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A simple and affordable membrane-feeding method for Aedes aegpyti and Anopheles minimus (Diptera: Culicidae)

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ABSTRACT

This study developed an artificial feeding (AF) method to replace direct host feeding (DHF) for the maintenance of Aedes aegypti and Anopheles minimus mosquito colonies. The procedure can be adopted by all laboratories due to its simple and affordable materials and design. The apparatus consists of heparinized cow blood contained in a 5cm diameter glass petri dish with 5cm^2 Parafilm M (Bemis ®) stretched thinly over the top, with a pre-heated bag of vegetable oil placed underneath to keep the blood warm. Both parts are contained within an insulated Styrofoam^{TM} box with a hole in the lid for mosquitoes to access the membrane. Mosquitoes are fed by AF for 15 minutes at a time. Feeding rate and fecundity of Ae. aegypti mosquitoes feeding on the AF device were compared to those feeding on a live rat (DHF(r)), and of Anopheles minimus mosquitoes feeding on the AF device compared to those feeding on a human arm (DHF(h)). Aedes aegypti mosquitoes fed by AF or DHF(r) had similar feeding rates (38.2±21.5% and 35.7±18.2%, respectively) and overall egg production (1.5% difference). Anopheles minimus mosquitoes fed by the AF method had a lower feeding rate (52.0±1.0% for AF compared to 70.7±20.2% for DHF(h)) and overall egg production

(40% reduction compared to DHF(h)). However, the number of eggs produced by AF-fed mosquitoes (1808 eggs per 100 mosquitoes) was still sufficient for colony maintenance, and with increased feeding time both parameters are expected to increase. Reduced feeding rate and overall egg production was observed when Ae. aegypti mosquitoes were fed on blood refrigerated for over two weeks. In conclusion, an AF device has been developed which can replace DHF for Ae. aegypti and An. minimus colony maintenance when using blood refrigerated for a maximum of two weeks.

Keywords: Aedes aegypti, Anopheles minimus, membrane feeding

INTRODUCTION

Pressure to implement the '3Rs' principle, to replace, reduce and refine the use of experimental animals, has increased in recent years. In Thailand this year, specifically, litigation has become much more stringent, making the use of animals ever more expensive and inconvenient. These issues jeopardize the future of research requiring animals in laboratories with limited resources, such as those investigating vector-borne disease in Thailand. Mosquitoes transmit pathogens which cause "several million deaths and hundreds of millions of cases [of disease] every year" (World Health Organization, 2015). One such pathogen is dengue fever; transmitted mainly by Aedes aegypti mosquitoes and resulting in around 20 million cases each year. The situation of dengue fever is particularly bad in Thailand due to the combination of Southeast Asia having the highest incidence of this disease (World Health Organization, 2009), with the emergence of numerous insecticide resistant populations of Ae. aegypti in Thailand (Somboon et al., 2003; Yanola et al., 2009). Consequently, it is imperative that research into understanding the mechanisms of resistance, developing control measures and research into dengue fever itself continues in Thailand in the future.

Animals are used in the above research areas to maintain large colonies of hematophagous mosquito species, including those that transmit dengue fever and malaria. Such species require a blood meal for egg production (Foster, 1995). Customarily, this blood meal was provided by direct host blood feeding (DHF), but the aforementioned animal welfare pressures and economic disadvantages have prompted the development of methods that do not involve a live animal. Moreover, very anthropophagic species require a human blood meal. This practice causes great discomfort to the volunteer and raises concerns about accidental transmission of disease, urging the replacement of this method also.

Numerous studies have developed artificial feeding (AF) methods and document their ability to replace DHF protocols for maintaining mosquito colonies and investigating pathogen infection rate (e.g. Benzon and Apperson, 1978; Yu and Wang, 2001; Tseng, 2003; Rampersad and Ammons, 2007; Deng et al., 2012; Costa-da-Silva et al., 2013; Luo, 2014). However, none of the existing methods are entirely appropriate for laboratories mass-rearing mosquitoes which have limited financial resources. The basic design that the various AF devices follow is that of the original Rutledge feeder, consisting of: a blood reservoir, a membrane through which mosquitoes access to the blood, and a method to keep the blood warm (Rutledge et al., 1964). Most membranes proposed, including collagen membrane casing, Parafilm-M or a condom are, in fact, inexpensive, accessible and feed mosquitoes effectively, but the equipment options for keeping the blood warm are usually expensive (Benzon and Apperson, 1987; Hagen and Grunewald, 1990; Novak et al., 1991; Deng et al., 2012; Costa-da-Silva et al., 2013; Luo, 2014). Deng et al. (2012), alongside other studies, proposed using an electric hotplate to keep the blood reservoir warm, while another method uses a specialised glass water-jacket to surround and heat the blood reservoir (Chemglass©, Yu and Wang, 2001; Phasomkusolsil et al., 2014). These devices are appropriate for feeding small numbers of mosquitoes, but in a mass-rearing situation where many cages need feeding simultaneously, a large number of devices will be required, becoming increasingly expensive. A commercially available AF method, developed by Hemotek Ltd, feeds up to 5-6 cages at once using an electric heating device; however this apparatus costs about $3000. Each of these methods is beyond the budget for many laboratories.

