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Full Length Research Paper

An evaluation of the response of *Anopheles gambiae s.s.* mosquitoes towards varying doses of crude aqueous neem extracts

***[1]Odinga V. Edward, Ruto A. Awori[1], Justice Murumbi[2] and Jeffery Saitoti[1]**

[1]Laboratory of Entomology, Delft University of Technology, Mekelweg 2, 2628 CD Delft, Netherlands.
[2]Department of Microbiology, Faculty of Sciences, Kenyatta University, Nairobi, Kenya.

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More focus is given to mosquito larval control due to the necessity to use several control techniques together in integrated vector management programmes. Botanical products are thought to be able to provide effective, sustainable and cheap mosquito larval control tools. However, bio-larvicides like *Azadirachta indica* (neem) could repel adult mosquitoes from laying their eggs in the treated larval habitats. In this study the response of *Anopheles gambiae s.s.* mosquitoes towards varying doses of crude aqueous neem extracts was examined. Non-choice oviposition tests were used to measure the proportion of mosquitoes laying on the first or second night, or not laying at all, when compared to the control. For each individual mosquito, the number of eggs laid and/or retained in the ovary was counted to determine the relationship between wing length and egg production. Larger female mosquitoes produced larger egg batches. The results show that at a dose of 0.1 g/l, a concentration previously found to be effective at controlling mosquito larvae, the oviposition behaviour of adult female mosquitoes was not significantly affected. The results indicate that the mosquitoes would expose progeny to this neem control tool, making the use of these simple neem wood extracts effective and potentially sustainable.

Key words: Neem, *Azadirachta indica, Anopheles gambiae,* oviposition, malaria, egg laying, wing length, fecundity.

INTRODUCTION

Malaria is arguably the most important tropical parasitic disease in the world. Transmission is centred on the tropics and globally it is estimated that half of the world's population is at risk (Hay et al., 2004, World Health Organisation, 2009). Almost one million people were estimated to have died from malaria in 2008, and there were over 240 million cases (World Health Organisation, 2009). Human malaria is transmitted by female *Anopheles* spp. (Diptera: Culicidae) mosquitoes when they take a blood meal.

The process of taking a blood meal, egg maturation and oviposition (egg laying) in mosquitoes is called the gonotrophic cycle (GC); females can have multiple GCs in their lifetime. Once eggs are mature, *Anopheles gambiae* Giles mosquitoes have a peak of flight activity at dusk, thought to be associated with oviposition-site selection (Jones and Gubbins, 1978); oviposition itself

*Corresponding author. E-mail: ovedward@gmail.com

occurs at night (McCrae, 1983) over a 2-4 h period (Fritz et al., 2008). In the field, A. gambiae are exposed to various different biotic and abiotic factors in natural (Gimnig et al., 2001; Minakawa et al., 2004) and man-made habitats (Mutuku et al., 2006; Howard and Omlin, 2008). Ovipositing mosquitoes can discriminate between these different biotic and abiotic factors using visual, semiochemical and physicochemical cues (Takken and Knols, 1999). Mosquito larvae (McCrae, 1984; Munga et al., 2006), competitors (Munga et al., 2006), predators (Angelon and Petranka, 2002; Blaustein et al., 2005), botanical extracts (Dhar et al., 1996; Elimam et al., 2009) and some types of bacteria (Huang et al., 2006) can repel mosquitoes from ovipositing, whilst other types of bacteria (Lindh et al., 2008), fungi (Sivagnaname et al., 2001) and low levels of conspecific larvae (Sumba et al. 2008) can attract ovipositing mosquitoes.

Due to widespread insecticide resistance in adult mosquitoes (Hemingway and Ranson, 2000), attention has been refocused towards the pre-DDT era control tools including larval control and environmental management (Killeen et al., 2002; World Health Organisation, 2009). These methods are getting more focus because the World Health Organisation recommends that malaria be tackled using integrated vector management (IVM) which uses all available control techniques that are locally appropriate and sustainable (World Health Organisation, 2004). Non-chemical larval control can either use natural predators (Ghosh and Dash, 2007; Howard et al., 2007), entomopathogenic fungi (Bukhari et al. 2010) or botanical larvicides (Shaalan et al., 2005). However, for whichever method is to be used, it is important to determine whether mosquitoes will continue to oviposit in treated larval habitats. This is because a larval control tool cannot be sustainable and effective at controlling successive generations if it prevents female mosquitoes from exposing their progeny to the control tool, especially when untreated oviposition sites are available. If a treatment does not repel mosquitoes then the females will still expose their progeny to the larval control tool.

