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Courtship activity, copulation & insemination success in a mosquito vector fed a herbal aphrodisiac: Implications for sterile insect technology

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***Background & objectives*: In sterile insect technology (SIT), mating competitiveness is a pre-condition for the reduction of target pest populations and a crucial parameter for judging efficacy. Still, current SIT trials are being hindered by decreased effectiveness due to reduced sexual performance of released males. Here, we explored the possible role of a herbal aphrodisiac in boosting the mating activity of *Aedes aegypti*.**

***Methods*: Males were fed one of two diets in this study: experimental extract of *Eurycoma longifolia* (MSAs) and sugar only (MSOs). Differences in life span, courtship latency, copulation activity and mating success were examined between the two groups.**

***Results*: No deaths occurred among MSA and MSO males. Life span of MSOs was similar to that of MSAs. The courtship latency of MSAs was shorter than that of MSOs ($P<0.01$). MSAs had greater copulation success than MSOs ($P<0.001$). In all female treatments, MSAs mated more than MSOs, but the differences in rate were significant only in the highest female density ($P<0.05$). In MSAs, mating success varied significantly with female density ($P<0.01$), with the 20-female group ($P<0.01$) having the lowest rate. Single MSA had better mating success at the two lowest female densities. In MSOs, there were no significant differences in mating success rate between the different female densities.**

***Interpretation & conclusions*: Our results suggested that the herbal aphrodisiac, *E. longifolia*, stimulated the sexual activity of *Ae. aegypti* and may be useful for improving the mating competitiveness of sterile males, thus improving SIT programmes.**

Key words *Aedes aegypti* - aphrodisiac - *Eurycoma longifolia* - mosquito vector - sexual behaviour - sterile insect technique

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Mosquito-transmitted illnesses have become a major global public health threat worldwide[1]. In 2016, the primary dengue vector, *Aedes aegypti*, was found to be responsible for many recent outbreaks of both dengue and Zika virus infections[2]. Insecticide use, the main strategy to combat dengue vectors has been ineffectual due to the development of resistance in mosquito populations[3]. The vector *Ae. aegypti* has developed resistance to nearly all classes of insecticide used to date[4]. Therefore, it is necessary to search for alternative vector control strategies.

The sterile insect technique (SIT) has been widely adopted as a sustainable method for control of mosquito-borne diseases[5]. The technique consists of mass-rearing, sterilization and release of sterile males into the wild, where they will compete for mates with their wild counterparts[5]. As they are sterile, such matings will produce no offspring and thus reduce the size of the target population[5]. However, most of these programmes also showed reduced sexual competitiveness of the released insects compared to their wild counterparts[6]. The Food and Agriculture Organization[7] argued that the triumph or failure of this control strategy would be directly related to the ability of the released laboratory-produced insects to effectively mate with wild females. In SIT, larvae of the target insect are reared in substantial numbers[8], a procedure that is known to affect adult quality and mating ability of the released adults[9]. Ionizing radiation, the main technique used for sterilization also reduces both competitiveness and life span[5]. Reduced longevity of released males can substantially decrease the effectiveness and increase the economic costs of SIT programmes[5]. Thus, the ability to produce laboratory insects with high sexual efficiency in the wild remains a major challenge in implementation of the SIT strategy.

SIT represents a promising strategy for dengue vector control[10]. Much of this optimism is based on the 72 per cent reduction in the wild population size obtained by Bellini *et al*[11]. There has been a marked increase in anti-dengue mosquito SIT trials in many parts of the world[12].

For many centuries, aphrodisiacs have been used to increase libido and arousal, especially in individuals with sexual problems[13]. Herbs such as *Ginseng*, *Rehmannia*, *Epimedium*, *Cordyceps*, *Lepidium meyenii* (maca), *Muira puama*, *Turnera diffusa* (damiana), *Ginkgo*, *Tribulus terrestris* and *Yohimbine* derived from the bark of the *Pausinystalia yohimbe* tree, have long been used as aphrodisiacs[14,15].

It has been shown that the scent of ripe and rotting fruit activates courtship-initiating brain pathways in the fruit fly, *Drosophila melanogaster*. These pathways, or networks of connected neurons, were previously shown to encourage male fruit flies to engage in courtship displays and begin mating[16]. Crickets infected with a viral aphrodisiac commence courtship at least twice as fast as healthy male crickets[17]. This study was undertaken to examine whether and to what extent the dengue vector, *Ae. aegypti*, survived after the uptake of a natural aphrodisiac. The impacts of such consumption on sexual behaviours, such as courtship latency, copulation activity and mating success, were also investigated.

