

Fluorescent markers rhodamine B and uranine for *Anopheles gambiae* adults and matings

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Research

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Abstract

Background:

Marking mosquitoes is vital for mark-release-recapture and many laboratory studies, but their small size precludes the use of methods that are available for larger animals such as unique identifier tags and radio devices. Fluorescent dust is the most commonly used method to distinguish released individuals from the wild population. Numerous colors and combinations can be used, however, dust sometimes affects longevity and behavior so alternatives that do not have these effects would contribute substantially. Rhodamine B has previously been demonstrated to be useful for marking adult *Aedes aegypti* males when added to the sugar meal. Unlike dust, this also marked the seminal fluid making it possible to detect matings by marked males in the spermatheca of females. Here we evaluate marking in *Anopheles gambiae s.s.* with rhodamine B and uranine to estimate their potential contribution.

Methods:

Two fluorescent markers, rhodamine B and uranine, were dissolved in sugar water and fed to adult *Anopheles gambiae*. Concentrations that are useful for marking individuals and seminal fluid were determined. The effects on adult longevity, the durability of the marking and detection of the marker in mated females was determined. We also evaluated effects on mating competitiveness.

Results:

Rhodamine B marking in adults is detectable for at least three weeks, however uranine marking declines with time and at low doses can be confused with auto-fluorescence. Both can be used for marking seminal fluid which can be detected in females mated by marked males, but, again, at low concentrations uranine-marking is more easily confused with the natural fluorescence of seminal fluid. Neither dye affected mating competitiveness.

Conclusions:

Both markers tested could be useful for field and laboratory studies. Their use has substantial potential to contribute to a greater understanding of the bio-ecology of this important malaria vector. Rhodamine B has the advantage that it appears to be permanent and is less easily confused with auto-fluorescence. The primary limitation of both methods is that sugar feeding is necessary for marking and adults must be held for at least 2 nights to ensure all individuals are marked whereas dusts provide immediate and thorough marking.

Background

Determining the characteristics of wild populations of mosquitoes such as population size, survival, dispersal and mating is challenging due to their small size, nocturnal habits and short life-spans. Some life-history traits can be determined in the laboratory, but the similarity of the parameter values to those

observed in the field is often doubtful because the artificial environments do not reflect the stresses of existence in natural environments.

One technique to determine some of these characteristics, mark-release-recapture (MRR), consists of releasing marked mosquitoes into wild populations and observing them in subsequent collections [1], however the number of marking methods is limited. Dyes were one of the earliest methods, in one case sprayed as an aqueous mist on caged adults [2], which the author claimed was easily detected. Radioisotope methods that were once used for marking adults [3] and matings [4] have fallen out of use, presumably for safety reasons. The rare earth mineral rubidium has been used to mark individuals and eggs [5] but its use is limited by the detection methods which include mass spectroscopy and X-ray fluorescence [6]. Stable isotopes have also been used to detect matings by marked males but similarly [7], detection requires mass spectroscopy. Paint marking patterns [1] have been applied in unique patterns and colors for individuals in at least one case [8] and can reportedly be performed quickly by skilled staff but do not allow detection of matings.

By far, after Day-Glo™ pigment powders became widely available, the most commonly used method employs various types of this easily detected fluorescent dust [9]. Dusts can be applied quickly to adults and all individuals in a single cage can be marked rapidly and detected directly either using white light or, more sensitively, far blue or near-ultraviolet (UV) light. Several contemporary alternative indirect methods for studying life history characteristics described in the context of mark-release-recapture (MRR) have been expanded upon [10], and most of the methods have been described in detail [11].

Building upon the work of Welch et al. [12], the usefulness of rhodamine B for marking *Aedes aegypti* has been demonstrated [13]. Of particular note is that this red dye could be detected in seminal fluid that was transferred during mating. Given the apparent low toxicity and absence of an effect on mating competitiveness in *Ae. aegypti*, we considered whether it might be similarly useful in *Anopheles gambiae*. The current interest in field studies of *An. gambiae* is largely related to genetic control [14, 15]. This is an application for which a new marker that could easily detect matings would be extremely useful, but we anticipated that the more delicate *An. gambiae* might respond differently to marking than *Ae. aegypti* and that the methods might need to be modified.

