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Effect of Temperature and pH on Egg Viability and Pupation of *Anopheles arabiensis* Patton (Diptera: Culicidae): Prospect for Optimizing Colony Reproduction Procedures

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Abstract

Optimizing procedures for mass rearing mosquitoes for practices akin to the sterile insect techniques or routine laboratory activities is crucial. The present study evaluated the impact of nutrients, temperature, egg storage period and pH on egg hatchability and pupation rate, respectively, of *Anopheles arabiensis* mosquitoes. First, twenty eggs, collected from female mosquitoes and raised on different diet types (*Rastrineobola argentea*, Tetramin® Baby fish food and Bakers' active yeast) in their larval stages, were stored at different time periods at two temperature regimes; 22 – 23^{O}C and 28 – 29^{O}C and later dispensed in plastic cups (4.0 cm top × 3.5 cm bottom × 2.7 cm height) containing 25cm^3 of water and left to hatch. Secondly, twenty L4s were placed individually in 100 Ml of larval rearing media of different pH regimes in plastic cups (7.5 cm top × 5.0 cm bottom × 8 cm height) and left to pupate and emerge as adults. The media were of pH 6, 6.8 (clean borehole water), 7, 8, 9 and cow dung solution. It was found that eggs incubated at 28-29^{O}C were less viable than those incubated at 22-23^{O}C ($p < 0.05$). Eggs remained hatchable for 8 days. Mean pupation time for L4 larvae maintained in untreated tap water (pH 6.8) differed significantly compared to other rearing media ($p p < 0.05$). Mean pupation time was neither influenced by sex ($p = 0.124$) or size ($p = 0.801$) of emerged mosquitoes. It was concluded that pH (6.8) and temperatures of 22-23^{O}C were optimal for pupation and egg hatchability, respectively.

Keywords: Temperature, pH, Storage period, Eggs hatchability, *An. Arabiensis*, Pupation.

1. Introduction

Ninety percent of mosquito's life history is aquatic. Eggs, larvae and pupae, referred to as immature stages, are aquatic and thrive differently in different habitat types (Munga *et al*., 2006; Ndenga *et al*., 2011). This is because habitats differ in physical, chemical and biological characteristics (Edillo *et al*., 2006). Emerged mosquitoes or adults are terrestrial. Typically less than 10% of laid eggs emerge as adult (Munga *et al*., 2007; Mwangangi *et al*., 2006; Okogun, 2005).

To optimize on survival, it has been observed that *Anopheles arabiensis*, *Anopheles funestus* (Lyons *et al*., 2013) and *Anopheles gambiae* (Bayoh and Lindsay 2003; Kirby and Lindsay, 2009; Bayoh and Lindsay, 2004; Rocca *et al*., 2009) mosquitoes choose to breed in open, sunlit pools. In such habitats it is believed, temperatures (Small *et al*. 2003; Hoffmann 2010; Parham *et al*., 2012), oxygen (Okogun, 2005), nutrient and pH (Russel, 1999; Tiimub *et al*., 2012) are optimal (Piyaratne *et al*., 2005) for the development of the immatures.

The replication of optimal condition for the generation of mosquito immatures is crucial for mass generation of malaria vectors for studies that require large numbers of mosquitoes for use in the laboratory or for procedures akin to the Sterile Insect Techniques (SIT) that would require millions of mosquitoes (Robinson *et al*., 2009) for purposes of irradiation and subsequent release to inundate and manage nuisance insect population (Knipling, 1955).

A challenge for mass production of anopheline mosquitoes is the fact that freshly laid anopheline eggs are sticky and therefore handling and counting them is tedious and impractical. Moreover, the eggs remain viable for a short time only when kept on wet substrates (Clements, 1992). This means efforts must be directed towards determining the drying and storing conditions that would make it easy to manipulate and count the eggs without compromising viability.

Like all poikilotherms, mosquitoes' biochemical and physiological processes depend on ambient environmental temperature (Couret and Benedict, 2014). Temperature influences the duration and rate of development (Dixon *et al*., 2009), timing of maturation (Yoshioka *et al*., 2012) and body size (Evans *et al*., 2012). It also dictates on humidity (Focks *et al*., 1993) that determines whether the adult mosquitoes aestivate or migrate to other areas of favourable temperatures (Lehmann *et al*., 2010; Adamou *et al*., 2011).