Options for affordable feeding methods are limited. One possibility is simply heating a condom containing blood before feeding, but in this situation the blood temperature drops quickly leading to lower feeding rates, or requiring several re-heating cycles per feed (Hagen and Grunewald, 1990; Novak et al., 1991). Tseng (2003) noted that warming a blood packet

in a water bath results in increased permeability of the Parafilm and subsequent leakage of blood. These issues make the methods too inefficient for mass-rearing purposes. Costa-da-Silva et al. (2013) designed an AF method in which pre-heated glycerol keeps the blood reservoir warm and does not use expensive or specialised equipment. Even so, in this procedure mosquitoes are removed to a small container before feeding, as in various other AF methods, and thus it is time-consuming and inefficient on a mass-rearing scale. The lack of appropriate AF device for mass-rearing mosquitoes prevents many laboratories from adopting AF to replace DHF in mosquito maintenance protocols, so the issues caused by DHF remain.

Consequently, this paper aimed to develop an AF method that can feed Ae. aegypti and the malaria vector Anopheles minimus, while being sufficiently simple and affordable to be used by all laboratories. The fitness of mosquitoes fed on AF or DHF were compared to determine whether DHF can be replaced. Three parameters were measured to represent mosquito fitness: feeding rate, oviposition rate and fecundity. This study also observed the effect of length of blood refrigeration time (~age of blood) on the three fitness parameters of Ae. aegypti mosquitoes. This will be used to determine how long blood can be refrigerated, while still being a suitable blood source.

MATERIALS AND METHODS

Mosquitoes

Ae. aegypti (PMD strain) and An. minimus (CM strain) larvae, pupae and adults were reared and maintained in an insectary in the Department of Parasitology, Faculty of Medicine, Chiang Mai University (CMU), Thailand, at 25±2°C, relative humidity of 80±10% and 12:12 light:dark photoperiod. Both strains originate from Chiang Mai Province and have been maintained in our insectary for over 10 years, routinely using Wistar rats or a human volunteer for the blood meal. For Ae. aegypti, larvae were hatched in large plastic tubs (40×25×10cm) and fed ground dog biscuit (Tesco®) daily, the water was changed when it became murky. Pupae were collected daily in small plastic cups, and placed in holding cages (30×30×30cm) where they emerged and remained as adults until testing. A damp cloth covering the cage was changed daily, to create humidity. Adult mosquitoes were maintained on a solution of 10% sucrose and 10% multivitamin syrup to promote longevity, these were also changed daily. Adult females 5-8 days post-eclosion were used for this study. Mosquitoes were fasted of sugar and vitamin solution ~24 hours prior to testing.

Larvae of An. minimus species were hatched in large plastic tubs (40×25×10cm), using 40W lamp overnight, and fed on ground fish food (Tetra®) once daily. After three days they were separated to ~700 larvae per tub and fed three times daily. After another two days the larvae were separated into tubs containing 80-100 larvae and fed three times daily. Each day pupae were collected into small plastic cups and put into holding cages (30×30×30cm). These were covered with two damp towels, to create humidity, and maintained on a solution of 10% sucrose and 10% vitamin, both of which were changed daily. Adult females 11-15 days old were used in this study. Mosquitoes were fasted of sugar and vitamin solution ~24 hours prior to testing.

Rats

Wistar rats weighing 300-400g were used for DHF(r). They were reared in the Laboratory Animal House, Faculty of Medicine, CMU. Non-anaesthetized rats were constricted in cages, and the rat-cage was placed in the centre of the cage containing mosquitoes for the allotted feeding time. Due to the restriction of blood feeding using laboratory animals, a maximum 15 min feeding time was allowed, which is sufficient for analysis of data. Once testing is complete the rat was marked with blue to ensure it is not used again the same day. The rat hair was maintained at a short length. The blood feeding protocol has been approved by the Animal Ethics Committee, the Faculty of Medicine, CMU (Protocol Number 05/2558).

AF apparatus and conditions for feeding Aedes aegypti

A range of inexpensive and accessible materials were investigated to develop a method which gave a suitable feeding rate (data not shown). The final AF apparatus consists of a blood reservoir where blood is held in a circular, glass petri dish (5cm diameter), with 5cm^2 Parafilm M (Bemis®) membrane stretched thinly over the top. This is placed on top of a sealed plastic bag containing ~130ml vegetable oil acting as the heating element. Preliminary tests showed that this volume of vegetable oil, glycerol or water, heated to 50°C, retained a high enough heat to keep the blood between 35-37°C for at least 15 minutes (feeding time). The blood reservoir and oil heating element are contained in a Styrofoam^{TM} insulated box, with a hole in the lid for mosquitoes to access the membrane (Fig. 1). Bovine blood was collected directly from healthy cows by a veterinarian at the Faculty of Veterinarian Medicine, CMU, during the practice of veterinary students. It was then immediately treated with 5U/ml heparin (5000U/ml stock concentration, 200U/ml working concentration) to prevent clotting. The blood is warmed to 37-38°C before testing, and ~12ml

was used per AF test. Air conditioning is turned off in the insectary for the duration of feeding, any cover on top of the mosquito cage is removed and the AF apparatus is placed in the centre of the cage for each AF experiment. To stimulate feeding activity, carbon dioxide is blown from exhalation into the feeding cage at the start of AF. Care was taken to prevent biohazard by using plastic gloves and disinfection after each experiment.

Feeding rate, oviposition rate and fecundity of Ae. aegypti fed by AF and DHF(r)

Two feeding cages (30x30x30cm) were prepared, each containing 100 female mosquitoes collected from the stock colony. The AF apparatus (as described above) was placed in one cage, while the DHF(r) method (rat-cage, as above) was placed in the other. Blood feeding occurred for 15 minutes, with the AF apparatus gently tilted every 5 minutes to prevent sedimentation. At the end of 15 minutes both feeding methods were removed, and the engorged (fed) females were removed and counted. Engorged mosquitoes were identified by visual observation of a swollen abdomen. Six parallel feeding rate replicates (1x AF cage and 1x DHF(r) cage) were carried out in total: on four consecutive days, followed by a break, and then on two consecutive days.