One botanical larvicide that has received much attention recently is derived from *Azadirachta indica* A. Juss (Meliaceae) (the neem tree). Extracts of different parts of this tree have been effective at killing mosquito larvae both in the laboratory (Okumu et al., 2007; Howard et al., 2009) and field (Awad and Shimaila, 2003; Gianotti et al., 2008). Furthermore, this tree grows in many African countries and could potentially be a sustainable component of IVM programmes. However, in the laboratory neem has been found to be an oviposition deterrent for mosquitoes (Dhar et al., 1996). A study with Anopheles stephensi Liston and Anopheles culicifacies Giles using a range of neem extracts found that 7 day old gravid mosquitoes exposed to neem volatiles for 90 min exhibited oviposition suppression, with neem-exposed females retaining significantly more eggs than control mosquitoes (Dhar et al., 1996). Females that were exposed to neem-derived volatiles immediately after mating and were left exposed to these volatiles for several days did not fully develop eggs either in that or successive GCs (Dhar et al., 1996). Similarly, when neem was fed to *A. stephensi* mosquitoes either before or during a blood meal, egg maturation and oviposition were adversely affected (Lucantoni et al., 2006).

Previously we have shown that a dose equivalent to 0.1 g of neem wood per litre of water causes a significant increase in larval *A. gambiae* Giles *s.s.* development time, and was also able to cause significant levels of mortality (Howard et al., 2009). In the current study, *A. gambiae s.s.* mosquitoes were used in non-choice experiments to test whether the 0.1 g/l and other doses of crude aqueous extracts of neem affected mosquito oviposition behaviour.

MATERIALS AND METHODS

Preparation of aqueous insecticidal extracts

Neem extracts were prepared as previously described (Howard et al., 2009). Briefly, wood and bark from neem trees collected from Mbita Point in Western Kenya were fed into a basic wood chipping machine to produce wood chippings (roughly 1 x 3 x 0.2 cm), which were left to dry in the shade. These dry chippings were then soaked in distilled water for five days after which time the water was filtered, removing the neem chippings and leaving just the aqueous extract into which the neem phytochemicals had leached. This simple method was used because it is more likely to provide sustainable control in the field than refined extracts requiring complex equipment and infrastructure. The different concentrations used in the oviposition experiments (equivalent to 0.1, 1 and 10 g neem wood per litre water) were made by serial dilution from a stock solution. Distilled water was used for the controls.

Mosquitoes

The Kisumu strain of *A. gambiae s.s.* was used. This strain has been maintained as a colony at the Kenya Medical Research Institute (KEMRI), Kisumu, for 17 years. After standard rearing, pupae were separated and placed into an adult cage for emergence. The following day any live pupae that had not emerged during the night were removed from the cage to ensure all adults were the same age. Both male and female adults were kept in the cage to allow mating to occur. *A. gambiae s.s.* host seeking peaks at day 4 post emergence (Takken et al., 1998), so once adults were four days old, females were blood fed on a live rabbit for 30 min. One hour after feeding, female mosquitoes that had ingested a full blood meal were moved to a new cage along with a number of male mosquitoes to allow unmated females to mate. Mating can increase the chance of egg maturation (Klowden and Russell, 2004) and females mating after a blood meal are as likely to oviposit as those that mate before a blood meal (Chambers and Klowden, 2001).

Two days after the first blood feed, female mosquitoes were again allowed to feed from a live rabbit because sometimes anophelines require multiple blood meals to develop their first batch of eggs (Clements, 1992; Briegel and Horler, 1993; Takken et al., 1998) and host seeking is still peaking at day 6 post emergence (Takken et al., 1998). One hour after this second feed, females that had blood fed or that were already semi-gravid from the first feed were further separated into another cage. Males were also placed

into the new cage. These mosquitoes were left for a further three days before the females were used in the oviposition experiments. Although leaving mosquitoes that had first fed five days previously without an oviposition site may seem a long time, previous research has shown that retention of mature eggs by *A. gambiae* females until an oviposition site is available does not adversely affect oviposition (Chambers and Klowden, 2001).