Humidity and temperature influence desiccation and therefore the rate of mosquito egg survival (Juliano *et al*., 2002). It follows that desiccation-resistant eggs will not

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only increase the potential of mosquito colony establishment in non-native habitats but also provide extended shelf life for mosquito eggs meant for procedures akin to the sterile insect techniques

Additionally, anopheline mosquitoes are known filter feeders and under suitable temperatures, pH and oxygen diffusion, acquire optimal nutrients from their aquatic habitats (Sanford, 2005). This, however, is dictated by the amount of Dissolved Oxygen (DO) within the aquatic habitat in such a manner that the higher the DO, the more unsuitable the habitat is to the larvae. Additionally, pH has been observed to alter dissolved oxygen content (Gilvear and Bradley, 2000) by increasing the amount of free ammonia. It follows that pH of larval habitat must be within optimal range because if this is not the case mosquito development is reduced (Curtis, 1996).

Based on this information, it was hypothesized that nutrient, temperature, egg storage period and pH, under which the mosquito parental stocks were exposed, had no effect on egg hatchability and pupation rates of *An. arabiensis* respectively. This study was conducted to:

(1) Evaluate the temperature regime that leads to minimal desiccation of *An. arabiensis* eggs;

(2) evaluate the pH that offers optimal pupation and emergence of *An. arabiensis* adults; and

(3) evaluate the effect of nutrients on *An. arabiensis* size.

2. Materials and Methods

2.1. Mosquito Colony Origin

The present study was carried out at the laboratories and insectaries of the School of Biological Sciences, University of Nairobi, Kenya. The *Anopheles arabiensis* mosquito strain used in the present study was originally from Dongola in northern Sudan but was sourced from the International Atomic Energy Agency (Seibersdorf laboratories) in Vienna, Austria. The mosquitoes were cultured at a temperature of 28 – 30 ^{O}C, relative humidity of 70 - 80% and photoperiod of 12:12 (L:D).

2.2. Larval Diet Types

Three larval diet types were used to rear *An. arabiensis* mosquitoes for the present study. *Rastrineobola argentea* also known as sardine in English was bought from a local market, oven dried, crushed into powder using a food blender and put in a glass vial. Tetramin® Baby fish food was obtained from Seibersdorf laboratories in The Netherlands. Bakers' active yeast was bought from the local supermarket. All the food types were refrigerated at 4^{O}C.

2.3. Mosquito Stock Culture

The mosquitoes used in the studies were from the F5 generation onwards and were reared following standard procedure (Dominic *et al*., 2005). The larvae were fed thrice daily, at 09.00, 13.00 and 17.00 hours. Each larva was given an approximate of 0.03 mg of a diet type. On emergence, the adults were offered 10% sugar solution soaked in cotton pads placed on the cages. The sugar solution served, as a source of energy. The energy sources were offered on a daily basis. Two days after emergence, the females were offered bovine blood provided via Hemotek® membrane feeding apparatus. The blood was obtained from a local abattoir and mixed with EDTA to prevent coagulation. On the second day after blood feeding, the females were provided with an oviposition dish to lay eggs. The eggs were then dispensed, larvae fed, larval water changed under similar conditions described by Yugi *et al*., (2014) as already stated.

2.4. Cow Dung Collection and Preparation

Fresh cow dung was collected every morning between 08.00-08.30 hours from the College of Agriculture and Veterinary Sciences of the University of Nairobi, Kenya. The dung was sourced from a lactating seven year old hybrid (Hereford and Borana) cow. The cow and others grazed on kikuyu, star, oat and *Hyparhania* grass *spp.* within the college grazing grounds. The animals were later provided with hay from Rhodes and Sateria in the evening during milking. After collection, the dung was placed in a plastic bag and immediately transported to the laboratory at the School of Biological Sciences, University of Nairobi, Kenya for further processing and refrigeration at temperatures of 4^{O}C.

2.5. Larval Rearing Media Preparation

Six larval rearing media of different pH (6, 7, 8 and 9), cow dung and clean untreated borehole water (pH 6.8) were used to rear L4s to pupation. All the media solutions contained 0.5 g of Potassium Hydrogen Carbonate ($KHCO_3$) except for solution of pH 6, clean borehole water and media made from cow dung. In particular, the rearing solutions were prepared as follows: (i) pH 6: two l of distilled water only, (ii) pH 7: 1 ml of 0.1 M hydrochloric acid and 0.5 g of $KHCO_3$ in 2 l of distilled water, (iii) pH 8: 0.5 g of $KHCO_3$ in 2 l of distilled water, (iv) pH 9: 0.5 Ml of 0.1M sodium hydroxide and 0.5 g of $KHCO_3$ in 2 l of distilled water, (v) Cow dung solution: 20 g of cow dung dissolved in 2 l of distilled water and (vi) clean borehole water: 2 l of untreated borehole water.