After feeding, 20-25 engorged Ae. aegypti females were randomly selected from each of the AF and DHF(r) cages. Each mosquito was separated into an isolated oviposition cup. These were lined with filter paper, upon which the eggs were eventually laid, and enclosed with a net. Cotton soaked in 10% sucrose solution was placed on the top of the cup and changed every two days. Three days post-feeding ~12 ml of part distilled, part grass-fermented water was added to the cups to stimulate oviposition. At six days post-feeding the mosquitoes were removed from the cups. The water was carefully poured out and the filter paper left to air dry for 1-2 days. Once dried, the number of eggs on each filter paper was

counted, by eye or stereomicroscope, noting the number of mosquitoes that did or did not oviposit in the given time.

Feeding rate, oviposition rate and fecundity of An. minimus fed by AF and DHF

Several observations were made when the developed AF method was initially used to feed An. minimus during daytime. These mosquitoes did not actively seek and could not locate the blood source when the AF device was placed in the centre of the cage, even when activated by CO_2 or when flying directly over the top. In comparison, once activated by a small amount of CO_2 the Ae. aegypti mosquitoes immediately searched for and located the blood source. The anopheles species was much less active than Ae. aegypti in general; resting on the side of the cages rather than flying around. To achieve an acceptable level of feeding, adaptations of the AF method were required, as follows. Two small cages (15×15×15cm) were prepared, each containing 100 female mosquitoes collected from the stock colony. Two AF devices were positioned up against the opposite sides of one feeding cage so that the blood reservoir membranes were flat against the net and faced inwards. For comparison, the forearm of one of the authors (CF) was placed against one side of the other feeding cage (DHF(h)). Feeding was carried out for 15 minutes, tilting the AF apparatus every 5 minutes. After removing the feeding mechanisms, the engorged females were removed and counted. This was repeated three times on one day.

After feeding, ~30 engorged females were randomly selected from each of the AF and DHF(h) cages and removed to a large cup. The cups were lined with paper and enclosed with net. A piece of sugar-soaked cotton was placed on top and changed daily, and a plastic bag was placed over the top to maintain humidity. Four days post-feeding the mosquitoes were separated to isolated oviposition cups; lined with filter paper and pre-filled with ~12 ml distilled water. These cups were sealed with a net and covered with black plastic. The

198 mosquitoes were allowed to lay eggs for 2 days. The laid eggs were counted, noting the
199 number of mosquitoes that did or did not oviposit in the given time.
200
201 **Testing the viability of refrigerated blood for AF Ae. aegypti**
202 Cow blood was collected and refrigerated at 4°C until used for testing. Fresh cow
203 blood was collected on the same day as testing and used as a control. Two comparisons were
204 carried out: Ae. aegypti mosquitoes fed by fresh blood or to two week refrigerated blood, and
205 Ae. aegypti mosquitoes fed by fresh blood or three week refrigerated blood. Feeding rate,
206 oviposition rate and fecundity were measured in both comparisons, following the above
207 methods for this species. Each comparison had three parallel replicates that were completed
208 on one day.
209
210 **Stastical analyses**
211 GraphPad Prism 6 and Microsoft Excel 2010 programmes were used for figure
212 generation and data analysis. Mantel-Haenszel chi^2 test was used to analyse feeding rate and
213 oviposition rate of Ae. aegypti and An. minimus mosquitoes fed by AF or DHF methods. Chi^2
214 for trend was applied to feeding rate data from Ae. aegypti fed on different ages of blood. To
215 analyse the average number of eggs per mosquito Paired t-test was used. Significance was
216 determined when $p<0.05$.

RESULTS

Feeding rate, oviposition rate and fecundity of Ae. aegypti fed by AF and DHF(r) methods

Females of Ae. aegypti visited the AF device immediately after it was placed in the cage. There was no significant difference between the feeding rates (mean±s.d.) of Ae. aegypti mosquitoes fed by AF or DHF(r), 38.2±21.5% and 35.7±18.2%, respectively, (Fig. 2a). When considering the separate parallel tests, in three tests AF had the higher feeding rate and in three tests DHF(r) had the higher feeding rate (Fig. 2b). Very low mortality of engorged females from AF and DHF(r) was observed until oviposition. There was no significant difference in the oviposition rate of engorged mosquitoes from the groups fed by AF or DHF(r), 98.5±2.3% and 97.3±3.2%, respectively. Among the mosquitoes that fed, on average the DHF(r)-fed group laid significantly more eggs per mosquito than the AF-fed group (paired t-test, $t = 2.76$, d.f.= 5, $p<0.05$); DHF(r)-fed mosquitoes laid 95.3±11.2 eggs per mosquito, and AF-fed mosquitoes laid 86.7±7.8 eggs per mosquito. Despite the difference in egg production per mosquito, overall egg output (calculated average egg output per 100 females) showed a negligible 1.5% fewer eggs were produced by AF than DHF(r); mosquitoes fed by AF produced 3262 eggs, and those fed by DHF(r) produced 3310 eggs (calculated average egg output per 100 females= average fecundity per female x feeding rate x oviposition rate). There was no difficulty of larval hatching in both feeding methods (data not shown).