In order to develop another marker with a different color, we also studied the usefulness of a related dye, uranine, which has yellow-green fluorescence. The results demonstrate that the different characteristics of the dyes expand the options for *An. gambiae* marking. While we believe that under certain circumstances releases of females can safely be performed [10], our primary focus in these experiments was on marking males.

Materials And Methods

All mosquitoes used were *An. gambiae* 'G3' strain (MR4 number MRA-112, BEI Resources, Manassas, VA). Mosquitoes were cultured using the Damiens larval diet [16] fed according to a standard operating procedure [17]. The light cycle was 12:12 light:dark with 30 m sunrise and sunset during the 12 h light

period. The observed air temperature and percent relative humidity (%RH) during the period of the observations were 27.4 °C and 80.4%RH (stdev 0.78 and 7.0 respectively). Adults were placed in aluminum cages [18] covered with tubular gauze (TG12 Stockinette, Bandages Plus, Doral FL) and allowed to feed on sugar water containing marker for 3 nights except during preliminary observations as noted below.

Two candidate fluorescent markers were tested, rhodamine B (Product no. R6626-25G, Sigma-Aldrich, St. Louis MO, hereafter called simply 'rhodamine') and uranine (Product no. 108462, Millipore-Sigma, Burlington MA, USA). These were dissolved in 10% w/v food-grade sucrose containing 0.1% w/v methylparaben (ME163, Spectrum, New Brunswick NJ, USA [19]) which will be described simply as 'sugar water'. Dyes were prepared at various concentrations (all % w/v) and supplied to mosquitoes on cotton balls soaked with 20 ml of each solution in 59 ml polypropylene cups (DuraHome Product no. X001QYETQV, Amazon, Seattle WA USA). Adults were immobilized by chilling on ice for examination and sorting. Adult marking was detected with an SZX-ZB7 microscope (Olympus Waltham, MA) using an XCite 120Q illuminator (Model no. C182979, Excelitas, Waltham, MA) using filter cubes for detection of DsRed, GFP or both simultaneously (Nos. 49004, 49020 and 59004 respectively, Chroma, Marlborough, MA USA). Adults were observed at magnifications from 8-56X.

Statistical analyses were performed using R 3.5.1 "Feather Spray" [20]. Where the data were a single value, or a proportion derived from numbers in each case (dead / alive, mated / not mated) per replicate, generalized linear models (GLM) were used and the contribution of fixed effects was assessed by deletion tests using F or χ^2 as appropriate to the dispersion pattern seen in the data.

Mixed effects models, which account for the pseudoreplication inherent with repeat measures from within experimental units / replicates, were used to analyze the effects of dye concentration on longevity. These used the 'nlme' package [21]. Further details are provided below.

Survival of males in the absence of sugar water

Because vitality and marking via sugar water requires males to consume dyed sugar water, we determined how long males could live without sugar water. This would indicate the period of time that might be necessary for feeding dye that would ensure all living males had consumed the dye. Male pupae (n = 48) were placed in a cage that contained no sugar water. Using the light schedule of this insectary, adults generally emerge shortly after the light period on the evening from pupae that form before approximately 10:00 a.m., therefore, males on the following day were considered to be 1 d old. The pupa cup was removed the following day (day 1) to ensure the males could not drink water in the pupa cup.

Acute toxicity

To determine the range of dye concentrations at which marking was detectable but which caused no acute mortality, tests were performed with concentrations we expected to be on the high and low ends of a useful range. The previous report [13]. described marking *Ae. aegypti* adults using rhodamine at

concentrations ranging from 0.1–0.8% w/v. Based on those values, we performed preliminary observations on rhodamine acute toxicity and marking at 0.1, 0.2, and 0.8% (w/v). Three cages of 20 males each were set up at a concentration of 0.8% and two at 0.1 and 0.2%, mosquitoes were allowed to feed for 3 nights and mortality recorded on the fourth day.

