Effects of elevated temperatures on the development of immature stages of *Anopheles gambiae (s.l.)* mosquitoes

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Abstract

Objective: This study investigated the effects of temperature on the development of the immature stages of *An. gambiae (s.l.)* mosquitoes.

Methods: Mosquito eggs were obtained from laboratory established colonies and reared under eight temperature regimes (25, 28, 30, 32, 34, 36, 38 and 40 °C), and 80 ± 10% relative humidity. Larvae were checked daily for development to the next stage and for mortality. Pupation success, number of adults produced, and sex ratio of the newly emerged adults were recorded. Larval survival was monitored every 24 hours, and data were analyzed using Kaplan Meier survival analysis. Analysis of variance was used where data followed normal distribution, and a Kruskal-Wallis test where data were not normally distributed. Larval and pupal measurements were log-transformed and analyzed using ordinary least square regression with robust standard errors.

Results: Increasing the temperature from 25 to 36 °C decreased the development time by 10.57 days. Larval survival ($X^2(6) = 5353.12$, $P < 0.001$) and the number of adults produced ($X^2(5) = 28.16$, $P < 0.001$) decreased with increasing temperature. Increasing temperatures also resulted in significantly smaller larvae and pupae ($P < 0.001$). At higher temperatures disproportionately more male than female mosquitoes were produced.

Conclusions: Increased temperature affected different developmental stages in the life cycle of *An. gambiae (s.l.)* mosquitoes, from larval to adult emergence. This study contributes to the knowledge on the relationship between temperature and *Anopheles* mosquitoes and provides useful information for modelling vector population dynamics in the light of climate change.

Keywords: *Anopheles gambiae*; Development time; Immature stage; Larval and pupal size; Survival; Temperature

Introduction

Anopheles mosquitoes are responsible for transmitting several diseases such as malaria and lymphatic filariasis. They are among the well-known vector species due to their crucial role in transmitting *Plasmodium falciparum* – a malaria parasite [1]. The ecology of mosquitoes is essential as conditions present can affect the development of mosquitoes [2, 3]. Biotic and abiotic factors and their interaction could influence the ecology of mosquitoes and subsequently affect the growth and development of mosquitoes [4, 5]. Biotic factors such as larval nutrition, competition for resources, predation by other species can influence larval development and survival [6-8]. Similarly, abiotic factors such as temperature, humidity and precipitation can influence the development and population dynamics of mosquitoes [9]. Abiotic factors such as temperature have been linked with significant variation in both the immature and adult-stage characteristics (such as larval development rates, fecundity, body size, and longevity) of insects [10, 11]. Temperature is generally the most important abiotic factor influencing an insect's behavior, development, survival, distribution, and reproduction [12]. *Anopheles* mosquitoes are sensitive to both mean and fluctuating environmental temperatures [13]. Temperature affects all three (egg, larval and pupal) stages of immature mosquitoes [1, 14, 15].

Projected elevated temperatures for Africa are anticipated to affect malaria transmission by affecting key life history characteristics of *Anopheles* mosquitoes [16]. One of the critical factors determining insect population growth rate is the proliferation rate of new individuals, which is critically dependent on the growth characteristics of the immature stages [17]. Furthermore, the number of mosquitoes in a population is dependent on the number of adults that enter and exit the population. Environmental temperature may significantly affect the population of mosquitoes [1, 16, 18].

