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1 **A simple and affordable membrane-feeding method for *Aedes aegypti* and**  
2 ***Anopheles minimus* (Diptera: Culicidae)**

3  
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9

10 ABSTRACT

11

12 This study developed an artificial feeding (AF) method to replace direct host feeding (DHF)  
13 for the maintenance of *Aedes aegypti* and *Anopheles minimus* mosquito colonies. The  
14 procedure can be adopted by all laboratories due to its simple and affordable materials and  
15 design. The apparatus consists of heparinized cow blood contained in a 5cm diameter glass  
16 petri dish with 5cm<sup>2</sup> Parafilm M (Bemis ®) stretched thinly over the top, with a pre-heated  
17 bag of vegetable oil placed underneath to keep the blood warm. Both parts are contained  
18 within an insulated Styrofoam<sup>TM</sup> box with a hole in the lid for mosquitoes to access the  
19 membrane. Mosquitoes are fed by AF for 15 minutes at a time. Feeding rate and fecundity of  
20 *Ae. aegypti* mosquitoes feeding on the AF device were compared to those feeding on a live  
21 rat (DHF(r)), and of *Anopheles minimus* mosquitoes feeding on the AF device compared to  
22 those feeding on a human arm (DHF(h)). *Aedes aegypti* mosquitoes fed by AF or DHF(r) had  
23 similar feeding rates (38.2±21.5% and 35.7±18.2%, respectively) and overall egg production  
24 (1.5% difference). *Anopheles minimus* mosquitoes fed by the AF method had a lower feeding  
25 rate (52.0±1.0% for AF compared to 70.7±20.2% for DHF(h)) and overall egg production

26 (40% reduction compared to DHF(h)). However, the number of eggs produced by AF-fed  
27 mosquitoes (1808 eggs per 100 mosquitoes) was still sufficient for colony maintenance, and  
28 with increased feeding time both parameters are expected to increase. Reduced feeding rate  
29 and overall egg production was observed when *Ae. aegypti* mosquitoes were fed on blood  
30 refrigerated for over two weeks. In conclusion, an AF device has been developed which can  
31 replace DHF for *Ae. aegypti* and *An. minimus* colony maintenance when using blood  
32 refrigerated for a maximum of two weeks.

33 **Keywords:** *Aedes aegypti*, *Anopheles minimus*, membrane feeding

34

## INTRODUCTION

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

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

58 Numerous studies have developed artificial feeding (AF) methods and document their  
59 ability to replace DHF protocols for maintaining mosquito colonies and investigating  
60 pathogen infection rate (e.g. Benzon and Apperson, 1978; Yu and Wang, 2001; Tseng, 2003;  
61 Rampersad and Ammons, 2007; Deng et al., 2012; Costa-da-Silva et al., 2013; Luo, 2014).  
62 However, none of the existing methods are entirely appropriate for laboratories mass-rearing  
63 mosquitoes which have limited financial resources. The basic design that the various AF  
64 devices follow is that of the original Rutledge feeder, consisting of: a blood reservoir, a  
65 membrane through which mosquitoes access to the blood, and a method to keep the blood  
66 warm (Rutledge et al., 1964). Most membranes proposed, including collagen membrane  
67 casing, Parafilm-M or a condom are, in fact, inexpensive, accessible and feed mosquitoes  
68 effectively, but the equipment options for keeping the blood warm are usually expensive  
69 (Benzon and Apperson, 1987; Hagen and Grunewald, 1990; Novak et al., 1991; Deng et al.,  
70 2012; Costa-da-Silva et al., 2013; Luo, 2014). Deng et al. (2012), alongside other studies,  
71 proposed using an electric hotplate to keep the blood reservoir warm, while another method  
72 uses a specialised glass water-jacket to surround and heat the blood reservoir (Chemglass©,  
73 Yu and Wang, 2001; Phasomkusolsil et al., 2014). These devices are appropriate for feeding  
74 small numbers of mosquitoes, but in a mass-rearing situation where many cages need feeding  
75 simultaneously, a large number of devices will be required, becoming increasingly expensive.  
76 A commercially available AF method, developed by Hemotek Ltd, feeds up to 5-6 cages at  
77 once using an electric heating device; however this apparatus costs about \$3000. Each of  
78 these methods is beyond the budget for many laboratories.

