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

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Abstract

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

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

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

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

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

Introduction

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

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

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

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

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

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

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

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

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

Methods

Description of study sites

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

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

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

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

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

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

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

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

Ethics

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

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

Results

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

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

Comparison between methods

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

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

Sac stages among live or dead mosquitoes

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

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

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

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

Discussion

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

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

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

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

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

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

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

Conclusions

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

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

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

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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)

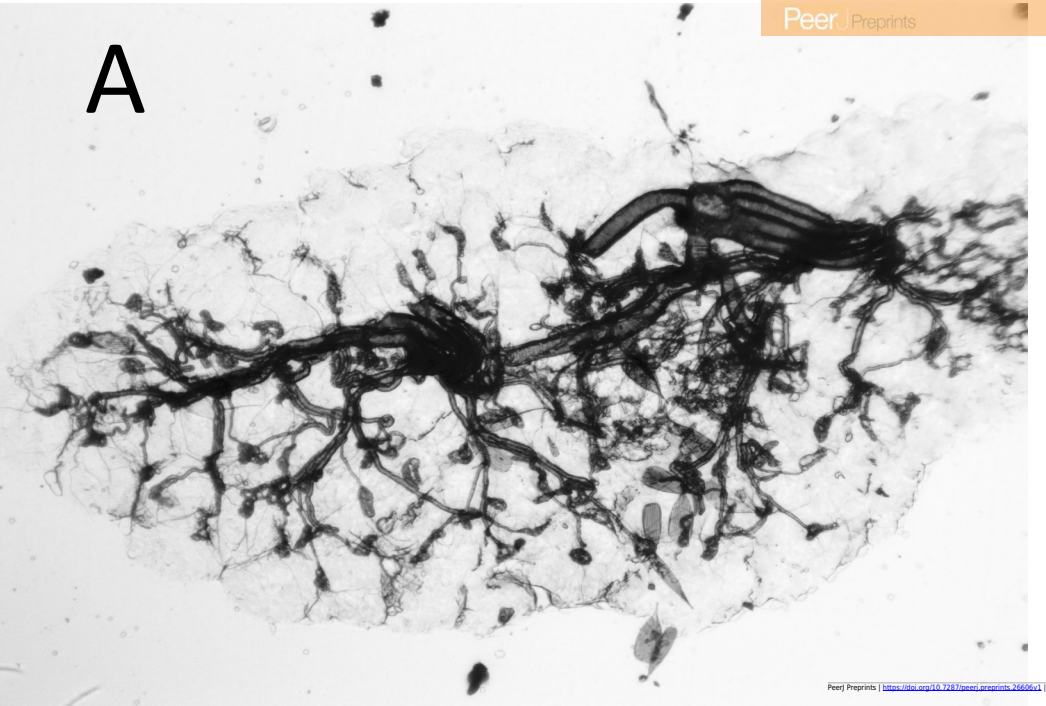
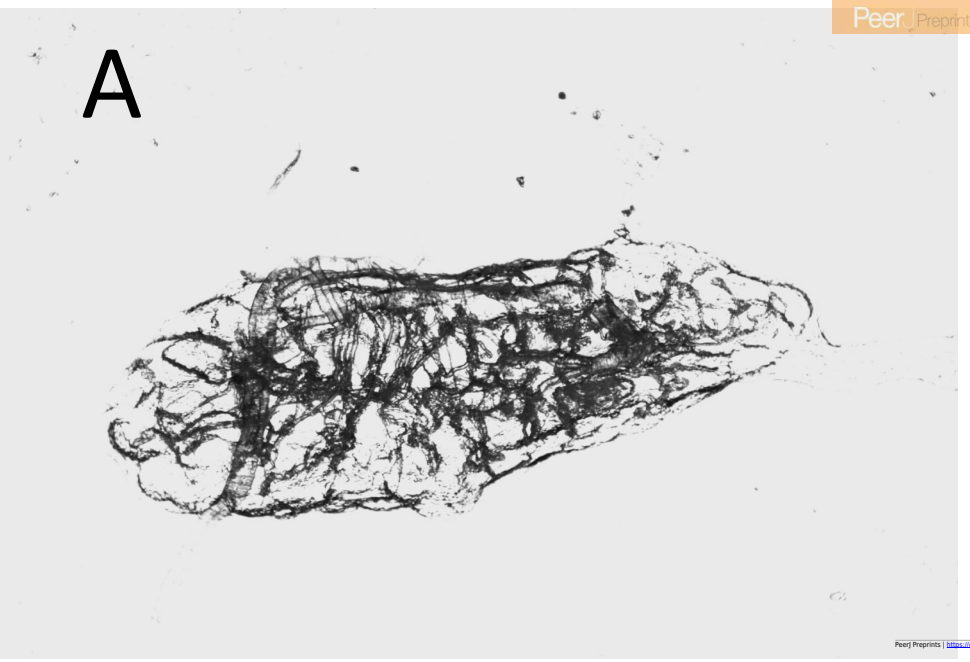