It is well known that fluctuations in temperatures affect the life expectancy or completion of a mosquito's life cycle [9] and can significantly increase pathogen proliferation [11]. Growth and development characteristics (such as development time, larval survival, and pupation success) of immature stages of *Anopheles* mosquitoes are adversely affected by increasing temperature [13, 14, 16-22]. However, information on these characteristics alone may not be sufficient to predict the population dynamics of mosquitoes. Production capacity (number of adults produced), which plays a vital role in estimating adult population produced from the immature stages, is understudied. Despite the importance of production capacity, evidence on the effects of temperature on the production capacity of *Anopheles gambiae (s.l.)* mosquitoes remains scarce. In addition, previous studies have not considered the impact of increasing temperature on the sex ratio (proportion of male to female) of *An. gambiae (s.l.)* mosquitoes. There is evidence that the availability of female mosquitoes plays a crucial role in determining population dynamics and disease transmission. While a few studies have demonstrated a link between temperature and sex ratio [23-25], they were done on *Aedes* rather than *Anopheles gambiae (s.l.)* mosquitoes. Therefore, this study investigated the effects of temperature on the development of the immature stages of *An. gambiae (s.l.)* mosquitoes, especially on the number of adults produced and sex ratio of the emerged adults.

Methods

Mosquito colony maintenance

This study used *Anopheles gambiae (s.l.)* mosquitoes (Tiassalé strains). The Tiassalé strain (a mixture of *An. gambiae (s.s.)* and *An. coluzzii*) originated from Tiassalé, Cote d'Ivoire [26] and has been maintained in the Vestergaard – Noguchi Memorial Institute for Medical Research Vector Labs (VNVL) insectary since 2010 in Ghana [27]. The eggs on filter paper were obtained from the insectary of VNVL. Mosquitoes were reared in climate incubators (RTOP-1000D,

Zhejiang, China) at the African Regional Postgraduate Program in Insect Science (ARPPIS), University of Ghana. Ghana has tropical temperature conditions with an average annual temperature ranging between 25 and 30 °C [28]. Hence, for experimental purposes, three temperatures were initially selected within this range; 25, 28 and 30 °C. Additionally, to evaluate the effects of elevated temperatures on immature mosquitoes, 2 °C increments from 30 °C were added to up to 40 °C was reached.

Mosquito eggs (about 400 per group) were placed in round plastic bowls (28 cm top diameter, 18 cm bottom diameter, 9 cm height) with 1 liter of de-chlorinated tap water and 1.5 ml of yeast to induce hatching [29]. The bowls were lined with A4 white paper to prevent eggs from sticking to the sides and drying out. Enough fresh de-chlorinated water kept at the respective temperatures was added daily to each bowl to maintain a volume of 1 liter due to loss of water to evaporation. Larval density was the same for all temperature regimes, and the incubators were programmed to have a photoperiod of 12:12 (light:dark) hours and relative humidity of 80 ± 10%. A HOBO MX1102 CO_2 logger (Onset Computer Corp., Cape Cod, MA, USA) was placed in each incubator and the main insectary to monitor daily temperature and relative humidity [30]. The temperature of the rearing water in each incubator was monitored using HOBO Pendant Temperature (UA-001-08) loggers (Onset Computer Corporation, Pocasset, MA) [31]. Data were downloaded daily between 10:00 and 11:00 am to ensure that temperature and humidity remained stable throughout the experiment (Table S1).

Larvae were fed daily on 10 mg of TetraFin goldfish flakes (Tetra Werke, Melle, Germany) sprinkled evenly on the water surface. The larval bowls were covered with nets to prevent pupae that may emerge into adults from flying away. Larval bowls in each temperature regime were checked daily, and pupae collected into cups and placed into cages (30 × 30 × 30 cm) until adult emergence. The position of the cages was rotated daily to control for the effects of position within the incubators.

Developmental time, larval survival and pupation success

Eggs on wet filter paper were placed in larval bowls with de-chlorinated tap water in each temperature regime to measure the development time (from egg hatching to adult emergence). On hatching, 160 first instar larvae from each egg tray were distributed into larval bowls containing de-chlorinated tap water and observed for development to pupae and then to the adult stage. Larval bowls were of the same size as the egg bowls, except they had no paper lining. The developing larvae were checked daily for the number of death, which was used to calculate mortality, and discarded. Larvae that did not move after being touched with pipette tip were recorded as dead. The time from egg hatching to adult emergence was recorded as the development time (in days). To calculate pupation success, pupae were collected daily using a Pasteur pipette. The number of larvae that pupated daily was noted and recorded. Pupation success was calculated for each temperature regime as the proportion of larvae that pupated from the total number of larvae. All experiments were repeated five times.