For uranine, three cages of 25 males were set up at each of 0.1, 0.2, 0.4 or 0.8% concentration in sugar water. As with rhodamine, mortality was recorded on the fourth day. A binomial generalised linear model (GLM) was used to estimate any pattern in variation of mortality between concentrations.

Detection by various methods

Starting with a 0.1% solution of both rhodamine and uranine, 5-fold dilutions were performed. Five μ l of each dilution was pipetted onto filter paper, dried and the spots were observed using various methods: a UV lamp (MRL-58, Cambridge, UK), LED flashlight (UV01, TATTU), the Olympus stereomicroscope with the broad emission filter, and the naked eye. All observations were made by three people and the lowest concentration of detectable dot was recorded in a dark location except when observing with the naked eye. This was an observation and minimal observable levels are illustrated, but no statistical analysis was required.

Detecting marker when fed at different concentrations

After observing very distinct marking at doses

0.2% rhodamine, three lower concentrations were added, 0.1%, 0.05% and 0.025%.

Durability of marking

To determine how long the markers were detectable in adults, mosquitoes that had been marked for 3 nights (50 females and 50 males) with 0.1 and 0.2% rhodamine or with 0.1, 0.2, 0.4 or 0.8% uranine were held in separate cages along with three unmarked control cages for each dose. Samples were removed at weekly intervals for 3 weeks and the number in which the marker was detectable was determined. Ten of each sex were examined at the end of weeks 1 and 2. In some cases there were not 10 of each sex remaining alive by week 3.

For each sample, the numbers marked and unmarked were bound together in a weighted binomial response variable. A quasibinomial GLM, with the cage identity fit as a factor, was used to explore any pattern in variation of the proportion of mosquitoes detectable as marked as a function of the sex of the mosquitoes, the dye concentrations, time since marking (in weeks – fit as a factor rather than a linear term) and their two-way interaction terms on the proportion identified as marked was estimated. The cage identity was also fit to the model to account for any cage effects. Effects were assessed by deletion testing of factors, interactions and factor-level simplifications.

Detection of mating by marked males

Cups of approximately 100 male pupae were placed in cages and the resulting adults were exposed to 0.05, 0.1, or 0.2% rhodamine or 0.1, 0.2 or 0.4% uranine in sugar water for 3 nights. On the following day, the adults were chilled and examined to ensure the marker was visible in all individuals. Thirty virgin females were added to each cage. Adults were provided an opportunity to mate for 4–7 nights. Matings were observed by examining spermathecae in Fluoroshield (F6057-20 ml, Sigma) and were observed sequentially for sperm and fluorescence on an Olympus BX60 at 40-200x magnification and then on an Evos Fl (ThermoFisher, Grand Island, NY) at 10-20x using the RFP filter cube for rhodamine (AMEP 4625, ThermoFisher) or the GFP filters for uranine (AMEP 4651, ThermoFisher).

Spermatheca marking durability

To determine whether spermatheca marking persisted, spermathecae were observed at intervals after females were given the opportunity to mate and males removed to determine whether fluorescence remained visible. Males that were confirmed as marked after having been fed either 0.8% uranine or 0.2% rhodamine were mated separately to pools of approximately 100 G3 females for 4 (rhodamine) or 3 (uranine) days. Males were then removed and spermathecae dissected at intervals.

Competitive matings

Competitive matings were performed by combining 20 confirmed rhodamine-marked and 20 unmarked males with 40 virgin G3 females. Males had been maintained on either sugar water or 0.1 or 0.2% rhodamine sugar water for 2 nights or 0.1, 0.2 or 0.4% uranine. Spermathecae were observed as described above, usually within 2 days of the end of the mating period.

