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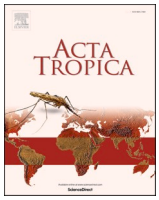

Optimisation of laboratory-rearing parameters for *Anopheles funestus* larvae and adults

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ABSTRACT

Anopheles funestus is one of the major malaria vectors in Africa. As with the other main vectors, insecticide resistance in this species threatens existing vector control strategies. Unfortunately, scientific investigations, which could improve understanding of this vector species or lead to the development of new control strategies, are currently limited by difficulties in laboratory rearing of the species. In an attempt to optimise laboratory-rearing conditions for *An. funestus,* the effect of an artificial blood-feeding system for adults, different larval diet doses, and a range of other rearing conditions on the life history traits of an existing colony were investigated. Firstly, fecundity and fertility in *An. funestus* adult females fed on either live guinea pigs or bovine blood supplied through an artificial membrane feeding system were assessed. Secondly, a life-table approach was used to assess the impact of larval food dose (mg/larvae), larval density (larvae/cm^2), and the depth of water used for larval rearing on life history traits. Fecundity was significantly higher when females were blood-fed on live anaesthetised guinea pigs than when fed on defibrinated bovine blood. However, the fertility of these eggs did not differ significantly between the two feeding methods or blood meal sources. Mosquitoes fed on defibrinated bovine blood using the artificial membrane feeding system showed an increase in egg production when the blood-feeding frequency was increased, but this difference was not statistically significant. The quantity of larval food influenced both time-to-pupation and pupal production. Increasing the larval densities resulted in reduced both time-to-pupation and pupal productivity. An optimal larval density of 0.48 larvae/cm^2 was vital in preventing overcrowding. Increased water depth in the larval trays, was associated with significantly lower pupal production and reduced pupal weight. In conclusion, these results show that *An. funestus* can be reared using defibrinated bovine blood delivered via an artificial membrane feeding system. The quantity of larval food, optimal larval density, and depth of water used for larval rearing are critical factors influencing colony productivity. These findings can be used to improve current guidelines for rearing *An. funestus* under insectary conditions.

1. Introduction

In 2020, malaria deaths increased by 12% compared to 2019, partly affected by disruptions to malaria control efforts during the COVID-19 pandemic (WHO, 2021). *Anopheles funestus* is recognised as a primary malaria vector that is highly anthropophagic (human feeding) and endophilic (resting indoors) (Gillies and De Meillon, 1968; Manga et al., 1997; Coetzee and Koekemoer, 2013). These behavioural traits make this species highly susceptible to indoor-insecticidal interventions, notably long-lasting insecticide-treated nets and indoor residual spraying. However, the rapid escalation of insecticide resistance has become a significant threat to the sustainability of these interventions (Fontenille

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et al., 1990; Coetzee and Koekemoer, 2013; Hancock and Hendriks, 2020; Moyes and Athinya, 2020). Because of this and other challenges, several alternative interventions are being developed. amongst these are genetic bio-control options such as the sterile insect technique (SIT) and genetically modified mosquitoes (Benedict and Robinson, 2003; James, 2005; Bourtzis et al., 2016). Some of these technologies will require a greater understanding of the biology of the target vector species and the colonisation of the vector species to support field release.

Unfortunately, for *An. funestus*, the scarcity of viable laboratory colonies and its refractoriness to colonisation presents a considerable challenge for developing bio-control strategies against this species (Service and Oguamah, 1958; Service, 1960; Gillies and De Meillon, 1968). There is limited knowledge on optimal laboratory rearing conditions for this species compared to other vector species, limiting the viability of bio-control technologies. Other than the early attempts in Nigeria in the 1950s (Service and Oguamah, 1958), only two stable *An. funestus* colonies, one from Mozambique in 2000 and the other from Angola in 2002, have been successfully established starting from wild material (Hunt et al., 2005; Zengenene et al., 2021). While these two colonies (which have been widely shared with multiple laboratories) enable key studies on this vector species, there remains a desire to colonise and study local *An. funestus* populations in different countries. To achieve this and potentially enable mass-rearing, the critical parameters that promote *An. funestus* proliferation under laboratory conditions need to be investigated and optimised.

One critical rearing parameter for successfully colonising mosquito species is having a sustainable method of providing a blood meal to adult female mosquitoes to facilitate egg development. Most *Anopheles* mosquitoes are anautogenous and depend on blood for reproductive success (Hansen et al., 2014). The blood provides key amino acids and proteins required for egg production (Briegel, 1990; Clements 1992; Takken et al., 1998; Olayemi et al., 2011; Gonzales and Hansen, 2016). *Anopheles funestus* feeds primarily on humans (Kahamba et al., 2022), a characteristic that may become a significant bottleneck to colonisation, especially in laboratories that rely on non-human vertebrates for the blood meal sources. Successful colonisation at scale may also require a change from direct human blood-feeding to animal or artificial membrane feeding systems (Gerberg, 1970; Benedict et al., 2007; Olayemi et al., 2011; LaFlamme, 2011). Current and past *An. funestus* colonies have been successfully maintained on either guinea pigs alone or both guinea pigs and human blood (Service and Oguamah, 1958; Hunt et al., 2005; Ngowo et al., 2021). This presents a challenge due to the large number of animals required, ethical concerns and cost implications associated with such blood delivery approaches (Gonzales and Hansen, 2016).