Larval and pupal weight and size of mosquitoes

Fifty (50) fourth instar larvae and 50 pupae were randomly selected from each temperature regime, immobilized at a fridge temperature of 4 °C, blot dried on a tissue paper towel and weighed, using an XS104 ultra-micro balance (Mettler Toledo Inc., Columbus, Ohio). A digital stereo microscope Leica EZ4 HD with LED and HD inbuilt camera (Leica Microsystems Limited, Switzerland) connected to a laptop was used to capture digital images of the pupae and larvae at a magnification of 16X. Body parts were measured using the Leica Application Software, version 3.4.0 (Leica

Microsystems Limited, Switzerland). Measurements included larval length (a proxy for body size) from the distal tip of the head (without considering the feeding brush, antennae, and caudal hair) to the end of the anal segment [32], length of the cephalothorax of the pupae, and used as a proxy for pupal size [25, 33].

Number of adults produced, and the sex ratio of mosquitoes

Adults that emerged from the 160 larvae under each temperature regime were counted and recorded. The number of adults produced was expressed as a proportion of emerged adults relative to total larvae. The sex ratio of adults emerging from the pupal stage under each temperature regime was determined as the ratio of adult males to adult females [34, 35]. All experiments were repeated five times.

Statistical methods

The assumptions of normality and homogeneity of variances were assessed using Shapiro-Wilk and Bartlett's tests, respectively, in Stata version 15.1 (StataCorp LLC, Texas, USA). Analysis of variance (ANOVA) was used to explore the relationship between temperature and development time. In cases where the overall model showed statistically significant differences, the Tukey post hoc test was further used to determine where the differences existed. Pupation success, number of adults produced, sex ratio, larval and pupal measurements failed to meet the normality criteria. Kruskal-Wallis tests with Dunn test pairwise comparison test were used to assess the effect of temperature on pupation success, the number of adults produced, and sex ratio of emerged mosquitoes.

Larval and pupal measurements were log-transformed. Ordinary least square (OLS) regression analysis with robust standard errors was used to determine whether significant differences existed among the different temperature regimes. Sensitivity analysis was further conducted using quantile and robust regression methods to determine how the regression coefficients and their respective standard errors change with respect to using different statistical models to assess the effects of temperature on larval and pupal weights and length. Survival analyses were performed using Kaplan-Meier survival analysis. Log-rank test and Cox proportional-hazards model were used to test the null hypothesis that larval survival did not change across the different rearing temperatures. The log-rank test compared the overall survival trend for the seven temperature regimes, while the Cox proportional-hazards model was used for two-sample comparisons at one temperature against survival at the baseline temperature (25 ^{o}C). Continuous variables with normal distribution were presented as mean (standard deviation [SD]), and non-normally distributed variables were reported as median (interquartile range [IQR]). In all statistical analyses, a *p-value* of less than 0.05 was considered significant.

Results

Development time of the immature stage

To assess the relationship between temperature variability and development time of immature stages of mosquitoes, the mosquitoes were reared at different temperature regimes (25, 28, 30, 32, 34, 36, 38 and 40 ^{o}C). Eggs incubated at 40 ^{o}C failed to hatch even after seven (7) days, and those that hatched at 38 ^{o}C died before pupation. Therefore, the development times at temperatures 38 and 40 ^{o}C could not be calculated. The results showed that the mean development time (the time it takes eggs to hatch into adults) of mosquitoes decreased with increasing temperatures (Table 1). When the temperature was increased from 25 to 36 °C, the mean development time decreased by 10.57 days. There was a statistically significant decrease (One-way ANOVA; $F(5, 24) = 133.55$, $P < 0.001$) in development time

with increasing temperature. Post hoc tests showed significant differences in the development time among the various temperature comparisons (Table S2).