2.6. Microscope Calibration

A light microscope (Unico) was calibrated to enable determination of mosquito sizes. This was done in the following manner. First, an ocular micrometer was mounted in one of the eyepiece lenses of the microscope followed by placement of a graticule slide on the stage of the microscope. Secondly, with the required objective lens in place (×40) the scale was adjusted until the zero line of the eyepiece scale aligned exactly with the zero line of the calibration scale on the graticule slide. This distance was 0.5 mm. The number of division between the distance between the two zero lines and where the next exact alignment between the ocular micrometer and graticule-slide scales occurred was also determined. This was 20µm. These measurements were then used to calculate the conversion factor with which direct wing length measurements were multiplied to estimate mosquito wing lengths. The conversion factor was calculated as follow:

$$\text{Conversion factor} = \frac{\text{Distance on graticule}}{\text{Divisions on ocular that matches graticule distance}}$$

$$\text{Conversion factor} = \frac{0.5\text{mm}}{20\mu\text{m}} = 0.025\text{mm}$$

1µm = 0.025 mm

2.7.1. Eggs Hatching Rates of An. Arabiensis

This experiment was conducted to determine the most suitable temperature regime (22 - 23°C or 28 - 29°C) for extended *An. arabiensis* egg storage period. The temperature regimes were settled on by slightly modifying the temperature regimes used in studies done by Meola, 1964 and Trpis 1972 on intraspecific variations in desiccation resistance by *Ae. aegypti* eggs. To facilitate this, *An. arabiensis* eggs were collected from *An. arabiensis* mosquitoes raised on different diet types (*Rastrineobola argentea*, Tetramin® Baby fish food and Bakers' active yeast) in their larval stages. The eggs were counted in two batches using fine tipped camel hair brushes under dissecting microscope (Leica Zoom 2000 at × 10 magnification) and placed in batches of 20s in paper towels in Petri dishes labeled with the date of preparation. One batch was placed on a table in a room maintained at 22 - 23°C and the other in a room maintained at 28 - 29°C. This procedure was repeated every two days 16 times to yield enough eggs to be stored for different period of time [zero days (eggs collected and used on the day of experiment), 2 days, 4 days, 6 days, 8 days, 10 days, 12 days, 14 days, 16 days, 18 days, 20 days, 22 days, 24 days, 26 days, 28 days, and 30 days].

On the experimental day, all the 32 sets of egg batches of 20 eggs each were dispensed each in separate conical oviposition cups. Each cup measured 4.0 cm top × 3.5 cm bottom × 2.7 cm height and contained 25 cm^3 of untreated borehole. Each cup was labeled with the temperature regime the eggs had been stored and length of time the eggs had spent in the room. The set ups were replicated five times (Table 1) and were left on tables in the insectary for two days to allow incubation and hatching.

Table 1. Experimental set up showing food type used to raise parental stock, eggs used per set up, number of replicates and total number of *An. arabiensis* eggs used per diet type

Type of food	Number of eggs used per oviposition cup		Replicates per day	Total number of eggs used
	22 - 23°C	28 - 29°C		
Crushed silver cyprinid fish	20	20	5	200
TetraMin® fish food	20	20	5	200
Baker's yeast	20	20	5	200

Forty-eight hours after the start of the experiment, the oviposition cups were carefully observed and larvae hatching from the eggs noted and counted. For cups where the number of hatched larvae were not equal to the number of dispensed eggs, all the unhatched eggs and shells were examined under a dissecting microscope and classified as either 'still viable but delayed in hatching' if it showed signs of hatching (eggs with open operculum) or 'non-viability' if it was non-hatched or with unopened opercula. The 'still viable' eggs were given more time to hatch. The numbers of larvae were recorded per temperature regime and diet type fed to the parental group at larvae stage and percentage mean egg hatchability determined using the formula;

$$\%\ \text{Mean egg hatchability} = \frac{\text{Number of counted L1}}{\text{Total number of eggs dispensed}} \times 100\%$$

2.7.2. Pupation of An. arabiensis

This experiment was conducted to determine the most suitable pH regime for expediting metamorphosis in mosquito production chain. Six sets of larval holding cups each measuring 7.5 cm top 5.0 cm bottom and 8 cm height consisting of ten larval holding cups were used. Each set of ten cups contained a particular rearing medium: (i) the first set contained solutions of pH 6, (ii) the second, pH 7, (iii) the third, pH 8, (iv) the fourth, solutions of fresh cow dung, (v) the fifth, pH 9 and (vi) the sixth untreated borehole water (pH 6.8). Each larval holding cup contained 100 Ml of the said larval medium.