The data consisted of the numbers mated and unmated, and, of those mated, by which male type (marked or not marked) in each replicate. These were bound together as binomial response variables and a quasibinomial GLM, was used to assess effects of marker and dose on these measures (weighted proportion of females mating or mating with fluorescent males). This method allowed for the overdispersion that was observed in the data (residual deviance was observed to be > residual df). Because the design was not fully orthogonal (rhodamine had fewer dose levels than uranine), dose was treated as a factor with three levels rather than as a linear measure.

Longevity

For longevity studies, only adult males and females in which the marker had been observed were used for tests. Adults were allowed to feed on 0.05 or 0.1% rhodamine in sugar water for 3 nights, anesthetized on ice and examined as described above. Three cages of 30 confirmed marked individuals at each concentration or of unmarked males and females were set up and adults were provided sugar water. Mortality was observed daily except some Saturdays until all adults were dead.

The data arising from this experiment are a daily-interval time series for each cage. Because different doses were considered appropriate based on the acute lethality for rhodamine and uranine, their data were considered separately. To allow for the temporal pseudoreplication arising from repeated measurement of sequentially-linked cohorts (cage replicates), mixed effects models were used to identify

whether there was a significant effect on the proportion surviving over time as a function of mosquito sex, the dose of marker received and whether the other sex they were caged with was itself dosed with marker. The interactions of sex and dose were also estimated. Random effects were used represent the pseudoreplication of within-cage trajectories. The survival was compared over the period of the experiment (42 days maximum) and assessment of the main effects and their interactions was by model simplification using L-Ratio tests at $p < 0.01$ to avoid over-interpretation, with Akaike Information Comparisons (AIC) to evaluate model fit.

Results

Survival in the absence of sugar water

All adults successfully eclosed and were alive in the cage on day 1. On day 2, 12 males were dead and on day 3 the remaining 36 males had died. We concluded that a minimum of 2 nights of feeding is necessary to ensure all males have imbibed the dyed sugar water or that they would die under the experimental environmental conditions. As a precaution, we routinely marked males for 3 nights.

Acute dye toxicity

Rhodamine acute toxicity

Of the three cages of males exposed to 0.8% rhodamine, after 3 nights of exposure, all males in two of the cages had died and this dose was not pursued further. In observations of the three cages of 20 males treated at 0.2%, 11, 4, and 5 males average mortality = 33%) were dead after 2 nights of exposure and we continued observations at this and lower doses.

Uranine Acute Toxicity

After 4 days, little mortality was observed at any concentration ($n = 0, 3, 3, 2 / 50$ at 0.1, 0.2, 0.4 or 0.8% uranine, respectively) and there was no indication that mortality varied with concentration ($F=1.65$, $d.f.=8,11$, $p=0.18$).

Detecting marker when fed at different concentrations

Marking of males after 3 nights of feeding on 0.025% rhodamine was difficult to detect with certainty and no further observations were made at that concentration. Marking was easily detectable at 0.05 and 0.1%.

Uranine was tested for marking at concentrations of 0.025, 0.05, 0.1, 0.2, 0.4 and 0.8% for 2 nights. No marking was detectable at the two lowest concentrations, 0.05 or 0.025%, and those were discontinued.

Detection by various methods

Both markers were more sensitively detected using the fluorescence microscope and, consistent with the other observations made elsewhere, rhodamine could generally be detected at lower concentrations regardless of the method (Fig. 1).

Durability of marking

When adults were marked with 0.1 and 0.2% rhodamine, marker was detected every week for 3 weeks after marking in all individuals that were alive (0.1%, 93 females, males 79; 0.2%, 89 females, 76 males). In the 0.1% treatment cages, 33 females and 19 males remained alive at week 3. By the final week of the 0.2% cages, 29 females and 16 males were alive. No marker was identified among the living rhodamine controls (69 females, 53 males). Because all living treated adults remained marked, no statistical analysis was warranted.

