

Oviposition interval and gonotrophic concordance in Southeast Asian anophelines

Mosquito survival and oviposition interval are important disease vector's determinants that may vary within species depending on the environment. The length of the oviposition interval may be estimated by the proportion of mosquito ovaries with sacs. Fourteen species of Anopheles were collected from four locations in Cambodia and 1965 specimens were dissected. Both the number and predominant species collected varied according to location and trapping method. Five species were collected in sufficient numbers to allow comparisons between locations. Sac rates differed between species but not within species between locations. *Anopheles minimus* had a higher sac rate in weeks leading up to a full moon. The more preserved environments supported more diverse anopheline fauna than the degraded environments. Despite the fact that many of the species occurred at very low densities there was no evidence of gonotrophic dissociation in any of the species. The principal malaria vector, *An. dirus*, was only common in one location where it was collected in miniature light-traps. It is suggested that a nationwide survey using such traps may help in determining patterns of malaria transmission in Cambodia.

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12 Introduction

13 Survival and oviposition interval are two of the most important factors in
14 determining whether a mosquito will be a vector of disease, since small
15 changes in either of these can have large effects on the insect's vectorial
16 capacity (Charlwood et al, 1986a). Since activities such as blood feeding
17 carry more risks for the mosquito than resting, mosquito survival is probably
18 more accurately expressed per entire gonotrophic cycle than per day (Burkot
19 et al, 1990). In contrast, pathogen development depends on ambient
20 temperature and progresses daily. Depending on the relative risks associated
21 with feeding, resting, and oviposition, infected mosquitoes that have a longer
22 cycle are more likely to survive to become vectors. One way the duration of
23 the gonotrophic cycle can be increased is for the mosquito to delay returning
24 to blood-feed after oviposition. Some species, such as *Chagasia bonnae* from
25 Brazil, tend to return to feed immediately after oviposition (Wilkes and
26 Charlwood, 1979), whilst in others, such as *Anopheles farauti* from Papua
27 New Guinea, the time spent between oviposition and re-feeding is largely
28 dependent on environmental conditions, in particular the moon phase
29 (Charlwood et al, 1986b, Birley and Charlwood, 1989, Kampango et al.,
30 2011), and the distance to the oviposition site (Charlwood et al, 1986a).
31 Factors affecting post-oviposition behaviour, however, remain poorly
32 understood and whether external, environmental factors or intrinsic ones are
33 more important is not always clear.

34 Another determinant of vectorial capacity is the number of blood meals taken
35 per gonotrophic cycle. Anophelines, including those from Southeast Asia (S.
36 E. Asia), are generally gonotrophically concordant, in other words, each blood
37 meal results in an egg batch laid the night that the mosquito becomes
38 completely gravid in a regular cycle. Under conditions of environmental
39 stress, however, especially during hot and dry conditions when potential
40 oviposition sites dry up, some species may delay oviposition and take several
41 feeds during each oviposition cycle which can then take weeks or months

42 instead of days to complete (Omer and Cloudsley-Thompson, 1970, Lehmann
43 et al, 2010, Adamou et al, 2011, Charlwood et al, 2013a). Such gonotrophic
44 discordance was described more than fifty years ago among *Anopheles*
45 *maculatus*, *Anopheles culicifacies*, *Anopheles annularis* and *Anopheles*
46 *aconitus* from India and Southeast Asia (Büttiker 1958, Rao 1947, Roubaud
47 1923). Since they are so long lived, even low-density populations of such
48 gonotrophically discordant mosquitoes are epidemiologically dangerous. As a
49 result of deforestation, hot, dry environments of the sort where
50 gonotrophically discordant mosquitoes may be found are now common for
51 much of the year in Cambodia. Gonotrophic discordance may be one survival
52 strategy for the local anophelines. The phenomenon has, however, not been
53 investigated in recent years. Ovaries of mosquitoes that are collected while
54 attempting to blood feed provide an indication of whether they are
55 gonotrophically discordant since gonotrophically discordant females have
56 ovarioles at an advanced stage of development, many being gravid, when
57 they (re)feed (Omer and Cloudsley-Thompson 1970). Examination of the
58 females' ovaries therefore allows for the estimation of mosquito post
59 oviposition behaviour, an assessment of survival rate and provides an
60 indication of whether they are gonotrophically discordant.

61 Of the many anophelines biting humans in Cambodia, only a few are vectors
62 of malaria and only one, *Anopheles dirus*, is a vector of any consequence
63 (Durnez et al, 2013). Differences among species in post-oviposition behaviour
64 may be one factor responsible for these differences. Post-oviposition
65 behaviour has, however, not previously been studied among S.E. Asian
66 mosquitoes and studies determining survival rates are limited (Catangui,
67 1971, Zalutskaya, 1959, quoted in Clements and Paterson 1981). One way of
68 determining if post-oviposition behaviour is, as suggested by Obsomer et al
69 (2007), environmentally determined or if it differs intrinsically between
70 species is to examine the behaviour of the same species of mosquito
71 sampled from a variety of locations.

