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1 A comparison between 'wet' and 'dry' dissections for the assessment of parity in
2 *Anopheles arabiensis* and determination of sac stage in mosquitoes alive or dead on
3 collection.

4

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23

24 [Abstract](#)

25

26 **Background.** The determination of parous rates in mosquitoes, despite numerous shortcomings,
27 remains a tool to evaluate the effectiveness of control programs and to determine vectorial
28 capacity in malaria vectors. Two dissection techniques are used for this. For one, the tracheoles
29 of dried ovaries are examined with a compound microscope and in the other the follicular stalk
30 of ovaries is examined, wet, with a stereomicroscope. The second method also enables the sac
31 stage of parous insects (which provides information on the duration of the oviposition cycle) and
32 mated status of insects to be determined. Despite widespread use the two techniques have not
33 previously been compared.

34 **Methods** We compared the two dissection techniques using *Anopheles arabiensis*, collected with
35 a tent-trap in Eritrea. The paired ovaries were removed in water and one was examined by each
36 method. From a separate set of dissections from Tanzania, we also determined if the sac stages of
37 *A. gambiae* s.l. (83% of 183 identified by PCR being *Anopheles arabiensis*) that were alive on
38 collection were different to those that died on collection and what the implications for vectorial
39 capacity might be.

40 **Results** 389 host-seeking, mosquitoes, from Furvela tent-traps in Eritrea and 1823 live and 1416
41 dead from Furvela tent-traps, CDC light-trap and window-trap collections were dissected from
42 Tanzania. Seven per cent of the dry ovaries could not be classified due to granulation (yolk) in
43 the ovariole that obscured the tracheoles. The sensitivity of the dry dissection was 92.74 % (C.I.
44 86.67-96.63%) and the specificity was 88.51 % (C.I. 79.88-94.35%) among the 211 ovaries that
45 could be classified by the dry technique and compared to the ovaries dissected wet. In collections
46 from Tanzania parous insects were more likely to die compared to nulliparous ones. The
47 proportion of parous mosquitoes with 'a' sacs (indicative of recent oviposition) was significantly

48 greater in insects that were dead (0.36) on collection in the morning compared to those that were
49 alive (0.12) (Chi square 138.9259, $p < 0.001$). There was a preponderance of newly emerged
50 virgin insects in the outdoor collection (Chi sq =8.8413, $p= 0.003$).

51 **Conclusions** The examination of mosquito ovaries using transmitted light in a ‘wet’ dissection is
52 a more useful and informative technique than examination of dry ovaries. In order to correctly
53 estimate the duration of the oviposition cycle mosquitoes should be dissected as soon as possible
54 after collection. Younger insects were more likely to attempt to feed outdoors rather than
55 indoors.

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59 Introduction

60
61 Despite its many shortcomings the measurement of parous and nulliparous rates (i.e. the
62 proportion of insects in a population that have, or have not, laid eggs) in mosquito vectors is
63 commonly evaluated as part of malaria control programs. The shortcomings include the
64 requirement that measurements are made over a complete population cycle, that nulliparous and
65 parous insects are sampled without bias and (for survival rate estimation) that survival is
66 independent of age (Clements & Paterson, 1981). Although they may not provide much more
67 than an approximation of survival (Gillies, 1989), an estimation of parous rates is useful in
68 control trials for comparisons between intervention and control areas (where they are expected to
69 be lower in interventions that target adult mosquitoes but higher in those that target larvae) and,
70 independent of survival estimation, they also provide information on the behaviour of young
71 insects that may themselves become a target for specific interventions.

72

73 Parity is determined by dissection. Following the maturation of the first batch of eggs
74 irreversible changes occur in the ovaries of female mosquitoes. The tightly packed and coiled
75 tracheolar system characteristic of nulliparous insects becomes stretched and uncoiled as the
76 eggs develop and never return to their previous state (Detinova, 1962). In newly emerged teneral
77 mosquitoes meconium, the remains of larval midgut epithelium can be seen as a green an opaque
78 mass inside the midgut (Rosay,1961 Romoser et al. 2000). This is excreted either following an
79 initial blood meal or within 48hrs of emergence. The tracheolar system can be seen in ovaries
80 that are dissected in distilled water and allowed to dry. Once dry the ovary can be examined
81 under a compound microscope. The 'dry' technique is simple and has been widely used.