Uranine produces a yellow-green color that is similar to natural autofluorescence and, at low treatment doses, these can be difficult or impossible to distinguish (Fig. 2). The proportion identified as uranine-marked in control cages did not vary among the different trials and all control cages were considered as one level ($F=0.03$, d.f.=55,61, $p=0.99$). The level of false positives in control treatments led us to ask at what dose would treatment effects be clearly distinguishable from these. In both sexes, the proportion identified as marked did not differ from controls in either dose levels 0.1 or 0.2% ($F=0.88$, d.f.=61,63, $p=0.42$ and $F=2.28$, d.f.=67,68, $p=0.14$ respectively).

A higher proportion of male mosquitoes were identified as 'marked' ($F=14.45$, d.f. 66,67, $p=0.0003$), but male and female mosquitoes responded similarly to the dose received and through time (sex:week $F=2.72$, d.f.=65,66, $p=0.10$; sex:dose $F=1.22$, d.f.= 66,68, $p=0.30$). There was, however, an interaction between dose and week ($F=4.48$, d.f.= 68,70, $p=0.015$); the proportion identifiable as marked declines differently through time as a function of dose.

At no dose was the marking visible in all individuals for the full 3 weeks in either sex though all mosquitoes marked at 0.8% were detectable after 1 and 2 weeks. All males marked at 0.4% were detectable for 2 weeks.

Detection of mating by marked males

Results of the detection of mating are shown in Table 1. Only one test at the dose rate of 0.05% rhodamine was conducted after it was determined that the marker could not be reliably detected in males

when males were fed at this rate. In spite of this, the marker could be detected in all matings from males marked with 0.05% rhodamine; though the number was small, as a mating marker it appears useful at concentrations that are not high enough for detecting the marker in the male body. At all doses of rhodamine, matings with the marker could be easily detected in almost all females.

At the lowest dose of uranine (0.2%), the marker could be detected in the spermathecae although the marker could not be reliably detected in the adult males themselves.

Spermatheca marking durability

Spermathecae that were positive for sperm were all positive for rhodamine at 3, 11, 17 and 24 days after mating (positive/negative, 8/14, 6/4, 8/2, 1/0). In the other experiment using uranine marking, spermathecae that were positive for sperm were all positive for uranine when dissected and observed at 4, 11, 18 and 25 days (positive/negative, 7/15, 9/1, 9/1, 8/2 respectively).

Competitive matings

A slightly greater proportion of females were mated by marked males than with unmarked males (mean=0.57, 95%CI=0.51-0.63, $\chi^2=23.93$, d.f.=14, $p=0.047$, Fig. 3).

The treatments, however, did not affect this proportion. The effect of dosage did not depend on the marker used ($F=2.79$, d.f.=10,11, $p=0.13$). The dose received itself did not affect choice of male type ($F=1.97$, d.f.=11,13, $p=0.19$) nor did the marker used ($F=1.18$, d.f.=13,14, $p=0.30$).

Longevity

In neither marker trial, did the marker status of the accompanying mosquitoes in the cage affect survival patterns (uranine: L.Ratio=0.48, d.f.=9,10, $p=0.49$; rhodamine: L.Ratio=0.10, d.f.=9,10, $p=0.76$).

With uranine, the two mosquito sexes responded differently to dosage; males generally had shorter lives than females, but were not as sensitive to increasing dye concentrations as were females (Table 2, L.Ratio=58.38, d.f.=8,9, $p<0.001$).

With rhodamine, longevity in control males was also shorter than for control females and, although the range of doses was low, longevity also declined less precipitously with increasing dye concentration than seen in females (Table 2, L.Ratio=14.37, d.f.=8,9, $p<0.001$).

Discussion

Detecting marker when fed at different concentrations

Recommended concentrations for *An. gambiae* of rhodamine are 0.1 and 0.2% and for uranine 0.2, 0.4 and 0.8%. At these concentrations, mortality is not substantial and rhodamine can be detected permanently in adults and easily in the spermathecae of females mated to marked males. These results do not represent experience with all strains so researchers should consider testing the markers on the specific strains that they plan to use. We have received one report (L Facchinelli, pers. comm.) that the “Banfora” (*Anopheles coluzzii*) strain suffers significant mortality after 72 h of 0.2% rhodamine exposure in their hands whereas the “Kisumu” (*An. gambiae*) strain did not. Therefore, those who are considering the use of either rhodamine or uranine are advised to test for possible toxicity effects before applications are developed.