Each day, a total of 60 L4s were randomly picked from the larval rearing trays and placed one in each cup for all the six sets of larval holding cups. Each larva was fed 0.03 mg of Tetramin® baby fish food per day. The experiments were started at 10.00 hours each day and allowed to continue until all the larvae pupated. The set ups were observed at an hourly interval from the start until 19.00 hours each day. The numbers of pupae developing were noted and recorded per the time duration taken for the L4 to pupate. The experiment was replicated five times. Any pupae that developed after 19.00 hours were reported to have developed after 24 hours. Every cup with a pupa was covered with mosquito netting and secured tightly at the top with a rubber band to prevent emerged adult from escaping. The pupae were left in their respective larval holding cups until the adults emerged. The sex of the emerged adult was determined by observing the antennae (male mosquitoes have highly feathered (plumose) antennae while female mosquitoes are sparsely feathered (pilose) antennae. A wing was removed from each emerged mosquito using a pair of fine tip forceps and measured to determine the size of the adult fly as described below.

2.7.2.1. Estimating Mosquito Size

Mosquito size was estimated by measuring the length of one of the mosquito wing (Zahiri and Rau, 1998). The wing was placed over a drop of water on a clean microscope slide, covered with a cover slip and then its length measured under a ×40 magnification from the distal end of alula to the tip, excluding the fringe scales.

2.8. Statistical Analysis

To study the effect of storage period, temperature and storage condition on hatching rate, correlation between percent hatch and storage period for each temperature/storage condition was tested and compared. The effects of temperature on egg viability and pH on pupation of L4s and the sex of emerging adult mosquitoes were analyzed as a function of mosquito size (based on wing length). Pearson correlation coefficient were calculated and tested for significance of each relationship at $p < 0.05$. Least-Squares regression lines were then determined and slopes and intercepts of lines were tested with Analysis of Variance in General Linear Model (Neter et al., 1996). All analyses were done using the Statistical Package for Social Scientists (SPSS) for windows version 11.5.

3. Results

3.1. Effects of Temperature and Storage Time on Egg Hatchability

The experiment was conducted for a month. It was observed that egg hatchability was significantly reduced when stored at a higher temperature: eggs incubated at 28-29 ^{o}C were less hatchable than those incubated at 22-23 ^{o}C ($p < 0.05$). Egg hatchability was affected significantly by the duration of storage time ($p < 0.05$). Eggs, from mosquito parental stock raised on crushed silver cyprinid fish and TetraMin® baby fish food larval diets, were hatchable even after 8 days as opposed to 6 days for mosquitoes raised on baker's active yeast (Table 2). In all cases, egg hatchability reduced as the number of days progressed ceasing altogether after day eight for the best performing larval diet.

Table 2. Mean percentage of *Anopheles arabiensis* eggs hatching out of batches of 20 eggs kept under two different temperature regimes (22-23^{o}C and 28-29^{o}C)

Time (days)	Crushed silver cyprinid fish		Tetramin® baby fish food		Baker's yeast	
	22-23^{o}C	28-29^{o}C	22-23^{o}C	28-29^{o}C	22-23^{o}C	28-29^{o}C
0	71	64	68	24	71	70
2	12	31	62	15	20	10
4	13	0	13	0	12	0
6	7	0	8	0	1	0
8	4	0	1	0	0	0
10	0	0	0	0	0	0
12	0	0	0	0	0	0
14	0	0	0	0	0	0
16	0	0	0	0	0	0
18	0	0	0	0	0	0
20	0	0	0	0	0	0
22	0	0	0	0	0	0
24	0	0	0	0	0	0
26	0	0	0	0	0	0
28	0	0	0	0	0	0
30	0	0	0	0	0	0

3.2. Effects of pH on Pupation of L4s

The experiment was conducted for ten days. It was found that the mean pupation time of L4 larvae maintained in untreated borehole water (pH 6.8) differed significantly compared to L4s maintained in other rearing media ($p < 0.05$) (Table 3). Mean pupation time was neither influenced by sex ($p = 0.124$) or size ($p = 0.801$) of emerged mosquitoes.