Besides blood-feeding, optimal larval rearing conditions are also necessary to ensure the consistent production of "high-quality" healthy adults (Damiens et al., 2012; Somda et al., 2017). The quality and quantity of larval food affect the developmental time and the number of emerging adults (Damiens et al., 2012; Somda et al., 2017). Harold (1930) initiated the first research on larval food for *Anopheles, Aedes* and *Culex* mosquitoes (*Culicidae*) almost a century ago. Since then, various studies to optimise larval rearing conditions have been conducted on multiple anophelines, including *An. darlingi* (Araujo et al., 2012), *An. stephensi* (Reisen et al., 1975) and *An. arabiensis* (Gilles et al., 2011; Damiens et al., 2012; Somda et al., 2017). Increased larval densities can also lead to extended larval development periods (Zengenene et al., 2022). This phenomena of extended larval development time and smaller *An. gambiae* adults, was also observed when water-rearing depths were increased (Phelan and Roitberg, 2013). However, there remains a limited number of larval rearing studies conducted on *An. funestus* (Ngowo et al., 2021; Zengenene et al., 2021, 2022).

To address the gaps, blood-feeding and larval rearing of *An. funestus* were investigated. These studies constituted an attempt to optimise laboratory-rearing conditions for this vector species.

2. Materials and methods

2.1. Biological material

All experiments were conducted using mosquitoes from the FUMOZ (*An. funestus* from Mozambique) colony (Hunt et al., 2005). Aquatic stages were maintained on a standard powdered larval diet consisting of crushed dog biscuits (Beeno®, South Africa, http://www.beeno.co.za) and brewer's yeast (Vital®, Vital Health Foods, South Africa), mixed at a 3:1 ratio respectively. The nutritional composition of the dog biscuits is provided in Zengenene et al. (2022). Adult mosquitoes were sustained on a 10% sucrose solution, and blood meals were provided using anaesthetised guinea pigs. Adult females were fed twice a week, and egg plates were provided after at least two blood meals.

2.2. Assessing the effect of different blood meal sources on fecundity and fertility

A total of 200 adults (100 virgin males and 100 virgin females) were allowed to mate in a BugDorm-4S3030 cage (32.5 cm x 32.5 cm x 32.5 cm; BugDorm®, Megaview Science Co., Ltd, Taichung, Taiwan, hereafter referred to as BugDorm cage) for 10 consecutive days with ad libitum access to 10% sugar solution. After the mating period, the 10-day-old females were sugar-starved for 24 h and provided with either defibrinated bovine blood (for 30 min) via an artificial membrane feeding system (Hemotek®, Hemotek Ltd, United Kingdom) or a live shaven anaesthetised guinea pig (for 15 min). The use of live guinea pigs, being the standard blood-feeding method used to maintain *An. funestus* colonies at the Botha de Meillon insectary, at the National Institute for Communicable Diseases, Johannesburg, was considered as the control cohort.

Artificial membrane blood feeding was done by adding 5 ml of defibrinated bovine blood to a preheated (37 °C) aluminium heating plate (3.7 cm in diameter and 1.3 cm thick) covered with hog casing membrane and connected to the Hemotek® membrane feeding system (Cosgrove et al., 1994; Damiens et al., 2013).

Feeding occurred in a dark room when adults were 11 days old (first blood meal) and again when they were 14 days old (second blood meal). Blood-fed females were not removed after first blood feeding to mimic routine rearing procedures for the colony. However, males and unfed females were removed after the second blood meal. The blood-fed females were counted to determine the feeding success. Subsequently, fecundity of these blood-fed females and fertility of resultant eggs was determined. These experiments were repeated three times using different biological material (biological repeats). Each biological repeat consisted of three replicates (for the artificial membrane feeding system) or one replicate (guinea pig feeding to limit the number of guinea pigs needed) (Appendix Table A1a).

Egg harvesting: Egg plates containing purified water were provided 48 h after the second (last) blood meal to induce females to oviposit. Eggs were harvested 24 h later. Water used was purified using reverse osmosis, which filters ionic contaminants, most organic compounds and all particulates from the water. However, the hardness of the water used for larval rearing was not analysed.

Percentage insemination: After egg harvesting, the spermathecae of 10 randomly-selected females from each cage were dissected to determine the percentage of inseminated females.

Fecundity: Fecundity (F) for the first gonotrophic cycle was calculated as the number of eggs per female after adjusting the total number of females (fn) with percentage insemination rate (i), as follows: Adjusted number of females (afn) = fn x i and Fecundity (F) = number of eggs (ne)/afn. The number of females used to calculate fecundity was the number alive in a cage when the cage was egg plated. It was not possible to determine whether females that died 24 hrs post egg plating contributed to egg production or not, therefore, no adjustment for female mortality was done post egg platting.

Fertility: Harvested eggs were transferred into small round larval bowls (9 cm base radius x 12 cm top radius with a height of 6 cm) filled with 100 ml of purified water. The eggs were monitored for hatchlings daily for 14 days. Fertility was calculated as the number of larvae hatched divided by the total number of eggs used (Munhenga et al., 2016).

2.3. Assessing the effect of blood feeding frequency using the artificial membrane feeding system on fecundity and fertility

Female *An. funestus* were either fed twice (Monday and Thursday) or four times (Monday, Tuesday, Thursday, and Friday) on defibrinated bovine blood using the artificial membrane feeding system as described in Section 2.2. Fecundity and fertility were estimated using the same cages and adult density as described in Section 2.2 above (see Appendix Table A1b).

2.4. Assessing the effects of larval diet dose on time-to-pupation, pupal production, adult emergence, pupal weight and wing length

One hundred first instar larvae were placed into standard larval trays (21 cm (length) × 15 cm (width) × 8 cm (height), containing 750 ml of purified water, resulting in a larval density of 0.32 larvae/cm^2. Five food weights (doses) (0.02; 0.03; 0.04; 0.06; 0.08 mg/larva) of the standard larval diet described in Section 2.1 were evaluated. Larvae were fed twice daily, with food quantity increasing proportionally daily until pupation (Table 1). One biological repeat comprising of three technical replicates and a total of three biological repeats were conducted.

Time-to-pupation: larvae were monitored daily until pupation. The number of days between the first instar stage of larvae to pupae was recorded and survival analysis was used to calculate the median time to pupation. As the ambient temperature was controlled and recorded, the water temperature was not recorded.