Throughout this whole process mosquitoes had access to 10% sugar solution soaked in cotton wool that was placed onto the roof of the cage and refreshed daily.

Oviposition experiment

Non-choice experiments were carried out to investigate the effects of water treatment on whether mosquitoes chose to lay their eggs and if so, if the mosquito laid at the first opportunity or waited until it became obvious no other option was available. Standard (30 x 30 x 30 cm) wire frame cages covered in cotton netting were used for the experiments. The wooden bottoms of these cages were painted black because more *A. gambiae s.l.* eggs are laid over dark than light areas (McCrae, 1984). For the oviposition sites, 40 ml of neem-treated or control water was soaked onto cotton wool in a Petri dish. A 90 mm filter paper was then placed on top of this wet cotton wool. At 5 pm a single gravid female mosquito and one Petri dish were randomly allocated to each cage. Cotton wool soaked in 10% sugar solution was placed onto the roof of the cage and refreshed daily. The mosquitoes were exposed to a natural dusk and left in a natural 12:12 h L:D cycle. The mean (±SE) maximum and minimum temperatures during the study were 30°C (±0.10) and 25°C (±0.11) respectively; the mean (±SE) humidity was 80% RH (±0.11).

The next morning, any mosquitoes that had died or were stuck to the filter paper were removed from the experiment. For mosquitoes continuing with the experiment, Petri dishes were removed from the cages and the number of eggs on each was counted using a dissection microscope. The Petri dishes were then put back into the cages. Mosquitoes were left in the cage to allow them to oviposit during the second night of the experiment. The following morning any mosquitoes that had died or were stuck to the filter paper were removed from the experiment. The Petri dishes were removed and the number of eggs counted again using a dissection microscope. Mosquitoes were removed for dissections as described below. Thirty replicates were carried out per water treatment (not including mosquitoes failing to complete the experiment).

Mosquito dissections

The morning after the second experimental night, mosquitoes were individually removed from the cages, knocked down in the freezer for 5-10 min and then dissected. Dissections were carried out on glass slides using hypodermic needles under a dissection microscope. Firstly, a dry dissection was carried out and one wing was randomly selected and removed from each mosquito and placed on a separate glass slide. Wings were measured from the tip (excluding fringe scales) to the axillary incision using a compound microscope and ocular micrometer.

For the wet ovary dissections, 0.85 g AnalaR salt (NaCl) was put into 100 ml distilled water to make a saline solution. A few drops of this saline solution were used to aid mosquito ovary removal. Ovaries were then gently opened and the number of eggs remaining inside was counted using a dissection microscope.

Statistical analysis

s.s. do not mature eggs (Hogg et al., 1996). Therefore, as well as the 9.7% (14/144) mosquitoes that died or stuck to the filter paper, the 5.5% (8/144) mosquitoes that had not developed eggs were also discarded from both types of analyses.

Effect of neem on oviposition

The purpose of this study was to see if neem treatments would affect oviposition behaviour by causing the mosquitoes to retain their eggs either for oviposition on the second night or in their ovaries at the end of the experiment. Therefore, the interest was in looking at whether the treatments had caused the number of mosquitoes that laid/retained their eggs to vary, rather than examine the number of eggs laid in each treatment. To analyse whether the number of mosquitoes laying or retaining eggs significantly differed between the four water treatments, the mosquitoes having laid (1^{st} or 2^{nd} night) or retained eggs were coded, and these coded data were analysed using chi-square tests. Since this involved three different statistical tests, all involving the control, the significance level was adjusted using the Bonferroni method. Therefore, the behaviour was reported as significantly different from the control if the p value was less than 0.017 (0.05/3).

The relationship between wing length and egg production

After testing to see if the data sets (wing lengths and total number of eggs produced (laid plus retained) per mosquito) were normally distributed, single factor ANOVA was used to test for any significant differences in the number of eggs produced by females exposed to each treatment. Similarly, single factor ANOVA was used to test for any differences in the wing length of females exposed to each treatment. No significant differences were found so the data were pooled and the correlation between wing length and number of eggs produced was analysed using simple linear regression. To see if there was a significant difference between the numbers of eggs that small and large mosquitoes produced, a two-sample t-test assuming equal variances was carried out. Analyses were carried out in SPSS 17.0 (SPSS Inc, 2008) with α set at 0.05.