72 We therefore examined the ovaries of anopheline mosquitoes attempting to
73 feed on people or domestic animals from four locations in Cambodia (three
74 of which at different times were hot and dry) to see if species showed
75 evidence of being gonotrophically discordant if post oviposition intervals and
76 mosquito survival rates differed among locations.

77 **Methods**

78 **Description of study locations**

79 Collections of host seeking mosquitoes were undertaken between February,
80 2012 and December, 2013 in the four locations shown in Figure 1 and
81 described below

82 ***Khum Otavao, Pailin Province***

83 Khum Otavao (N12.789 E102.690) (Fig 2) is a village on a shallow ridge
84 15kms from Pailin town. Pailin Province is the centre of delayed *Plasmodium*
85 *falciparum* clearance by artemisinin (Noedl et al, 2008). Historically Pailin
86 Province was densely forested, and malaria transmission was relatively
87 intense. In recent years, the province has been deforested and transmission
88 has decreased markedly (Maude et al., 2014). People grow cassava as a cash
89 crop. In Khum Otavao, a man-made pond is used to store water for use
90 during the dry season. During the rainy season, jars and pots are used to
91 collect rainwater close to houses. A small seasonal stream, that tends to
92 become a marsh in the wet season, runs to the west of the village.

93 ***Krorhom Krom, Veal Veng, Pursat Province***

94 In the study location Krorhom Krom (N12.215 E 103.080) (Fig 3), 25km out of
95 Veal Veng town (Pursat Province), people live in isolated, separate houses
96 built alongside the road. They grow maize as a cash crop. In the dry season,
97 much of the vegetation in the area is burnt. Pigs, buffalo, cows and dogs are
98 common in Krorhom Krom. Active deforestation is ongoing in the area and

99 reductions in forest cover were visible between study visits. Pursat Province is
100 also an area where malaria parasites are being cleared slowly when treated
101 with artemisinin (Amaratunga et al, 2012).

102 ***Kroh Salau, Khoh Khong Province***

103 The village of Kroh Salau (N 11.460 E 103.049) (Fig 4) is on an island in the
104 Krasaop Wildlife Sanctuary, Khoh Kong Province. The island is surrounded by
105 mangrove forest but rises to circa 200m above sea level where there is a
106 pagoda. Fishing is the main occupation. The village consists of numerous
107 closely packed wooden houses on stilts either built over the water or close to
108 the shore. Dogs are reasonably common but other domestic animals are rare.

109 ***Ou Chrar, Mondolkiri Province***

110 The village of Ou Chrar (N 12. 237, E106.848) (Fig 5) is situated in a rice
111 growing area, which, at the time of the study, was still surrounded by
112 secondary forest (Charlwood et al., 2014). The village consists of 32 houses
113 made of wood or bamboo, many of them on stilts. Pigs, buffalo and dogs are
114 common. Active logging and forest clearance is ongoing in Ou Chrar. The
115 forest remains closer to the village than in the other locations. People also
116 use the forest for hunting and collection of more sustainable resources. It is
117 the only one of the four study locations where *Anopheles dirus* is common
118 and active malaria transmission is occurring.

119 **Mosquito collection**

120 A variety of hosts, baits and trapping methods were used. Landing collections
121 were the main collection method used in Pailin, Khoh Kong and Pursat, but
122 this method was not used in Mondolkiri. Mosquitoes were collected with an
123 aspirator as they landed on the exposed lower legs of adult men. Landing
124 collections were largely undertaken for the first four hours after sunset

125 although all night collections were also performed in Pailin. Miniature Centers
126 for Disease Control and Prevention (CDC) light-traps were run in each of the
127 study locations inside and outside houses with both humans and domestic
128 animals as bait. When humans were used as bait they slept under a mosquito
129 net. MMX traps (Kline, 2006) and 'Furvela' tent-traps (Govella et al, 2009,
130 Charlwood et al, 2012, 2013b) were also used in the three mainland
131 locations, but not in Kroh Salau (Khoh Khong).