Determination of larval survival time and pupation success

Eggs kept at 40 °C failed to hatch; therefore, larval survival was estimated at seven (7) temperature regimes (25, 28, 30, 32, 34, 36, and 38 °C). Pupation success was estimated at all temperature regimes except 38 and 40 °C. Larvae reared at 38 °C died before pupation. Survival curves were plotted and presented as Kaplan-Meier plots based on the number of larvae that died before pupating (n = 3699) (Table S3). From the results, larval survival decreased with increasing rearing temperature (Figure 1). Temperature regimes had significantly different survival functions (Log-rank test; $X^2(6) = 5353.12$, $P < 0.001$). Compared to the baseline temperature (25 °C), larval survival statistically decreased with increasing temperature to 28, 30, 32, 34, 36 and 38 °C (Table S4).

On pupation success, the results showed that the highest median pupation success was recorded at 28 °C and the lowest at 36 °C (Table 1). There was a statistically significant decrease (Kruskal-Wallis test; $X^2(5) = 27.23$, $P < 0.001$) in pupation success with increasing temperature. Post hoc tests revealed statistically significant differences between five temperature comparisons (Table S2).

Measurement of larval and pupal weight and size

Larval and pupal weight and size were calculated for *An. gambiae (s.l.)* mosquitoes reared at 25, 28, 30, 32, 34, and 36 °C. The results showed that the median larval weight decreased with increasing temperature from 25 °C [2.1 (IQR, 0.6) mg] to 36 °C [1.1 (IQR, 0.3) mg]. Similar trends were observed for larval size, with larger larvae being recorded at 25 °C [4.94 (IQR, 0.39) mm] (Table 2). In addition, the median pupal weight decreased with increasing temperature, from 25 °C to 36 °C. Similarly, pupal size decreased with increasing temperature. At 25 °C, pupal size was 1.73 (IQR, 0.10) mm but decreased with increasing temperature (Table 2). A change in temperature from 25 to 28 °C significantly decreased larval weight by 0.15 (95% CI; 0.26, 0.05, $P = 0.001$). Increasing the temperature from 25 to 36 °C significantly decreased larval size ($\beta_{\text{larval size}} = 0.11$, 95% CI; 0.14, 0.09, $P < 0.001$), pupal weight ($\beta_{\text{pupal weight}} = 0.34$, 95% CI; 0.40, 0.28, $P < 0.001$) and pupal size ($\beta_{\text{pupal size}} = 0.12$, 95% CI; 0.14, 0.10, $P < 0.001$). Overall, sensitivity analysis using quantile and robust regressions showed consistent coefficients with the ordinary least square regression (Table 3).

Temperature, number of adults produced, and sex ratio of An. gambiae (s.l.) mosquitoes

The number of adults produced (proportion of larvae that emerged as adults), and sex ratio of mosquitoes were estimated at 25, 28, 30, 32, 34 and 36 °C. Mosquitoes failed to develop at temperatures above 36 °C. The number of adults produced decreased to a stistically significant degree (Kruskal-Wallis test; $X^2(5) = 28.16$, $P < 0.001$) as temperature increased from 28 °C [66.25 (IQR, 3.13) %] to 36 °C [10.63 (IQR, 3.13) %] (Table 1). Post hoc tests showed significant differences in only four temperature comparisons (Table S2).

Furthermore, the number of male adult mosquitoes produced increased with increasing temperature: the median sex ratio of male/female (M/F) ranged from 0.89 (IQR, 0.04) at 25 °C to 2.60 (IQR, 0.60) at 36 °C (Table 1). The increase in the sex ratio of mosquitoes with increasing temperature was statistically significant (Kruskal-Wallis test; $X^2(5) = 28.07$, $P < 0.001$), with fewer females emerging at higher temperatures. Post hoc tests showed that sex ratio differed at 25 vs 34 °C, 25 vs 36 °C, and 28 vs 36 °C (Table S2).