RESULTS AND DISCUSSION

Effect of neem on oviposition

There were no significant differences in the total number of eggs produced (laid plus retained) ($F=2.39$, $df=3,118$, $p=0.07$) between mosquitoes exposed to the four treatment types, indicating that when fully gravid mosquitoes are exposed to neem, the exposure does not significantly affect egg production (that is, by making mosquitoes reabsorb eggs (Clements, 1992).

It was found that mosquitoes either laid all of their eggs on one night, or retained all of their eggs. Only 4.1% (5/122) of mosquitoes laid their eggs over a number of nights, and these were distributed between the four treatment groups. In addition, most of the mosquitoes laid their eggs did so on the first night (Figure 1), just 10.7% (13/122) of the mosquitoes laid their eggs on the second night. Of these, only one was exposed to the control treatment and four mosquitoes came from each of the neem treatments. However, there was no significant difference between the four water treatments with respect to the day mosquitoes laid their eggs ($\chi^2 = 1.1$, $df=3$,

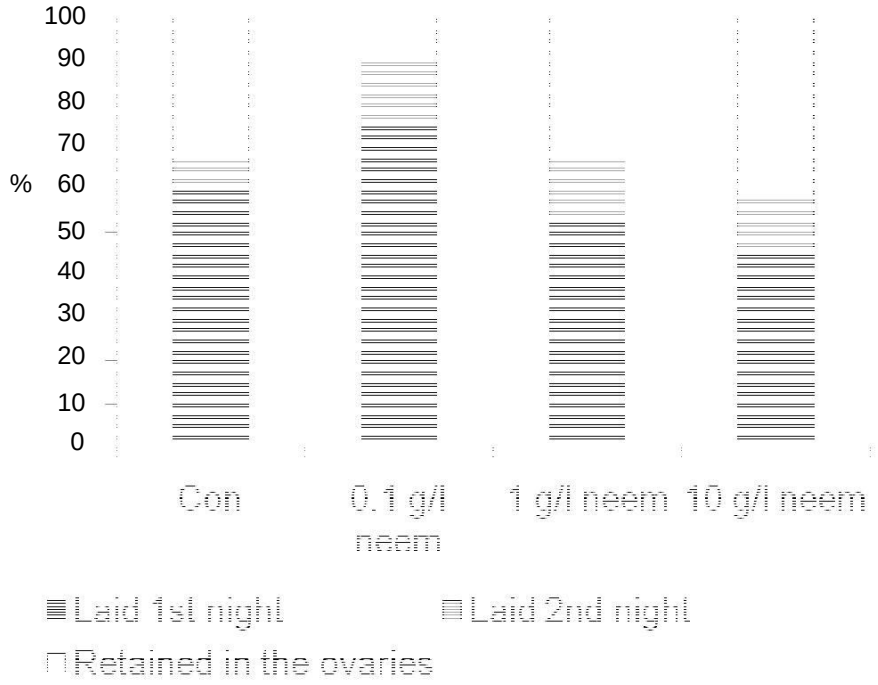

Figure 1. Proportional breakdown of mosquitoes laying eggs on the first (black) or second (grey) nights, and those retaining eggs in the ovaries (white) after being exposed to control water (N=30) or aqueous neem extracts at concentrations of 0.1 g/l (N=29), 1 g/l (N=30) or 10 g/l (N=33).

p=0.77).

Sixty percent of the mosquitoes exposed to control water laid their eggs on the first night, and a further 6.7% laid on the second night. This left 33.3% of the

mosquitoes with eggs retained in their ovaries (Figure 1). Chambers and Klowden (2001) had a similar finding with just two-thirds of their *A. gambiae s.s.* females ovipositing their eggs in two consecutive nights. For the lowest neem concentration of 0.1 g/l, 75.8% of the mosquitoes laid eggs on the first night, a further 13.8% laid on the second night and just 10.4% of mosquitoes retained eggs in the ovaries (Figure 1). When comparing mosquitoes that laid eggs with those that retained their eggs, and after adjustment for the Bonferroni method, there was no significant difference between the number of mosquitoes that laid their eggs when exposed to the low neem dose of 0.1 g/l when compared to the control-exposed mosquitoes (χ^2=4.5, df=1, p=0.033). For both the 1 g/l (χ^2=0.0, df=1, p=1.0) and 10 g/l (χ^2=0.5, df=1, p=0.458) doses there were also no significant differences in the number of mosquitoes either laying or retaining their eggs when compared to the control mosquitoes.