132 **Mosquito processing**

133 Collected mosquitoes were identified morphologically using the keys of
134 Rattanaarithikul and Panthusiri (1994) and Rattanaarithikul and colleagues
135 (2010) to species or species complex. Each hour's collection was kept in
136 individual containers and an haphazard sample of insects from the first
137 four hours of landing collection (18:00–21:00) was dissected in eye-
138 drops (Optrex). Females were separated into the following classes:

139 *Nulliparous I*. Ovarioles at Stage I, with tracheoles visibly coiled and the ovary
140 small and transparent. If freshly killed they were also examined for the
141 presence of mating plugs in the common oviduct. These mosquitoes were
142 searching for their first blood meal when caught.

143 *Nulliparous II*: Ovarioles at Stage II, (i.e., with some yolk present in the
144 developing follicle) the ovary transparent and clean. These mosquitoes may
145 have been searching for their first or second blood meal when caught.

146 *Parous with a-c sacs*: The ovariole stalk distended, indicating that the
147 mosquito had returned to feed shortly after oviposition. Ovaries and oviducts
148 with colour, and tracheoles not visible. From the middle of the study period
149 onward, sacs were further classified into 'a', 'b' or 'c' sac subclasses
150 according to the scheme given by Wilkes and Charlwood (1979).

151 *Parous with d sacs:* The sac from the previous oviposition contracted,
152 indicating that there had been a delay between oviposition and returning for
153 re-feeding. Ovaries and oviducts with colour, and tracheoles not visible.

154 The presence of retained Stage V eggs in parous females, according to the
155 sac stage, was also noted.

156 Unfortunately, the great majority of mosquitoes were dead and dry upon
157 examination. In order to dissect dry mosquitoes, the abdomen was punctured
158 with needles so that the saline solution entered and re-hydrated the internal
159 organs. After being left for 10 or 15 minutes they could, with care, be
160 dissected in the usual manner although it was not possible to determine the
161 presence of mating plugs or spermatozoa in the spermatheca with any
162 confidence.

163 **Data analysis**

164 Differences in sac rate between species within locations and between
165 locations within species were calculated with Fisher's exact test.

166 With A the sac rate, or the proportion of parous host searching mosquitoes
167 that laid eggs earlier that day, out of all parous host seeking mosquitoes and
168 with τ the length of the gonotrophic cycle of a mosquito (from blood feeding
169 to the start of searching for a new blood meal) and with some further
170 assumptions, u , the average length of the feeding cycle in a mosquito
171 population can be estimated.

172 Assuming that all mosquitoes succeed in blood feeding when host searching
173 (Charlwood et al., 1997), but that some mosquitoes (A) take an extra day to

174 rest,
$$u_1 = \tau + (1 - A)$$

175 Assuming that all mosquitoes search for a host after τ days of resting, but
 176 have a daily probability of $P_A = 1 - A$ of surviving a day of host searching but
 177 not finding a host,
 178 (Chitnis et al., 2008, equation 23).

$$u_2 = \theta_f = \tau + \frac{P_A}{1 - P_A} = \tau + \frac{1 - A}{A}$$

179 We did not collect information on the value of τ but assumed a value of 3
 180 days. Confidence intervals for A and u_1 (due to uncertainty in A , but not
 181 taking uncertainty in τ into account) were calculated with Fisher's exact test.
 182 Confidence intervals for u_2 (due to uncertainty in A , but not taking
 183 uncertainty in τ into account) were estimated with a simple Bayesian model
 184 (See Additional file 1).

185 The Simpson diversity index and the Shannon index for the anophelines
 186 collected were calculated for landing collections from each of the locations
 187 where they were conducted and according to trapping method in Pailin, Veal
 188 Veng (Pursat) and Ou Chrar (Mondolkiri). The Simpson index equals the
 189 probability that two mosquitoes taken at random from the dataset belong to
 190 the same species:

$$l = \frac{\sum_{i=1}^R n_i(n_i - 1)}{N(N - 1)}$$

191 where n_i is the number of entities belonging to the i^{th} type, R is the number
 192 of different types of mosquitoes collected, and N is the total number of
 193 entities in the dataset.

194 Larger values of the Simpson index represent lower diversity.

195 The Shannon index provides an indication of the evenness in distribution
196 among the species. The idea is that the more species there are, and the more
197 equal their proportional abundances in the collection, the more difficult it is
198 to correctly predict which species will be the next one collected. The Shannon
199 index quantifies the uncertainty (entropy) associated with this prediction. It is
200 most often calculated as follows:

$$H' = \sum_i \left(\frac{n_i}{N} - \log_{10} \left(\frac{n_i}{N} \right) \right)$$

201 The Shannon index therefore quantifies the uncertainty in predicting the
202 species identity of an individual that is taken at random from the dataset. As
203 evenness decreases (from all species being equally common to their being a
204 single predominant species) so does the probability of being able to predict
205 the next species collected, and hence the value of the index increases.