82

83 An alternative technique is to examine the follicular stalk at the time of dissection with
84 transmitted light using a stereomicroscope. The dissection is performed in isotonic saline (to
85 avoid swelling of tissues) but can also be performed with water. This dissection has the
86 advantage that it can provide information on both the mated status and the duration of the
87 oviposition cycle in parous insects

88

89 Irreversible changes also occur following oviposition in the pedicel that connects the ovarioles to
90 the lateral oviduct (Hoq & Wilkes, 1995). In this case granulation occurs in the basal body,
91 small areas in the calyx wall enclosed by the ovariolar sheaths consisting of six to nine
92 specialized epithelial cells, making them visible when examined with light coming through the
93 preparation. A large egg sac remains in the ovarioles immediately after ovulation. The sac
94 gradually contracts and, 12-24 h after ovulation, consists of heavily folded tunica above the
95 calyx. In nulliparous females, there is no coloration of the pedicel. The tracheolar system is also
96 visible in this dissection so that its appearance can also assist in interpretation of the preparation.

97 Examination of the pedicel in parous females also enables the duration of the oviposition cycle to
98 be estimated since an insect with a large sac would have been caught shortly after oviposition
99 whereas one with just a basal body would have oviposited approximately a day earlier. The
100 duration of the oviposition cycle has a major impact on the proportion of mosquitoes that might
101 be vectors. For example, a change from a two-day cycle to a three-day one produces a four-fold
102 increase in the potential numbers of vectors.

103

104 The dissection also enables the mated status of newly emerged nulliparous insects to be
105 determined. In particular, examination of the spermatheca and oviducts is possible. Virgin
106 insects do not have sperm in the spermatheca and, in recently mated anophelines, there is a male
107 donated mating plug (Gillies 1956, Baldini et al., 2013) visible in the common oviduct. This is
108 absorbed over the following 12-24 hours. Thus, with this dissection it is possible to separate
109 nulliparous insects into three categories: virgins, recently mated insects and those that have
110 mated 24 hours, or more, earlier. In practise, the overall appearance of the ovary is used to assess
111 parous status: transparent and small ovaries with coiled tracheoles are indicative of nulliparity,
112 whilst larger, darker, ovaries enlarged ampullae (Gillies, 1956) and an uncoiled tracheolar
113 system indicate that the mosquito is parous.

114

115 Anopheleines and Aedines differ in that in the former oogenesis is an ‘all or nothing’
116 phenomenon that requires a complete blood meal to proceed whilst in the latter, individual
117 follicles may develop following partial blood meals. This makes estimates of age more difficult
118 in the latter group.

119 Hugo et al (2008) compared these and more sophisticated techniques using laboratory reared
120 *Aedes vigilax* and *Culex annulirostris*. They considered that the dry technique (when allied to
121 the observation of the presence of meconium in the stomach of the mosquito) was the most
122 suitable for parity determination.

123 The two methods of dissection have not previously been compared in anophelines, nor have they
124 been compared using wild insects whose life conditions differ from insects reared and
125 maintained in the laboratory. Here, therefore, we compare these methods with wild caught
126 *Anopheles arabiensis*. We also compare estimates of the duration of the oviposition cycle from
127 insects that died shortly after collection with those that remained alive up to the time of
128 dissection.

129 Methods

130 Description of study sites

131

132 *Anopheles arabiensis*, collected between the 7th and 23rd of October 2017 with a Furvela tent-trap
133 (Charlwood et al., 2017) below the village of Adi Bosco (15° 41' 41.67" N 38° 38' 54.59" E at
134 an altitude of 1536m above sea level) in Anseba province, Eritrea, were dissected in bottled
135 drinking water. Mosquitoes, that were alive upon collection, were killed in a freezer and were
136 then used for these dissections. One ovary was placed on a slide to dry for subsequent
137 examination and the other was assessed directly for parity and sac stage. Insects from the latter
138 dissection were classified according to the scheme outlined by Charlwood et al., (2003). The sac
139 stage in parous insects was determined according to the scheme outlined in Wilkes & Charlwood
140 (1975).

141 Each mosquito was given a unique identifying number and subsequently comparisons between
142 assessments of parity were determined. A number of *Culex quinquefasciatus* collected with a

143 CDC light-trap from a bedroom in Asmara, Eritrea, where potential hosts slept under mosquito
144 nets were also dissected for a comparison of the appearance of the ovaries of the two species.