Table 3. Mean pupation time and pupation rates (proportion of larvae that pupated within eight hours) of L4s maintained in different rearing media. The proportion of eclosed male mosquitoes is shown in parenthesis

Medium	N	Pupation time (h)	Pupation rate	Eclosion rate	Females	Wing size (mm)
Untreated borehole water (pH6.8)	60	3.28 ± 0.25 ^{a}	0.88	0.82	0.66 (0.34)	3.12 ± 0.02^{b}
Cow dung solution	100	5.6 ± 0.47 ^{b}	0.72	0.71	0.69 (0.31)	2.96 ± 0.02^{b}
PH 6 solution	100	7.09 ± 0.62 ^{b}	0.73	0.73	0.65 (0.35)	2.97 ± 0.17^{b}
PH 7 solution	100	6.67 ± 0.44 ^{b}	0.74	0.72	0.66 (0.34)	3.03 ± 0.17^{b}
PH 8 solution	100	6.54 ± 0.63 ^{b}	0.74	0.73	0.58 (0.42)	2.99 ± 0.02^{b}
PH 9 solution	100	5.99 ± 0.47 ^{b}	0.79	0.66	0.66 (0.34)	2.98 ± 0.02^{b}

Notes:

1. Mean pupation time in hours of mosquito larval stages followed by different letter superscripts in the same row differ significantly.
2. Mean wing size in millimeters of adult mosquitoes followed by same letter superscripts in the same row do not differ significantly.

4. Discussion

In the present study, temperature and length of storage period as contributors to desiccation for mosquito eggs and pH as a determinant of rearing water quality for the larval stages of the mosquitoes were experimented on. The purpose of this step was to obtain information as to their contribution and mechanisms of manipulation towards optimizing mass rearing of the malaria vector *An. arabiensis* mosquitoes.

In the present study, it was found that higher temperatures were unfavorable to egg hatchability, an observation replicated by Bayoh and Lindsay (2003), Lyons *et al*., (2012) and Khan *et al*. (2013). Egg hatchability, in the present study, was found to be inversely proportional to storage time that is hatchability reduced with increased storage time a finding that was similarly reported for *An. gambiae* in Eritrea (Shililu *et al*., 2004).

An. arabiensis eggs in the present study remained viable for only 8 days. This time was similar to that reported for similar species (Khan *et al*., 2013) but shorter than 10 days reported for *An. gambiae* complex eggs kept in drying sandy loam (Shililu *et al*., 2004) and 12 days in dry soils (Beier *et al*., 1990). However, the observed dramatic reduction in the rate of egg hatchability especially after day zero (for freshly laid eggs) is consistent with that observed in other studies (Beier *et al*., 1990; Shililu *et al*., 2004; Khan *et al*., 2013).

There are many studies on the influence of temperature at the early developmental stages of *Anopheles gambiae* (Koenraadt *et al*., 2003), *Anopheles albitarsis*, *Anopheles*

aquasalis (Benedict, 1991), and *Aedes aegypti* (Farnesi *et al*., 2009). Indeed, microscopic observations on *An. gambiae* embryos showed that extreme high temperatures affect humidity that influences desiccation rates. This impacts normal mosquito egg development (Impoinvil *et al*., 2007). This might have been the case in the present study where a low rate of hatchability was observed for temperatures of 28-29^{o}C.

It is known that conditions experienced by juvenile mosquitoes determine mosquito adult characteristics (Lyimo *et al*., 1992; Beck-Johnson *et al*., 2013). In the present study the forth larval instars (L4s) were used to simulate the effect of pH on development and size of emerged adult *An. arabiensis* mosquitoes. Pupation was observed to be more rapid in untreated borehole water (pH 6.8) though mean pupation time for the different emerging sexes (male and female) were not affected by the pH of the rearing solution. The former findings differ while the latter agree with finding of Edillo *et al*. (2006) and Pelizza *et al*. (2007) who found no significant influence of pH on anopheline aquatic stages. Earlier however, water of near neutral pH (pH of 6.8 - 7.2) was observed to be most optimal for the weakening of the egg shells for the first instar larvae stage to emerge (Okogun *et al*., 2003). Indeed, *An. arabiensis* have been observed to associate with waters with low acidity (Robert *et al*., 1998), apparently via the use of ion exchange mechanisms, especially Na+/H+ exchangers, to move acid/base equivalents (Havas,1981). It is most probable that in the present study untreated borehole water (pH of 6.8) was less acidic and approximated the above conditions providing the most optimal condition for the weakening of L4s' and pupae exuvia leading to rapid pupation and emergence of adults mosquitoes, respectively.

Members of *An. gambiae* complex including *An. arabiensis* breed in clean shallow waters that are sunlit (McCrae, 1984; Kweka *et al*., 2012). These are the preferred optimal habitat conditions. In this study, the use of cow dung in the preparation of a rearing medium was to test if the mosquitoes could prefer otherwise and as the results showed this was the case as there was no significant effect of contamination on the development of *An. arabiensis* mosquitoes.

The present study concludes that pH did not contribute to the pupation rate of the L4s though near neutral pH (pH 6.8) was observed to be optimal for pupation. It is also noted that *An. arabiensis* eggs remained hatchable for 8 days when incubated under temperatures of 22-23^{o}C.

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