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

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

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

99

## MATERIALS AND METHODS

100

### 101 **Mosquitoes**

102 *Ae. aegypti* (PMD strain) and *An. minimus* (CM strain) larvae, pupae and adults were  
103 reared and maintained in an insectary in the Department of Parasitology, Faculty of  
104 Medicine, Chiang Mai University (CMU), Thailand, at 25±2°C, relative humidity of 80±10%  
105 and 12:12 light:dark photoperiod. Both strains originate from Chiang Mai Province and have  
106 been maintained in our insectary for over 10 years, routinely using Wistar rats or a human  
107 volunteer for the blood meal. For *Ae. aegypti*, larvae were hatched in large plastic tubs  
108 (40×25×10cm) and fed ground dog biscuit (Tesco®) daily, the water was changed when it  
109 became murky. Pupae were collected daily in small plastic cups, and placed in holding cages  
110 (30×30×30cm) where they emerged and remained as adults until testing. A damp cloth  
111 covering the cage was changed daily, to create humidity. Adult mosquitoes were maintained  
112 on a solution of 10% sucrose and 10% multivitamin syrup to promote longevity, these were  
113 also changed daily. Adult females 5-8 days post-eclosion were used for this study.  
114 Mosquitoes were fasted of sugar and vitamin solution ~24 hours prior to testing.

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

124 **Rats**

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

134

135 **AF apparatus and conditions for feeding *Aedes aegypti***

136 A range of inexpensive and accessible materials were investigated to develop a  
137 method which gave a suitable feeding rate (data not shown). The final AF apparatus consists  
138 of a blood reservoir where blood is held in a circular, glass petri dish (5cm diameter), with  
139 5cm<sup>2</sup> Parafilm M (Bemis®) membrane stretched thinly over the top. This is placed on top of  
140 a sealed plastic bag containing ~130ml vegetable oil acting as the heating element.  
141 Preliminary tests showed that this volume of vegetable oil, glycerol or water, heated to 50°C,  
142 retained a high enough heat to keep the blood between 35-37°C for at least 15 minutes  
143 (feeding time). The blood reservoir and oil heating element are contained in a Styrofoam™  
144 insulated box, with a hole in the lid for mosquitoes to access the membrane (Fig. 1). Bovine  
145 blood was collected directly from healthy cows by a veterinarian at the Faculty of  
146 Veterinarian Medicine, CMU, during the practice of veterinary students. It was then  
147 immediately treated with 5U/ml heparin (5000U/ml stock concentration, 200U/ml working  
148 concentration) to prevent clotting. The blood is warmed to 37-38°C before testing, and ~12ml

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

154

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

156 Two feeding cages (30x30x30cm) were prepared, each containing 100 female  
157 mosquitoes collected from the stock colony. The AF apparatus (as described above) was  
158 placed in one cage, while the DHF(r) method (rat-cage, as above) was placed in the other.  
159 Blood feeding occurred for 15 minutes, with the AF apparatus gently tilted every 5 minutes to  
160 prevent sedimentation. At the end of 15 minutes both feeding methods were removed, and the  
161 engorged (fed) females were removed and counted. Engorged mosquitoes were identified by  
162 visual observation of a swollen abdomen. Six parallel feeding rate replicates (1x AF cage and  
163 1x DHF(r) cage) were carried out in total: on four consecutive days, followed by a break, and  
164 then on two consecutive days.

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

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

175

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

177         Several observations were made when the developed AF method was initially used to  
178 feed *An. minimus* during daytime. These mosquitoes did not actively seek and could not  
179 locate the blood source when the AF device was placed in the centre of the cage, even when  
180 activated by CO<sub>2</sub> or when flying directly over the top. In comparison, once activated by a  
181 small amount of CO<sub>2</sub> the *Ae. aegypti* mosquitoes immediately searched for and located the  
182 blood source. The anopheles species was much less active than *Ae. aegypti* in general; resting  
183 on the side of the cages rather than flying around. To achieve an acceptable level of feeding,  
184 adaptations of the AF method were required, as follows. Two small cages (15×15×15cm)  
185 were prepared, each containing 100 female mosquitoes collected from the stock colony. Two  
186 AF devices were positioned up against the opposite sides of one feeding cage so that the  
187 blood reservoir membranes were flat against the net and faced inwards. For comparison, the  
188 forearm of one of the authors (CF) was placed against one side of the other feeding cage  
189 (DHF(h)). Feeding was carried out for 15 minutes, tilting the AF apparatus every 5 minutes.  
190 After removing the feeding mechanisms, the engorged females were removed and counted.  
191 This was repeated three times on one day.