206 **Ethics**

207 The ethical committees of the National Centre of Malariology (CNM) in Phnom
208 Penh, (Cambodia) and of the Liverpool School of Tropical Medicine (UK)
209 approved the study. The mosquito collectors and householders were informed
210 about the objectives, processes and procedures of the study and oral
211 informed consent was sought from them. Collectors were recruited among
212 the adult village population on the understanding that if they wanted to
213 withdraw from the study they could do so at any time without prejudice.
214 Access to malaria diagnosis and treatment was guaranteed throughout the
215 study.

216 **Results**

217 A total of 14 species of anophelines were collected from the four study
218 locations. *Anopheles minimus* was the most common mosquito in Khum

219 Otavao (Pailin), *An. maculatus* predominated in Krorhom Krom (Pursat) and
220 *Anopheles epiroticus* was the predominant one of the seven species collected
221 on the island of Kroh Salau. *Anopheles dirus* predominated in Ou Char
222 (Mondolkiri) where fourteen species of anophelines were collected.
223 *Anopheles dirus*, previously common (CNM unpublished data), was rare or
224 absent in the other three study locations. Although many of the anophelines
225 identified belong to known species complexes, to date, only single members
226 of the freshwater species complexes identified from Cambodia (*An. minimus*,
227 *An. maculatus* *An. barbirostris*, *An. aconitus*) have been found (Sinka et al.,
228 2011). In all cases, these have been the 'type' species. Thus, we considered
229 that the mosquitoes belonging to these complexes were the 'type' species
230 indicated.

231 Diversity indices for the different locations and the different trapping
232 methods are given in Table 1. As diversity increases values of the Simpson
233 index are reduced hence larger values represent lower diversity whilst larger
234 values of the Shannon index indicate that the evenness of the sample
235 increases.

236 The smallest value of the Simpson index, hence the location with the greatest
237 diversity, in landing collections, was observed in the location where there was
238 still a minimal amount of forest cover, Krorhom Krom (Pursat), and the least
239 diversity was observed in Khum Otavao (Pailin), the mainland location that
240 was almost completely deforested. The island location of Kroh Salau (Khoh
241 Kong) had an intermediate index. The MMX trap collected the most diverse
242 group of anophelines compared to other collection methods (as evidenced by
243 the lowest Simpson index). Light-traps hung underneath houses or close to
244 humans and tent-traps had similar diversity indices whilst light-traps hung
245 close to pigs or cows in Krorhom Krom (Pursat) collected a less diverse group
246 of mosquitoes than did the light-trap hung close to people although numbers
247 of mosquitoes collected were considerably higher (Table 2). With the
248 exception of this trap the Shannon index, as expected, varied in tandem with

249 the Simpson index (excluding the pig trap r^2 between the two indices was
250 0.848, but including it was only 0.236).

251 Table 3 shows the parous rates and sac rates, with derived estimates of the
252 oviposition intervals of the fourteen species dissected, according to the
253 location. Overall, *An. aconitus* had the shortest and *Anopheles sinensis* the
254 longest estimated cycles.

255 In Mondolkiri, sac rates were significantly lower for *An. barbirostris* and *An.*
256 *dirus* as compared to *An. kochi* and *An. philippinensis*. In Pailin, sac rates
257 were significantly lower for *An. barbirostris* as compared to *An. maculatus*
258 and *An. minimus*. In Pursat, sac rates were significantly lower for *An.*
259 *aconitus*, *An. jamesi*, *An. maculatus* and *An. vagus* as compared to *An. kochi*.
260 For none of the species, comparisons between locations showed significant
261 differences in sac rates. Therefore, we could not find evidence for a 'nurture'
262 effect on the length of the oviposition cycle that might have been caused by
263 differences in the suitability of the environment.

264 Sac stages for 135 mosquitoes from eleven species dissected in 2013 are
265 shown in Table 4. With the exception of *An. jamesi* and *An. barbirostris* (two
266 of the species with the lowest rate of mosquitoes returning to feed with sacs)
267 sacs were largely considered to be 'a' or 'b' (i.e. large to medium sized)
268 indicating that the mosquitoes had returned to feed on the night following
269 oviposition.

270 In the week prior to a full moon sacs rates in *An. minimus* from Pailin
271 increased (indicating a shorter oviposition cycle) but moon phase did not
272 appear to affect the duration of the cycle in *An. dirus* from Ou Charar
273 (Mondolkiri) (Table 5).