Figure 2(on next page)

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)



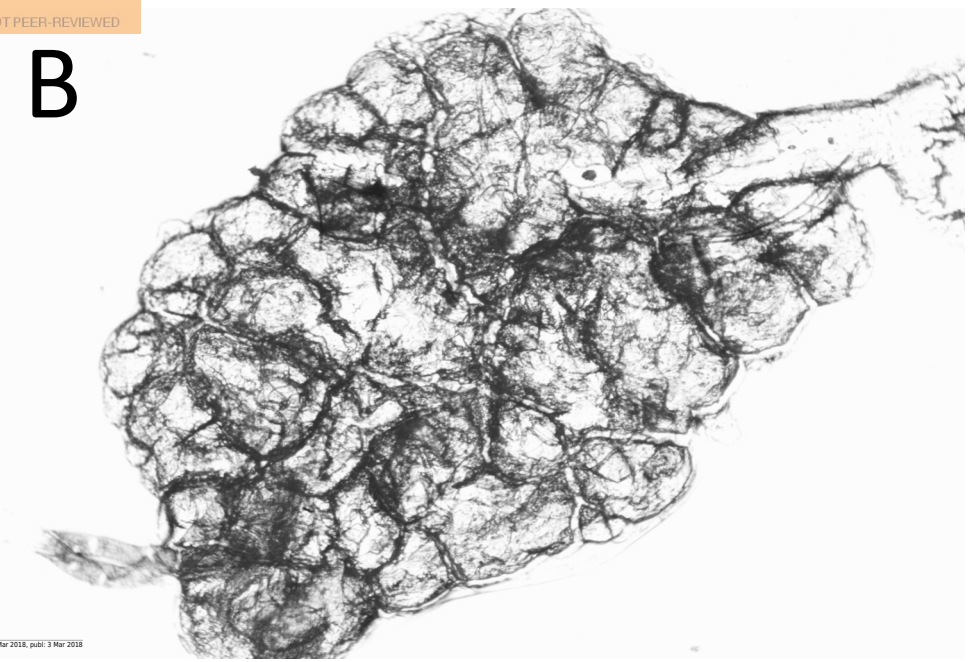


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Figure 3(on next page)