192         After feeding, ~30 engorged females were randomly selected from each of the AF and  
193 DHF(h) cages and removed to a large cup. The cups were lined with paper and enclosed with  
194 net. A piece of sugar-soaked cotton was placed on top and changed daily, and a plastic bag  
195 was placed over the top to maintain humidity. Four days post-feeding the mosquitoes were  
196 separated to isolated oviposition cups; lined with filter paper and pre-filled with ~12 ml  
197 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

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### **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

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

## DISCUSSION

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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.

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

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

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## CONCLUSION

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

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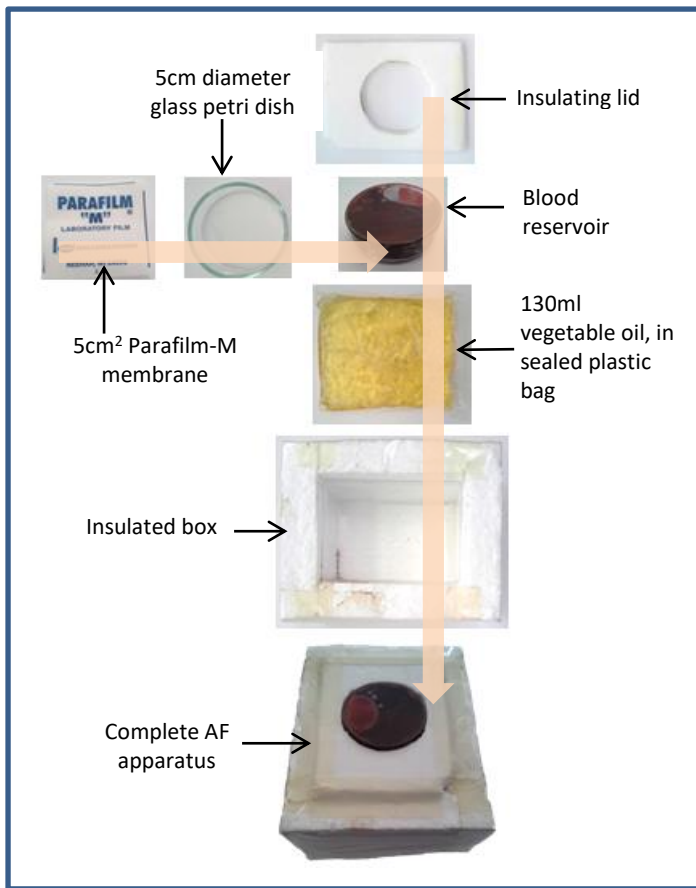
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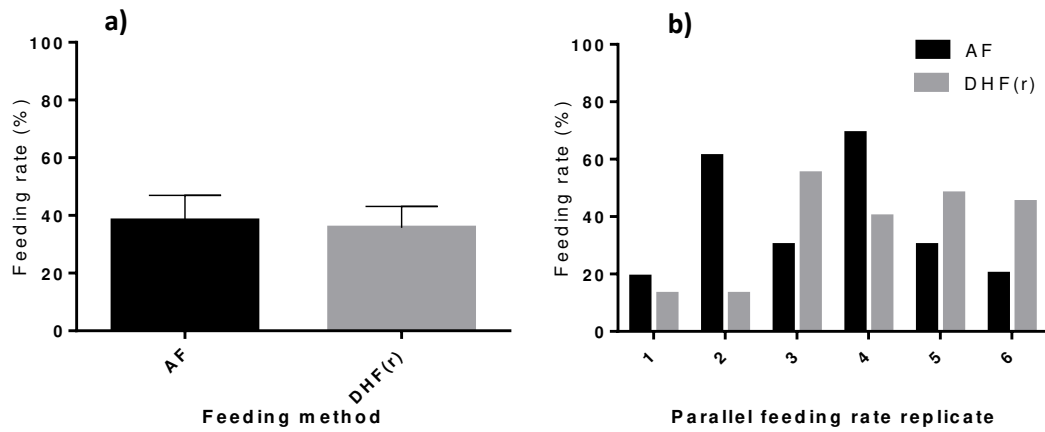
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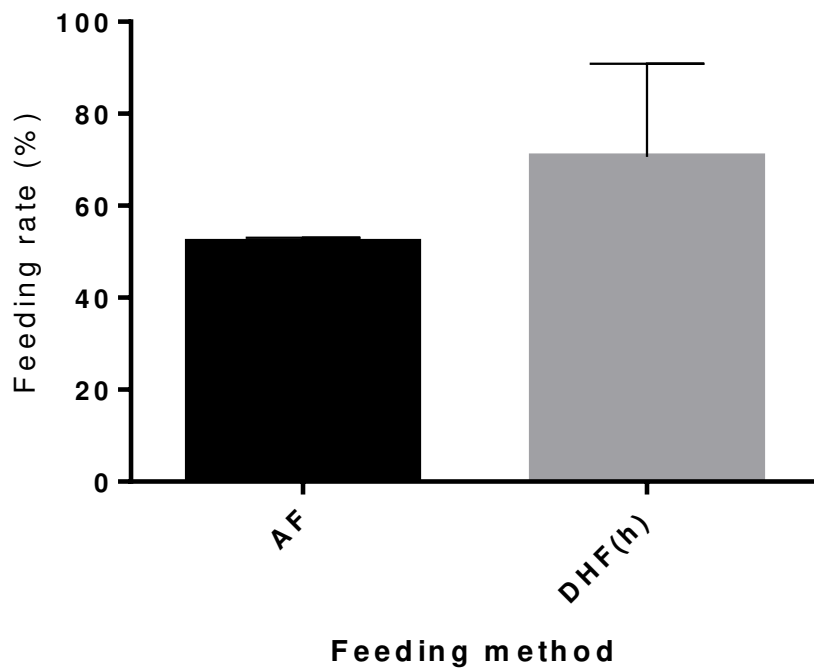
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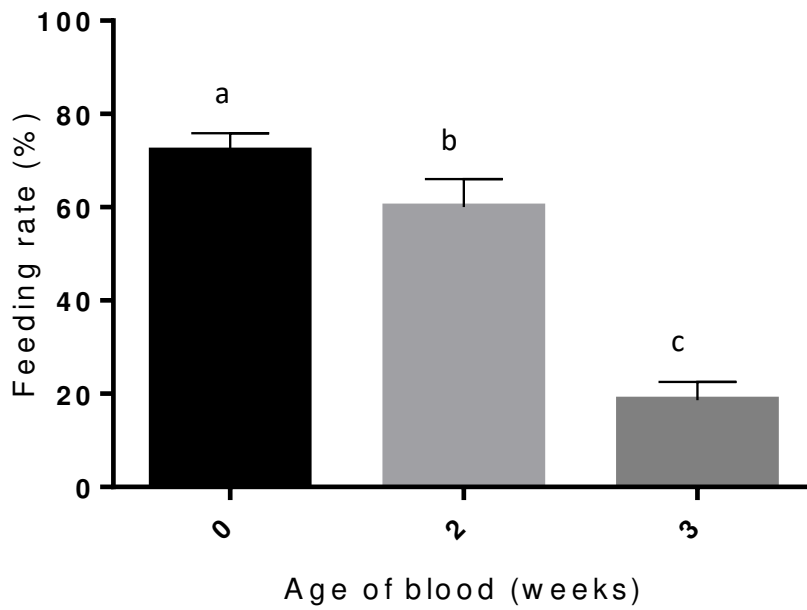
**Fig. 1.** Components of AF apparatus and assembled device. Diagram showing blood reservoir and entire feeding apparatus assembly.



**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.