In a previous study the same type of neem-treated water and the same mosquito strain was used, and it was found that at 0.1 g/l, larvae exposed during the first three instars had significantly increased development times when compared to larvae reared in control water (Howard et al., 2009). In addition, the concentration that inhibited 90% of adult emergence (IE90) was around 0.15 g/l for early instar mosquito larvae (Howard et al., 2009). These oviposition results show that if neem wood was applied to water bodies at a concentration of around 0.1 g/l then not only would mosquito larvae take significantly longer to develop into adults, with significantly fewer surviving to adulthood (Howard et al., 2009), but these preliminary results suggest that the adult mosquitoes would not be significantly deterred from laying their eggs in the neem-treated water, so successive generations of mosquitoes would keep being exposed to the botanical larvicide. In addition, ovipositing *A. gambiae s.s.* adults have been shown to exhibit a memory (Sumba et al., 2004) because they prefer to oviposit in the same water type in which they were reared, when compared to water in which another *A. gambiae s.s.* strain was reared (Ogbunugafor and Sumba, 2008). This preference for “known” water has even been found when mosquito repellents were placed in water (Kaur et al., 2003); *Aedes aegypti* L. mosquitoes reared in water containing citronella and neem exhibited reduced repellence towards ovipositing in the treated water than mosquitoes reared in clean water (Kaur et al. 2003). The results suggest that at 0.1 g/l, *A. gambiae s.s.* larvae can be considerably controlled (Howard et al. 2009) and females will still oviposit in the water. Given previous findings about mosquito memory (Kaur et al., 2003; Sumba et al., 2004; Ogbunugafor and Sumba, 2008), any mosquitoes emerging from the

neem-treated water may preferentially return to oviposit in that water, exposing their progeny to the control measure.

Previously, neem has been shown to repel mosquito oviposition. Dhar et al. (1996) used short exposures to show that gravid 7 day old Anopheles mosquitoes laid significantly more eggs in the control water when compared to mosquitoes exposed to broken neem seed kernels, purified neem oil and neem volatile fractions (Dhar et al., 1996). The current results show no repellency caused by neem exposure, and this lack of a repellent effect is likely due to the lack of azadirachtin in our neem water (Howard et al., 2009). To our knowledge no previous work has been published showing the oviposition response of mosquitoes to pure azadirachtin. However, azadirachtin has been found to repel oviposition by other insects including the sweetpotato whitefly (Kumar and Poehling, 2007) and diamondback moth (Lui and Lui, 2006). It is therefore likely that the oviposition-repellent constituent in neem is azadirachtin.

It is promising that repellent properties were not found in this study when a simple application method was used. The expectation is that community involvement in mosquito control will increase as IVM programmes spread across Africa (World Health Organisation, 2004; van den Berg and Takken, 2007). Communities are more likely to use mosquito control methods that require the least sophisticated equipment and infrastructure, and this will be especially true in resource-poor rural areas. Therefore, the finding that when raw neem wood is placed into water at a relatively low dose the proportion of mosquitoes ovipositing is not affected, is encouraging. In addition, no repellent effects were seen even at a dose 100 times that required for successful mosquito control (Howard et al., 2009). If this simple application of the control tool is to be used by rural communities, then the dose may not always be controlled. This could lead to overtly high doses being used, but evidence suggests that even these very high doses will not adversely affect mosquito oviposition behaviour. However, these laboratory results need to be verified in the field, because it is possible that the oviposition response to neem is different in natural water bodies that produce a range of volatile signals. In addition, choice tests need to be carried out to determine how mosquitoes would react when given a choice of oviposition substrates.

The relationship between wing length and egg production

Whilst neem has been shown to affect egg development in mosquitoes when given before or with the blood meal (Lucantoni et al., 2006), the mosquitoes in the current study had developed their eggs before being exposed to neem. As a result, no significant difference between the number of eggs produced by mosquitoes in the four treatment groups was found ($F = 2.39$, $df = 3,118$ and $p=0.07$). In addition, there were no significant differences between the wing lengths of the mosquitoes in the four treatment groups ($F=0.05$, $df=3,118$ and $p=0.98$) so the data were pooled for the purpose of examining the relationship between wing length and egg development.

