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Standardising Bioassays for Passive Spatial Emanators against Mosquito Vectors

Walter Fabricio Silva Martins

Fabricio.Martins@lstmed.ac.uk

Liverpool School of Tropical Medicine

Timothy Hill

Liverpool School of Tropical Medicine

Rosemary Susan Lees

Liverpool School of Tropical Medicine

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Abstract

The increasing use of passive spatial emanators (SEs) for vector control has highlighted the need for standardised laboratory methods for bioefficacy testing. Peet–Grady (PG) chamber assays are widely used for volatile insecticides; however, key methodological parameters remain poorly characterised. Here, we investigated how ventilation (airflow and baffle configuration), mosquito holding cage design, and cage mesh structure influence mosquito responses to a transfluthrin (TF) passive emanator. Susceptible and pyrethroid-resistant Anopheles gambiae mosquitoes were exposed to TF, and responses were assessed using flight inhibition (FI) and 24-hour mortality. The assay was highly reproducible, with 83% agreement among replicate comparisons in FI dynamics. Ventilation had the greatest effect, with airflow differences producing 6- to 19-fold changes in the odds of FI. Time to FI thresholds was up to 4-fold shorter under medium airflow without a baffle than under low airflow with a baffle. Cage mesh structure influenced FI, with larger mesh apertures producing 10- to 20-fold higher odds of FI, whereas cage geometry had little effect, supporting higher-throughput dual-cage configurations. These findings demonstrate that ventilation, mosquito holding cage geometry, and cage mesh structure strongly influence mosquito exposure in PG-chamber assays and should be standardised for passive spatial emanator bioassays.

Introduction

Mosquito-borne diseases remain a persistent global public health challenge, particularly malaria and *Aedes*-borne arboviral diseases such as dengue, chikungunya, and Zika. Recent epidemiological data indicate sustained transmission across endemic regions. In 2023, the estimated global burden of malaria reached 263 million cases, a 4% increase on the previous year.[1] Meanwhile, dengue transmission has risen markedly over recent decades, doubling to nearly 59 million cases in 2021.[2] Multiple factors have contributed to this increasing burden, including rapid urbanisation, climatic changes, international travel, and the expanding geographical distribution of mosquito vectors.[3,4] At the same time, the effectiveness of current vector control programmes is increasingly challenged by widespread insecticide resistance and behavioural adaptations in mosquito populations, which reduce the efficacy of conventional indoor interventions, including insecticide-treated nets (ITNs) and indoor residual spraying (IRS).[5]

To address persistent challenges faced by vector control programmes, the WHO recommended spatial emanators (SEs) in 2025 as a novel intervention for malaria prevention.[6] SEs belong to a broader group of volatile insecticide products traditionally referred to as spatial repellents (SRs).[7] These interventions release airborne insecticides that disrupt host-seeking and biting behaviours.[8] Volatile anti-mosquito products based on these principles, including mosquito coils, aerosol sprays and liquid vapourisers, have long been used in households for self-protection in regions endemic for vector-borne diseases.[9]

However, the development of long-lasting passive emanator systems has enhanced the feasibility of large-scale SE deployment for public health. Currently, most SE systems are pyrethroid-based, utilising transfluthrin or metofluthrin as active ingredients. To date, however, the WHO conditional recommendation for spatial emanators is based on evidence generated using transfluthrin-based products.[6] Although the use of pyrethroid-based SEs may seem counterintuitive given widespread mosquito resistance to other pyrethroid interventions, recent epidemiological and entomological studies indicate that transfluthrin-based SEs containing transfluthrin or metofluthrin can significantly reduce mosquito landing, blood-feeding, and malaria infection risk in pyrethroid-resistant *Anopheles* and *Aedes* populations.[10-13]

Collectively, these findings indicate the growing potential of SEs as complementary vector-control tools for wider implementation in vector-control initiatives. Nevertheless, the recent adoption of SEs for public health applications highlights the need for robust, standardised laboratory methodologies to evaluate their efficacy under controlled conditions. Unlike traditional contact insecticides, volatile chemicals can elicit a wide range of behavioural and toxicological responses. Consequently, no single laboratory assay to date captures the full spectrum of biological responses relevant to SEs. In light of these methodological challenges, current WHO guidelines outline several complementary approaches for evaluating SEs and volatile insecticides, including Y-tube olfactometers for assessing host-attraction and movement responses, free-flight systems for evaluating landing and feeding inhibition, and Peet–Grady chamber (PG-chamber) assays for characterising insecticidal activity, such as knockdown and mortality.[7,14]