Material & Methods

Mosquito rearing and stocks: Samples of eggs originating from a colony of *Ae. aegypti* from the University Sains Malaysia, Penang, Malaysia, were used to establish a colony at the Entomology laboratory (External Laboratories of the Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, Kota Samarahan). Eggs were hatched in dechlorinated water and reared at a density of 200-250 in 4 l plastic trays (As One Corporation, Osaka, Japan) filled with 800 ml of aged tap water as delineated elsewhere[18]. Larval food consisted of powdered cat food pellets (ProDiet Cat Food, Malaysia) provided in an amount of 0.10-0.15 g every two days. The rearing medium was replaced with fresh medium before the third food supply. Pupae were collected from rearing trays and placed in 250 ml plastic vials containing 10-15 ml of water, which in turn were transferred into mosquito breeding cages measuring 30×30×30 cm (BugDorm; MegaView Science Co., Ltd., Taichung, Taiwan). Adults had access to a 10 per cent sucrose solution and females were blood-fed on hamsters 4-5 days post-emergence. Eggs were air-dried and stored as a stock colony as described[19]. The laboratory environmental conditions were 21-34°C, 60-86 per cent relative humidity and photoperiod 13:10 h (light:dark) with one hour of dusk.

Production of experimental virgin subjects: For colony establishment, egg samples taken from the stores were submerged in dechlorinated water. Twenty four hours later, newly hatched larvae were raised in a 4 l plastic tray (Tray 1) at a density of 1000 and supplied with 0.15 g of ProDiet cat food every two days, with the rearing medium being replaced with fresh before

the third food provision. To ensure the isolation of males from females, pupae were kept in individual 1.5 ml Eppendorf tubes containing 0.15 ml of water. On emergence, adults' sex was determined under a stereomicroscope (SZ-LED; Kenis, Osaka, Japan). To prevent mating before bioassays, males and females were pooled in separate breeding cages (30×30×30 cm) and provided with 10 per cent sucrose solution. To produce blood-fed females, a restrained hamster was placed within the cage for one hour. Fully blood-fed females were transferred into another cage where they were allowed to digest the blood meals for 24 h. These females were referred to as blood-fed virgin females.

Experimental aphrodisiac and experimental extract: The natural aphrodisiac selected in this study was the root of the Borneo strain of *Eurycome longifolia*. This *Simarouba* species is known as a supplement for enhancing sexual performance[20,21]. Conventionally, the plant is used as a decoction[21]. Several animal and human trials have been conducted to evaluate the properties of the roots using either water or ethanol extracts[21,22]. A sample of the root of the plant obtained from the Faculty of Resource Science and Technology (Universiti Malaysia Sarawak) was cut into slices of 0.5-1 cm in thickness. A sample of 0.25 g weighed using a Vibra analytical balance (Shinko Denshi Co. Ltd., Tokyo, Japan) was soaked in 50 ml of water in a 250 ml plastic container and allowed to decay. After five days of disintegration, 10 g of sucrose was added to the filtered solution and the volume was adjusted to 50 ml. The solution thus obtained after complete dissolution of the sugar was designated as the experimental aphrodisiac extract.

Male survival and longevity following uptake of Eurycoma longifolia extract: As *E. longifolia* root extract can be toxic at certain doses[22], the impact of its uptake was examined on adult longevity. Briefly, 30 males that emerged on the same day were placed in a standard adult mosquito cage (30×30×30 cm) where they were allowed to feed on 10 per cent sucrose solution. After two days of sugar feeding, 15 males were singly placed in an experimental unit consisting of a glass tube (2×8 cm) with its cover modified to hold a 1.5 ml Eppendorf tube with the bottom cut out. All males had access to 10 per cent sucrose-aphrodisiac solution through cotton wicks placed through the cover crossing the Eppendorf tube. The remaining 15 males were individually transferred into similar tubes but with cotton wicks saturated with sugar solution (control). For convenience, the males fed sugar-aphrodisiac were designated as MSA and males fed sugar only were designated as MSO. All 30 tubes (15 MSA tubes and 15 MSO control tubes) were inspected after 24 h for adult death.