Discussion

Development time of mosquitoes decreases with increasing temperature

This study found that the development time of mosquitoes decreased with increasing temperature. However, larval development differed among the four larval instars – the fourth instar had the longest developmental time. The observed decrease in development time with increasing temperature is probably due to a rise in mosquito body temperature, which increases respiration and metabolism rates and causes mosquitoes to develop faster, thereby shortening the development time [25]. This observation is consistent with previous studies [7, 11, 17, 20], where development times of immature mosquitoes decreased with increasing temperature. The fourth instar had the longest developmental time, probably unsurprising since this stage precedes the pupal stage and likely possesses a significant amount of nutrient reserves needed to transition to adulthood [6]. This observation agrees with the findings of Loetti et al. [36], who observed differences in the development times for the immature stages of *Culex eduardoi*, with the fourth having the longest development time. Similar observations have been made with regards to development times for *Aedes albopictus,* where the fourth instar took the longest time to develop, accounting for 25 – 36% of total development time [37]. The fact that temperatures are predicted to rise in the future means time to completion of the mosquito life cycle could shorten, as mosquitoes are likely to develop faster.

Survival time of* An. gambiae *(s.l.) larvae decreases with increasing temperature

The survival of mosquito larvae decreased with increasing temperature. A possible explanation for the decreased larval survival at higher temperatures might be due to denaturation of proteins, interactions with oxygen supply, increased metabolic rate, disruption of membrane structure, and dehydration [37-39]. Mosquito larvae likely increase their metabolic rates to overcome any thermal stress experienced in the breeding habitat, resulting in higher energy expenditure [40, 41]. The increased metabolic rates could exceed oxygen supply from the environment leading to reduced performance, lowered tolerance to thermal stress [42], and the death of the larvae. Other studies have linked high larval mortalities and reduced larval survival time with increasing temperatures in other mosquito species such as *An. gambiae (s.s.)* [17, 43], *An. arabiensis* and *An. quadriannulatus* [16], *Culex quinquefasciatus* [41], and *Aedes aegypti* [44]. In a future warmer temperature, it is possible that larval populations could decrease because of high larval mortalities. Estimating the effects of higher temperatures on larval survival could provide critical information in controlling larval populations in future warmer temperatures.

Larval and pupal size decreases with increasing temperature

Larval and pupal size decreased with increasing temperatures. This could be due to the short duration in the development of immature stages; there is reduced food intake by the larvae [45], resulting in decreased larval size and consequently pupal size. These are similar to the observation of previous studies [13, 25, 46, 47] in which rearing temperature significantly influenced larval and pupal size, with lower temperatures resulting in larger larvae and pupae and vice versa. According to Keena and Moore [48], temperature has an influence on larval weight and therefore on the ability of the larvae to pupate. This could suggest that the temperature of the larval environment may have a profound impact on the adult stage. It is possible that in a future warmer temperature, the size of mosquitoes could reduce, and this can affect almost all aspects of its physiology, performance, morphology, and fitness [49, 50].

Pupation success of mosquitoes decreases with increasing temperature

Pupation success, the proportion of larvae that pupated from the total number of larvae, decreased with increasing temperature. According to Clements [51], it is a prerequisite for mosquitoes and other holometabolous insects to reach a certain critical body mass in the course of larval development before they can pupate, and this mass decreases with increasing temperature [52]. In this current study, the highest pupation success was recorded at 28 ^{o}C, a finding similar to that of Mamai et al. [21], who reported higher pupation success of *An. arabiensis* mosquitoes at 27 ± 1^{o}C. The failed metamorphosis of larvae to pupae and adults could be attributed to the fact that higher temperatures increase development rate, therefore, resulting in rapid uptake of nutrients and faster metabolism [16]. This requirement may be physiologically demanding, leading to insufficient body mass needed for eclosion (emergence of the adult from the pupal stage) [52].