145

146 The sac stages of mosquitoes according to whether they were alive or dead upon collection were
147 determined from collections undertaken in the village of Kyamyorwa in Muleba district, Kagera
148 Province, Tanzania, from December 1 2015 to January 17 2016. Mosquitoes were collected in a
149 CDC light-trap, run inside a bedroom with two human and one canine host; a window-trap from
150 the same room and a Furvela tent-trap outdoors with a single sleeper (Le Clair et al., 2017). Live
151 mosquitoes were removed from the collection bags with an aspirator prior to being killed and
152 both recently killed and those dead on collection were identified to species or species group
153 using the keys of Gillies & De Meillon (1968) and Gillies & Coetzee (1975). Mosquitoes in
154 Tanzania were dissected in saline eye drops (Charlwood et al., 2016).

155 *Anopheles arabiensis* is the only member of the *A. gambiae* complex that has been identified
156 from previous collections in Eritrea (Shilulu et al., 2003) and so it is assumed that this was the
157 member of the complex that was collected. A sub-sample of the *A. gambiae* s.l. from
158 Kyamyorwa were identified to species by multiplex real-time PCR Taq Man assay (Bass et al.,
159 2008).

160 In order to determine if the different age groups were caught in similar proportions indoors (in
161 light-trap and window-trap combined) and outdoors (in the tent-trap) the number of the different
162 ages collected live and dead were estimated by multiplying the total by the proportion in each
163 category and then summing the estimated totals. The overall proportion of each age group
164 (indoors and outdoors) was then estimated and indoor and outdoor collections compared by Chi-
165 Square test (at a significance level of 0.05).

166 We also assume that gonotrophic development (from blood feeding to becoming gravid) takes 2
167 days in Kyamyorwa; hence mosquitoes with 'a' or 'b' sacs were considered to have a 2-day
168 feeding cycle and those with 'c' or 'd' (no sac) to have added an extra day (i.e. to have a 3-day
169 cycle). Estimates of the population mean duration of the feeding cycle (μ) in live and dead
170 parous insects were therefore determined according to the proportions of Sac and No-sac
171 mosquitoes in the collection where μ is the mean feeding frequency of parous insects in days:

$$\mu = [(n \text{ Sac} * 2) + (n \text{ No-sac} * 3)] / (n \text{ Sac} + n \text{ No-sac})$$

176 Ethics

177
178 The collections conducted in Tanzania were done as a component of the Pan African Malaria
179 Vector Research Consortium project 'Evaluation of a novel long lasting insecticidal net and
180 indoor residual spray product, separately and together, against malaria transmitted by pyrethroid
181 resistant mosquitoes' which received ethical clearance from the ethics review committees of the
182 Kilimanjaro Christian Medical College (certificate number 781 on the 16 September 2014), the
183 Tanzanian National Institute for Medical Research (20 August 2014), and the London School of
184 Hygiene and Tropical Medicine (reference 6551 on 24 July 2014). The trial was registered with
185 ClinicalTrials.gov (registration number NCT02288637) on 11 July 2014.

186 Collections in Eritrea were undertaken by the first author in his tent during supervision of
187 students from the College of Health Sciences, Asmara, undertaking their fieldwork as part of a
188 course entitled 'The ecology of malaria vectors'.

189 Results

190 Ovaries of *Cx. quinquefasciatus* were clearer and the tracheoles easier to see than was the case
191 with the *A. arabiensis* (compare Figs 1A, B with Fig 2A, B).
192
193

194 In almost 10% (23 of 238) of the *A. arabiensis* dissected the deposition of yolk in the follicles
195 made assessment of the age difficult or impossible from the dry dissections. In some cases,
196 wetting the preparation again temporarily enabled the tracheoles to become visible for
197 assessment (Fig 3A and B)

198 Comparison between methods

199
200 Four (1.4%) of the 286 insects dissected 'wet' were gravid. There were 211 ovaries that could be
201 classified by the dry technique and compared to the ovaries dissected wet (Table 1). There was a
202 91.5% (C.I. 86.30-94.49%) concordance between the methods. Nevertheless, 18 of 211 (10
203 parous and 8 nulliparous) were given different classifications by the two methods. Thus,
204 assuming that the wet dissection was correct, the sensitivity of the dry dissection was 92.74 %
205 (C.I. 86.67-96.63%) and the specificity was 88.51 % (C.I. 79.88-94.35%).