Pupal production: The total number of pupae obtained was recorded and represented as a proportion of the total number of first instar larvae used.

Pupal weight: Due to limited pupal numbers, a subset of 30 male and 30 female pupae were randomly selected from each treatment. Each pupa was placed onto filter paper to absorb and remove excess water before being weighed using an analytical balance (KERN ABT 120–5DM, Balingen, Germany). Overall mean weight was calculated.

Adult emergence: Adult emergence was monitored and recorded for all pupae after excluding those used for weight measurements. Percentage emergence was calculated as the number of emerged adults divided by the number of pupae for each diet dose.

Wing length: Upon emergence, a subsample of 60 adults (30 females and 30 males) per treatment were immobilised in a refrigerator (4 °C), and used for wing length measurements (as a proxy for adult size). The right wing was gently dissected from the body using forceps, placed onto a glass slide, and then viewed under a microscope (SZ2-ILST, Olympus Corporation Tokyo, Japan) fitted with a camera (S2 × 7). Wing length was measured using Olympus Stream Essentials 1.9.4 from the distal edge of the allula to the end of the radial vein, excluding the fringe scales (Marina et al., 1999).

2.5. Assessing the effects of larval densities on time-to-pupation, pupal production, adult emergence, pupal weight and wing length

Five different larval densities (0.25, 0.32, 0.48, 0.64, 1.27 larvae/cm^2) were evaluated under standard rearing conditions described in Section 2.1. Larvae were fed twice daily at a dose of 0.04 mg/larva of the standard larval diet (Table 1), based on the diet dose experiment above. The experiments were divided into two stages. Stage one evaluated 0.25, 0.32 and 0.48 larvae/cm^2, while stage two investigated 0.32, 0.64 and 1.27 larvae/cm^2. One biological repeat comprised of three technical replicates, and a total of three biological repeats were conducted. The parameters assessed were the same as in the diet dose experiment (Section 2.4).

2.6. Assessing the effects of water depth on time to pupation, pupal production adult emergence, pupal weight and wing length

Three different water depths (1, 3, and 5 cm) were compared. Larvae were reared as described above (Section 2.5) with a larval density of 0.48 larvae/cm^2 and were fed 0.04 mg/larva of larval food twice a day. The evaluation consisted of three biological repeats, each comprising five technical replicates. The parameters assessed were the same as in the diet dose experiment (Section 2.4).

2.7. Data analysis

Statistical analyses were completed using statistical software StataCorp, version 14 (2015) at a 0.05 significance level. The Shapiro-Wilk normality test was used to assess normality of variables (Shapiro and Wilk, 1965). Two-sample t-tests were used to compare fecundity and fertility. Kaplan-Meier survival analysis (Kaplan and Meier, 1958) was used to estimate the time-to-pupation. Log-rank tests were used to test for equality of survivor functions between the different treatments. A one-way analysis of variance (ANOVA) (Anscombe, 1948) was used to compare proportions of pupae produced, proportions of adults produced, wing length and pupal weight between treatments and controls. Where a significant difference was observed, Tukey's "honestly significant difference" (HSD) *post hoc* test was used to determine differences between pairs. For data that did not fit the assumptions of normality, Kruskal-Wallis tests (Kruskal and Wallis, 1952) were used to determine differences for each variable. Differences between pairs were then analysed using pairwise χ^2 tests.

2.8. Ethics

The blood-feeding protocol for routine colony rearing was approved by the National Health Laboratory Service, Animal Ethics Committee (AESC: 1993–047).

3. Results

3.1. Effects of different blood meal sources on fecundity and fertility

The percentage of females that were successfully fed (% blood-fed ±

Table 1
Anopheles funestus larval feeding regimen using standard larval diet. Larvae were fed the amounts indicated in mg/larva twice a day i.e. once each in the morning and afternoon.

Diet dose / feeding	Treatment	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13 until end
	T1	0.02	0.03	0.04	0.06	0.08	0.10	0.12	0.14	0.20	0.26	0.32	0.40	0.50
	T2	0.03	0.04	0.05	0.07	0.09	0.12	0.15	0.20	0.25	0.30	0.35	0.40	0.50
	T3	0.04	0.05	0.06	0.08	0.10	0.14	0.18	0.22	0.28	0.32	0.40	0.45	0.50
	T4	0.06	0.08	0.10	0.14	0.18	0.24	0.30	0.40	0.50	0.60	0.70	0.80	1.00
	T5	0.08	1.00	0.12	0.16	0.20	0.28	0.36	0.44	0.56	0.64	0.80	0.90	1.00

Diet dose / feeding (mg/larva): larvae were fed twice a day using the quantity of food indicated in the table.
T: Treatment; D1: Day one, etc.

S.D.) did not differ significantly between females provided with anaesthetised guinea pigs (53.00% ± 4.58; $n = 300$) and those provided with defibrinated bovine blood using the artificial membrane feeding system (48.78% ± 11.04; $n = 900$) (Independent t-test, $t_{(10)} = 0.63$, $p = 0.5442$) (Table 2; Appendix Table A1a). Furthermore, the percentage of females inseminated (% insemination ± S.D.) amongst mosquitoes fed on guinea pigs was 83.33% ± 15.27, ($n = 30$), and 62.22% ± 23.33, ($n = 90$) for those fed on bovine blood using the artificial membrane feeding system. These differences were not statistically significant (independent t-test, $t_{(10)} = 1.44$, $p = 0.1799$).

Females fed on guinea pigs produced significantly more eggs per female (10.94 ± 0.77; $n = 1460$) than those fed on bovine blood via the artificial membrane feeding system (2.88 ± 2.09; $n = 664$); ($t_{(10)} = 6.37$, $p = 0.0001$). However, the egg fertility (% of eggs hatching into larvae) did not differ between blood sources (guinea pigs (79.56% ± 9.34; $n = 826$); bovine blood (77.33% ± 14.44; $n = 664$); two sample t-test ($t_{(10)} = 0.25$, $p = 0.8105$)).