Feeding rate, oviposition rate and fecundity of An. minimus fed by AF and DHF(h) methods

241 The average feeding rate of An. minimus mosquitoes fed by DHF(h) was significantly
242 higher than those fed by AF (70.0±20.2% compared to 52.0±1.0%) (Mantel-Haenszel X^2 test,
243 $X^2 = 23.45$, d.f.=1, $p<0.001$) (Fig. 3). The mortality of engorged females was very low until
244 oviposition in both methods. Of the fed mosquitoes, the two groups had comparable
245 oviposition rates; 84±8.0% for the AF fed An. minimus mosquitoes and 90.43±6.0% for the
246 DHF(h) fed mosquitoes. Mosquitoes fed by DHF(h) laid more eggs per mosquito than those
247 fed by AF, 48.6±3.8 and 41.4±6.1, respectively (paired t-test, t = 1.01, d.f.= 72, p = 0.038).
248 The overall egg output (calculated average egg output per 100 females) of An. minimus
249 mosquitoes fed by AF was 1808 eggs and by DHF was 3062 eggs, a 40% reduction when
250 feeding by AF.
251
252 **Testing the viability of refrigerated blood for AF**
253 Ae. aegypti feeding rate was highest on fresh blood, followed by 2-week-old blood,
254 and lowest on 3-week-old blood; 72.0±8.6%, 60.0±8.5%, and 18.7±6.7%, respectively (X^2 for
255 trend, $X^2 = 43.25$, d.f. = 1, $p<0.001$), (Fig 4). Very few engorged females died until
256 oviposition. Among the fed mosquitoes, the oviposition rate when fed on fresh blood did not
257 differ significantly to mosquitoes fed on 2 week- or 3-week old blood; 91.5±4.8% for fresh
258 blood compared to 96.0±4.0% for 2-week-blood, and 100.0±0.0% for fresh blood compared
259 to 98.7±2.3% for 3-week old blood. There was no significant difference in average number of
260 eggs produced per mosquito when fed on fresh (101.4±3.3 eggs) or 2-week-old blood
261 (94.8±0.3 eggs), and between mosquitoes fed on fresh (92.2±3.5 eggs) or 3-week-old blood
262 (84.2±4.0 eggs). As a whole, overall egg output (calculated average egg output per 100
263 females) of mosquito groups fed on fresh blood (6680) was about 18% higher than
264 mosquitoes fed on 2-week-old blood (5460) and was about 76% higher than mosquitoes fed
265 on 3-week-old blood (6638 for fresh blood and 1554 for 3-week-old blood). Visual

observations also noted the colour of the blood change from bright red to a darker red over the three weeks.

DISCUSSION

The results from this study demonstrate that the herein developed AF apparatus can successfully maintain two species of mosquito, while remaining affordable and efficient. This combination makes AF a reality for laboratories with limited resources. One of the main findings which supports AF replacing DHF(r) is that there was no significant difference in the feeding rate of Ae. aegypti mosquitoes fed by AF or DHF(r). This is an improvement on some existing AF devices where mosquitoes fed at a higher rate on DHF, but there are also AF devices that have achieved a higher feeding rate than DHF (Deng et al., 2012; Costa-da-Silva et al., 2013; Phasomkusolsil et al., 2013). According to the Laboratory Animal Center of CMU, the cost of 1 Wistar rat is about $6.5 and a maintenance room $1000/month. The cost of our feeding apparatus was less than $1 and each device will last for several months.

Although some may argue that further tests are needed to confirm the feeding rate results on account of the large variation, such variability is not unique to this paper, suggesting that this variation may be expected (Costa-da-Silva et al., 2013; Phasomkusolsil et al., 2013). These results could also be seen as the most appropriate for justifying the replacement of DHF(r) with AF because this study was conducted in conditions representative of routine blood feeding large colonies of mosquitoes. In this situation it is not always possible to control each variable. External variables that could have caused variable feeding rate include using rats of different ages throughout the tests. Newer rats are generally more agitated during the mosquito feeding process, causing more disturbance to mosquito feeding than older, less active rats. This results in the large variation in DHF(r) mosquito feeding rate. Large variation in AF feeding rate may result from different conditions on each day of testing, such as the age of the blood, to be discussed later. Moreover, slight discrepancy in the age of mosquitoes used for each parallel test is likely to account for

293 variation in feeding rate on both AF and DHF(r) methods. Even so, still no significant
294 difference in feeding rate was found between the AF and DHF methods, validating the
295 replacement of DHF(r). Equally, such variables could be controlled in additional tests to
296 obtain more consistent results, if necessary.

297 There is also potential for further development of this AF method to achieve the
298 higher feeding rates seen in other studies (Deng et al., 2012; Phasomkusolsil et al., 2014). For
299 instance, increasing membrane surface area may reduce competition between mosquitoes,
300 adding odour cues might improve detection of the blood source, and increasing the volume of
301 heat pack filled with water which has higher specific heat capacity (~4.2 kJ/kg.K) than
302 vegetation oil and glycerol (~2.0 kJ/kg.K) can maintain the required blood temperature for
303 longer.