With uranine, the slight difference between detection using the LED flashlight and the fluorescence microscope indicates that field use of the simple flashlight is almost as useful as microscopic examination. Admittedly, detection in mosquitoes is different from detection on filter paper, but both methods are useful in evaluating the ability to identify a mark. Overall, rhodamine can be more sensitively detected and is less easily confused with auto-fluorescence in mosquitoes.

In our hands, rhodamine adult marking at 0.1 and 0.2% is permanent for at least 3 weeks. In contrast, uranine gradually becomes undetectable and cannot be distinguished reliably from autofluorescence at the lowest doses and thus must be used at concentrations of at least 0.4% for male marking up to 2 weeks and at 0.8% for females. While this might be considered a disadvantage for most uses, in some cases, the persistence of previously marked adults interferes with subsequent releases using the same marker in which case this decline in detectability could be advantageous.

Both uranine and rhodamine are useful mating markers. Rhodamine is more specific and sensitive than uranine as the male accessory gland fluid has weak yellow-green fluorescence that is easily confused with low-dose uranine marking. Therefore, at low feeding concentrations, it requires practice to distinguish normal fluorescence from uranine. In many cases, we examined the spermatheca and did not detect sperm upon the initial examination but after observing rhodamine fluorescence, re-examined the spermatheca under white light and located them. Therefore, rhodamine is possibly a more sensitive marker for mating than observing sperm directly, but we do not know whether seminal fluid is sometimes transferred with no, or few, sperm.

No effect of either marker on male mating competition was detected. This is similar to the observations of Johnson[13] and indicates real promise for this marking purpose and these dyes in *An. gambiae* s.l. When uranine is used at low concentrations however, interpreting outcomes may be difficult. While no difference was identified, it is possible that some non-marked uranine male matings were mistaken for uranine marked male matings, particularly at the lowest dose. Moreover, multiple matings, which are known to occur, by both a marked and unmarked male would likely be classified as only being due to a marked male.

Conclusions

Both rhodamine and uranine fed at appropriate concentrations for each dye are extremely promising and useful for durable marking of *An. gambiae* adults and for detecting matings. Their use in both laboratory and field studies of these mosquitoes has substantial potential to contribute to a greater understanding of the bio-ecology of this important malaria vector.

Researchers who are considering either of these markers should confirm that the chosen marker does not affect the life-history traits in their study strains or local wildtype mosquitoes that are most important to the outcomes of their experiments.

Declarations

Ethics approval and consent to participate

The activities described here did not include human subjects. Bloodfeeding for stock-keeping used an anesthetized rabbit under CDC protocol 2863DOTRABC.

Consent for publication

All authors have approved the content of this manuscript.

Availability of data and materials

All data are available upon request from the corresponding author.

Competing interests

The authors declare they have no competing interests in the work described here nor in its interpretation.

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Authors' contributions

RDR and EIA performed all technical work. CMC performed statistical analysis, prepared most figures and guided interpretation and drafting of the results. MQB drafted the manuscript and oversaw the editing and revisions. EBD assisted drafting and reviewing the manuscript. All authors reviewed and approved of the final manuscript.

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Abbreviations

GLM
general linear model
GFP
Green fluorescent protein
RH
Relative Humidity
Stdev
Standard Deviation
UV
Ultraviolet

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Tables

Table 1. Sperm in spermathecae (n) with (Fluor.) and without (Neg.) fluorescent spermathecae

Marker dose %	Exps. (n)	With Sperm		No sperm	
		Fluor.	Neg.	Fluor.	Neg.
rhodamine					
0.05	1	7	0	0	10
0.1	3	55	0	0	20
0.2	3	35	0	0	47
Total	7	97	0	0	77
uranine					
0.1	2	6	0	0	12
0.2	1	7	3	0	5
0.4	3	60	0	0	20
0.8	3	59	0	0	27
Total	6	73	3	0	37