3.2. Effects of blood feeding frequency using the artificial membrane feeding system on fecundity and fertility

The percentage of females that took a blood meal (% blood-fed ± S.D.) when fed twice using the artificial membrane feeding system (46.33% ± 13.65, $n = 300$) did not differ significantly from those fed four times (51.44% ± 10.98, $n = 900$), (independent t-test, $t_{(10)} = -0.66$, $p = 0.5223$), (Table 2, Appendix Table A1b). The percentage of females inseminated (% insemination ± S.D.) were also similar between the two groups (two meals: 90.00% ± 10.00 ($n = 30$); four meals: 71.11% ± 24.72 ($n = 90$); independent t-test ($t_{(10)} = 1.26$, $p = 0.2377$)). However, the number of eggs produced per female was doubled in females fed four times (4.61 ± 2.67; $n = 1554$) compared to females fed twice (2.3 ± 1.58; $n = 317$), even though this difference was not statistically significant (independent t-test, $t_{(10)} = -1.39$, $p = 0.0977$). The mean fertility (% of eggs hatching ± S.D.) of eggs laid by females that were blood-fed twice was 81.28% ± 16.06 ($n = 317$) compared to 83.85% ± 13.13 ($n = 1554$) in eggs from females blood-fed four times, this difference was statistically insignificant ($t_{(10)} = -0.28$, $p = 0.7829$).

3.3. Effects of larval diet dose on time-to-pupation, pupal production adult emergence, pupal weight and wing length

Time-to-pupation: Larval development time (time-to-pupation) was calculated as the time taken by the first instar larvae to develop into pupae. The median times-to-pupation in days (IQR: Q1 - Q3) for *An. funestus* reared on 0.02, 0.03, and 0.04 mg/larva of the larval diet were 16 (15 - 17), 15 (14 – 17), and 15 (14 – 17) days respectively (Table 3). Further analysis of larval survival distributions using the log-rank test showed that the time-to-pupation differed significantly between the three larval doses ($\chi^2_{(2, n = 2189)} = 79.45$, $p < 0.0001$). The larvae fed on the lowest diet dose (0.02 mg/larvae) had the longest time-to-pupation, i.e. 16 (15 - 17) days (Table 3). Subsequent pairwise comparisons showed a significant difference in time-to-pupation between 0.02 mg/larva and either 0.03 mg/larva ($\chi^2_{(1, n = 1389)} = 31.68$, $p < 0.0001$) or 0.04 mg/larva ($\chi^2_{(1, n = 1467)} = 83.93$, $p < 0.0001$). This was also true when comparing time-to-pupation between 0.03 mg/larva and 0.04 mg/larva ($\chi^2_{(1, n = 1522)} = 10.18$, $p = 0.0014$). In the second stage experiments, the median times-to-pupation were 17 (16 - 17), 12 (12 – 15) and 13 (12–16) days when larvae were fed 0.04, 0.06 and 0.08 mg/larva of the diet, respectively (Table 3). However, no subsequent statistical analysis was conducted due to low pupal production (see below).

Pupal production: Pupal production between the diet doses differed significantly ($F_{(2, 24)} = 6.22$, $p = 0.0067$) in the first stage experiments. The highest pupal production (% pupae produced ± S.D.) was from larvae fed at 0.04 mg/larva (88.89% ± 5.86, $n = 800$), and the lowest from larvae fed at 0.02 mg/larva (74.11% ± 10.07, $n = 667$) (Table 3). In the second stage experiments, the 0.06 and 0.08 mg/larva doses yielded only 0.67% and 9.11% of pupae, respectively (Table 3), and were considered unsuitable for rearing *An. funestus*. Downstream analysis was impossible and therefore excluded in subsequent data analyses.

Pupal weight: The mean pupal weight (± S.D.) was 1.34 ± 0.10; 1.36 ± 0.11 and 1.35 mg ± 0.12 mg for larvae fed at 0.02, 0.03 and 0.04 mg/larva respectively. These were not statistically different (Kruskal-Wallis; $H_{(2)} = 2.518$, $p = 0.2840$; $n = 540$).

Adult emergence: Adult emergence ranged from 93.76% ± 3.76 ($n = 487$) for larvae fed at a dose of 0.02 mg/larva to 96.82% ± 2.59 ($n = 542$) for those fed at 0.03 mg/larva. Feeding larvae at a higher dose of 0.04 mg/larva produced 96.58% ± 2.96 ($n = 599$) emergence of adults from the pupae. There were no significant differences in emergence between the larval diet doses (ANOVA: $F_{(2, 24)} = 2.65$, $p = 0.0915$).

Wing length: Mean wing lengths (± S.D.) for larvae reared at 0.02 ($n = 180$), 0.03 ($n = 180$) and 0.04 mg/larva ($n = 180$) of the diet were 3.28 ± 0.16, 3.27 ± 0.12 and 3.31 mm ± 0.17 respectively (Table 3). The Kruskal-Wallis analysis showed no significant differences in wing lengths between these diet doses ($H_{(2)} = 3.529$, $p = 0.1713$).