304 While AF-fed Ae. aegypti mosquito groups laid significantly fewer eggs per mosquito
305 than those fed by DHF(r), there was only the smallest difference in overall average egg
306 production. Consequently, this concludes that AF is suitable for replacing DHF(r) for mass-
307 rearing purposes as there is no significant negative effect on colony maintenance ability.
308 Again, if deemed necessary, there are solutions to increase the number of eggs laid per
309 individual which will further increase the overall number of eggs. Egg production is
310 proportional to amount of protein engorged (Foster, 1995), therefore it can be assumed that
311 either there is lower protein content in a blood meal taken from the AF method, or AF-fed
312 mosquitoes took a smaller blood meal. Gonzales et al. (2015) compared the egg-producing
313 capacity of Ae. aegypti fed on whole blood, serum, red blood cells (RBCs), or artificial diet
314 containing bovine serum albumin (BSA) and haemoglobin. The study concluded that whole
315 blood gives the highest engorgement rate compared to serum or RBCs, but that mosquitoes
316 fed on whole blood, serum and BSA could produce eggs, while those fed on RBCs or
317 haemoglobin could not. Luo (2014) demonstrated that the egg production of Ae. aegypti fed

318 on pig plasma was significantly lower than pig whole blood. This means that mosquitoes fed
319 by AF in our study may give a lower egg production if they imbibe mainly plasma or serum
320 instead of whole blood if any sedimentation occurs.

321 Many studies have discussed the variance of fecundity of mosquitoes after feeding on
322 different hosts, as summarised by Lyimo and Ferguson (2009). Further investigations have
323 found this variation to be caused by different levels of amino acids in blood from different
324 hosts; some amino acids are necessary for egg production, while others are limiting factors
325 for egg production (Dimond et al., 1956, Spielman and Wong, 1974). Chang (1976) reported
326 that isoleucine was the amino acid limiting the egg production of mosquitoes fed on human
327 blood compared to those fed on pig blood, as human blood has a much lower concentration of
328 this amino acid. The content of isoleucine in bovine blood is as low as human blood, but
329 about 3 times lower than rat blood, while protein content is not so different (Clements, 1992).
330 It is possible, therefore, that the Ae. aegypti mosquitoes fed on rat or pig blood by AF
331 consumed a better composition of amino acids for egg production. It is equally possible that
332 Ae. aegypti mosquitoes take a larger blood meal from the rat than the AF device containing
333 cow blood as they have become adapted to feeding by this method and on this particular
334 blood (Deng et al., 2012; Takken and Verhulst, 2013). Testing alternative blood sources to
335 find which produces the optimal number of eggs may, therefore, be worthwhile. For the
336 situation in the insectary used for this study, however, the method and blood source proposed
337 are sufficient.

338 Previous studies have successfully fed a range of species using their AF device, but a
339 'forced feeding' technique is usually applied (Yu and Wang, 2001; Phasomkusolsil et al.,
340 2013). This procedure requires the mosquitoes to be held in a small container and positioned
341 directly under the blood source. The original AF method developed herein, however, is
342 simply placed in the centre of a large cage for 'free feeding', i.e. mosquitoes have to actively

343 locate the blood source. This has proved effective for feeding Ae. aegypti, but the results for
344 An. minimus are less convincing. After adaptation of the apparatus, An. minimus was able to
345 feed, but at a much lower rate than by DHF(h), questioning whether the AF apparatus can
346 feed this species. In this case, though, the feeding rate measured does not represent the
347 maximum percentage of mosquitoes that will feed on the AF device. At the end of the 15
348 minutes feeding, a high density of this species is still attempting to engorge through the
349 membrane, unlike when feeding Ae. aegypti. Thus, it is feasible that a considerably higher
350 percentage of mosquitoes would engorge on the AF mechanism if given a longer time to feed,
351 potentially reaching the same feeding rate as that on the DHF(h) method. Even without
352 increasing feeding time, however, 52% feeding success is still sufficient for colony
353 maintenance as there was only a small difference in number of eggs laid per mosquito, and
354 still a relatively high overall egg production. Hence, the AF apparatus can justifiably replace
355 DHF(h) for maintaining An. minimus colonies, especially if feeding time is increased.
356 Furthermore, these results show it is possible to use the apparatus for feeding other
357 laboratory-maintained mosquito species than just Ae. aegypti, but alteration of the protocol
358 may be necessary.

359 The AF method developed herein simulates a live organism mainly by heat cues and
360 there is no attempt to simulate other factors known to influence host location, including: host
361 odour, shape of the hosts or a continual supply of carbon dioxide (Takken and Verhulst,
362 2013). An. minimus struggling to detect and locate the blood source compared to Ae. aegypti,
363 therefore, indicates that An. minimus is more sensitive to other host signals such as odour, a
364 longer-range host-location factor, than heat (Takken and Knols, 1999; Tisgratog et al., 2012;
365 McBride et al., 2014). Hence, adding real or simulated host odour to the AF apparatus has the
366 potential to improve feeding rate by the anopheline mosquito species.

Results from this paper investigating the effect of the age of blood infer that mosquitoes may use 'taste', or 'a system to judge the quality of a blood (food) source' (Gonzales et al., 2015), alongside heat and odour cues, for host preference and location (Takken and Verhulst, 2013; McBride et al., 2014). It has been shown that Ae. aegypti mosquitoes have the capacity to choose whether to imbibe a blood meal or to continue probing (Gonzales et al, 2015). Luo (2014) demonstrated that the feeding rate of Ae. aegypti on pig plasma using cattle collagen sausage-casing membrane was only 4.8%, but was increased to 91.5% when fed on plasma-added ATP. Any changes in the composition of a host's blood, therefore, may mean a mosquito no longer detects it as belonging to their preferred host. In this study the colour of bovine blood changed over increasing refrigeration time, suggesting that the composition of the blood had indeed changed. This could arise, firstly, by blood compounds becoming increasingly oxidised, or due increasing numbers of hemolysed RBCs emptying their contents into the plasma (Hess, 2010). If mosquitoes do detect and decide to feed on a host according the specific chemical composition of its blood (i.e. taste), when the mosquitoes were given older blood they may not have recognised it and thus did not engorge. This trend is supported by another paper which found a 50% reduction in feeding rate of Ae. aegypti fed on blood after it was refrigerated for 20 days, it suggested that blood becomes less attractive as a food source to mosquitoes as it ages due to fractionation (Pothikasikorn et al., 2010). Similarly, Luo (2014) noted that female mosquitoes fed with preserved pig blood that was stored for more than 40 days had near zero percent engorgement. In preliminary tests, poor feeding rate and similar behaviour to mosquitoes fed on refrigerated blood (i.e. increased probing without engorging) were observed by mosquitoes fed on defrosted blood (data not shown). This implicates the 'taste' phenomenon again, suggesting that mosquitoes do not recognise the defrosted host blood due to RBC hemolysis changing the chemical composition. In summary, the greatly reduced