Number of adult mosquitoes produced decreases with increasing temperature

The number of adults produced (production capacity) decreased with increasing temperature, with fewer female mosquitoes emerging at high temperatures, compared to males. These results are consistent with those of Bayoh and Lindsay [17], who reported that the number of *An. gambiae (s.s.)* mosquitoes produced decreased with increasing temperature. Our findings on sex ratio are in agreement with the findings of Monteiro et al. [23], where there was an equilibrated male-female ratio at 25 °C. However, at 30 °C, the production of males was about two (2) times that of females. It is possible that a large proportion of female larvae and pupae were killed before becoming adults, leading to the production of fewer female mosquitoes at high temperatures.

Further investigations are needed to provide greater insight into the mechanism underlying the production of fewer female mosquitoes at higher temperatures. It is likely that mosquito populations could reduce in a future warmer temperature because there would be few female mosquitoes available for mating and egg-laying. Naturally, biotic and abiotic factors could affect the development and population dynamics of mosquitoes. For instance, biotic factors such as the availability of food for mosquito larvae could increase their developmental rates, but the presence of predators can reduce the abundance of mosquitoes [53]. In addition, other abiotic factors such as humidity and precipitation can influence the growth and development of mosquitoes. Excessive precipitation could affect the survival of immature mosquitoes by overflowing larval breeding sites. Humidity could also affect the hatching rate of mosquito eggs [54].

Conclusion

Increasing temperature affected the different developmental stages in the life cycle of *An. gambiae (s.l.)* mosquitoes, from larvae to adult emergence. Larvae kept at higher temperatures (32 – 38 ^{o}C) developed faster and produced smaller larvae and pupae. Fewer adults were produced from larvae kept at 32 and 36 ^{o}C, with the majority of them being males. The reduced production of females at higher temperatures could decrease the mosquito population. This study only altered temperature while keeping other parameters constant (such as relative humidity, larval density, food quantity, and photoperiod). Varying some of these factors could likely affect the development of immature mosquitoes in the natural environment; therefore, as a next step, further studies should consider varying some of these factors in addition to temperature to determine their composite effect on the development of mosquitoes. This is likely to be an arduous task but will be necessary to advance the understanding of future warmer climate or climate change on mosquito development. The current study contributes to the knowledge on the relationship between

temperature and *Anopheles* mosquitoes and provides useful information for modelling vector population dynamics in light of warmer temperatures expected as a result of climate change.

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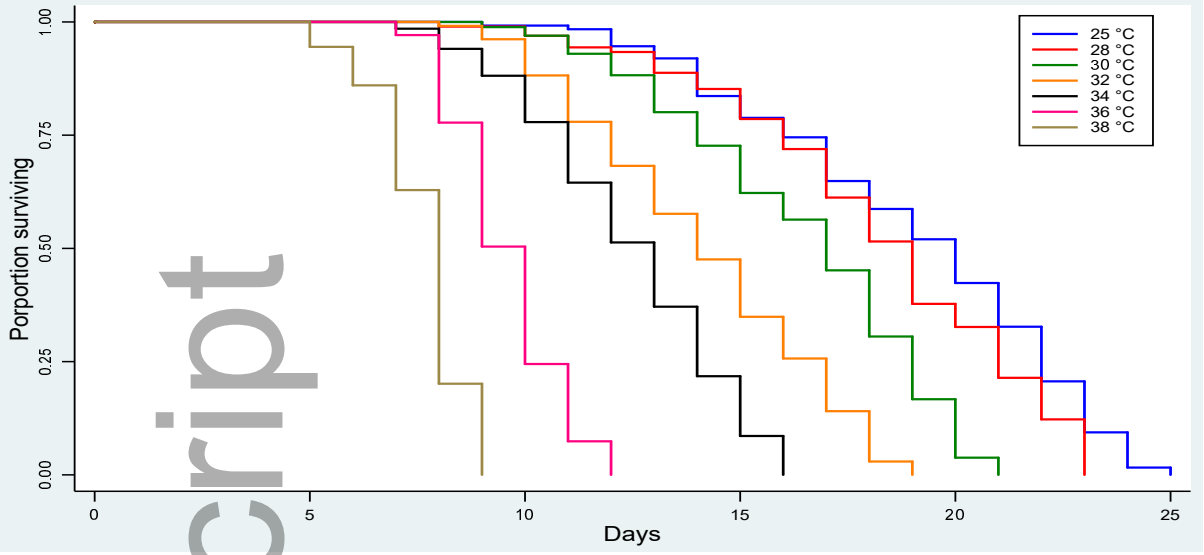