The mean (±SE) wing length was 3.09 mm (±0.01), and the mean (±SE) number of eggs produced was 53.6 (±2.9). The number of eggs produced by individual mosquitoes was significantly ($n=122$; adjusted $r^2=0.25$ and $p<0.0001$) and positively correlated in a linear fashion with wing length (Figure 2). Thus, 25% of the variation in the number of eggs produced is explained by the mosquito wing length. This positive correlation between wing length and the number of eggs produced has previously been found in laboratory colonies (Briegel, 1990; Takken et al., 1998) and wild caught mosquitoes from Tanzania (Lyimo and Takken, 1993), The Gambia (Hogg et al., 1996) and Mali (Yaro et al., 2006).

It was also found that providing two blood meals was sufficient to get even small mosquitoes to mature eggs. It has been previously suggested that A. gambiae females with wing lengths shorter than 3 mm are unable to start oogenesis after the first blood meal (Briegel, 1990; Lyimo and Takken, 1993). In this study, 27% (33/122) of mosquitoes that produced eggs had wings shorter than 3 mm (Figure 2).

Wing length is used as a measure of mosquito body size. Larger *A. gambiae* females have been shown to have higher levels of lipid, protein and carbohydrate at eclosion (Briegel, 1990). They also take larger blood meals (Briegel, 1990), are better able to utilize the meal (Takken et al., 1998) and are therefore able to produce more (Briegel, 1990; Lyimo and Takken, 1993; Hogg et al. 1996; Takken et al., 1998) and larger (Takken et al., 1998) eggs. In addition, larger blood meals lead to a higher protein content per egg (Briegel, 1990). Larger female mosquitoes therefore have a higher reproductive efficiency (fecundity) than smaller mosquitoes. In agreement with this, when mosquitoes from the present study were categorised as being small (wing length <3.15 mm) or large (wing length ≥3.15 mm), there was a significant difference in the mean number of eggs that each group produced ($t=6.26$, $df=120$, $p<0.0001$) with small females producing a mean (±SE) of 40.7 (±2.9) eggs compared to 72.9 (±4.7) for large females. As well as producing more eggs, larger females also tend to live longer, host seek more (Takken et al., 1998) and require fewer blood meals to become fully gravid (Lyimo and Takken, 1993).

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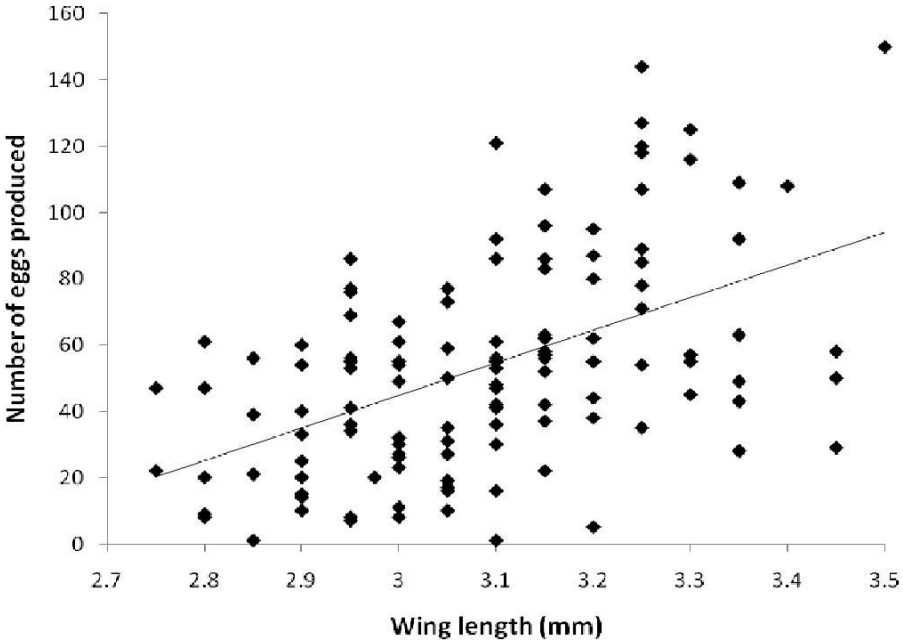

Figure 2. Number of eggs produced (laid plus retained) per mosquito in relation to wing length in *A. gambiae s.s.* mosquitoes. Line represents linear regression (adjusted r^2=0.25; p<0.0001).

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