3.4. Effects of larval densities on time-to-pupation, pupal production adult emergence, pupal weight and wing length

Time-to-pupation: Larvae reared at a very high density of 400 larvae per tray (1.27 larvae/cm^2) had the shortest time-to-pupation. The median (IQR: Q1 – Q3) times-to-pupation were 19 (17 - 21), 17 (16 - 19), and 16 (15 - 17) days for the three rearing densities 0.25, 0.32 and 0.48 larvae/cm^2, respectively (Table 4). Log-rank test analysis indicated significant differences in time-to-pupation between the rearing densities ($\chi^2_{(2, n = 2001)} = 405.21$, $p < 0.0001$). Larvae reared at the lowest density (80 larvae/tray or 0.25 larvae/cm^2) had the longest time-to-pupation from first instar larvae to pupae. Subsequent pairwise comparisons showed there were significant differences in time-to-pupation when 0.25 larvae/cm^2 were compared to 0.32 larvae/cm^2 ($\chi^2_{(1, n = 973)} = 149.23$, $p < 0.0001$) and 0.48 larvae/cm^2 ($\chi^2_{(1, n = 1367)} = 369.83$, $p < 0.0001$). Time-to-pupation of larvae reared at 0.32 larvae/cm^2 was significantly longer than larvae reared at a density of 0.48 larvae/cm^2 ($\chi^2_{(1, n = 1662)} = 78.78$, $p < 0.0001$). Similarly, analysis for stage two showed significant differences in time-to-pupation between the three larval rearing densities (Log-rank test: $\chi^2_{(2, n = 2454)} = 26.93$, $p < 0.0001$). Subsequent pairwise analyses also showed significant differences between 0.32 larvae/cm^2 and 0.64 larvae/cm^2 ($\chi^2_{(1, n = 1467)} = 4.73$, $p = 0.0297$), 0.32 larvae/cm^2 and 1.27 larvae/cm^2 ($\chi^2_{(1, n = 1639)} = 20.30$, $p < 0.0001$), and 0.64 larvae/cm^2 and 1.27 larvae/cm^2 ($\chi^2_{(1, n$

Table 2
Summary of blood feeding outcome of *An. funestus* females fed on A) live animal host (control) and artificial blood feeding system (treatment) and B) two blood meals vs four blood meals through an artificial blood feeding system.

	Blood feeding approach (N)	% females blood fed ± S.D. (n)	% females inseminated ± S.D. (n)	Number of eggs laid/ female ± S.D.	% Egg fertility ± S.D. (n)
A	Anaesthetised guinea pig (300)	53 ± 4.58 (159)	83.33 ± 15.28 (30)	10.94 ± 0.77	79.56 ± 9.34 (826)
	Artificial feeding using Hemotek feeder (900)	48.78 ± 11.04 (439)	62.22 ± 23.33 (90)	2.88 ± 2.09	77.33 ± 14.44 (664)
B	Artificial feeding using Hemotek feeder for two blood meals (300)	46.33 ± 13.65 (139)	90 ± 10.00 (30)	2.30 ± 1.58	81.28 ± 16.06 (317)
	Artificial feeding using Hemotek feeder for four blood meals (900)	51.44 ± 10.98 (463)	71.11 ± 24.72 (90)	4.61 ± 2.67	83.85 ± 13.13 (1554)

N= total number of females used; *n*= number of females that fed, inseminated or number of laid eggs and hatched.

Table 3
Summary of developmental parameters of *An. funestus* larvae reared at different larval diet doses.

	Diet dose (mg/larva)	Total number of L1 used	Developmental parameter monitored: Median time-to-pupation in days (IQR)	Total number of pupae from L1	Mean proportion of L1 developing into pupae (S.D.)%	Mean pupal weight (S.D.) mg	Total number of pupae used for adult emergence *	Total number of adults emerged	Mean proportion of pupae developing into adults (S.D.)%	Mean wing length (S.D.) mm
Stage 1	**0.02**	900	16 [15 - 17][a]	667	74.11 (10.07)[a]	1.34 (0.10)	487	455	93.76 (3.76)	3.28 (0.16)
	0.03	900	15 [14 - 17][b]	722	80.22 (10.18)[ab]	1.36 (0.11)	542	524	96.82 (2.59)	3.27 (0.12)
	0.04	900	15 [14 - 17][c]	800	88.89 (5.86)[b]	1.35 (0.12)	620	599	96.58 (2.96)	3.31 (0.17)
Stage 2	**0.04**	900	17 [16 −17]	678	75.33 (18.01)	NA	NA	NA	NA	NA
	0.06	900	12 [12 - 15]	6	0.67 (1.12)	NA	NA	NA	NA	NA
	0.08	900	13 [12 - 16]	82	9.11 (17.95)	NA	NA	NA	NA	NA

* indicates the total number of pupae available for adult emergence after pupae were removed for pupal weight.

Table 4
Summary of the effects of different larval rearing densities on the developmental parameters of *An. funestus* larvae.

	Larval rearing density (larvae/cm^2), (larvae/tray)	Total number of L1 used	Developmental parameter monitored: Median time-to-pupation in days (IQR)	Total number of pupae from L1	Mean proportion of L1 developing into pupae (S.D.)%	Mean pupal weight (S.D.) mg	Total number of pupae used for adult emergence□	Total number of adults that emerged	Mean proportion of pupae developing into adults (S.D.)%	Mean wing length (S.D.) mm
Stage 1	**0.25 (80)**	720	19 [17–21][a]	339	47.08 (24.28)[a]	1.36 (0.18)	161	128	81.30 (20.35)	3.36 (0.15)
	0.32 (100)	900	17 [16–19][b]	634	70.44 (12.29)[b]	1.37 (0.16)	454	392	86.07 (9.85)	3.33 (0.14)
	0.48 (150)	1350	16 [15–17][c]	1027	76.07 (14.53)[b]	1.37 (0.16)	757	696	91.85 (6.66)	3.33 (0.15)
Stage 2	**0.32 (100)**	900	15 [14–16][#]	652	72.44 (15.95) [#]	1.31 (0.15)[a]	472	421	89.18 (7.74)	3.30 (0.13)[a]
	0.64 (200)	1800	14 [13–15]*	815	45.28 (26.08) [#]*	1.35 (0.14)[b]	509	406	76.03 (20.54)	3.28 (0.12)[b]
	1.27 (400)	3600	12 [11–13][&]	987	27.42 (29.41)*	1.29 (0.12)[a]	570	501	86.44 (8.92)	3.24 (0.11)[c]

□ before pupae were taken for weighing

Superscript letters and symbols indicate significant differences between treatments, if there are no letters or symbols there were no significant differences between treatments.

