negative correlation between larval rearing density and resultant adult sizes (Ng'habi *et al.*, 2005; Muriu *et al.*, 2013; Epopa *et al.*, 2018). The result of this study indicates that rearing *An. funestus* larvae at high density negatively impact both female and male sizes. This indirectly affects the potential of *An. funestus* laboratory colonisation because smaller females are known to be less fecund (Clements, 1992; Lyimo and Takken, 1993). Low fecundity levels are unfavourable during laboratory rearing, leading to colony collapse. Similarly, larger males have been shown to have a reproductive advantage over their smaller counterparts, at least in *An. gambiae* and *Ae. aegypti* (Helinski and Harrington, 2011; Sawadogo *et al*., 2013). However, no such data exist for *An. funestus*, which offers future research avenues.

Rearing *An. funestus* larvae at low and high larval densities did not bias any adult gender production. These observations are congruent with the findings of Mamai *et al.* (2018) where no differences in the sex ratio of *An. arabiensis* adults were observed at alternating densities. Balanced sex ratio is a desirable trait when colonising mosquitoes.

Laboratory colonised *An. funestus* larvae anchor on the edges of the rearing container (personal observation), probably as adoption from natural behaviour. It was therefore hypothesised that adding an extra anchoring surface to larvae reared at high densities could increase the density of mosquitoes that can be bred per surface area. Adding an extra anchoring surface reduces the surface area available to larvae and this potential confounding effect increased the larval density from 3.33 larvae/cm^2 (no anchoring surface) to 3.44 larvae/cm^2. This might explain the extended larval developmental time, but it increased the proportion of larvae surviving to pupation and resulted in larger adults. Shorter larval developmental time in larvae reared without extra anchoring substance could be ascribed to high early instar larval mortalities due to competition for anchoring surface. This later resulted in more food and less crowding on the remaining larvae, subsequently reducing time to pupation. This was previously observed by Yadav *et al.* (2017). It should be noted that there was a five-day difference in larval developmental time between larvae reared at 3.33 larvae/cm^2 as part of the larval density experiments (Table 1) and the larvae reared at 3.33 larvae/cm^2 as part of the anchoring surface experiments (Table 2). Although the experimental set-up was the same, these two experiments were conducted four months apart, and the variation could be ascribed to unknown food quality variation, temperature, humidity fluctuations or other unknown factors.

Significant decrease in larval mortality after adding an extra anchoring surface probably resulted from reduced competition for anchoring surface. This resulted in reduced early instar mortality, a

bottleneck during rearing larvae at high densities. The significant relationship between adult size and available anchoring surface strengthens the theory that anchoring surface is more important than water surface area during *An. funestus* larval development. This is particularly important in mass rearing and designing equipment, where large quantities of larvae can be reared with low space requirements. Larval crowding negatively affected larval development and consequently had an impact on adult sizes. In the anchoring experiments, it can be speculated that the additional anchoring surface could have prevented the overcrowding effects leading to the emergence of larger adults. It was not possible with the current experimental design to have a consistent larval density (larvae/cm^2) between the control (without an anchoring surface) and treatment (with an additional anchoring surface) experiments, which is a limitation of this study.

In summary, this study helped to understand the relationship between larval density and several mosquito life-history traits. Under standard laboratory conditions, density-dependent competition and alterations negatively influenced the development and adult size of *An. funestus* in ways that have consequences for successful laboratory colonisation. The addition of an extra anchoring surface subsequently altered the harmful effects of overcrowding and resulted in increased larval survival and larger adult sizes. It is therefore ideal to rear larvae at 0.83 larvae/cm^2 to ensure optimal pupal production; alternatively the addition of anchoring surface can be used at higher larval densities. Furthermore, results from this study indicate that the anchoring surface is more important than the surface area at high larval densities. This information will help to standardise rearing of *An. funestus* under laboratory conditions in different geographical areas. The longevity and survival of the resultant adults was not investigated in this study as previous studies have revealed negative effects of high larval density on post-emergence adult longevity and survival (Ombok *et al*., 2002; Reiskind and Lounibos, 2009; Ukubuiwe *et al*., 2019). However, this needs

to be confirmed in this species. Further studies on the impact of larval density on fecundity, fertility and adult longevity, exploring different material, and size of the extra anchoring surface feasible for laboratory rearing are recommended.

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Author contributions

MPZ: Assisted with project design, acquisition of data, analysis and interpretation of data. Drafted the first and subsequent versions of the manuscript. GM: Assisted with project design, interpretation of data, data analysis, provided critical revisions on the manuscript. FO: provided critical revision on the manuscript. LLK: Conception and design of the study, interpretation of data, provided critical revision on the manuscript and approved the final version to be submitted.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Figure Legends

Figure 1: Experimental set up used during the assessment of the impact of adding an anchoring substance on reducing overcrowding: a = 3.33 larvae/cm^2 with a total anchoring perimeter of 640mm without an anchoring surface (Control); b = 3.33 larvae/cm^2 with a wax paper anchoring surface constituting a total of 1,672mm anchoring perimeter.

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