Another experiment was conducted to examine the longevity of males. Briefly, 24 males from the previous experiment were divided into two groups of 12 individuals each. Each group was placed in one of the subsequent environments: (*i*) cage holding a 250 ml feeding device containing 200 ml of sugar-aphrodisiac solution, or (*ii*) cage holding a 250 ml feeding device containing 200 ml of sugar solution. Six days later, the apparatuses (sugar and sugar-aphrodisiac) were removed from the cages and both groups were given continuous access to 10 per cent sucrose solution. The two cages were inspected daily and the number of dead individuals was recorded until all 24 individuals had died. Both survival and longevity experiments were conducted under the laboratory environmental conditions: 21-34°C, 60-86 per cent relative humidity and photoperiod 13:10 h (light:dark) with one hour of dusk.

Courtship activity following uptake of E. longifolia extract: Ten virgin MSAs (3-4 days old) were placed in a mating cup, which consisted of a 250 ml transparent plastic container with two opposite mesh net-screened windows (each 1 cm^2) and covered with a lid at the middle having an aperture filled with a 1.5 ml Eppendorf tube; the bottom of the tube was cut out and filled with a cotton wick saturated with 10 per cent sucrose solution. After 10 min of adaptation to the plastic container environment, five sugar-fed virgin females (4-5 days old) were released together into the cup. Immediately upon release, an assistant sitting nearby began recording the time to first copulation attempt (courtship latency) and the number of successful copulations (copulations) within 30 min. Similarly, ten virgin MSOs (3-4 days old) and five sugar-fed virgin females (4-5 days old) were placed in another cup and observed as described above. For MSA and MSO, ten and eight replicates, respectively, were observed on different days. All of the observations were performed between 1600 and 1800 h.

Mating success following uptake of E. longifolia extract: One virgin MSA (3-4 days old) and five blood-fed virgin females (3-5 days old) that had digested blood meals for two days were placed together in a standard adult mosquito cage and allowed to

cohabit in the presence of 10 per cent sucrose solution. After one day of cohabitation, the females were singly placed in an oviposition device consisting of a glass tube (2×8 cm), identical to the experimental unit used in the male survival study, except it was lined with a section of filter paper as an egg deposition site. After a three-day oviposition period, the eggs were allowed to dry up in the laboratory environment for five days. The dried eggs were flooded by fully filling the glass tube with water (48 ml) enriched with 0.03 g of yeast, as described elsewhere[23]. Flooding was repeated two more times with a five-day drying period between the two bouts of flooding. This bioassay was repeated a second time but with one old virgin MSO (3-4 days old) and five blood-fed virgin females (4-5 days old) (control). The bioassays '1 MSA×5 blood-fed females' and '1 MSO×5 blood-fed females' were replicated nine and eight times, respectively. Experimental procedures similar to those described above for '1 MSA×5 blood-fed females' were also carried out for: (*i*) 1 MSA×10 blood-fed females/1 MSO×10 blood-fed females, and (*ii*) 1 MSA×20 blood-fed females/1 MSO×20 blood-fed females. There were 10 replicates for each of the bioassays '1 MSA×10 blood-fed females' and '1 MSO×10 blood-fed females'; four and three replicates for '1 MSA×20 blood-fed females' and '1 MSO×10 blood-fed females', respectively. In all bioassays, the tubes were monitored for 24 h after flooding for the presence or absence of newly hatched larvae, which was used to score successful mating.

Data collection: In the survival bioassay, the status (live or dead) of both MSO and MSA individuals was recorded in each glass tube replicate. These numbers were used to compute the survival rate for MSO and MSA groups. This rate was considered as the total number of males that survived after one day of exposure to a given adult experimental feeding source/15×100. In the longevity bioassay, the number of days between egg hatching and adult death was scored as the life span. In the courtship behaviour study, immediately after the collective release of the five females into the cup, the assistant used a stopwatch to begin recording the courtship latency, which was defined according to Eastwood and Burnet[24] as the time taken by a male to initiate courtship of a female. With reference to Roth[25], we considered initiation of courtship when a male tried to attach itself to a flying female and was noted for each cup replicate. Copulation was defined as any effective genital contact of a formed copula, which persisted for at least 10 sec, following Roth's[25] definition. The mean values of courtship latencies and numbers of copulations were used as parameters of courtship behaviour. In the mating success study, a female that had cohabited with either MSAs or MSOs of which at least one egg has hatched during one of the three flooding events was judged as mated. Such females were counted and the resulting numbers were used to calculate mating success rate as the number of females with at least one hatched egg/initial number of virgin females cohabited with males ×100.