Figure 1: Kaplan-Meier survival plots of *An. gambiae (s.l.)* larvae reared at different temperatures. 25 °C (blue) was set as baseline against which survival at other temperatures were compared; 28 °C (red), 30 °C (green), 32 °C (orange), 34 °C (black), 36 °C (pink), 38 °C (brown).

Table 1: Development time, pupation success, number of adults produced and sex ratio of mosquitoes reared at different temperatures

Temperature regime (ºC)	Development time (in days) Mean (±SD)*	Pupation success (%) Median (IQR)**	Adults produced (%) Median (IQR)**	Sex ratio (M/F) Median (IQR)**
25	20.17 (±0.75)^{a}	53.75 (3.12)^{a}	41.88 (1.87)^{a}	0.89 (0.04)^{a}
28	18.40 (±0.89)^{b}	75.00 (0.63)^{ab}	66.25 (3.13)^{ab}	1.08 (0.04)^{ab}
30	16.08 (±1.03)^{c}	34.38 (1.87)	28.75 (2.50)	1.88 (0.00)
32	14.54 (±0.38)^{d}	28.13 (2.50)^{ac}	23.13 (2.50)^{ac}	2.08 (0.00)
34	13.01 (±0.61)^{e}	24.38 (1.88)^{d}	20.00 (1.88)^{ac}	2.20 (0.02)^{b}
36	9.60 (±0.55)^{f}	22.50 (3.12)^{d}	10.63 (3.13)^{c}	2.60 (0.60)^{c}
38	-	-	-	-
40	-	-	-	-

NB: Larvae at 38 ºC died before pupating and eggs kept at 40 ºC did not hatch; SD = Standard Deviation; IQR = Interquartile range; M = Male; F = Female; Within a column, different lowercase letters indicate significant difference at $P < 0.05$; *p-values* for outcomes indicated by single asterisk (*) were generated using One-way ANOVA test and those with double asterisk (**) were generated using Kruskal-Wallis test.

Table 2: *An. gambiae (s.l.)* larval and pupal weight and size at different temperature regimes

Temperature regime (ºC)	Larval weight (mg) Median (IQR)	Larval size (mm) Median (IQR)	Pupal weight (mg) Median (IQR)	Pupal size (mm) Median (IQR)
25	2.10 (0.60)	4.94 (0.39)	2.10 (0.70)	1.73 (0.10)

28	1.60 (0.50)	4.59 (0.61)	2.00 (0.40)	1.65 (0.14)
30	1.50 (0.50)	4.72 (0.36)	1.80 (0.30)	1.68 (0.08)
32	1.50 (0.40)	4.71 (0.48)	1.70 (0.50)	1.61 (0.06)
34	1.30 (0.30)	4.63 (0.38)	1.70 (0.50)	1.59 (0.08)
36	1.10 (0.30)	4.42 (0.44)	1.50 (0.10)	1.52 (0.17)
38	-	-	-	-
40	-	-	-	-

NB: Larvae at 38 °C died before pupating and eggs kept at 40 °C did not hatch; IQR = Interquartile range.