$_{= 1802)} = 4.32$, $p = 0.0377$).

Pupal production: The percentage of pupae (mean% pupae produced ± S.D.) produced per treatment from stage one experiments ranged from 47.08 ± 24.28 ($n = 339$) to 76.07% ± 14.53 ($n = 1027$) (Table 4). The mean percentage pupal production differed significantly depending on the larval rearing density for stage one (Kruskal Wallis; $H_{(2)} = 7.308$, $p = 0.0259$). Pairwise comparisons within stage one showed that the pupal production from larvae reared at 0.25 larvae/cm^2 was significantly different from those reared at 0.32 larvae/cm^2 ($p = 0.0202$, 95% C.I. = [3.78 −38.69]) and 0.48 larvae/cm^2, ($p = 0.0073$, 95% C.I. = [4.50 - 24.49]). Pupal production did not differ between larval densities of 0.32 and 0.48 larvae/cm^2 ($p = 0.3865$).

The pupal production (% ± S.D.) from the second phase evaluations was lowest (27.42% ± 29.41) when larvae were reared at the highest density (1.27 larvae/cm^2) (Table 4). There were statistically significant differences in mean percentage pupal production between the different larval rearing densities (Kruskal Wallis; $H_{(2)} = 10.31$, $p = 0.0058$). Pairwise comparisons within stage two, revealed significant differences in mean percentage pupal production between larvae reared at a density of 0.32 larvae/cm^2 (72.44% ± 15.95) and 0.64 larvae/cm^2 (45.28% ± 26.08) ($p = 0.0169$, 95% C.I. = [−27.09, −3.09]). This was also true when comparing 0.32 larvae/cm^2 (72.44% ± 15.95) and 1.27 larvae/cm^2 (27.42% ± 29.41) ($p = 0.001$, 95% C.I. = [−24.52, −7.64]). Generally, there was a weak but significant relationship between larval rearing density and pupal production (r^2 (53) = 0.1379, $p = 0.0057$); pupal production decreased as the larval rearing density increased.

Pupal weight: The weight (mean weight ± S.D.) of pupae reared at different larval densities during stage one experiments were generally similar (1.37 mg ± 0.16 observed in the 0.48 and 0.32 larvae/cm^2 trays and 1.36 mg ± 0.18 in the 0.25 larvae/cm^2 tray). Kruskal-Wallis test showed no significant differences ($H_{(2)} = 0.71$, $p = 0.7014$).

However, in the second stage experiment, the lowest weight was obtained from the 1.27 larvae/cm^2 trays (1.29 mg ± 0.12, $n = 417$). The mean pupal weights for larvae reared at 0.32 and 0.64 larvae/cm^2 were 1.31 mg ± 0.15 ($n = 180$) and 1.35 mg ± 0.14 ($n = 306$) respectively, these differences being statistically significant (Kruskal-Wallis, $H_{(2)} = 30.59$, $p = 0.0001$). Pairwise comparisons showed a statistical difference between pupae reared at 0.64 and 0.32 larvae/cm^2 ($\chi^2_{(1,\ n\ =\ 486)} = 7.38$, $p = 0.0066$). Pupal weight from larvae reared at 0.64 larvae/cm^2 and 1.27 larvae/cm^2 also differed significantly ($\chi^2_{(1,\ n\ =\ 723)} = 30.94$, $p = 0.0001$). Comparisons of the pupal weight when larvae were reared at a density of 0.32 larvae/cm^2 and 1.27 larvae/cm^2 did not differ significantly ($\chi^2_{(1,\ n\ =\ 597)} = 2.79$, $p = 0.0952$). There was no correlation between larval density and pupal weight from stage two experiments ($r^2_{(902)} = 0.0037$, $p = 0.0680$).

Adult emergence: In stage one, the percentage of adult emergence ranged from 81.30 to 91.85% (Table 4), and there were no significant differences between the three larval rearing densities (Kruskal-Wallis; $H_{(2)} = 1.3256$, $p = 0.5372$). In stage two, the mean percentage of adult emergence ranged between 76.03 and 89.18% (Table 4), with no significant difference between these cohorts ($F_{(2,16)} = 1.82$, $p = 0.1944$).

Wing length: The mean wing length of adults emerging from larvae

reared at 0.25 larvae/cm^2 was 3.36 mm ± 0.15 (n = 92), 3.33 mm ± 0.14 for those reared at 0.32 larvae/cm^2 were (n = 180) and 3.33 mm ± 0.15 for those reared at 0.48 larvae/cm^2 (n = 2700). There were no statistically significant differences in wing lengths between the different cohorts (Kruskal-Wallis, $H_{(2)} = 2.30$, $p = 0.3169$). However, in the stage two experiments, there were significant differences in wing lengths between the cohorts (Kruskal-Wallis, $H_{(2)} = 39.74$, $p = 0.0001$) (Table 4). Pairwise comparisons showed that wing lengths in adults emerging from larvae reared at 0.32 larvae/cm^2 (3.30 mm ± 0.13, n = 180) differed significantly from those reared at 0.64 larvae/cm^2 (3.28 mm ± 0.12, n = 253), ($\chi^2_{(1,\ n\ =\ 433)} = 5.30$, $p = 0.0213$) and those reared at 1.27 larvae/cm^2 (3.24 mm ± 0.11, n = 295), ($\chi^2_{(1,\ n\ =\ 475)} = 38.36$, p = 0.0001). This was also true for wing lengths of adults emerging from larvae reared at 0.64 larvae/cm^2 and compared to wing lengths of adults from larvae reared at 1.27 larvae/cm^2 ($\chi^2_{(1,\ n\ =\ 548)} = 15.34$, p = 0.0001). There was a significant relationship between larval rearing density and wing length for stage two ($r^2_{(727)} = 0.0395$, $p < 0.0001$), with wing length decreasing as larval rearing density increased.