Table 2. Adult longevity of dye-treated adults

Median Day of 50% Mortality		Concentration of fluorescent dye (%)				
		0.0	0.1	0.2	0.4	0.8
Uranine	Female	28	26	24	24	21
	Male	21	20	24	20	19
Rhodamine	Female	27	27	21	ND ¹	ND
	Male	19	9	8	ND	ND

¹ Not done

Figures

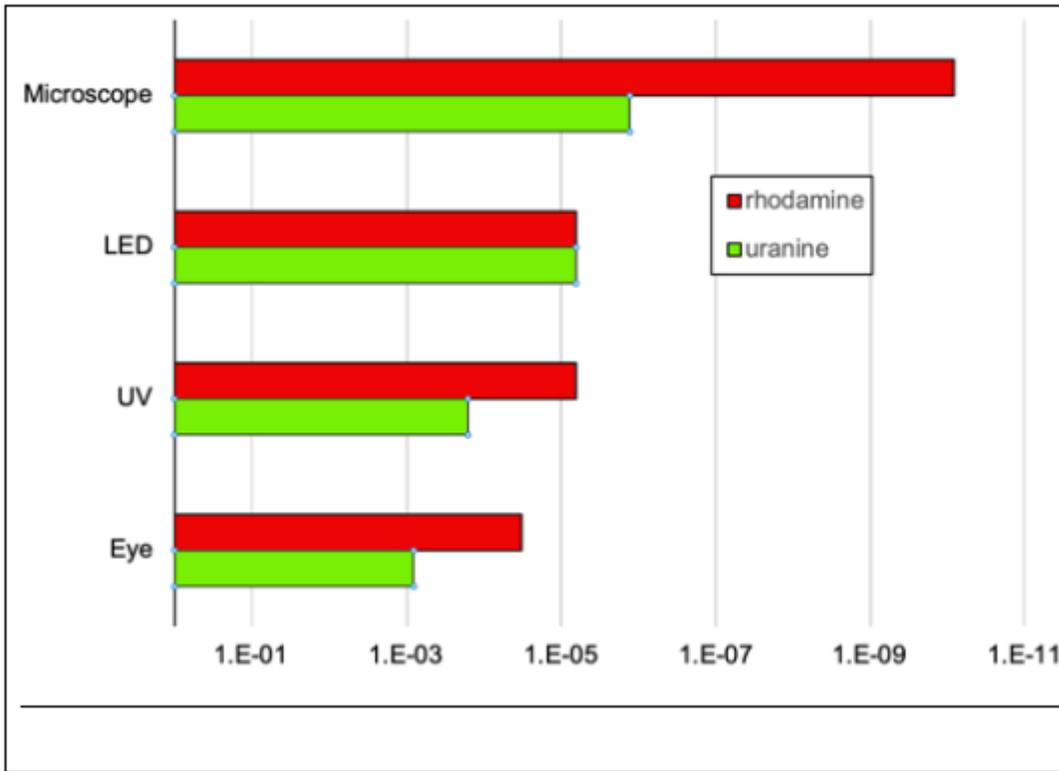


Figure 1

Sensitivity of detection by method. The minimum concentration (%) at which the two markers were detected on filter paper when using different methods after drying the spots.