feeding rate of mosquitoes fed on three week refrigerated blood produced a considerably lower number of eggs overall, causing a reduction in the mass-rearing capacity. Therefore, only blood refrigerated for less than two weeks should be used for AF with the aim of maintaining mosquito colonies. However, taking blood from living animals or freshly slaughtered animals (e.g. cows and pigs) can be hazardous. Use of pathogen free animal blood from commercial products may be an alternative way, if it is available. In addition, serum, plasma, and artificial diet (BSA) supplemented with ATP, all of which can be frozen, may be used instead of whole blood (Luo, 2014; Gonzales et al., 2015).

CONCLUSION

This paper demonstrates that the herein developed AF apparatus can justifiably replace DHF for feeding two mosquito species, Aedes aegypti and Anopheles minimus, saving laboratories money and making procedures safer and easier. Furthermore, there is room for improvements of the AF method to obtain higher feeding rates and fecundity, if desired. This particular AF method is unique as it can be employed by all laboratories irrespective of available resources due to consisting of common materials which are inexpensive to produce and assemble, while the procedure remains simple and efficient. Blood used for AF can be stored in the refrigerator for up to two weeks without considerable effect on overall egg production, but any longer may reduce the capacity to maintain mosquito colonies.

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424 REFERENCES

425

426 Benzon, G., Apperson, C., 1987. An electrically heated membrane blood-feeding device for
427 mosquito colony maintenance. J. Am. Mosq. Control Assoc. 3, 322-324.
428 Chang, Y. H., 1976. The effect of various blood components on oogenesis of the yellow fever
429 mosquito, Aedes aegypti Linnaeus (Diptera: Culicidae). Ph.D. dissertation, University
430 of California, Davis.
431 Clements, A.N., 1992. The biology of mosquitoes, volume 1: Development, nutrition and
432 reproduction. Chapman & Hall, London, 509 pp.
433 Costa-da-Silva, A., Navarrete, F., Salvador, F., Karina-Costa, M., Ioshino, R., Azevedo, D.,
434 et al., 2013. Glytube: A conical tube and Parafilm M-based method as a simplified
435 device to artificially blood-feed the dengue vector mosquito, Aedes aegypti. PLoS
436 ONE 8, 53816.
437 Deng, L., Koou, S., Png, A., Ng, L., Lam-Phua, S., 2012. A novel mosquito feeding system
438 for routine blood-feeding of Aedes aegypti and Aedes albopictus. Trop. Biomed. 29,
439 169-174.
440 Dimond, J., Lea, A., Hahnert Jr., W., DeLong, D., 1956. The amino acids required for egg
441 production in Aedes aegypti. Can. Entomol., 88, 57-62.
442 Foster, W.A., 1995. Mosquito sugar feeding and reproductive energetics. Annu. Rev.
443 Entomol. 40, 443-474.
444 Gonzales, K. K., Tsujimoto, H., Hansen, I. A., 2015. Blood serum and BSA, but neither red
445 blood cells nor haemoglobin can support vitellogenesis and egg production in the
446 dengue vector Aedes aegypti. PeerJ 3:e938; DOI 10.7717/peerj.938.
447 Hagen, H., Grunewald, J., 1990. Routine blood-feeding of Aedes aegypti via a new
448 membrane. J. Am. Mosq. Control Assoc. 6, 535-536.

449 Hess, J., 2010. Red cell changes during storage. Transfus. Apher. Sci. 43, 51-59.
450 Luo, Y.P., 2014. A novel multiple membrane blood-feeding system for investigating and
451 maintaining Aedes aegypti and Aedes albopictus mosquitoes. J. Vector Ecol. 39, 271-
452 276.
453 McBride, C., Baier, F., Omondi, A., Spitzer, S., Lutomiah, J., Sang, R., et al., 2014.
454 Evolution of mosquito preference for humans linked to an odorant receptor. Nature
455 515, 222-227.
456 Novak, M., Berry, W., Rowley, W., 1991. Comparison of four membranes for artificially
457 blood feeding mosquitoes. J. Am. Mosq. Control Assoc. 7, 327–328.
458 Phasomkusolsil, S., Pantuwatana, K., Tawong, J., Khongtak, W., Monkanna, N., Kertmanee,
459 Y., et al., 2014. Factors influencing the feeding response of laboratory-reared Aedes
460 aegypti. Southeast Asian J. Trop. Med. Public Health 45, 40-46.
461 Phasomkusolsil, S., Tawong, J., Monkanna, N., Pantuwatana, K., Damdangdee, N.,
462 Khongtak, W., et al., 2013. Maintenance of mosquito vectors: effects of blood source
463 on feeding, survival, fecundity, and egg hatching rates. J. Vector Ecol. 38, 38-45.
464 Pothikasikorn, J., Boonplueang, R., Suebsaeng, C., Khaengraeng, R., Chareonviriyaphap, T.,
465 2010. Feeding responses of Aedes aegypti and Anopheles dirus (Diptera: Culcidae)
466 using out of date human blood in a membrane feeding apparatus. J. Vector Ecol. 35,
467 149-155.
468 Rutledge, L.C., Ward, R.A., Gould, D.J., 1964. Studies on the feeding response of
469 mosquitoes to nutritive solutions in a new membrane feeder. Mosq. News 24, 407-
470 419.
471 Somboon, P., Prapanthadara, L., Suwonkerd, W., 2003. Insecticide susceptibility tests of
472 Anopheles minimus s.l., Aedes aegypti, Aedes albopictus, and Culex quinquefasciatus
473 in northern Thailand. Southeast Asian J. Trop. Med. Public Health 34, 87–93.