Table 3: Relationship between temperature and *An. gambiae (s.l.)* larval and pupal weight and size

Outcome	Temperature regime (°C)	Ordinary Least Square regression with robust standard errors β [95% CI]	Sensitivity Analysis	
			Quantile regression β [95% CI]	Robust regression β [95% CI]
Larval weight (mg)	25	Ref	Ref	Ref
	28	-0.15 [-0.26, -0.05]**	-0.27 [-0.38, -0.16] ***	-0.21 [-0.31, -0.12] ***
	30	-0.26 [-0.36, -0.15] ***	-0.34 [-0.45, -0.22] ***	-0.31 [-0.40, -0.22] ***
	32	-0.27 [-0.36, -0.18] ***	-0.34 [-0.45, -0.22] ***	-0.32 [-0.41, -0.23] ***
	34	-0.35 [-0.45, -0.25] ***	-0.48 [-0.59, -0.37] ***	-0.41 [-0.50, -0.32] ***
	36	-0.52 [-0.61, -0.42] ***	-0.65 [-0.76, -0.54] ***	-0.57 [-0.66, -0.48] ***
Pupal weight (mg)	25	Ref	Ref	Ref
	28	-0.10 [-0.17, -0.03] **	-0.05 [-0.15, 0.05]	-0.09 [-0.17, -0.02] **
	30	-0.17 [-0.24, -0.10] ***	-0.15 [-0.25, -0.05] **	-0.17 [-0.24, -0.10] ***
	32	-0.20 [-0.27, -0.12] ***	-0.21 [-0.31, -0.11] ***	-0.19 [-0.26, -0.12] ***
	34	-0.22 [-0.30, -0.15] ***	-0.21 [-0.31, -0.11] ***	-0.23 [-0.30, -0.16] ***
	36	-0.34 [-0.40, -0.28] ***	-0.34 [-0.43, -0.24] ***	-0.34 [-0.40, -0.28] ***
Larval size (mm)	25	Ref	Ref	Ref
	28	-0.04 [-0.08, -0.01]*	-0.07 [-0.11, -0.04] ***	-0.06 [-0.09, -0.03] ***
	30	-0.04 [-0.07, -0.02] ***	-0.04 [-0.08, -0.01] *	-0.04 [-0.07, -0.01] **
	32	-0.05 [-0.08, -0.02] ***	-0.05 [-0.08, -0.01] *	-0.05 [-0.08, -0.02] **
	34	-0.07 [-0.09, -0.04] ***	-0.07 [-0.10, -0.03] ***	-0.07 [-0.10, -0.04] ***
	36	-0.11 [-0.14, -0.09] ***	-0.11 [-0.15, -0.07] ***	
Pupal size (mm)	25	Ref	Ref	Ref

28	-0.02 [-0.04, -0.00] *	-0.04 [-0.06, -0.02] ***	-0.03 [-0.05, -0.02] ***
30	-0.03 [-0.05, -0.02] ***	-0.03 [-0.05, -0.01] **	-0.03 [-0.05, -0.02] ***
32	-0.06 [-0.08, -0.05] ***	-0.07 [-0.09, -0.05] ***	-0.07 [-0.09, -0.05] ***
34	-0.08 [-0.10, -0.07] ***	-0.08 [-0.10, -0.06] ***	-0.09 [0-.10, -0.07] ***
36	-0.12 [-0.14, -0.10] ***	-0.12 [-0.15, -0.10] ***	-0.13 [-0.15, -0.11] ***

Larval weight, pupal weight, larval size, and pupal size were log-transformed; single asterisk (*) represents significant difference at *P < 0.05*; double asterisk (**) means *P < 0.01*; triple asterisk (***) means *P < 0.001*; Ref means Reference. β means regression coefficients, 95% CI means 95% Confidence interval; *p-values* were generated using OLS with robust standard errors.