206 Since the discrepancies were almost equally distributed between nulliparous and parous insects
207 an overall estimate of survival would be similar. During the experiment in Adi Bosco, the
208 number of *A. arabiensis* collected decreased from a mean of 126 per tent per night to 34 per
209 night whilst the parous rate (determined by the wet dissection) increased from 0.28 to 0.56
210 (correlation between the number collected and parous rate = -0.71). Nevertheless, since the
211 population was changing and collections did not cover the complete population cycle any
212 estimates of survival from the present data would be imprecise and possibly incorrect.

213 Sac stages among live or dead mosquitoes

214
215 Among 183 *A. gambiae* s.l. from Kyamyorwa identified to species by PCR 152 (83.1%) were *A.*
216 *arabiensis* (LeClair et al., 2017). Thus, the great majority of insects from Kyamyorwa were also
217 *A. arabiensis*.

218 Between November 30 2015 and January 17 2016, 1921 live (273 from the light- trap, 436 from
219 the window trap and 1209 from the tent-trap) and 1728 dead (711 from the light-trap and 705
220 from the tent-trap) *A. gambiae* s.l. were dissected (Table 2). The smaller numbers of live insects
221 dissected from the light-trap was due to the low survival of the mosquitoes in the trap (LeClair et
222 al, 2017). All insects collected from the window trap were alive. 574 (29.9%) of the live insects
223 and 689 (39.9%) of the dead insects were parous (Chi-Square 10.0308 $p = 0.002$). Thus, parous
224 insects were more likely to die compared to nulliparous ones. Among the nulliparous insects,
225 virgins survived better than those with mating plugs (Chi-Square 5.4373, $p = 0.020$). The
226 estimated total proportion of the different age groups (combining estimated numbers of both live
227 and dead insects) were also different between mosquitoes collected indoors (light and window-
228 trap combined) and those collected outdoors (Table 3). Virgin insects predominated in the
229 outdoor collection (Chi-Square 19.138. $p = 0.000012$) whilst parous insects, even excluding
230 teneral insects (virgins and those with mating plugs), predominated indoors (Chi-Square for all
231 insects 41.9559, $p < 0.001$ and 8.8546, $p = 0.002923$ excluding teneral insects). Hence, newly
232 emerged insects were more likely to attempt to feed outdoors rather than indoors.

233
234 Among parous insects the proportion with 'a' sacs was significantly greater in insects that were
235 dead (0.36) on collection in the morning compared to those that were alive (0.12) (Chi-Square
236 138.93, $p < 0.001$) (Fig 4). The estimated duration of the oviposition cycle among live insects,
237 based on equation 1, was 2.7 days and among dead ones was 2.4 days. The proportion of parous
238 insects dissected from Adi Bosco (that were all alive on collection) with large sacs was also
239 significantly different to those from Kyamyorwa (68 of 91 compared to 113 of 424) (Chi-Square
240 75.97, $p < 0.001$).

241

242 Parous rates were lower in the mosquitoes that had remained alive at the time of capture (Chi-
243 Square = 39.46, $p < 0.05$). There was no significant difference in the parous rates of mosquitoes
244 collected in the window trap compared to the light-trap (Chi-Square =2.57, $p = 0.109$ n.s.) nor
245 between virgin and plug rates among newly emerged insects from these two types of collection
246 (Chi-Square = 0.0002, $p = 0.98$. n.s.) but there was a difference between tent and window trap
247 (Chi-Square =21.76, $p = < 0.001$).

248 Discussion

249
250 Ovaries of *Cx. quinquefasciatus* were much easier to classify using the dry technique than were
251 the *An. arabiensis*. Our results indicate that almost 10 per cent of the *A. arabiensis* had
252 unreadable ovaries using this technique, which would affect assessments of survival. A similar
253 proportion of unreadable ovaries of *Aedes vigilax* and *Culex annulirostris* was observed by Hugo
254 et al. (2008). As with the *A. arabiensis* this was apparently due to the deposition of material
255 (yolk) in the follicles that obscured the tracheoles. Thus, despite its ease, the dissection of
256 Anopheles ovaries in water and their subsequent examination with a compound microscope
257 when dry, is not as good, or useful, as examination of the ovaries using a stereomicroscope with
258 transmitted light from a mirror. A mirror is better than an artificial light source since by altering
259 its position the contrast of the preparation can be changed so that the visibility of structures
260 within the ovaries changes making assessment easier.