474 Spielman, A., Wong, J., 1974. Dietary factors stimulating oogenesis in Aedes aegypti. Biol.
475 Bull. 147, 433-442.
476 Takken, W., Knols, B., 1999. Odour-mediated behaviour of Afrotropical malaria
477 mosquitoes. Annu. Rev. Entomol. 44, 131-157.
478 Takken, W., Verhulst, N., 2013. Host preferences of blood-feeding mosquitoes. Annu. Rev.
479 Entomol. 58, 433-453.
480 Tisgratog, R., Tananchai, C., Juntarajumnong, W., Tuntakom, S., Bangs, M., Corbel, V., et
481 al., 2012. Host feeding patterns and preference of Anopheles minimus (Diptera:
482 Culicidae) in a malaria endemic area of western Thailand: baseline site description.
483 Parasites Vectors 5, 114.
484 World Health Organisation, 2009. Dengue: Guidelines for diagnosis, treatment, prevention
485 and control. WHO/HTM/NTD/DEN/2009.1.
486 World Health Organization, 2012. Global strategy for dengue prevention and control 2012 -
487 2020. Geneva.
488 World Health Organization, 2015. WHO | Executive summary. [online] Available at:
489 http://www.who.int/whr/1996/media_centre/executive_summary1/en/index9.html
490 [Accessed 26 May 2015].
491 Yanola, J., Somboon, P., Walton, C., Nachaiwieng, W., Prapanthadara, L.A., 2010. A novel
492 F1552/C1552 point mutation in the Aedes aegypti voltage-gated sodium channel gene
493 associated with permethrin resistance. Pestic. Biochem. Physiol. 96, 127–131.
494 Yu, C., Wang, J., 2001. Culicoides arakawae (Diptera: Ceratopogonidae) efficiently blood-
495 fed and infected with Leucocytozoon caulleryi through a natural membrane. Vet.
496 Parasitol. 99, 297-303.

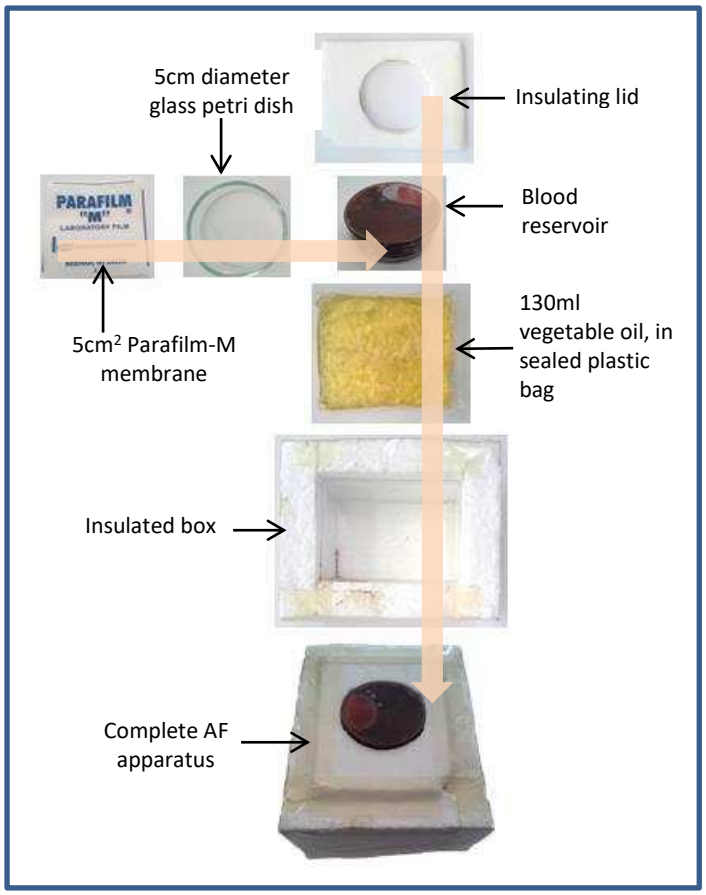

Fig. 1. Components of AF apparatus and assembled device. Diagram showing blood reservoir and entire feeding apparatus assembly.