3.5. Effects of water depth on time-to-pupation, pupal production, adult emergence, pupal weight and wing length

Time-to-pupation: The median time-to-pupation (IQR: Q1 – Q3) for larvae reared at a depth of 1 cm was the longest at 18 days (14 - 19), followed by 16 days (15 - 19) and 16 days (15 - 17) for larvae reared at 3 cm and 5 cm depths respectively (Table 5). A log-rank test showed that time-from first instar larvae to pupation differed significantly between the different rearing water depths ($\chi^2_{(2,\ n\ =\ 1915)} = 58.10$, $p < 0.0001$). Subsequent pairwise comparisons showed time-to-pupation differed significantly when larvae were reared at a water depth of 1 cm compared to those reared at a water depth of 3 cm ($\chi^2_{(1,\ n\ =\ 1110)} = 22.29$, $p <$ 0.0001), and between larvae reared at water depths of 3 cm and 5 cm ($\chi^2_{(1,\ n\ =\ 1334)} = 70.08$, $p < 0.0001$). There was no significant difference in time-to-pupation for larvae reared at a water depth of 1 cm compared to those reared at a depth of 5 cm ($\chi^2_{(1,\ n\ =\ 1386)} = 0.64$, $p = 0.4187$).

Pupal production: Percentage pupal production (% pupae produced ± S.D.) was highest when larvae were reared at a depth of 3 cm (63.51% ± 25.68, n = 1429) followed by a depth of 1 cm (56.39% ± 30.23, n = 1269), and the lowest pupal production was obtained at a depth of 5 cm (28.62% ± 23.69, n = 669) (Table 5). Statistical analysis showed a significant difference in pupal production between the three treatments (Kruskal-Wallis, $H_{(2)} = 11.06$, $p = 0.0040$). Pairwise comparisons revealed that the differences in pupal production were between larvae reared at water depths of 1 cm and 5 cm ($\chi^2_{(1,\ n\ =\ 392)} = 5.688$, p = 0.0171), as well as between water depths of 3 cm and 5 cm ($\chi^2_{(1,\ n\ =\ 434)} = 10.602$, $p = 0.0011$). There was no significant difference between the pupal production when larvae were reared in water 1 cm or 3 cm deep ($\chi^2_{(1,\ n\ =\ 209)} = 0.190$, $p = 0.6632$).

Pupal weight: Pupal weights (Mean weight in mg ± S.D.) were higher for larvae reared in water 1 cm deep (1.25 mg ± 0.22, n = 270) compared to those reared at depths of 3 (1.16 mg ± 0.18, n = 270) and 5 cm (1.09 mg ± 0.16, n = 270). Pupal weight differed significantly between treatments (Kruskal-Wallis, $H_{(2)} = 16.15$, $p = 0.0003$).

Subsequent pairwise comparisons showed that pupal weight from larvae reared in water 1 cm deep differed statistically from those reared at water depths of 3 cm ($\chi^2_{(1,\ n\ =\ 120)} = 4.419$, $p = 0.0347$) and 5 cm ($\chi^2_{(1,\ n\ =\ 120)} = 15.126$, $p = 0.0001$). The pupal weight of larvae reared at a water depth of 3 cm also differed statistically from those reared at a water depth of 5 cm ($\chi^2_{(1,\ n\ =\ 120)} = 4.665$, $p = 0.03$). There was a significant relationship between water depth and pupal weight ($r^2_{(180)} = 0.1029$, $p < 0.0001$), with pupal weight decreasing as water depth increased.

Adult emergence: The proportion of adults emerging from pupae from the three water depths did not differ statistically (ANOVA: $F_{(2,\ 6)} = 1$, p = 0.4227) and ranged from 90.18 to 94.41% across the different treatments (Table 5).

Wing length: The wing sizes of adults emerging from larvae that were reared at a depth of 1 cm (2.49 mm ± 0.13, n = 270) were similar to those of larvae reared at depths of 3 (2.51 mm ± 0.13, n = 270) and 5 cm (2.49 mm ± 0.11, n = 270). Statistically, there were no significant differences in the wing lengths regardless of the depth of the water in which the larvae were reared ($H_{(2)} = 0.48$, $p = 0.7876$).

4. Discussion

This study reports on crucial laboratory rearing parameters for *An. funestus* and was an attempt to contribute towards the optimal rearing conditions of the species. It is the first report evaluating the impact of blood meal sources on fecundity and fertility and the effect of larval diet and water depth on *An. funestus* growth and development.

The study compared the fecundity and egg fertility of *An. funestus* females that were offered blood meals from two different delivery systems. Blood was provided to *An. funestus* females via a live host (anaesthetised guinea pig) or an artificial membrane feeding system using defibrinated bovine blood. The females' blood-feeding rates when using artificial system and live animals were comparable. However, the number of eggs per female (fecundity) obtained from the two blood-fed cohorts differed significantly. Fecundity in females blood-fed using the artificial membrane feeding system was considerably lower than in females fed on live animals (guinea pigs). The higher fecundity in females that fed on guinea pigs could be an adaptation of the strain to guinea pig blood feeding. However, it cannot be excluded that the difference in fecundity might be due to the use of defibrinated blood in the artificial membrane feeding system. It is established that defibrination results in a loss of a protein component, mainly fibrinogen, and might explain the reduction in fecundity reported here (Johnstone and Thorpe, 1987; Wotkuh-Wocadkuu, 1970). Fecundity was improved by increasing blood-feeding frequency using the artificial membrane feeding system,

Table 5
Summary of different developmental parameters of *An. funestus* larvae reared at three different water depths.