261

262 Results from Tanzania indicate that young *A. arabiensis*, in particular virgin insects, are more
263 likely to feed outdoors than older ones. This is similar to the behaviour of *A. coluzzii* from Ghana
264 (Charlwood et al., 2012) and indicates that mating has an effect on host seeking in a relatively
265 subtle fashion, at least in the *A. gambiae* complex. Whether the same behaviour occurs in other

266 endophilic mosquitoes is not known. Whilst this might mean that the risk of acquiring malaria
267 per bite is lower outside they imply that the risk of transmitting it to a mosquito that may survive
268 through the extrinsic cycle are greater outdoors. They also imply that a potential control
269 technique aimed specifically at young insects should work preferentially outdoors. Young, naïve
270 mosquitoes may be attracted to a wider range of potential hosts than older insects (which may
271 return to feed on hosts that they have successfully fed on previously, (Vantaux et al, 2003,
272 Vinauger et al, 2014). Odour baited traps that target such young insects may be one possible
273 approach.

274

275 The proportion of live parous mosquitoes with 'a' sacs from the tent-trap recorded from Eritrea
276 was significantly higher than that recorded from Kyamyorwa. The higher rates are probably
277 because the much lower temperatures in Adi Bosco (12° C minimum at night in Adi Bosco
278 compared to 27° C in Kyamyorwa) slowed contraction of the sacs. At the higher temperatures,
279 typical of the tropics, it behoves the entomologists to kill and dissect the mosquitoes as soon as
280 possible after collection. If there is a delay, sacs are likely to contract during the time that the
281 mosquito is collected and killed. This will tend towards an overestimation of the duration of the
282 cycle (in our case 2.7 days compared to 2.4 days) and as a result an overestimation of the
283 vectorial capacity of the population as a whole. Given the variation in age and the effect that
284 environmental conditions can have on the relative proportion of the population biting indoors or
285 outdoors (Charlwood et al.,2011) it also behoves the entomologist to undertake simultaneous
286 collections indoors and outdoors for population assessment.

287 Surprisingly, virgins survived better than recently mated insects. This may be because they were
288 collected later in the night than recently mated insects (and so had a shorter time in the stressful

289 environment of the trap). However, given that virgin and recently mated females of *A. coluzzii*
290 have similar patterns of activity in landing collections (Charlwood et al., 2003) and that the rates
291 were similar between light-trap (where the majority of mosquitoes had died) and window-trap
292 (where they were all alive, Le Clair et al, 2017) this is unlikely.

293

294 It is possible that dissections will in future be replaced by other techniques, notably assessment
295 of age based on reflectance of Near Infra-Red (NIR) light (Mayagaya et al., 2009, Krajacich et
296 al., 2017) or gene transcription (Cook et al. 2006, 2007). Nevertheless, the techniques remain
297 experimental and in the process of development. For the time being dissections remains the
298 method of choice.

299 Conclusions

300

301 The utility of examination of tracheolar coiling in dried ovarioles dissections for the assessment
302 of mosquito age differs between genera. Among anophelines the technique is less useful than
303 examination of ovaries wet with transmitted light.

304 The wet dissection also allows for determination of oviposition cycle duration. However, insects
305 need to be dissected shortly after capture for the information to be meaningful.

306 Recently emerged virgin *Anopheles arabiensis* are more likely to seek hosts outdoors rather
307 than indoors.

308

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310

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315

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407 mosquito *Aedes aegypti*. *The Journal of Experimental Biology* **217**: 2321-2330
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Figure 1(on next page)

Ovaries of A) nulliparous and B) parous *Culex quinquefasciatus* showing the 'textbook' appearance of the tracheoles.

The coiled ends of the tracheoles in the nulliparous insect (A) can be compared to the extended tracheoles of the parous insect (B)