Statistical analysis: The dissimilarities in survival, longevity, courtship latency, number of copulations and mating success were examined by analysis of variance (ANOVA) using Systat v.11 statistical software (Systat Software, Inc., Richmond, CA, USA). In the mating success study, means (±standard error) were analysed by Tukey's honestly significant difference test where needed.

Results

No deaths occurred among the males that were allowed to feed on sugar (MSOs) or sugar- *Eurycoma* extract (MSAs) for 24 h. However, an additional six days exposure of these two groups of males to their respective food sources followed by continuous maintenance on sugar resulted in different life spans. *Ae. aegypti* MSOs had a life span of 84.08±5.25 days (range: 33-100 days), while the mean life span of their counterparts maintained on the herbal extract (MSAs) was 68.25±6.29 days (range: 33-95 days).

The courtship latency of *Ae. aegypti* males varied significantly with feeding history ($P<0.01$). The time taken by MSOs to initiate courtship of females presented in groups of five individuals was 78.90±13.46 sec (range: 30-182 sec). For MSAs, the time to courtship initiation was 25.10±5.28 sec and ranged between 8 and 53 sec.

The mean number of copulations of *Ae. aegypti* males fed on sugar (MSOs) was 15.62±3.76 and ranged between 5 and 37. In the MSA group, the number of copulatory events ranged from 10 to 37 and averaged 23.20±7.89. The difference in number sexual intercourse events between MSAs and MSOs was significant ($P<0.001$).

The mating success patterns of *Ae. aegypti* male types engaged with different numbers of females are shown in the Figure. In the five-female treatment groups, the mean mating rate of MSAs

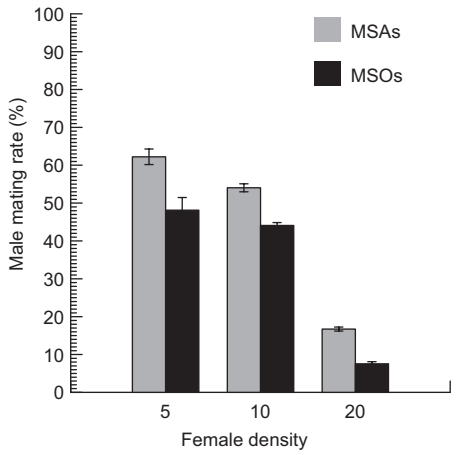

Figure. Mating success. Mean (±standard error) of successful inseminations of *Aedes aegypti* females under three different densities (5, 10, and 20) by males fed sugar solution only (MSO) and those fed sugar+*Eurycoma longifolia* extract (MSA).

(62.22±8.58%, range: 20-100%) was higher than that of MSOs (48.12±13.94%, range 0-100%), but the difference was not significant. In the 10-female treatment groups, the cohabitation of MSAs and sugar-fed females (54.05±4.42%, range: 33.3-70%) tended to result in greater mating success compared to that of MSOs with the same type of females (44.08±3.30%, range: 33.3-62.5%), but the difference between MSA and MSO mating rates was not significant. In the 20-female treatment groups, *Ae. aegypti* MSAs had a mean mating rate of 16.72±2.11 per cent (range: 11.1-20%), whereas their MSO counterparts mated at a mean rate of 7.54±2.12 per cent (range: 5-11.76%); these two mean values were significantly different ($P<0.05$). In MSAs, the mean mating rate differed significantly between female densities ($P<0.01$). The mean mating rate recovered from cages hosting one MSA and five sugar-fed females was 62.22±8.58 per cent, which was higher than that from cages where one MSA cohabited with ten females, but the difference was not significant. The single MSA showed a lower rate of mating when mixed with 20 females (16.72±2.11%) than with five females ($P<0.01$). The mating rate was higher in cages with one MSA and 10 females than in those with one MSA and 20 females ($P<0.01$). In MSOs, there were no significant differences in mating success rate between the different female densities.