Among these approaches, PG-chamber assays have become a primary laboratory method for evaluating aerosolised and volatile products under controlled environmental conditions.[7] However, PG-chamber assays are highly sensitive to the chamber's physical and operational conditions, making standardisation challenging.[15] Factors such as airflow configuration, fan placement, mosquito containment methods, cage structure and insecticide evaporation dynamics can influence airborne chemical distribution and mosquito exposure, thereby affecting behavioural and toxicological responses.[15,16] Furthermore, current WHO guidelines for testing volatile products were established before the development of long-lasting passive emanator systems and have not been substantially revised to address contemporary SE technologies.[7,14] As a result, several methodological parameters relevant to passive emanator testing remain poorly defined, limiting standardisation and complicating comparisons between laboratory studies evaluating SEs.

As part of ongoing efforts to standardise volatile insecticide bioassays, we recently developed a cage-based methodology for testing aerosolised insecticides in PG-chambers. The approach increased assay throughput and reproducibility while enabling simultaneous behavioural monitoring of exposed mosquitoes. Several features of this methodology, including the dual-cage design and video-assisted behavioural analysis, provided a standardised framework readily adaptable for evaluating passive emanators. However, unlike actively dispersed volatile insecticides, passive emanators rely on the

continuous evaporation and passive dispersion of insecticide from a treated substrate. Consequently, mosquito exposure may be more sensitive to airflow conditions, ventilation patterns, and cage characteristics. It remains unclear whether methodological parameters optimised for other volatile insecticide delivery systems are directly transferable to passive emanator bioassays.

Building on this framework, the present study systematically evaluated how airflow configuration, cage design and mesh structure influence mosquito responses to passive spatial emanators. Robustness of the bioassay to changes in these test parameters was assessed by exposing mosquitoes to transfluthrin and measuring two entomological endpoints: flight inhibition (FI) and 24-hour mortality. In this context, flight inhibition (FI) is defined as the transient inability to sustain normal flight during airborne exposure to a volatile insecticide. Although FI may overlap with classical knockdown, including loss of postural control and incapacitation, it is distinguished by its transient nature. Mosquitoes exhibiting FI may recover normal flight following removal from exposure, particularly pyrethroid-resistant mosquitoes, whereas knockdown typically persists beyond the exposure period.

By systematically evaluating methodological parameters that can affect the reproducibility of SE bioassays, this study aims to facilitate the development of more standardised laboratory evaluation systems for SEs and other volatile vector-control interventions.

Materials and Methods

Mosquito colonies

Two colonies of *Anopheles gambiae s.s.*, with distinct pyrethroid-resistance profiles determined by WHO tube bioassays and aerosolised insecticide assays,[15,17] were used to assess mosquito responses to volatile insecticides under various test conditions. The susceptible Kisumu strain and the pyrethroid-resistant Tiassalé strain were reared by the Liverpool Insect Testing Establishment (LITE).[18]

Mosquitoes were maintained under standard insectary conditions at 27 ± 2 °C, 80 ± 10% relative humidity, and a 12:12 h light:dark photoperiod. All bioassays were conducted on non-blood-fed female mosquitoes aged 3–5 days.

Transfluthrin emanator preparation

Technical-grade transfluthrin (TF; purity 99.2%, Sigma-Aldrich) was dissolved in analytical-grade acetone to prepare a solution containing 10 mg of TF in 3 mL of solvent. This solution was applied to Grade 1 Whatman filter papers (15 × 12 cm) within a fume hood. Coated papers were left uncovered for 5-10 minutes until the solvent had fully evaporated, then mounted on a custom 3D-printed support frame designed to minimise surface contact and suspend the papers at the centre of the PG-chamber (Fig. S1). A new TF-treated paper was prepared for each experiment to ensure a consistent exposure concentration, as the exact amount of active ingredient released in each assay could not be measured directly.

