BIOLGICAL ASSAY METHODS FOR MOSQUITO REPELLENTS
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ABSTRACT. Three biological assay procedures for repellents are currently documented in the literature: 1) ASTM E951-94, Laboratory testing of non-commercial repellent formulations on the skin. 2) ASTM E939-94, Field testing topical applications of compounds as repellents for medically important and pest arthropods. 1. Mosquitoes. 3) WHO/CTD/WHOPE/IC96.1, Report of WHOPE informal consultation on the evaluation and testing of insecticides. One public draft set of repellent-testing guidelines is available on the internet: 4) USEPA OPPTS 810.3700, Product performance test guidelines. Insect repellents for human skin and outdoor premises. In practice, the outcome of a repellent bioassay using any of these procedures is affected by the absorption, penetration, and chemical modification of repellent on skin and by evaporation, abrasion, and perspiration. Other abiotic factors that influence mosquito responses to repellent stimuli are light, temperature, humidity, repellent dose, exposure time, and test-cage shape and size. Biotic variables in repellent bioassays are larval nutrition, carbohydrate availability for adult mosquitoes, age and parity of females, and differences in the innate attraction/repellency of test subjects. Geographic location and seasonal and diel activity cycles in mosquitoes determine when and where repellents can be tested in the field. Critical knowledge of these sources of variation can be converted to improved precision and accuracy in repellent bioassays and the resulting information used to efficiently select new repellent compounds for toxicological evaluation and field testing.

INTRODUCTION
Testing of insect repellents, whether in the laboratory or the field, is performed using a process called biological assay (bioassay for short) (Robertson and Preisler 1992). Bioassays can be used to answer 3 questions about repellents:

* Is the candidate material repellent?
* What quantity of material is required for repellency?
* How long does repellency last?

Biological assays are an experiment that uses a living organism. Repellent bioassays typically involve mosquitoes or some other type of bloodsucking arthropod. In a bioassay, a stimulus is applied, a response is observed, and the process is repeated until enough observations have been made for a population response to be estimated with the desired level of precision. In repellent bioassays, the stimulus is normally a dosage of repellent applied to human skin, to the skin of an animal subject, or to an inanimate object such as fabric, membrane, or filter paper. The treated object is exposed to hungry mosquitoes in a cage (in laboratory tests) or to wild (and often mixed-species) populations of mosquitoes in the field. The response to the stimulus is categorized according to the needs of the experiment but typically comprises the number of mosquitoes that approach; land; land and probe; or land, probe, and bite the repellent-treated object.

Conventional repellent bioassays can be grouped according to whether they use in vitro or in vivo methods. In vitro systems use cloth, filter paper, animal membrane, and olfactometry (Lal et al. 1963, Rutledge et al. 1976, Frances et al. 1993, Chou et al. 1997). In vivo methods use animal and human subjects (Hill et al. 1979, Rutledge et al. 1994, Barnard et al. 1998). In vitro systems are fast, safe, inexpensive, and can be used to test many repellents, regardless of toxicity, but have poor comparability among methods and are of questionable relevance to repellent usage by humans. In vivo methods are slow, expensive, allow testing of only one repellent at a time, and preclude, or greatly complicate, the evaluation of toxic chemicals. They also require human and/or animal subject usage review board approvals. The comparability of test results between human and animal subjects is problematic, although it can be argued that results using the former are most accurate because they incorporate the repellent end user.

Basic requirements for a biological assay system include randomization of test subjects, randomization of treatments among test subjects, adequate replication, and the use of negative and/or positive controls. A negative control is the equivalent of an untreated control; for example, the untreated forearm of a human subject exposed to hungry mosquitoes in the field to determine biting rate during a repellent bioassay. Positive controls are used for comparison purposes. The forearm of a human subject treated with 25% ethanolic DEET and exposed to wild mosquitoes to determine biting rate on a standard repellent-treated arm is an example. The positive control is used to calculate the comparative effectiveness of a 2nd repellent (of unknown efficacy) that is tested at the same time on another subject.