Discussion

In the present study, males fed with the aphrodisiac solution (MSAs) initiated courtship earlier than their sugar-fed counterparts (MSOs), indicating that the aphrodisiac stimulated courtship behaviours of *Ae. aegypti* MSAs. Many neurotransmission features and transcripts that are involved in the sexual behaviour control in mammals have homologues in flies[26]. Nearly all courtship rituals in flies require gene expression[27]. *Ae. aegypti* possesses a homologue of the fruitless gene named *Aeafru*, which is expressed in a manner similar to that in flies and is closely linked to the neurochemical control of courtship rituals[28]. There is a close relationship between fruitless gene expression level and courtship intensity. Males that lack the fruitless gene do not court females[29] and low levels of fruitless gene expression hinder courtship activities[29]. In the present study, two solutions were orally administered to young virgin males and those that ingested 10 per cent sugar+*E. longifolia* showed markedly more courtship activities than those provided with the solution free of *E. longifolia.* The enhancing effects of *E. longifolia* on male mating motivation have been well established[30]. The root of *E. longifolia* contains many compounds, including quassinoids, which is known to be an important aphrodisiac molecule[31]. Although neurotransmission, gene expression or the chemistry of the *Eurycoma* extract were not assessed in the present study, the observed differences in courtship latency between MSAs and MSOs might be due to lower dopamine levels in MSOs. It was also likely that MSAs had higher expression levels of the *Aeafru* gene.

MSAs had more successful copulations than MSOs. In *Ae. aegypti* males, the act of copulation involves a series of behavioural actions before transfer of seminal material into the female[32], and this cascade of behaviours has been well described[33]. Both MSAs and MSOs mated with females, but insemination and fertilization success rates were better for the former than the latter. For successful insemination, male mosquitoes must release sperm and seminal fluid into the reproductive tract of the female[34]. Many factors such as mate body size and age[34] have been documented as major factors influencing sperm transfer. In the present study, experimental males were fed the same amount of larval food, emerged as adults on the same day and were 3-5 days old at the time of the bioassay. Therefore, differences in sperm count and quality between groups due to discrepancies in body size or age were unlikely. *E. longifolia* has been reported to improve sperm quality. Chan *et al*[35], working with rodents, investigated the effects of *Eurycoma* extract containing quassinoids. They orally administered different doses of the extract

to normal and infertile males for over one month and found that extract-fed males had significantly higher sperm count, which increased with concentrations of the extract. They reported that the infertile males fed the extract showed appreciable increase in sperm count and spermatozoa motility. In a related study performed in rats, Ambiye *et al*[36] evaluated the spermatogenic activity of a traditional Indian aphrodisiac, *Ashwagandha* (*Withania somnifera*) root. They orally administered *Ashwagandha* to oligospermic males and compared semen parameters between the treated group and a placebo control group. They observed a 167 per cent increase in sperm count, 53 per cent increase in semen volume and 57 per cent increase in sperm motility in the treated group. Increases in sperm number and motility have been reported to augment fertilization success[37]. Although sperm was not investigated in the present study, the increased insemination success rate observed in the MSA group was consistent with good sperm quality.

It was observed *Eurycoma* extract had little impact on survival or longevity of *Ae. aegypti*. The success of SIT programmes depends not only on mating performance but also on the released insects surviving at least as long as their wild counterparts[5]. SIT programmes require repeated release of sterile males into the target site. Sufficient longevity of the released insects would reduce the required frequency of release and therefore, decrease the associated economic costs[5]. In the present study, MSAs lived for about 68 days, which was just 16 days shorter than that in the group fed with 10 per cent sugar solution. The observed increases in courtship, copulation and mating capabilities of MSAs combined with their relative longevity will allow longer inter-release periods, thereby decreasing costs. Additional research is needed to determine whether sterilization by irradiation alters the effects of *Eurycoma* extract observed in the present study.

This study was carried out to evaluate the effects of the herbal aphrodisiac, *E. longifolia*, on the copulatory activities and mating success of *Ae. aegypti* with a view towards its prospective use to improve the mating ability of sterile males, thus benefiting SIT programmes against dengue vectors. Our results showed that the uptake of *Eurycoma* extract was not deleterious to the survival of *Ae. aegypti* males, as evidenced by their long life span. Furthermore, consuming the aphrodisiac shortened courtship latency. More importantly, males fed the aphrodisiac (MSAs) had more copulations and greater insemination success than those that did not (MSOs). In the field, increased courtship activities will tend to generate elevated sexual aggressiveness. With more sexual attacks, males have higher chances of acquiring mates and also increased chances of successful mating. The increased mating success will therefore, cause a reduction in the population size of the target insect as the eggs of the wild females will not hatch. Reduced courtship and mating activity remain major challenges in captive breeding, an important part of biodiversity conservation efforts. This approach is likely to reduce the costs associated with feeding as *E. longifolia* is a dietary supplement[37] and has high energy[30] and nutritional[38] value.

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***Conflicts of Interest*:** None.

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