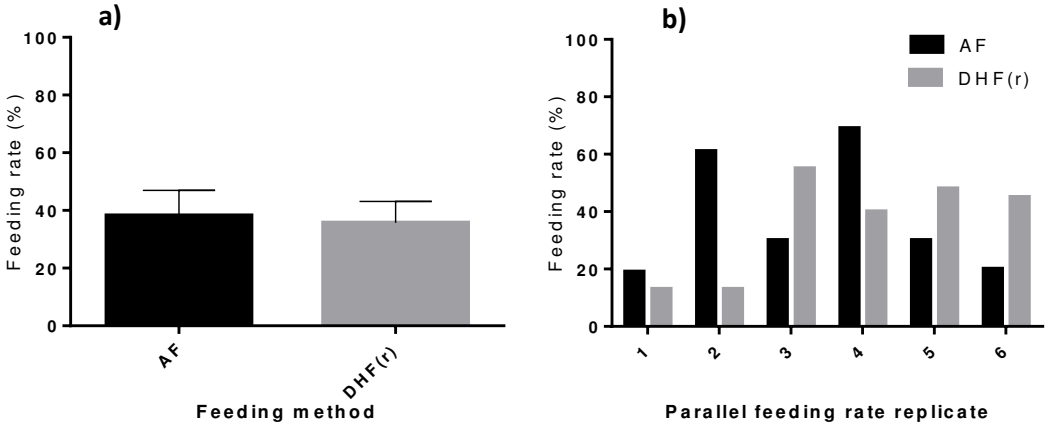

Fig. 2. Average feeding rate of female Ae. aegypti mosquitoes on AF and DHF(r) methods (100 females each) in 15 minutes. a) The average feeding rate on each method, there was no significant difference in average feeding rate. The bars represent standard error. b) The feeding rate on AF and DHF(r) across six replicates. Feeding rate= (number of females engorged after 15 minutes feeding/ total number of females in the feeding cage) x 100.

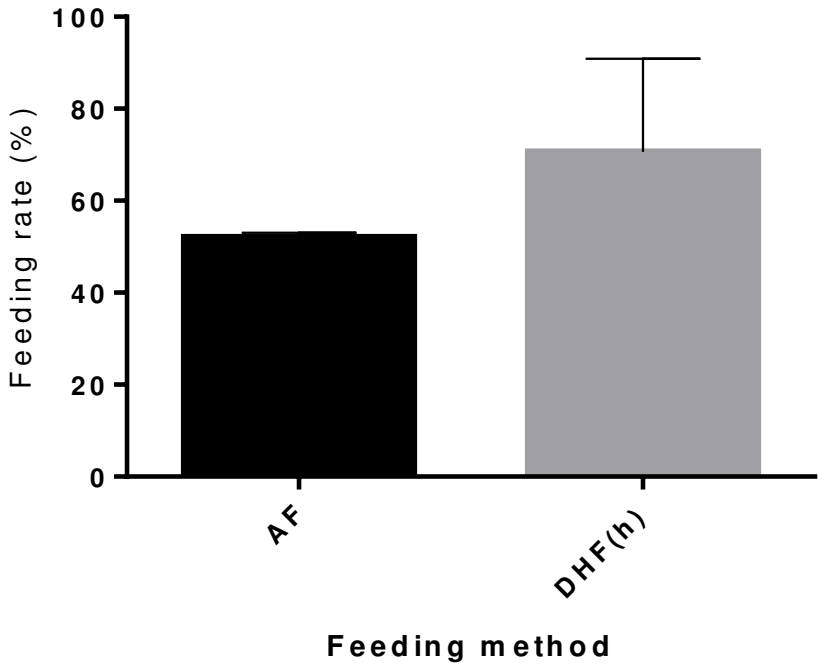

Fig.3. Average feeding rate of An. minimus on AF and DHF(h) apparatus (100 females each). Significantly more mosquitoes fed on DHF(h) than AF method ($p<0.001$). The bars represent standard error. Feeding rate= (number of females engorged after 15 minutes feeding/ total number of females in the feeding cage) x 100.

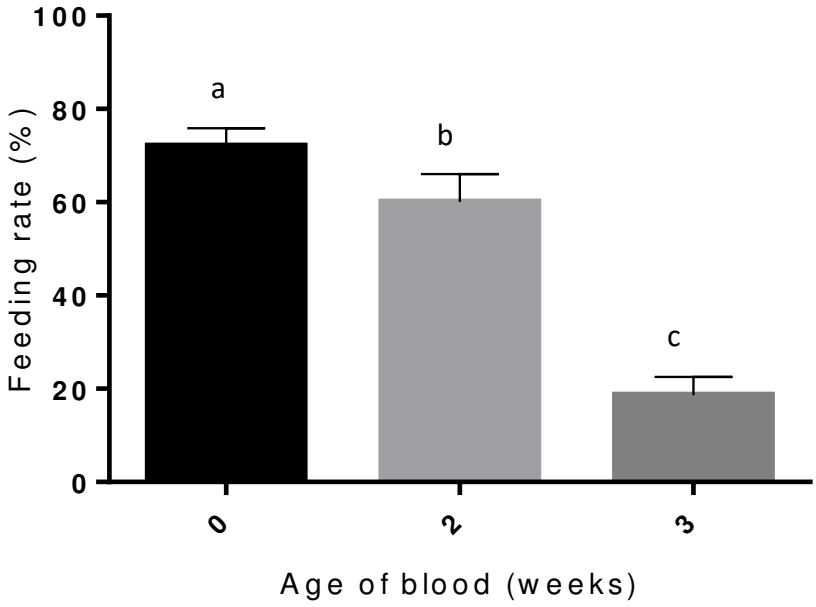

Fig. 4. Average feeding rate of Ae. aegypti female mosquitoes on AF apparatus containing cow blood which had been refrigerated for varying lengths of time (100 females each). The average feeding rates are significantly different from one another ($p<0.001$), letters indicating significantly different values. The age of blood is the number of weeks stored in the refrigerator and the bars represent standard error. Feeding rate= (number of females engorged after 15 minutes feeding/ total number of females in the feeding cage) x 100.