There are 3 repellent bioassay procedures documented in the literature and 1 set of repellent testing guidelines available on the internet:

Testing of Non-Commercial Repellent Formulations on the Skin.


A 5th repellent bioassay system, the screened cage method, is frequently cited in the literature (Gouck and Smith 1962, Smith et al. 1963, Schreck 1977, Barnard et al. 1998) and a 6th system, modified from ASTM E951-94, has also been published (Klun and Debboun 2000).

LABORATORY REPELLENT BIOASSAY METHODS

ASTM E951-94

This method comprises the use of a rectangular (18 cm long, 5 cm wide, 4 cm high) clear plastic test cage with five 29-mm diameter openings in the bottom. A template is used to place 4 repellent dosages and a control on the skin of a human volunteer in a pattern that matches the openings on the test-cage bottom. The cage is strapped to the arm or leg of a volunteer, bottom-side to the skin, with 10–15 nulliparous, 5–15-day-old female mosquitoes placed into the cage through a 13-mm opening in one end. A test commences when the plastic slide (0.3 mm thick) that blocks the openings in the test cage bottom is withdrawn, allowing mosquitoes access to the repellent-treated skin. The number of mosquitoes that land on and probe the skin in 2.5 min is recorded. The dose–response data obtained with ASTM E951-94 have been used to calculate median and 95% effective doses (ED) (Buescher et al. 1983, Rutledge et al. 1983) and to describe functional responses, in time, of mosquitoes to topical repellents (Buescher et al. 1983).

K&D module

An extension of ASTM E951-94 is the K&D module (Klun and Debboun 2000). This apparatus purportedly minimizes the likelihood of treatment interactions, increases the number of possible treatments per replicate, and permits large numbers of replicated observations for each human test subject. The authors propose the module for testing the responses of more than 1 mosquito species at a time, to 1 dose of repellent, or for evaluating repellent responses in the same species using specimens from geographically distinct locations.

Skill is required in the design of experiments that use small-cage testing methods, as treatment effects can be confounded with edge effects (Southwood and Henderson 2000), the latter as a consequence of position (of a feeding port or module). In addition, multiple replicates of treatments on the same human subject do not provide a basis for comparison of treatments among different subjects (Mead 1988), the attractiveness or repellency of which, to mosquitoes, can be highly variable (Schreck et al. 1990).

WHO/CTD/WHOPES/IC/96.1

Laboratory repellent bioassays based on the WHO protocol require a mosquito-filled screened cage and use DEET as a positive control. Human test subjects are preferred over laboratory animals or artificial membranes. Aedes aegypti, the normal test species, is used in variable numbers, but other mosquito species can be substituted, depending on the needs of the experiment. An area of skin ranging from that on the entire forearm to as little as 25 cm$^2$ is treated with repellent and exposed to caged mosquitoes. Untreated skin is covered with a glove or other protective material. For compounds of unknown toxicology, the repellent can be applied to a cotton stockinette sleeve and the treated sleeve pulled over a 2nd untreated stockinette on the arm to prevent skin contact with the repellent.

At least 5 variations of the WHO method have been developed to meet the testing needs of different institutions (WHO 1996). These emphasize either the determination of protection time after treatment with a single repellent dose or percent protection in relation to repellent dose. The protocols are as follows:

- A 25-cm$^2$ area on a subject’s forearm is treated with an ethanolic solution of repellent (treatment) and the same-sized area on the adjacent forearm is treated with alcohol (negative control). Both arms are simultaneously introduced into 1 cage and the numbers of mosquitoes biting each arm in 5 min is recorded. Percentage protection is calculated by comparing biting rates on the treatment and control arms.
- A subject’s feet and legs are treated with repellent, exposed to 25 female mosquitoes in a mosquito-proof enclosure (1 m × 1 m × 3 m), and the number of bites in 10 min recorded.
- A subject’s forearm is treated with 1 ml of a 25% ethanolic repellent solution and introduced into a mosquito-filled cage for 3 min once every 30 min. Protection time is the time elapsed between repellent application and the first mosquito bite.
followed by a confirmatory bite in the same, or next, exposure period.