Experimental overview

To assess the reproducibility of volatile insecticide bioassays conducted in PG-chambers, a standardised 1.80 x 1.80 x 1.80 cm chamber system used to evaluate volatile insecticides under controlled conditions,[7] we systematically evaluated the effects of ventilation configuration, cage design, and cage mesh structure on mosquito flight inhibition (FI) and 24-hour mortality. The study's experimental variables are summarised in Fig. 1.

The study comprised two experimental designs. The first evaluated standard single- and dual-cage configurations across four ventilation configurations, defined by two fan-speed settings (low and medium) tested with and without a baffle. The second experiment evaluated five cage mesh types that differed in pore structure (Fig. 1). All mesh configurations were tested at the medium fan speed, with and without a baffle. For both experimental designs, mosquito responses were assessed using behavioural and toxicological endpoints. Behavioural responses were quantified as: (i) FI measured at 30 min and at the end of a 60-min exposure, (ii) FI dynamics recorded at 5-min intervals over a 60-min exposure period, and (iii) time-to-threshold responses corresponding to 25%, 50%, and 80% FI (FI25, FI50, and FI80). FI was defined as the proportion of mosquitoes unable to sustain flight and resting on the bottom surface of the cage during the observation period. FI was assessed by manually counting affected mosquitoes through the chamber observation windows at each 5-min interval. Toxicological responses were assessed by recording mortality 24 h after exposure. Following exposure, mosquitoes were transferred to holding cups, maintained under standard insectary conditions with access to 10% sucrose solution, and scored for mortality after 24 h.

Ventilation configuration

Ventilation within the PG-chamber was generated by a centrally positioned 30-cm-diameter fan and a baffle mounted above the fan, following WHO guidance for volatile insecticide testing.[7] Four ventilation configurations were tested, comprising low- and medium-speed fan settings, each evaluated with and without a baffle.

Wind speed was measured using an anemometer (Thermo anemometer AV90, Martindale electronics) mounted on a tripod, with a horizontal support arm positioned 7.5 cm from the outer edge of the fan blades. Measurements were collected from three independent fan units of the same model, installed in both PG-chambers, to assess consistency across experimental setups. For each ventilation configuration, wind speed was measured at two standardised chamber locations: (i) 10 cm above the fan and (ii) 10 cm below the chamber ceiling, where the transfluthrin-treated paper was located. Measurements were recorded in triplicate, with each replicate representing the mean of 10 consecutive readings collected at fixed intervals over a 30-s period.

Throughout this study, the term ‘ventilation configuration’ refers to the experimental combination of fan speed and the presence or absence of a baffle. Wind speed is defined by anemometer measurements that quantify fan-generated air movement. The term airflow is used more broadly to describe air

movement within the chamber under a given ventilation configuration, encompassing differences in wind speed and changes in airflow direction and distribution associated with the presence or absence of the baffle. More detailed airflow characterisation, such as smoke visualisation, particle-tracking approaches, or computational fluid dynamics modelling, was beyond the scope of the present study and would require specialised instrumentation and analytical methods.

Cage designs and mesh configurations

Three cage designs were evaluated (Fig. 1). The single- and dual-cage configurations were used as the standard assay systems[15]. Both comprised a standard cage (20 × 20 × 20 cm), with the dual-cage featuring an internal mesh partition dividing the interior into two halves. The third cage design, the Modular Mesh Cage (MMC), was developed to investigate whether mesh structure influences mosquito exposure to volatile insecticides. While the standard BugDorm mesh used in the assay was broadly consistent with the mesh aperture recommended by WHO for volatile insecticide testing,[7] the MMC incorporated interchangeable mesh materials ranging from the standard BugDorm mesh (pore diameter ≈ 0.5 mm) to meshes with substantially larger openings (up to ≈ 2.7 mm; Table S1), allowing evaluation of how mesh properties affect insecticide dispersion and mosquito responses within the bioassay system

The MMC consisted of a disposable cardboard cage with interchangeable front and back mesh panels within a rectangular enclosure measuring 20 × 20 × 6 cm, allowing evaluation of alternative mesh configurations with larger apertures and differing structural characteristics (Fig. 1). The five mesh configurations evaluated with the MMC system were characterised using image-based analysis to quantify mean pore diameter, pore area, and pore-size variability. For each mesh type, images were collected from three independent mesh regions at a standardised magnification (Ultrazoom 3 GT vision). For each image, a custom R script randomly selected 10 pore regions for parameter extraction, and the resulting measurements were averaged across replicates.