A



B

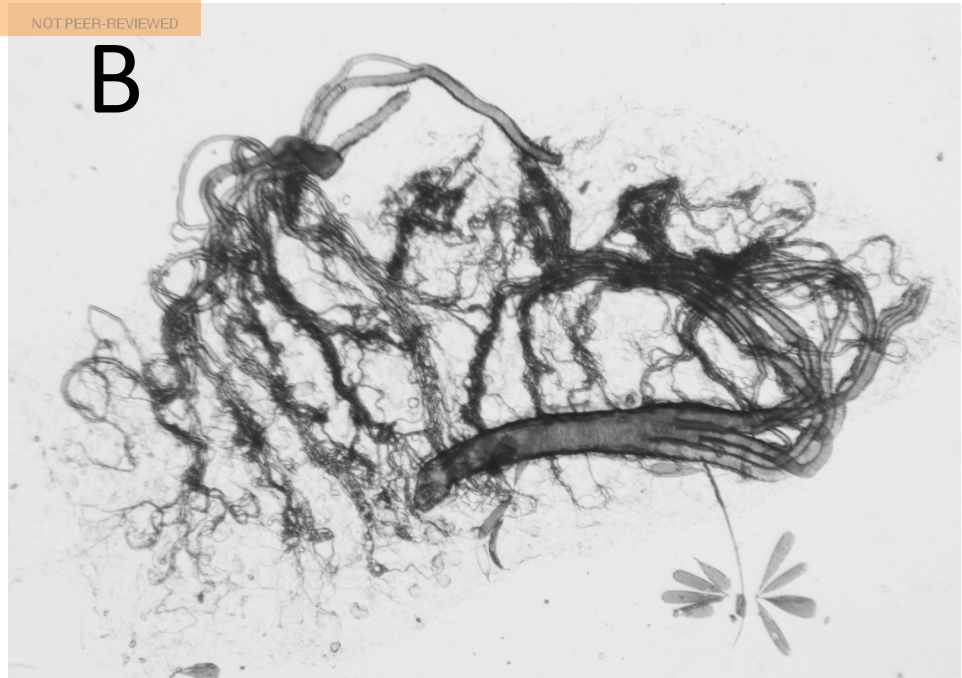


Figure 2 (on next page)

Ovaries of A) nulliparous and B) parous *Anopheles arabiensis*

Compared to Figure 1 the tracheoles are more difficult to distinguish in the *Anopheles*, both in the nulliparous insect (A) and the parous one (B)

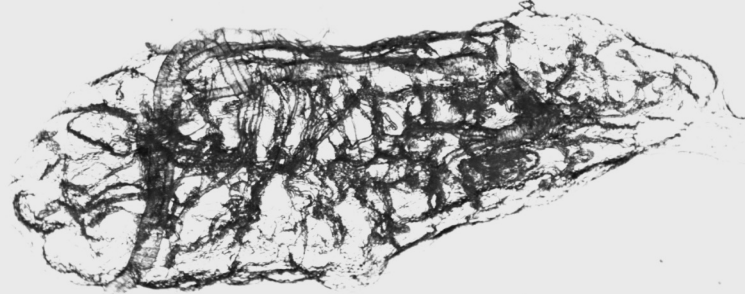
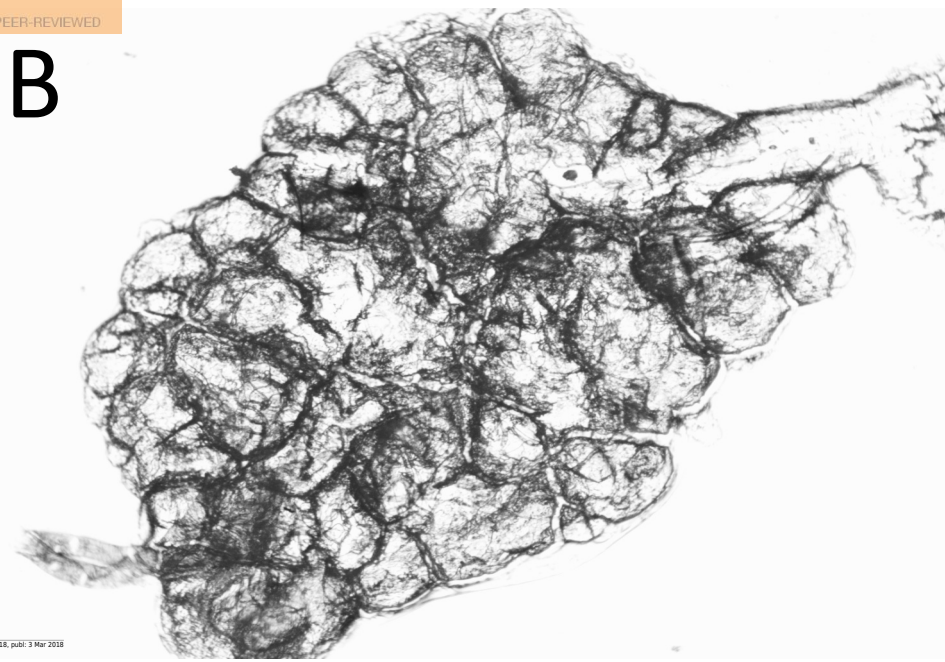
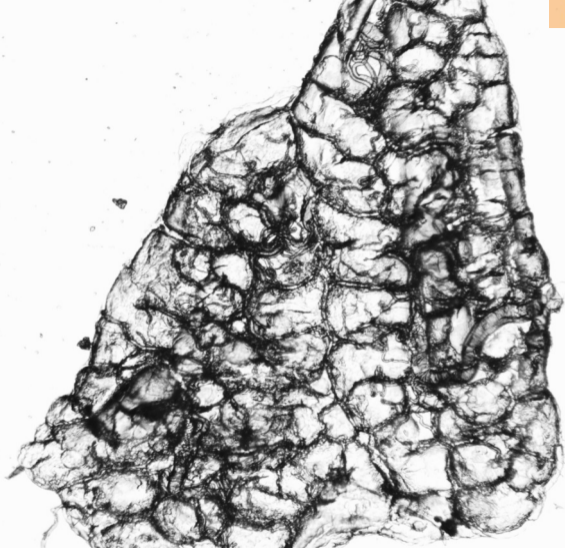
A**B**