- One gram of repellent is dissolved in sufficient acetone to saturate 280 cm² of cotton stockinette. The stockinette is drawn over the arm of a subject and exposed to 1,500 caged female mosquitoes for 1 min, at daily or weekly intervals, until 5 bites are obtained.

- A subject's untreated arm is exposed to 50 caged female mosquitoes, followed by repeated exposures of the same arm with increasingly higher doses of repellent. In each exposure, the arm is withdrawn before the mosquitoes can imbibe blood. Probit analysis is used to calculate the ED₅₀. When the dose giving 100% repellency is identified, the arm is re-exposed at 60-min intervals until repellency declines to 50%.

**Screened cage test method**

The screened cage bioassay method employs a 40-cm³ aluminum-frame cage with a metal bottom, screened top and back, clear acrylic sides (for viewing), and a front stockinette sleeve for access. Two-hundred human host-seeking (Posey and Schreck 1981) nulliparous, 7–8-day-old female mosquitoes are placed in the cage 1 h before the test. Treatment comprises a 25% ethanolic solution of repellent applied to the forearm of a volunteer (between the wrist and elbow) at the rate of 1 ml per 650 cm² of skin surface area. The treated forearm is inserted into the cage (a glove is used to protect the hand from mosquito bites) and the number of mosquitoes that land and probe the skin in 3 min is observed and recorded. The observations are repeated every 30 or 60 min. Two bites in one 3-min test, or one bite in one 3-min test, followed by 1 or more bites in a 2nd test 30 min later ends the test for the repellent. A 2nd cage of mosquitoes is used as a positive or negative control. Depending on the requirements of the experiment, protection time is calculated as the time elapsed between repellent application and the first confirmed mosquito bite or the time between repellent application and the observation period immediately preceding the first confirmed bite. Data obtained using this bioassay method can be used to calculate complete protection time (CPT).

The determination of CPT using the screened cage method is based on the responses of mosquitoes in the upper extreme of the frequency distribution for repellent tolerance rather than on the mean response of the population. Consequently, the technique does not measure the ED₅₀ of the test repellent by the mosquito population or other percentiles of interest (Rutledge et al. 1978). Additionally, it confounds variation in repellent activity (per unit concentration applied) with the rate of repellent loss from the skin (Curtis et al. 1987), although the latter appears to be a linear function over time (Rutledge et al. 1985).

**FIELD REPELLENT BIOASSAY METHODS**

**ASTM E939-94**

Using this method, 1.5 ml of repellent solution is applied to the forearm (between the wrist and elbow) or lower leg (between the knee and ankle) and the treated limb exposed continuously to biting mosquitoes as the subject moves through a mosquito-infested habitat. Biting mosquitoes are collected from treated and untreated skin (usually an exposed forearm) at regular intervals to determine mosquito biting rates and for species identification. This procedure is used to determine CPT, but percent repellency can be determined if a negative control is used.

Rutledge (1988) noted 3 shortcomings of the statistical methods described in ASTM 939-94 for data obtained using the incomplete block design (IBD). The first concerned the design itself, correction of which involves analysis of data from (what Rutledge termed) a "resolvable balanced IBD," rather than the balanced IBD. The 2nd shortcoming concerned inefficient evaluation of interblock information and, the 3rd, improper use of adjusted means for estimating treatment means. Rutledge (1988) provides details for correcting each problem.

**WHO/CTD/WHOPES/IC/96.1**

When using the WHO field method, repellent tests are made in the vicinity of human domiciles. Mosquito biting rate and the assessment of repellency are based on the capture of mosquitoes attacking human volunteers, thus, tests are timed to exploit the biting cycle of the target mosquito species. Test subjects are spaced ±10 m apart and rotated in a randomized manner throughout the experiment to minimize positional errors. Appropriate criteria for repellency include ±80% reduction in biting rate for 6–8 h without adverse user side effects.

