Free-flight room studies

Factors which may affect validity of **free-flight room studies**:

- study not conducted in compliance with Good Laboratory Practice (GLP)
- negative control mortality exceeds 10% after 24 hours holding
- failure to conduct power calculations to determine the required number of replicates and the number of mosquitoes released per replicate
- inadequate sample sizes
- selected species/strains not suitable for testing with the proposed spatial emanator product
- environmental or holding conditions outside of target range
- tests not conducted in alignment with the test system's circadian rhythm
- mosquito phenotypic resistance not characterized prior to the commencement of the study *(1)*.

1. Purpose of the study

For the purposes of the prequalification assessment, free-flight room studies are conducted to investigate the characteristics of the proposed spatial emanator product by means of:

- investigation of the biological activity of a spatial emanator product under controlled conditions by observing relevant effects on free-flying mosquitoes representative of a simulated interaction with the host, and
- chemical analysis to determine the retention/release of active ingredient(s) (AIs) during and after artificial and/or operational ageing.

2. Requirement for submission of free-flight room studies

A minimum of one free-flight room study is required for Module 5 submissions.

Free-flight room studies must be GLP-compliant.

3. Considerations for use of the method

3.1 Classification as a bioassay

The free-flight room test is best considered as a standardized bioassay which is useful in characterizing the entomological mode of action (MoA) of the AI(s) in a spatial emanator product and the intended effect of the product. The study:

- can be conducted in a consistent manner across testing facilities;
- allows for variations in test **sample preparation** in order to investigate how the efficacy of spatial emanator products may change throughout the intended useful life;
- allows for the investigation of multiple observable endpoints in the free-flying mosquito, and
- allows for the observation of additional endpoints in caged mosquitoes located in the study area, for example, mortality.

Free-flight studies can be used to characterize the intended entomological effect of proposed spatial emanator products with AI(s) that are intended to dispel, disorientate and/or kill mosquitoes. The use of a human volunteer in the study allows for the characterization of the proposed product using free-flying mosquitoes to mimic conditions found when mosquitoes are host-seeking in the wild.

The consistency of the method supports the analysis across samples, geographic locations and vector resistance profiles, thereby providing useful information about the changes in the biological performance of a spatial emanator product throughout the intended useful life.

The free-flight room study characterises the intended effect of the proposed spatial emanator product on free-flying mosquitoes under controlled conditions. The free-flight room study is designed to evaluate spatial emanator products using the breadth of the spectrum of vectors against which the product may be efficacious. Careful experimental design is required to ensure adequate and consistent measurement of outcomes in the mosquito.

4. Considerations for contract research organization (CRO) selection

Manufacturers should consider the availability of relevant mosquito species/strains, and the characteristics of these species/strains, in the selection of CRO(s) to conduct the free-flight room study(ies). The available species/strains at the selected site(s) should exhibit traits in alignment with the defined primary target(s) based on the MoA of the AI(s) and intended effects of the product. To assist with CRO selection, characterization data for the vector population's target traits, for example, WHO susceptibility tests, insecticide resistance intensity assays, genomic screening, etc., generated by the relevant CRO should be considered.

Additionally, manufacturers should consider potential requirements from other departments within WHO, and National Regulatory Authority requirements for product registration in order to prioritize generation of characterization data which can be used to support registration and/or selection decisions across multiple countries/organizations.

5. Considerations for chemistry method selection

Chemical analysis is conducted to quantify the AI content of spatial emanator products before and after use in free-flight room studies. The chemical analysis of samples at intermediate time points is useful in characterizing the rate of AI loss under the controlled conditions of the testing facility.

In supplementary chemical analysis, the total AI of the product should be measured using the available/validated enforcement analytical method (validation may be in-house and could require bridging to CIPAC or other methods if being validated concurrently).