274 A small number of gravid, and therefore possibly gonotrophically discordant,
275 insects were collected in the landing collections (Table 6). With the exception

276 of *An. vagus*, the proportion of gravid insects among those collected was
277 always very low. Females from rare species (i.e. where less than 10
278 individuals were collected) were not more likely to be gravid than females
279 from more common species. Throughout the study period, at both wet and
280 dry periods of the year, the great majority of insects were collected with
281 ovaries at Christopher's Stage I or Stage II. During all seasons (including the
282 wet season when potential breeding sites were common) *An. vagus* females
283 had ovaries at all stages of development including Stage III and Stage IV.

284 Females of the following species were collected at least once with a mating
285 plug (Gillies, 1956): *An. dirus*, *An. maculatus*, *An. minimus*, *Anopheles jamesi*,
286 *An. aconitus*, *Anopheles philippinensis* and *Anopheles tessellatus*. Females
287 with mating plugs, in particular *An. maculatus*, could often be recognized
288 from the distension of the last segment of the abdomen, due to the plug,
289 prior to dissection. At the height of the dry season in Ou Chrar (Mondolkiri)
290 one of three *An. dirus* dissected had Stage I ovaries and a mating plug
291 indicating that breeding was still occurring at that time.

292 **Discussion**

293 In the present study, the lowest diversity in anophelines from landing
294 collections was found in the deforested area of Pailin province and the
295 greatest observed in the location where there was still a minimal amount of
296 forest cover, Krorhom Krom, in Pursat. The rapid scale at which deforestation
297 is taking place in Krorhom Krom implies that diversity is likely to decrease in
298 the near future. *Anopheles dirus* was only found in the one mainland location
299 that had some forest left in the vicinity, albeit this was being (illegally) cut
300 down during the study. Indeed, the presence of *An. dirus* is an indication of a
301 healthy forest environment. It should, however, be possible to control
302 malaria, especially in and around houses (where during the study, malaria
303 was being transmitted (unpublished data)) without destroying the forest.

304 It is possible that the species collected in low numbers may be species that
305 are not attracted to humans and so were rare in the present study.
306 Nevertheless, numbers collected in the MMX trap were also low. Some
307 species, notably *An. kochi*, were caught in considerable numbers in light
308 traps hung close to cows or pigs. On the other hand, the anthropophilic *An.*
309 *dirus* was rare in areas where it had previously been common. In particular, in
310 earlier years, *An. dirus*, which had been the most common mosquito in
311 Krorhom Krom (CNM unpublished data), was rare there during the present
312 study, only 27 mosquitoes were caught in 2121 hours of landing collection.
313 Given that the environment is rapidly changing in the study locations, it is
314 possible that we are witnessing the gradual demise of a number of species,
315 including *An. dirus*, rather than rare species hanging on through a set of
316 effective survival strategies.

317 *Anopheles minimus* was not apparently a vector in Pailin despite the fact that
318 it fed on humans and was relatively long-lived. No cases of malaria were
319 reported from the study village in Pailin during the whole study period. Nor
320 were any oocysts seen on the stomachs of dissected mosquitoes. This
321 accords with the recent decline in malaria observed in Pailin province as a
322 whole (Maude et al., 2014).

323 Sac rates were similar within species that were collected and dissected in
324 sufficient numbers from more than one location although rates differed
325 between species. Thus, intrinsic factors may play a more important role in
326 determining the time at which parous mosquitoes return to feed following
327 oviposition than extrinsic ones. Moonphase, nonetheless, appeared to affect
328 the cycle in *An. minimus*. As with *An. farauti* from Papua New Guinea (Birley
329 & Charlwood, 1989), it was shorter when moonlight was present prior to, or
330 at sunset, compared to nights when no moonlight was present at this time.
331 Moonlight did not appear to affect the duration of the cycle in the forest
332 dwelling *An. dirus* even though, in their review of factors affecting the biology
333 of this species, Obsomer and Coosemans (2007) considered that moonlight

334 was considered to be important. It is possible that, in the forest environment
335 where most oviposition of *An. dirus* occurs, illumination is of less consequence
336 than other factors in oviposition site and host location.

337 As far as we know, this is the first report of the presence of mating plugs in
338 mosquitoes from S.E. Asia. The presence of a newly emerged *Anopheles dirus*
339 with a mating plug at the height of the dry season in Ou Charar (Mondolkiri) is
340 an indication that active breeding was continuing. The species is known to
341 withdraw into the deep forest in the dry season (Gingrich et al., 1992) and
342 this may have been the case in Ou Charar.