Additional metrics describing mesh tightness (rim) and a derived airflow index were calculated from image-based measurements. Because insecticide dose on the papers and ventilation configuration were standardised across assays, differences in mosquito responses between cage configurations were interpreted as evidence that mesh structure may influence the level of mosquito exposure.

Statistical analysis

The two study designs, one comparing ventilation configurations and one comparing mesh configurations, were analysed as independent experiments because ventilation conditions differed between study designs. All analyses were conducted in R (version 4.6.0)[19] and RStudio (version 2026.04.0). Statistical analyses were performed using functions from the stats package, Cox regression models implemented in the survival package,[20] and estimated marginal means and pairwise

comparisons from the emmeans package.[21] Data processing was conducted using packages from tidyverse,[22] and figures generated using ggplot2.[23]

Reproducibility across experimental replicates and between independent PG chambers was assessed using FI time-response trajectories. Differences in FI dynamics between replicates or chambers were evaluated using linear models with time-by-replicate or time-by-chamber interaction terms, respectively. Differences in FI dynamics across ventilation configurations were assessed using linear models with time-by-treatment interaction terms. Interaction coefficients (Δslope) quantified differences in the rate of change of FI over time between experimental conditions.

Fixed FI endpoints at 30 and 60 min were analysed with binomial generalised linear models (GLMs) to assess the effects of airflow configuration, cage design, mesh structure, and their interactions. Mortality at 24 h was also analysed with binomial GLMs to test the effects of strain, airflow configurations, and strain-by-airflow interactions. Effect sizes were estimated using odds ratios (ORs) with confidence intervals.

Time-to-threshold analyses of FI responses (FI_{25}, FI_{50}, and FI_{80}) were conducted using Cox regression analyses. Threshold times were estimated from FI time-course data, and replicates that did not reach the defined threshold within the 60-min exposure period were treated as censored.

P-values were adjusted for multiple testing using the Benjamini–Hochberg false discovery rate procedure when multiple comparisons were performed. Statistical significance was defined as an adjusted P-value < 0.05.

Results

Reproducibility of PG-chamber Bioassay

Within-replicate

Reproducibility at the replicate level was assessed across 36 assay configurations (N = 114 replicates) using a slope-based interaction model derived from time–response curves (%FI × replicate) (Fig. 2a,b, Table S2). Overall, replicate-level differences were minimal, with 83.3% (30 of 36) of cases showing no significant variation in time-response curves between replicates of the same assay configurations (Table S2). No significant replicate-level variability was observed in any assay involving susceptible mosquitoes, as all adjusted P-values were ≥ 0.99 across airflow, mesh, and cage setups (Fig. 2a,b). Among resistant mosquitoes, however, 6 of 17 (35%) instances of significant replicate variability were associated with either a low-speed fan (23.5%) or a baffle-equipped fan (11.76%) (Table S1).

Between-chamber

Reproducibility between PG-chambers was generally consistent, with minimal variability across experimental comparisons (Fig. S1, Table S3). Of 42 matched chamber comparisons—assessed in both

chambers and categorised into four datasets × strain groups—most showed no significant differences in %FI time-response patterns between chambers (75%; adjusted P ≥ 0.05).

Consistent with the overall trend, no significant chamber effects were detected in the MMC assay for either susceptible or resistant strains (adjusted P ≥ 0.05). Likewise, in both single- and dual-cage setups, susceptible mosquitoes showed no evidence of chamber-level variability (adjusted P ≥ 0.05). By contrast, resistant mosquitoes exhibited a notable time-by-chamber interaction (adjusted P < 0.001; Table S3), suggesting reduced reproducibility under these conditions. This pattern was primarily observed in experiments with different ventilation configurations and baffle treatments (Fig. 1A; Table S3), suggesting that the observed differences between chambers may reflect greater variability in these assay conditions rather than systematic differences between PG chambers.