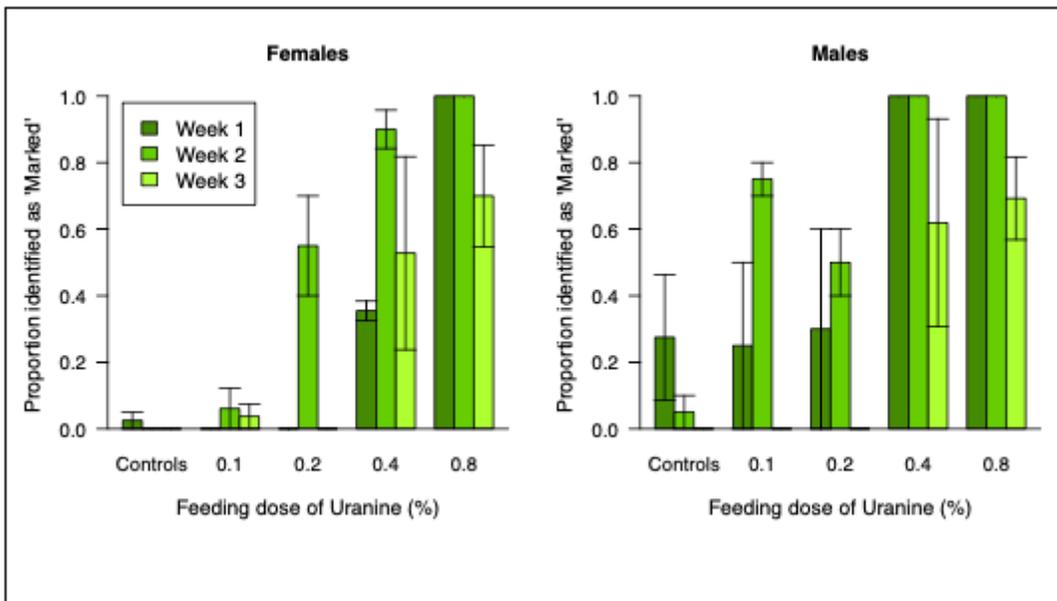


Figure 2

Durability of uranine marking. The proportion of female and male *An. gambiae* identified as 'marked' by feeding with increasing concentrations of uranine were evaluated for 3 weeks post-marking.

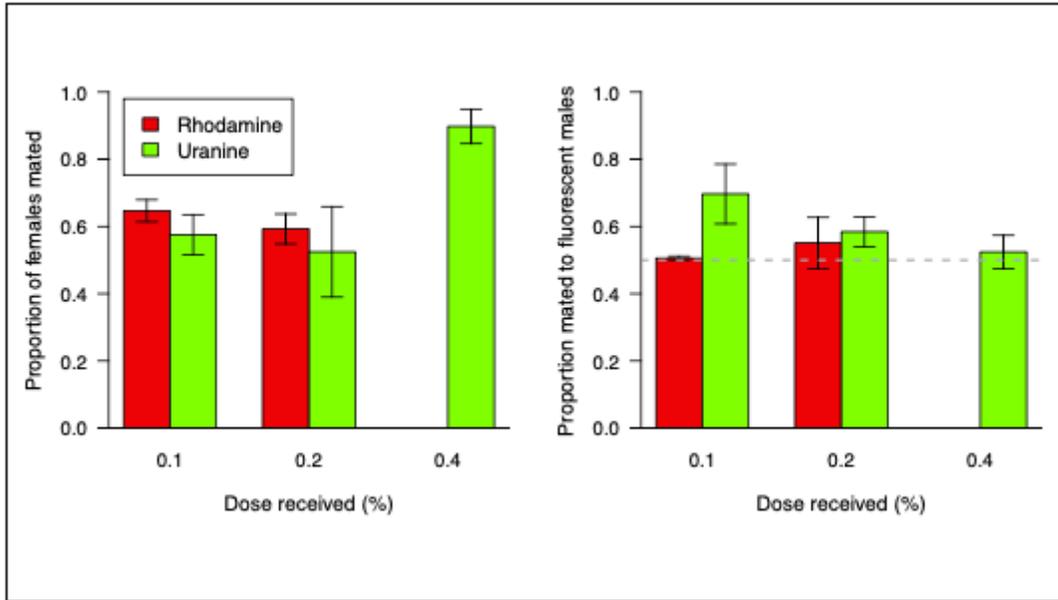


Figure 3

Competitive Mating. The mean proportion (+/-SEM) of female mosquitoes mated overall (left) and, (right) the proportion (+/-SEM) of those mating with fluorescent males as a function of the marker used and dose received. The dotted line represents the assumption of equal proportions of females mated by either type of male.