6. Study materials

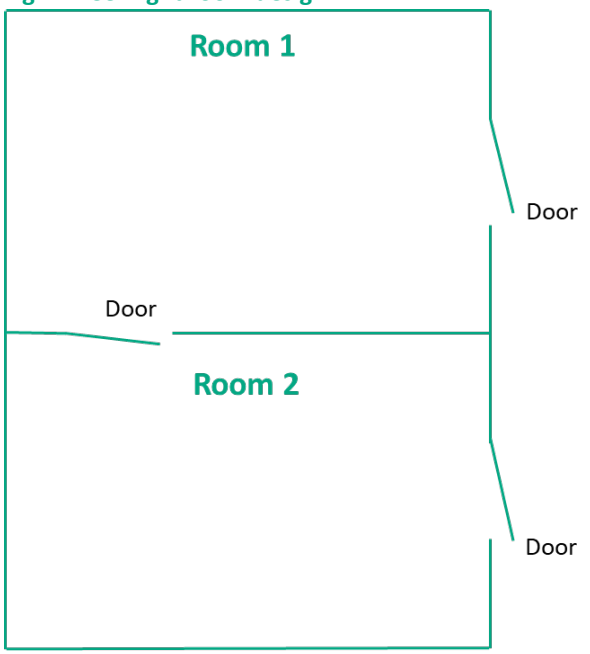
6.1 Free-flight rooms

Flight rooms allow evaluation of spatial emanator products under standardized, controlled conditions that resemble those in which mosquitoes interact with an area treated with a spatial emanator product and which contains humans. Flight rooms can be used for studies measuring landing inhibition, blood-feeding inhibition and, through the use of caged mosquitoes, additional endpoints such as mortality.

Flight rooms for spatial emanator studies should consist of two interconnected rooms, with a volume of approximately 30 m^3 per room (Fig 1) *(2)*. Flight rooms should be constructed to prevent mosquito ingress and egress, and the interior surfaces should be smooth, light-coloured surfaces that make it easy to see mosquitoes and which are non-permeable and easy to clean.

Flight rooms should be equipped with an extractor fan.

Fig 1. Free-flight room design



Free-flight rooms:

- Two rooms linked by an adjoining door
- Room volume = 30 m^3 (per room)
- Environmental conditions 27 ± 2 C°, 80% ± 20% RH
- Equipped with an extraction fan to clear vapours between replicates

6.1.1 Free-flight room preparation

Prior to use, free-flight rooms should be cleaned with detergent and ventilated to clear residual traces of cleaning solution. Contact bioassays should be conducted to ensure that no cleaning fluid residue that may affect mosquito behaviour or mortality is present on interior surfaces.

The environmental conditions in free-flight rooms (temperature and humidity) should be continuously monitored with a data logger.

6.2 Test and reference items

6.2.1 Test items

Free-flight room studies conducted for Module 5 data generation should include spatial emanator products from a minimum of three production batches that have been fully characterized as part of the data generation for Module 3. For the development of a new product dossier, it is critical that the batches used in the free-flight room study(ies) are the same as those used for other data generation, such as the characterization of chemical and physical characteristics and the semi-field efficacy studies.

Documentation of the source, receipt and handling and storage of spatial emanator products prior to testing is critical.

The environmental conditions of the storage room/area and the duration of storage prior to the use of products in studies should be documented and reported in the study report.

6.2.2 Reference items

The purpose of the **reference items** (positive and negative controls) is to validate the experimental procedures.

The means by which **reference items** were obtained and the storage conditions prior to testing should be documented, with certificate of conformity and batch numbers reported.

6.2.2.1 Negative control

Negative control samples should be untreated product samples/untreated product casings, depending on the design of the product. In the case of active spatial emanator products, an empty device can be used as the negative control.

6.2.2.2 Positive control

The positive control(s) should be selected based on the intent and design of the study, including the selection of method(s), endpoint(s), and species, in order to support the assessment of the validity of the study. All positive controls should be prequalified products that have an entomological MoA consistent with the entomological MoA and the intended effect of the product that is under investigation.

It is critical that the selected positive control(s) is used consistently in other studies for data generation.

The selected positive control should be as similar as possible with respect to AI(s), intended useful life and type of product (passive/active emanation).

7. Baseline quality check

There are no specific baseline quality check requirements for spatial emanator products used in free-flight room studies. CROs receiving products for use in studies should ensure that the Certificates of Analysis (COA) for the product batches used in the studies are provided and that the values reported in the COA are within the specified limits.

To ensure that product samples used in studies remain within the manufacturing release limits prior to the commencement of the study(ies), attention should be paid to the storage of products after receipt.

8. Sample preparation

The test materials to be used in the flight room study(ies) should be the finished product used in accordance with the intended directions for use. Ensure that all prepared samples are adequately labelled and stored appropriately, as improper storage may impact the results of the test and invalidate the study.

8.1 Sample ageing

Free-flight room studies conducted for Module 5 data generation use spatial emanator products that are unused, and samples that have been either artificially or operationally aged.

Products to be used as unaged samples should be kept in packaging until the first day of testing.