343 Gonotrophic discordance has recently been described, or implied, among
344 African malaria vectors including *An. arabiensis*, (Omer and Cloudsley-
345 Thompson 1970), *An. gambiae* (Lehmann et al, 2010, Adamou et al, 2011)
346 and *An. funestus* (Charlwood et al., 2013a). The small number of gravid
347 insects collected in landing or light-trap collections in the present study
348 implies that, for even the least common species, gonotrophic discordance
349 was not the survival strategy adopted during the long hot dry season.
350 Species, such as *An. maculatus* and *An. aconitus*, previously reported as
351 becoming discordant, were not apparently more likely to be so than others.
352 Thus, for all locations and all species, and at all times, gonotrophic
353 concordance appeared to be the rule. As Schapira and Boutsika (2012) point
354 out, Southeast Asian anophelines are physiologically adapted to humid
355 conditions, having wider spiracles than African ones. They may invest in large
356 numbers of eggs per batch to the detriment of longevity, and thereby
357 vectorial capacity (Schapira and Boutsika 2012). This is likely to reduce their
358 ability to survive in hot dry environments, typically associated with
359 gonotrophically discordant anophelines.

360 In forested areas of Bangladesh, *An. dirus* has been considered to be an
361 effective vector due to an 'exceptional survival' (Rosenberg and
362 Maheswary,1982). Our results indicate that in in Ou Charar (Mondolkiri), where

363 active transmission was occurring the mosquito had a relatively low parous
364 rate and the mosquito does not appear to survive any longer than many
365 other anophelines. Rather, its highly developed anthropophagy and
366 endophily is likely to be the cause of it being a malaria vector. Only a single
367 specimen was collected in the MMX trap but it predominated in light-traps
368 hung inside bedrooms in Ou Chrar, even in well-built houses built on stilts up
369 to three meters off the ground.

370 National malaria prevalence surveys are undertaken in Cambodia. These
371 provide information on the groups of people most at risk from the disease
372 but, because of the high rates of internal seasonal migration in Cambodia
373 (Maltoni 2007), do not provide information on the place where the infection
374 was acquired. Light-traps hung inside houses provide a suitable measure of
375 *An. dirus* densities. Given the relative ease of sampling with light-traps, a
376 national *An. dirus* survey is also worth doing. Sampling for other species
377 requires landing catches and is not necessary.

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512 **Figure legend**

513 Map of Cambodia showing the four locations where collections and
514 dissections were undertaken during the study. 1 - Khum Otavao, Pailin
515 Province; 2- Krorhom Krom, Veal Veng, Pursat Province, 3 - Kroh Salau, Khoh
516 Khong Province; 4 - Ou Chrar, Mondolkiri Province

Figure 1 (on next page)

Figure 1.

Map of Cambodia showing the four locations where collections and dissections were undertaken during the study. 1 - Khum Otavao, Pailin Province; 2- Krroh Krom, Veal Veng, Pursat Province, 3 - Kroh Salau, Khoh Khong Province; 4 - Ou Chrar, Mondolkiri Province



Figure 2

The village of Khum Otavao, Pailin Province. Annual Report of Artemisinin Resistant Malaria Research Programme Annual Report of Artemisinin Resistant Malaria Research Programme Khum Otavao, Pailin Province Khum Otavao, Pailin Province Annua



Figure 3

The study site at Krohom Krom, Veal Veng, Pursat Province, Cambodia.



Figure 4

The study site at Kroh Salau, Khoh Kong Province, Cambodia.



Figure 5

The house closest to the forest 2012, Ou Chra, Mondolkiri Province, Cambodia. In 2013 the trees were cut down.



Table 1 (on next page)

Table 1.

Simpson and Shannon indices according to trapping method and location.

Collection	Province	Location	Simpson index	Shannon index
Landing	Pailin	Khum	0.93	0.476
	Pursat	Otavao Krorhom	0.54	0.679
	Khoh	Krom Kroh Salau	0.79	0.523
Light	Pailin	Khum	0.88	0.470
human Light	Mondolki ri	Otavao Ou Charar	0.80	0.602
Tent	Pailin	Khum	0.87	0.525
MMX	Pailin	Otavao Khum	0.46	0.726
Light	Pursat	Otavao Krorhom	0.90	0.407
cow Light	Pursat	Krom Krorhom	0.72	0.540
human Light	Pursat	Krom Krorhom	1.00	0.368
pig		Krom		

Table 1. Simpson and Shannon indices according to trapping method and location.

Table 2 (on next page)

Table 2

Mean numbers by species when CDC light-traps were run close to two people sleeping outdoors under a net, an open stable with two cows and a pigsty with three (little) pigs, Veal Veng, Pursat Province

<i>Species</i>	Host		
	Two humans	Two cows	Three pigs
<i>An. aconitus</i>	0.42	0.24	0.45
<i>An. minimus</i>	0.62	0.59	0.72
<i>An. maculatus</i>	5.58	19.72	32.10
<i>An. kochi</i>	4.27	19.59	58.90
<i>An. jamesi</i>	0.23	0.62	1.00
<i>An. barbirostris</i>	0.00	0.17	0.72

Table 3 (on next page)

Table 3

Parous rates, sac rates and estimated duration of the oviposition cycle by location of collection and species. Legend: * Within a location, species for which the value is not significantly different ($\alpha=0.05$) share the same letter(s); 95%CI designates 95% confidence interval; NA designates where estimates are not available. Cycle lengths u1 and u2 as explained in the methods section.