**USEPA TEST GUIDELINES**

The OPPTS guidelines have been developed for laboratory and field evaluation of pesticides and toxic substances and for acquiring test data submitted to the Agency for review under the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601) and the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U.S.C. 136 et seq.). The product-performance test guidelines contained in OPPTS 810.3700 describe specific methods for evaluating insect repellents and reflect the USEPA's minimum recommendations for developing reliable repellent-product performance data. The guidelines are available electronically in portable document format (PDF) at www.epa.gov/epahome/research.htm under the heading "Researchers and Scientists/
Test Methods and Guidelines/OPPTS Harmonized Test Guidelines.”

**SOURCES OF VARIATION IN REPELLENT BIOASSAYS**

**Abiotic factors**

Many factors influence the outcome and interpretation of repellent bioassays. Skin-mediated effects comprise absorption and penetration of repellent on skin, but evaporation, abrasion (contact with clothing), washing or rinsing of treated surfaces, and perspiration also result in repellent loss (Gabel et al. 1976, Rutledge et al. 1985, Gupta and Rutledge 1989, Rueda et al. 1998). These physical factors are difficult to control in a bioassay, but their contribution to experimental error can be minimized by random selection of test subjects, the use of appropriate sample sizes in bioassays, and by recognizing and avoiding pseudoreplication. Loss of repellent by abrasion or by washing or rinsing from treated skin can be minimized by rigorous oversight of the test proceedings and by diligence on the part of the test subject.

Light, temperature, humidity, and air quality at the testing venue are important environmental influences in repellent bioassays (Frances et al. 1996, Gupta and Rutledge 1989, Reinfenrath and Spencer 1989). These factors can be manipulated to desired levels in the laboratory, but, in nature, their variation profoundly affects mosquito responses to repellent stimuli. Thus, field bioassays should be standardized with respect to season, geographic location, and the time of the diel period in which observations are made. When this is not possible, tests should be designed so that estimates of important physical and climatic parameters are included as treatment variables in the statistical analysis.

Additional environmental sources of variation in bioassays are repellent dose and exposure time (Rutledge et al. 1985), and test cage configuration (Lomax and Granett 1971, Schreck 1977, Barnard et al. 1998). In the latter case, research suggests relationships between protection time, mosquito test population size, and the mosquito biting rate. However, investigations using different test-cage configurations and mosquito population sizes (Bar-Zeev and Ben-Tamar 1971, Lomax and Granett 1971, Khan et al. 1975, Schreck 1977, Frances et al. 1993) have not led to a consensus regarding the optimal mosquito biting rate and density for repellency tests. One reason is that test-cage shape and size and mosquito density effects vary depending on the mosquito species. For *Ae. aegypti*, for example, repellent protection time is inversely related to cage size but is not affected by mosquito density, whereas, for *Anopheles quadrimaculatus*, protection time is short in large (125-liter) cages with high mosquito densities (640 cm³ per mosquito) and long in medium (65-liter) cages with low mosquito densities (640 cm³ per mosquito) (Barnard et al. 1998).

**Biotic factors**

Biological factors in repellent bioassays comprise larval nutrition, carbohydrate availability to adult mosquitoes, age and parity in female mosquitoes, partial blood engorgement, and innate differences among repellent-treated test subjects (Wood 1968, Khan et al. 1975, Barnard 1998, Xue and Barnard 1999). An important behavioral factor that affects bioassay results is the timing and intensity of mosquito biting activity (Gouck and Smith 1962, Xue and Barnard 1996). Ignorance of temporal feeding patterns can compromise estimates of protection time for repellents that have extended activity, as can poor knowledge of biting rate. In screened cage tests, biting patterns can vary with the size of the cage, and this factor can affect the determination of repellency (Barnard et al. 1998).

A comprehensive understanding of the parameters that affect repellent bioassays can minimize false-positive responses in the early stages of repellent screening. Rigorous bioassay standards in the later stages of testing facilitate identification of the most promising new repellents and provide a sound basis for selecting new repellents for toxicology testing and evaluation in field tests.

The selection of a repellent bioassay procedure should always be based on the biological relevance of the method and its capacity to yield precise experimental data. When these 2 outcomes are achieved, one can correlate the results from different bioassay techniques to obtain an accurate estimate of the repellency of any compound.

**REFERENCES CITED**