Product samples may be artificially aged for use in free-flight room studies. In these cases, it is recommended that a PQ200 protocol review application be submitted prior to the commencement of sample preparation.

Products that are aged in real-time should be aged in environmental conditions as similar as possible to those that will be found during the free-flight room study, for example, similar temperature and humidity conditions, and with standardized airflow/air exchange.

8.2 Product samples for chemical analysis

Product samples which are not immediately analysed for chemical content should be individually wrapped and stored in a manner which has been shown to limit changes in the product in advance of chemical analysis, for example, in aluminium foil and held at 4°C. In the case of shipment to an external testing facility for the determination of chemical content, samples should be shipped under conditions shown to limit changes in the product so as to ensure accurate and robust chemical analysis results.

9. Human volunteers

The free-flight room study(ies) requires the presence of a human volunteer as an attractant force. The volunteer should not smoke, drink alcohol or use perfumed skin care products for the duration of their involvement in the study as these factors can affect human attractiveness to mosquitoes.

Institutional ethical approval for the study must be sought from the institutional Ethical Review Board and/or local authorities. Written informed consent must be obtained from each volunteer prior to their participation in the study.

10. Environmental conditions

10.1 Environmental monitoring

The environmental conditions of the spatial emanator storage/ageing area(s), the free-flight testing room and insectaries should be continuously monitored and reported.

10.2 Test room environmental conditions

The temperature and humidity of the flight room and any rooms used for post-exposure holding should be maintained at 27 ± 2° C and 80 ± 20%, respectively.

All environmental conditions at which the studies are conducted should be documented and reported.

10.2.1 Air exchange and air flow/wind speed

The presence of air exchange mechanisms during testing prevents the 'build – up' of excessive emanated AI during the pre-conditioning and testing period. Flight rooms that are constructed within buildings with integrated ventilation systems should be maintained at an air exchange rate of approximately 110 m^3/h *(2)* or another air exchange rate typical of that found in constructed buildings *(3)*. The selection of the air exchange rate must be justified, and the justification presented in the study report.

A fan should be placed on the floor of the study room in which the volunteer sits, to mimic conditions in houses which may have open windows. The fan should point away from the volunteer and the installed products *(4)*. The wind speed of the fan should be set no higher than 4.5m/s, and the wind speed in front of each installed product and in front of the volunteer should be 0m/s *(5-6)*.

Prior to the release of mosquitoes, the air speed in front of each installed product and in front of the volunteer should be measured. Results should be reported in the study report.

10.3 Time of day

Free-flight room studies should, where possible, be conducted during the diel period of the test systems. This can be achieved by either conducting the tests during the time of day in which the test system is active and host-seeking or through the rearing of test systems using shifted light cycles to improve operational feasibility.

11. Test systems

The selection and preparation of test systems for use in free-flight room studies to characterize the presence and bioavailability of AI(s) emanated from the spatial emanator product should align with the entomological MoA of the product under investigation.

11.1 Test system species for use in free-flight room studies

Free-flight room studies should be conducted with at least three mosquito species (*Aedes*, *Culex* and *Anopheles*). Species/strain characteristics should be indicative of the target population(s) against which the product is intended to have an effect. At least one insecticide susceptible strain and one insecticide resistant strain should be used from each tested species.

Consideration should be given to the declared description of target vectors (Module 1), the declared diseases intended to be controlled with respect to target vectors (Module 1) and the planned locations of semi-field studies when selecting laboratory strains for use in free-flight room studies.

11.1.1 Age and physiological status of test systems

Free-flight room studies should be conducted using female test systems that are nulliparous and 5–8 days post-emergence.

Prior to commencement of the flight room study, test systems should be starved of sugar. The starvation should be conducted in the diel period that corresponds to the biting activity of the species.

The precise period of starvation will vary according to local conditions and must be standardized and reported in the study report.

11.2 Insecticide resistance characterization of selected test systems

Test systems to be used in free-flight room studies must be characterized for insecticide resistance prior to the commencement of the study (but not more than six months before the study starts). Results are reported in the matrix of selected mosquito strains.

12. Selection of sampling timepoints

The sampling timepoints to be used in free-flight room studies are dependent on the design of the product and the duration of the intended useful life. As an example, a product designed to be efficacious for 1 month might be tested weekly, while a product designed to be efficacious for 12 months might be tested monthly.