Rearing water depth (cm)	Total number of L1 used	Developmental parameter measured							
		Median time-to-pupation in days [IQR]	Total number of pupae from L1	Mean proportion (%) of L1 developing into pupae (S.D.)	Mean pupal weight (S.D.) mg	Total number of pupae for adult emergence*	Total number of adults emerged	Mean proportion of pupae developing into adults (S.D.)%	Mean wing length (S.D.) mm
1 cm	2250	18 [14–19]^a	1269	56.39 (30.23)^a	1.25 (0.22)^a	1209	1125	94.05 (3.06)	2.49 (0.13)
3 cm	2250	16 [15–19]^b	1429	63.51 (25.68)^a	1.16 (0.18)^b	1369	1235	90.18 (2.37)	2.51 (0.51)
5 cm	2250	16 [15–17]^a	669	28.62 (23.69)^b	1.09 (0.16)^c	609	589	94.41 (5.87)	2.49 (0.11)

L1= first instar larvae.
* indicates the total number of pupae available for adult emergence after pupae were removed for pupal weight (as per Table 2 in manuscript)
Superscript letters that differ indicate significant differences between treatments, if there are no letters there were no significant differences between treatments.

but this was not statistically significant.

Even where fecundity is low, it is possible to improve this through continuous selection. Mosquitoes are known to adapt to different blood-feeding sources depending on circumstances and the availability of the host blood meal (Bruce-Chwatt et al., 1966; Phasomkusolsil et al., 2013; Gunathilaka et al., 2017; Khan et al., 2021). This was recently confirmed by Zengenene (2021), who successfully increased fecundity in an *An. funestus* colony from Angola after six generations of selection on an artificial blood-feeding system using defibrinated bovine blood. After this study, a FUMOZ colony was sustained on bovine blood (Maharaj et al., 2022). Although the experimental procedures were different, from this study, Maharaj et al. (2022) reported a fecundity of 48.11 ± 4.12 eggs per female on the same FUMOZ colony blood-fed on defibrinated cattle blood. Supporting the notion that selection has improved the egg production in this species. However, it is unknown if defibrinated bovine blood will be suitable for establishing a new colony from wild females.

Another critical parameter for successful maintenance of *An. funestus* colonies under insectary conditions is optimising larval rearing conditions. This study investigated the impact of larval diet dose, density and water rearing depth on five key physiological parameters (time-to-pupation, pupal production, pupal weight, adult emergence success and adult wing length [as a proxy for body size]). Larval diet dose significantly affected the median larval time-to-pupation for *An. funestus*. Increasing larval food quantities initially had a significant positive impact on larval survivorship until a critical point where further increases became detrimental. Higher doses (0.06 and 0.08 mg/larva) resulted in significantly lower pupal production. The optimal larval food dose with the highest pupal productivity (almost 90% of larvae pupated) was 0.04 mg/larva. The decreased pupation at higher food doses is not peculiar to this study and was previously reported in *An. arabiensis* and *An. stephensi* (Reisen, 1975; Gilles et al., 2011; Damiens et al., 2012). The high larval mortality at high food doses might be attributed to larval suffocation as it will result in excess food on the water surface, making it impossible for anopheline larvae to breathe. It could be possible that using a liquid diet might mitigate this challenge; further investigation to explore this is recommended (Damiens et al., 2012)

Feeding larvae different food doses did not affect the mean pupal weight, adult emergence and wing length. However, the limited sample size used for measuring pupal weight and wing length might preclude this result. Never the less, this observation contrasts with results obtained for *An. arabiensis* where higher quantities of larval diet produced larger *An. arabiensis* adults (Damiens et al. (2012). Interestingly the wing sizes reported in this study were bigger than previously published studies on *An. funestus* (Mwangangi et al., 2004; Ngowo et al., 2021; Zengenene et al., 2022). Therefore, it is possible that the quantity of food provided was above the daily nutritional requirements for the larvae.

The ideal larval rearing density was investigated with faster time-to-pupation and high pupal productivity as the key indicators for successful rearing. These criteria were met at a larval density of 0.48 larvae/cm^2, and lower or higher densities decreased pupal production. The lower pupal production at higher larval densities might be explained by predation or, more likely larval suffocation due to overfeeding. This might be mitigated by maintaining the total larval food dose per day but feeding a lower dose (mg/larva at a time) at more frequent intervals (remaining with the same amount of food in a 24 h feeding cycle). It is difficult to explain the decrease in pupal production at low larval density. One hypothesis is that increased larval mortality resulted from first instar larvae perhaps being less mobile and unable to feed properly in large containers, possibly due to uneven or sporadic distribution of food on the water surface.

This study also concluded that a water depth of 3 cm fulfils the key criteria for successful larval rearing (fastest time-to-pupation and highest pupal production). Interestingly, the pupal weight decreased with increased water depth, although no differences in adult wing sizes were recorded. Tchuinkam et al. (2011) showed that water depths of 3 cm or less produced more *An. gambiae* pupae (>70%) compared to water depths of 6, 10, 15 and 30 cm. In deeper water, larvae and pupae most likely use more energy reserves when swimming to the surface, resulting in decreased resources available for development (Tchuinkam et al., 2011).

In conclusion, *An. funestus* females can produce viable eggs if fed on defibrinated bovine blood. Therefore, an artificial membrane feeding system is a possible alternative adult blood-feeding system to maintain colonies. Optimal larval feeding, density and water depth can ensure maximum pupal productivity in relatively short periods. These optimised parameters could be used in standard operating procedures for laboratory-reared *An. funestus* and provides vital information for the future design of mass rearing equipment for this species. However, these should be evaluated against local populations and other laboratory strains where and when available.

Declaration of Competing Interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

Author statement

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Disclosures

LLK conceptualised the project, LNF; GM; MLK and LLK designed the project; LNF and MPZ conducted the experiments; LNF, PMZ, MLK, GM and LLK analysed data; LNF, MLK, FO, GM and LLK wrote first and subsequent versions of the manuscript. All authors read and approved the final manuscript.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actatropica.2022.106785.

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