Figure 3(on next page)

A) dry and B) re-wetted ovaries of a nulliparous *Anopheles arabiensis*.

The addition of a thin layer of water can temporarily make the tracheoles visible sufficient for a diagnosis of insect age to be made, in this case a nulliparous insect

A



B

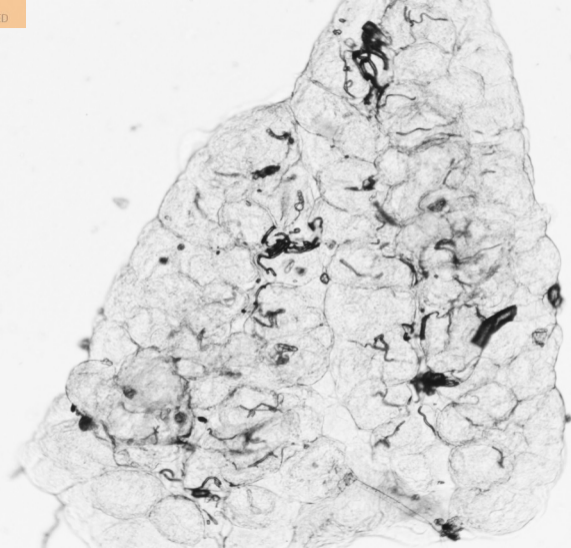


Figure 4(on next page)

Sac stages of *Anopheles arabiensis* that were alive or dead upon collection

Sac stages of mosquitoes collected dead were larger than in those insects that had remained alive up to the point of dissection.