13. Experimental procedures

13.1 Free-flight room set-up

Testing rooms for free-flight studies should be constituted as two adjoining rooms connected by an internal door (Fig 1). Fig. 2 illustrates considerations for free-flight room set-up.

13.1.1 Product sample installation

Product samples should be installed in Room 1 according to the declared product use pattern in the product application form (Module 1). Care should be taken to ensure that the product is installed in alignment with the label instructions.

Product samples are installed in Room 1 at a standardized time period prior to the beginning of the experiment. The standardized time period is dependent on the design of the product and must be justified and reported in the study report.

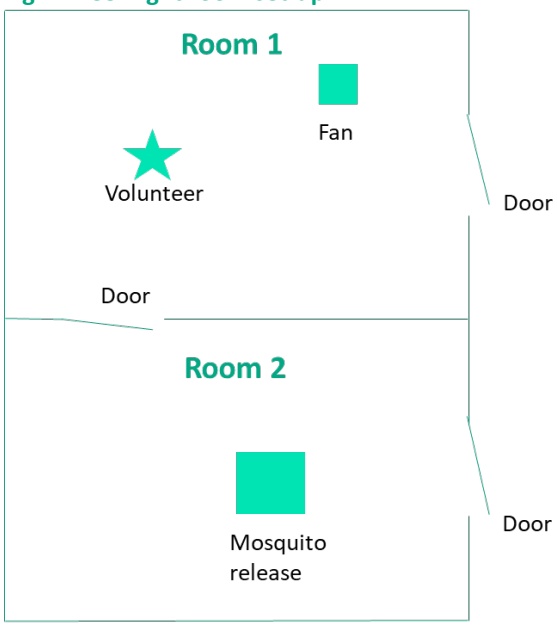
13.1.2 Volunteer placement

The volunteer sits in Room 1.

13.1.3 Mosquito release

Mosquitoes are released in Room 2 following approximately one hour of acclimatization in conditions similar to the free-flight rooms.

Fig 2. Free-flight room set-up



Free-flight rooms:

- Two rooms linked by an adjoining door
- Room volume = 30 m^3 (per room)
- Environmental conditions 27 ± 2 C°, 80% ± 10% RH
- Equipped with an extraction fan to clear vapours between replicates
- A fan is placed with the air flow pointing away from the volunteer and the installed product(s)
- Wind speed is measured at volunteer and product prior to the release of mosquitoes

Room 1:

- Spatial repellent product(s) are placed in Room 1. Placement and number is dependent on the description of product use pattern (to) be declared in the product application form (Module 1)
- The volunteer is located in Room 1

Room 2:

- Mosquitoes are released in Room 2

13.2 Sample size calculations for free-flight room studies

Sample sizes are based on the primary endpoint. A power calculation should be conducted to determine the required sample size, the number of replicates that are required and the number of mosquitoes to be released per replicate.

Sample size calculations should be conducted using simulations based on the primary endpoint, thus simplifying power calculations.

13.3 Selection of entomological endpoints

The potential **endpoint(s)** which may be selected for use in free-flight room studies must be representative of the intended effect of the product. Typically, free-flight room studies measure the protective efficacy of the proposed spatial emanator product, using either landing inhibition or blood-feeding inhibition endpoints.

Table 1 provides information pertaining to the relevant endpoints which may be observed and measured during free-flight room studies. The endpoint to be used for decision-making purposes must be selected based on the intended entomological MoA of the proposed product and be used consistently across all free-flight room and semi-field studies. Justification for the selection of the decision-making endpoint must be presented in the study report.

Regardless of the intended entomological effect of the product, 24-hour mortality (M24) should be observed and documented for the purpose of monitoring the experimental controls and thereby experimental acceptability.

Please note that it is the responsibility of the applicant to determine the relevant endpoints to be used in the free-flight room study(ies) and to propose scientific justifications, i.e. it is not a requirement to measure every endpoint that is listed in Table 1.

13.4 Duration of free-flight room studies

Each replicate of a free-flight room study typically lasts for one hour (not including product installation, pre-study emanation or test system acclimatization). The period of exposure can be product dependent. In cases where the design of the product is such that a different duration of exposure is indicated, it is recommended that a PQ200 protocol review application be submitted for review.