Site	Species	Dissected	Parous	Parous rate (95%CI)	Sac rate (95%CI) *	Cycle length (u1)	Cycle length (u2)
Khoh	barbirostris			0.75 (0.19-0.99)	0 (0-0.71) a	4 (3.29-4)	NA
Khoh	epiroticus	4	3	0.29 (0.22-0.38)	0.33 (0.19-0.49) a	3.68 (3.51-3.81)	5.08 (4.09-6.96)
Khoh	sinensis	137	40	0.25 (0.16-0.36)	0.16 (0.03-0.4) a	3.84 (3.6-3.97)	8.33 (4.64-19.72)
Khoh	umbrosus	77	19	0 (0-0.84)	NA	NA	NA
Khong	aconitus	2	0	0.67 (0.3-0.93)	0.83 (0.36-1) a-b	3.17 (3-3.64)	3.2 (3.04-4.39)
Mondolki	barbirostris	9	6	0.83 (0.63-0.95)	0.2 (0.06-0.44) a	3.8 (3.56-3.94)	7 (4.39-14.28)
Mondolki	dirus	24	20	0.58 (0.53-0.63)	0.34 (0.27-0.4) a	3.66 (3.6-3.73)	4.97 (4.5-5.62)
Mondolki	hodgkini	379	220	0 (0-0.52)	NA	NA	NA
Mondolki	jamesi	5	0	1 (0.03-1)	1 (0.03-1) a-b	3 (3-3.98)	3 (3.01-8.29)
Mondolki	kochi	1	1	0.66 (0.52-0.78)	0.6 (0.42-0.76) b	3.4 (3.24-3.58)	3.67 (3.34-4.31)
Mondolki	maculatus	53	35	0.6 (0.15-0.95)	0.33 (0.01-0.91) a-b	3.67 (3.09-3.99)	5 (3.24-16.72)
Mondolki	minimus	5	3	1 (0.03-1)	0 (0-0.98) a-b	4 (3.03-4)	NA
Mondolki	philippinensis	1	1	0.61 (0.42-0.78)	0.68 (0.43-0.87) b	3.32 (3.13-3.57)	3.46 (3.18-4.18)
Mondolki	tesselatus	31	19	0.2 (0.01-0.72)	0 (0-0.98) a-b	4 (3.03-4)	NA
Mondolki	vagus	5	1	NA	NA	NA	NA
Mondolki		0	0	NA	NA	NA	NA

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Pailin	aconitus	42	25	0.6 (0.43-0.74)	0.48 (0.28-0.69) a-b	3.52 (3.31-3.72)	4.08 (3.5-5.34)
Pailin	barbirostris	72	36	0.5 (0.38-0.62)	0.14 (0.05-0.29) a	3.86 (3.71-3.95)	9.2 (5.51-18.19)
Pailin	dirus	5	2	0.4 (0.05-0.85)	0.5 (0.01-0.99) a-b	3.5 (3.01-3.99)	4 (3.1-12.53)
Pailin	jamesi	1	0	0 (0-0.98)	NA	NA	NA
Pailin	maculatus	232	122	0.53 (0.46-0.59)	0.52 (0.42-0.61) b	3.48 (3.39-3.58)	3.94 (3.66-4.33)
Pailin	minimus	835	568	0.68 (0.65-0.71)	0.6 (0.56-0.64) b	3.4 (3.36-3.44)	3.67 (3.56-3.79)
Pailin	philippinensis	4	2	0.5 (0.07-0.93)	0.5 (0.01-0.99) a-b	3.5 (3.01-3.99)	4 (3.1-12.05)
Pailin	tesselatus	10	9	0.9 (0.55-1)	0.78 (0.4-0.97) a-b	3.22 (3.03-3.6)	3.29 (3.07-4.25)
Pailin	umbrosus	2	0	0 (0-0.84)	NA	NA	NA
Pailin	vagus	12	5	0.42 (0.15-0.72)	1 (0.48-1) a-b	3 (3-3.52)	3 (3-3.83)
Pursat	aconitus	33	25	0.76 (0.58-0.89)	0.32 (0.15-0.54) a	3.68 (3.46-3.85)	5.13 (3.93-7.84)
Pursat	barbirostris	19	7	0.37 (0.16-0.62)	0.29 (0.04-0.71) a-b	3.71 (3.29-3.96)	5.5 (3.54-13.99)
Pursat	dirus	11	2	0.18 (0.02-0.52)	1 (0.16-1) a-b	3 (3-3.84)	3 (3.01-5.46)
Pursat	jamesi	105	78	0.74 (0.65-0.82)	0.42 (0.31-0.54) a	3.58 (3.46-3.69)	4.36 (3.88-5.12)
Pursat	kochi	300	215	0.72 (0.66-0.77)	0.74 (0.68-0.8) b	3.26 (3.2-3.32)	3.35 (3.26-3.48)
Pursat	maculatus	389	214	0.55 (0.5-0.6)	0.49 (0.42-0.56) a	3.51 (3.44-3.58)	4.04 (3.8-4.35)
Pursat	minimus	27	21	0.78 (0.58-0.91)	0.48 (0.26-0.7) a-b	3.52 (3.3-3.74)	4.1 (3.48-5.53)