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

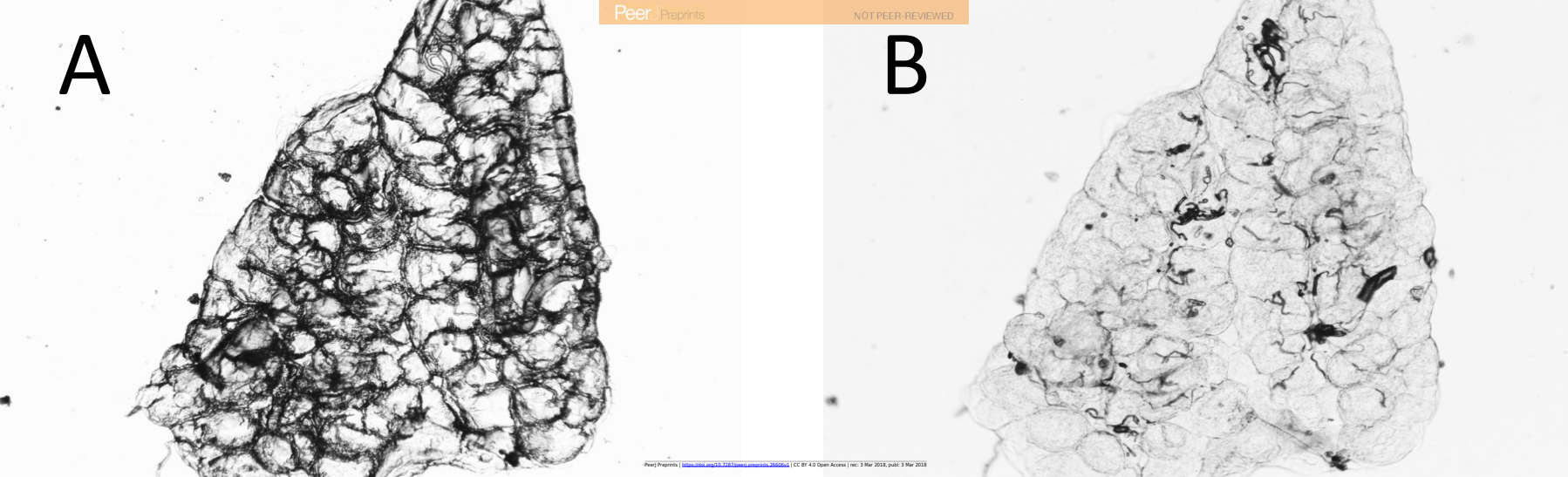

Figure 4(on next page)

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.

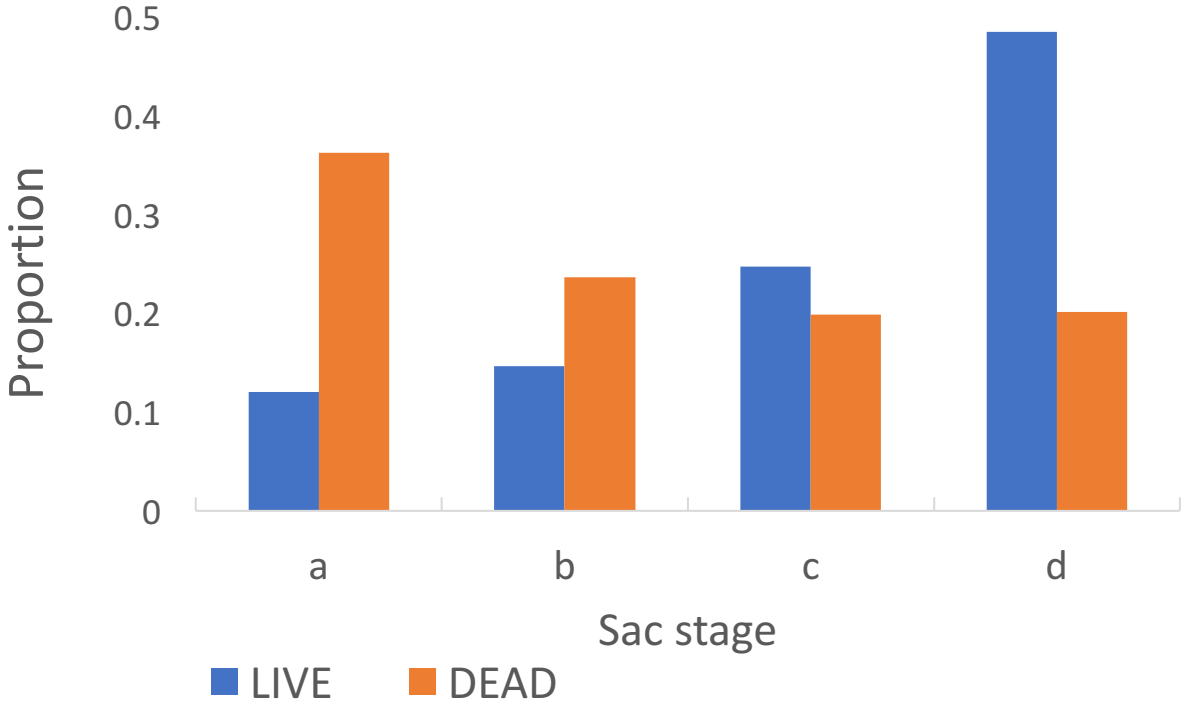

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Table 1(on next page)

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

	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)

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.

		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 (0.39-049)

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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