Table 1. Free-flight room study endpoints

Endpoint	Time of measurement	Purpose/Definition	Considerations
Blood-feeding inhibition	At the end of the exposure period	The proportion of unfed females. Blood-fed includes partially or fully blood engorged mosquitoes. Blood-feeding inhibition is the proportion of mosquitoes that are not fed.	
Landing inhibition	Continuously during exposure period	The reduction in mosquito landings in the treatment arm(s) relative to the control arm(s)	
Personal protection	At the end of the exposure period	The reduction in blood-fed mosquitoes in the treatment arm(s) relative to the control arm(s)	
Protective efficacy	At the end of the exposure period or continuously during the exposure period, depending on the selected calculation	The protection elicited by the proposed product relative to the control arm	Protective efficacy can be measured using either the reduction in landing or the reduction in blood feeding. The calculation used must be specified in the study report.
Knockdown	At the end of the exposure period	The proportion of mosquitoes knocked down at the end of the exposure period.	
Room entry	At the end of the exposure period	The proportion of mosquitoes that enter Room 1 from Room 2	

Endpoint	Time of measurement	Purpose/Definition	Considerations

Mortality at 24 hours (M24)	24 hours after the exposure period has ended	The measurement of mortality is an indicator of the lethal effects of the product (if any) and is additionally used to validate experimental procedures.
		
		Mortality is observed by the following indicators:
		
		• No sign of life; immobile; cannot stand.
		• Moribund mosquitoes are also classed as dead after 24 hours of holding, as it is unlikely that they would survive in nature, i.e.
		  • any mosquito that cannot stand, for example, has 1 or 2 legs.
		  • any mosquito that cannot fly in a coordinated manner.
		  • any mosquito that lies on its back, moving legs and wings but unable to take off
		  • any mosquito that can stand and take off briefly but falls down immediately.
			Extension of the exposure time, or inclusion of multiple exposure times, must be declared and scientifically justified in the context of the product being tested and study being conducted.
			
			The standard holding time post-exposure for free-flight room studies is 24 hours. Control mortality should not exceed 10% after 24 hours. Otherwise, the test is invalidated.
			
			Any extension of the post-exposure holding time must be declared and scientifically justified in the context of the product being tested and study being conducted.
			
			For example, Mortality at x hours after exposure - Mx
			
			Control mortality should not exceed 20% after extended holding times. Otherwise, the test is invalidated.

Other	Applicants may propose other endpoints to be measured by means of free-flight room studies with adequate justification.		

14. Results and data analysis

Results for the proposed product and controls (negative, positive) should be presented in both tabular and graphical format.

Descriptive and inferential statistics with appropriate error measurements should be used to present results.

14.1 Free-flight room study statistical analysis

14.1.1 Descriptive statistics

All endpoints should be presented with an appropriate measure of centrality and dispersion, for example, arithmetic mean % and 95% confidence intervals for percentages; median and interquartile range for count data. Data for the control arm provides data needed to appraise the quality of study conduct and should always be presented.

The number of replicates conducted and the number of mosquitoes per replicate must be presented.

14.1.2 Inferential statistics

To ensure standardization of analytical approaches, a specific model must be used when performing the analysis and presenting the results for assessment. To relate the outcome variables to the intervention and covariates, generalized linear regression models (GLMs) should be used. The choice of model will depend on the endpoint(s) under investigation. For binary endpoints, such as the proportion of mosquitoes dying, a logistic model is appropriate. For outcomes that are counts, a Poisson or negative binomial model may be more appropriate.

Full details of the statistical methodologies employed and the statistical results should be reported as part of the study report. The full outputs from statistical models and the statistical code in the format in which it was produced should be submitted as part of the product dossier.

14.2 Criteria for study validity and acceptance

Acceptance of chemical analysis results is based on the criteria for the selected available/validated enforcement analytical method.

Proposed spatial emanator products are expected to fulfil the following criteria in the free-flight room study in order to meet the requirements for prequalification:

1. Study power:
    - The study must demonstrate sufficient power for decision-making, based on the conducted power calculation and the sample sizes of mosquitoes used during the study.

2. Results for free-flying mosquitoes:
    - The results for the spatial emanator product at the end-of-life ageing at the selected endpoint must be statistically significantly higher than the results for the negative control, ***and***
    - The results for the spatial emanator product at the end-of-life ageing at the selected endpoint must ***either***:
        - » Be statistically non-inferior to the end-of-life aged positive control, or
        - » Demonstrate efficacy against the target vector that is statistically equal to or better than the selected epidemiologically relevant threshold, e.g. a 30% reduction in blood feeding.