Pursat	tesselatus	8	2	0.25 (0.03-0.65)	0 (0-0.84) a-b	4 (3.16-4)	NA
Pursat	vagus	25	23	0.92 (0.74-0.99)	0.39 (0.2-0.61) a	3.61 (3.39-3.8)	4.56 (3.69-6.52)

Table 3. Parous rates, sac rates and estimated duration of the oviposition cycle by location of collection and species.

Legend: * Within a location, species for which the value is not significantly different ($\alpha=0.05$) share the same letter(s); 95%CI designates 95% confidence interval; NA designates where estimates are not available. Cycle lengths u1 and u2 as explained in the methods section.

Table 4(on next page)

Table 4

Sac stage by species (ordered according to estimated duration of the gonotrophic cycle length, from the shortest to the longest) of anophelines dissected between 20 January and 23 December 2013.

Table 4. Sac stage by species (ordered according to estimated duration of the gonotrophic cycle length, from the shortest to the longest) of anophelines dissected between 20 January and 23 December 2013.

Species	Proportion parous with sacs	Sac stage		
		a	b	c
<i>An. aconitus</i>	0.43	2	0	2
<i>An. epirocticus</i>	0.33	0	1	0
<i>An. dirus</i>	0.34	22	12	11
<i>An. vagus</i>	0.50	1	0	0
<i>An. minimus</i>	0.60	15	18	8
<i>An. maculatus</i>	0.50	8	5	2
<i>An. kochi</i>	0.72	4	2	0
<i>An. philippinensis</i>	0.68	3	6	2
<i>An. tessellatus</i>	0.58	2	0	1
<i>An. jamesi</i>	0.42	0	2	5
<i>An. barbirostris</i>	0.20	0	0	1

Table 5 (on next page)

Table 5

Sac stages of *An. minimus* and *An. dirus* dissected grouped according to moon-phase. (Chi square for *An. minimus* with three degrees of freedom = 10.56, $p = 0.0144$; Chi square for *An. dirus*, with two degrees of freedom = 0.24; $p = 0.887$).

Table 5. Sac stages of *An. minimus* and *An. dirus* dissected grouped according to moon-phase. (Chi square for *An. minimus* with three degrees of freedom = 10.56, $p = 0.0144$; Chi square for *An. dirus*, with two degrees of freedom = 0.24; $p = 0.887$).

Moon phase	<i>An. minimus</i>			<i>An. dirus</i>		
	With sacs	Without sacs	Proporti on with sacs	With sacs	Witho ut sacs	Proporti on with sacs
New	152	97	0.61	-	-	-
First	91	88	0.51	37	66	0.36
Full	76	33	0.70	19	37	0.34
Last	32	21	0.60	21	44	0.32

Table 6 (on next page)

Table 6

Number dissected and number of those that were gravid from the four collection locations in Cambodia and best estimates (with adjusted Wald confidence intervals) of the proportion gravid. No gravid insects were recorded from the other species dissected.

Table 6. Number dissected and number of those that were gravid from the four collection locations in Cambodia and best estimates (with adjusted Wald confidence intervals) of the proportion gravid. No gravid insects were recorded from the other species dissected.

Species	Total dissected	Number gravid	Best estimate (Adjusted Wald C.I.)
<i>An. aconitus</i>	85	1	0.023 (<0.0001-0.07)
<i>An. epirocticus</i>	138	1	0.014 (<0.0001-0.04)
<i>An. jamesi</i>	108	1	0.018 (<0.0001-0.06)
<i>An. maculatus</i>	632	6	0.011 (0.004-0.021)
<i>An. minimus</i>	865	2	0.004 (0.0001-0.009)
<i>An. vagus</i>	44	7	0.174 (0.076-0.297)