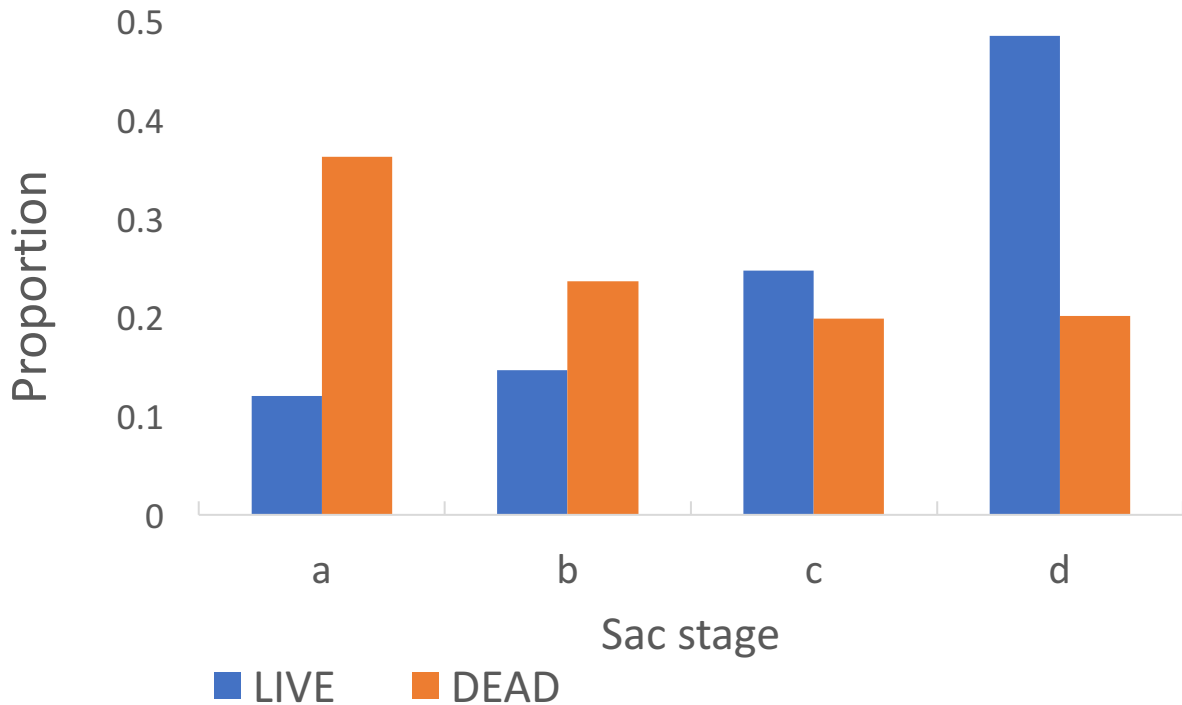


Table 1 (on next page)

Age of *Anopheles arabiensis* determined either by immediate 'wet' dissection using transmitted light or examined dry with a compound microscope

Age of *Anopheles arabiensis* from Elaboret, Eritrea, determined either by immediate 'wet' dissection using transmitted light or examined dry with a compound microscope

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	Nulliparous	Parous	Unreadable	Parous rate (Adj Wald C.I.)
Wet dissection	175	112	2	0.39 (0.34-0.45)
Dry dissection	165	104	20	0.39 (0.33-0.45)

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Table 2 (on next page)

Number of *A. arabiensis* dissected by age, collection type and mosquito condition (live or dead) on collection.

Number of *A. arabiensis* dissected by age, collection type and mosquito condition (live or dead) on collection. Note all mosquitoes in the window trap were alive on collection.

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		Virgin	Plug	NI	NII	a- sac	b- sac	c- sac	d- sac	Total dissected	Parous rate (Adj Wald C.I.)
Tent	Live	385	321	56	127	32	51	77	160	1209	0.27 (0.25-0.29)
	Dead	180	167	31	57	94	64	56	56	705	0.38 (0.35-0.42)
Light	Live	107	58	6	29	10	7	13	43	273	0.27 (0.22-0.32)
	Dead	131	199	31	50	114	82	64	40	711	0.42 (0.39-0.46)
Window	Live	75	78	10	29	24	34	36	55	341	0.44 (0.39-0.49)

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Table 3(on next page)

Number of *A. arabiensis* collected indoors (light-trap and window-trap) and outdoors alive or dead on collection and proportion in each age category.

Number of *A. arabiensis* collected indoors (light-trap and window-trap) and outdoors alive or dead on collection and proportion in each age category, Kyamyorwa, Tanzania, December 2015-January 2016

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Location	Condition	Total collected	Prop ⁿ Virgin (Adj Wald C.I.)	Prop ⁿ Plug	Prop ⁿ Null	Prop ⁿ Parous
Indoor*	Live	560	0.27 (0.23-0.31)	0.22 (0.19-0.26)	0.62 (0.58-0.66)	0.38 (0.34-0.42)
	Dead	3865	0.22 (0.19-0.25)	0.25 (0.23-0.29)	0.58 (0.54-0.61)	0.42 (0.39-0.46)
	All	4425	0.22 (0.21-0.23)	0.25 (0.24-0.26)	0.58 (0.57-0.60)	0.42 (0.40-0.43)
Outdoor	Live	2029	0.30 (0.28-0.33)	0.27 (0.25-0.29)	0.73 (0.71-0.75)	0.27 (0.25-0.29)
	Dead	1605	0.24 (0.21-0.27)	0.23 (0.21-0.26)	0.62 (0.59-0.65)	0.38 (0.35-0.41)
	All	3634	0.27 (0.26-0.29)	0.25 (0.24-0.27)	0.68 (0.67-0.70)	0.32 (0.30-0.33)

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