    Please note that the choice of using non-inferiority methods of statistical analysis or a threshold-based statistical analysis may be dependent on the availability of positive controls of sufficiently similar design to the proposed product. The selected statistical method of analysis (non-inferiority/threshold based) must be incorporated into the sample size calculations and the statistical analysis plan prior to the commencement of the study.

If mortality at 24 hours exceeds 10% the test should be discarded and repeated. If 24-hour mortality (M24) exceeds 5% then mortality should be control corrected using Abbotts Formula.

15. Study report

The study report must be a comprehensive description of the study, procedures and include justification for specific scientific approaches and/or deviations from standardized methods.

The suggested study report sections for free-flight room study reports are below. These sections are provided for guidance and do not need to be strictly followed.

- Cover page
- Table of contents
- GLP compliance statement
- Results summary
- List of abbreviations
- Background information
- Study rationale
- Study objectives
- Study endpoints
- Criteria for study acceptance
- Methods
    - » Test systems
        - Indicate the most recent date of insecticide resistance characterization (NB. The results of the characterization are presented in the matrix of mosquito strains.)

- » Test and reference items
  - Description of each test and reference item including:
    - Batch numbers
    - The number of test items received per batch
    - Source
    - Date of manufacture
    - Date of receipt
    - Storage conditions since receipt
    - Justification for choice of positive control(s)
- » Sample preparation
  - Sample storage/and or ageing conditions
  - Artificial ageing methodology (if employed)
- » Sample shipment details for chemical analysis (if required)
- » Chemical analysis methods (if chemical analysis was conducted on site at the testing facility)
- » Experimental set-up
- » Data analysis
  - Statistical analysis methods

- Results
  - » Free-flight room study
    - Summarized tabular results from the free-flight room test
    - Graphical presentation of results
    - Narrative description of results
  - » Data analysis and statistical results
    - Free-flight room study
      - Summary statistics
      - Inferential statistics
      - Non-inferiority analysis (if employed)
  - » Results interpretation and demonstrated study acceptance criteria
- Discussion and conclusions
  - » The study report must include an interpretive analysis of the results. Specific discussions on any methodological deviations, anomalies in results or other factors which may have impacted the results should be included.

16. Related documents

- WHO PQT/VCP Implementation guidance – Considerations for the selection of controls for use in spatial emanator product studies
- WHO PQT/VCP Implementation guidance – Semi-field studies for spatial emanator products
- WHO PQT/VCP Implementation guidance – Semi-field methods for spatial emanator products – experimental huts
- WHO PQT/VCP Implementation guidance – Matrix of selected mosquito strains
- WHO PQT/VCP Implementation guidance – Template MSMS

17. References

1. Manual for monitoring insecticide resistance in mosquito vectors and selecting appropriate interventions. Geneva: World Health Organization; 2022. Licence: CC BY-NC-SA 3.0 IGO (https://iris.who.int/bitstream/handle/10665/356964/9789240051089-eng.pdf?sequence=1, accessed 24 July 2025).
2. Guidelines for efficacy testing of spatial repellents. Geneva: World Health Organization & WHO Pesticide Evaluation Scheme; 2013 (https://www.who.int/publications/i/item/9789241505024, accessed 24 July 2025).
3. Update for Chapter 19 of the Exposure Factors Handbook: Building Characteristics. Washington, DC: U.S. Environmental Protection Agency; 2018: 19-1–19-9 (https://www.epa.gov/sites/default/files/2018-07/documents/efh_-_chapter_19_update.pdf, accessed 24 July 2025).
4. Guidelines for efficacy testing of household insecticide products – mosquito coils, vaporizer mats, liquid vaporizers, ambient emanators and aerosols. Geneva: World Health Organization & WHO Pesticide Evaluation Scheme; 2009 (https://www.who.int/publications/i/item/WHO-HTM-NTD-WHOPES-2009.3, accessed 24 July 2025).
5. Adeleke, E., Shittu, R., Beierkuhnlein, C., Thomas, S. High wind speed prevents the establishment of the disease vector mosquito *Aedes albopictus* in its climatic niche in Europe. Front. Environ. Sci.; 2022;10 (https://www.frontiersin.org/journals/environmental-science/articles/10.3389/fenvs.2022.846243/full accessed 24 July 2025).
6. Cardé, R., Multi-cue integration: how female mosquitoes locate a human host. Current Biology. 2015; 25 (18): R793–R795 (https://www.sciencedirect.com/science/article/pii/S096098221500891X?via%3Dihub, accessed 24 July 2025).