PG-chamber assay setup

Cage Configuration

Mesh Structure

Image-based analysis showed notable structural differences among five mesh types (Fig. 1; Table S1). The average pore diameter ranged from 0.50 mm (BugDorm) to 2.71 mm (MMC-3), a 5.4-fold pore size variation, while the mean pore area ranged from 0.196 to 5.79 mm², a 29.5-fold difference. Larger pore areas generally exhibited less size variability (Fig. S2). The derived airflow index, which integrates pore size and mesh tightness, exhibited an even greater range of variation, with a 43.7-fold difference between BugDorm and MMC-3 (Table S1)

Mesh differences were associated with variations in mosquito responses under medium-ventilation conditions (Fig. 2c). Variations of FI between mesh types were most apparent at 30 minutes under baffled conditions, especially in resistant mosquitoes, where responses in mesh-3 were approximately twice as high (25%) as in mesh-1. Without the baffle, differences among most mesh configurations diminished, although mesh-5 and the single-cage (mesh-5) setup remained distinct, with responses in mesh-5 about 2.5 times lower than in other MMC cage configurations (Fig. 2c). By 60 minutes, mosquito responses had become similar across most configurations, except for mesh-5 and single cage.

Cage Geometry

Across different configurations, responses in single- and dual-cage configurations were similar at both 30 and 60 minutes, with differences of FI generally within ~5–10% (Fig. S3a). No significant differences were observed between configurations across any condition or strain (Bonferroni-adjusted P ≥ 0.175; Fig. S3B).

In contrast, reducing cage depth from 20 cm to 6 cm (with the same mesh configuration, mesh-5) under medium ventilation without a baffle at both 30 and 60 minutes increased FI responses (Fig. 2d). FI was approximately 20–25% higher at 30 minutes and about 10–15% higher at 60 minutes compared with

standard-depth cages. Pairwise comparisons confirmed significant differences between MMC with mesh-5 and both single- and dual-cage configurations in resistant mosquitoes at 30 minutes, and in dual-cage comparisons at 60 minutes. No significant differences were observed in susceptible strains (Fig. S3c) since FI was always 100%.

Ventilation configuration

Wind speed measurements were highly consistent across fan units and PG-chambers (Fig. S4a; Table S4). Based on the measurements taken without a baffle, wind speed increased with fan intensity, ranging from approximately 3.7 m/s at low speed to 4.8 m/s at high speed (≈1.3-fold increase).

In this study, airflow refers to the movement of chamber air under a given ventilation configuration, encompassing both fan-generated wind speed and changes in airflow distribution associated with the presence or absence of a baffle. Airflow conditions differed markedly between baffle and no-baffle settings at the fan level with a difference of around 3-fold (Fig. S4b). At the ceiling, where the insecticide source is located, the variation was less pronounced, with approximately a 1.6-fold difference.

These differences in airflow conditions were reflected in FI dynamics, particularly among resistant mosquitoes (Fig. 3a,b). In susceptible mosquitoes, the time-response curves showed no significant differences in slope (all $P \geq 0.223$) across all cage setups, regardless of airflow condition (Fig. 3a). FI rapidly reached nearly complete levels within 10–15 minutes under all airflow scenarios (Fig. 3b).

Conversely, resistant mosquitoes showed significant differences in their time-response curves at both low and medium airflow ($P < 0.001$) in the dual- and single-cage assay setup (Fig. 3b). By contrast, no significant difference in curve slope was observed at medium airflow, with or without a baffle, in the MMC cage (Fig. 3a). In resistant mosquitoes, the FI response increased more gradually (Fig. 3b). In the dual/single-cage setup with a baffle-equipped fan, 50% inhibition was not achieved, whereas with unrestricted airflow (no baffle) it was reached by 45 minutes. In the MMC cage, both airflow conditions achieved 50% FI, despite a 10-minute difference between them. Notably, complete FI (100%) was reached only in the no-baffle setting for Meshes 1-4 (Fig. S5), which had larger pores than Mesh-5, the mesh used in single- and dual-cages.

Interaction Between Airflow and Cage Structure

The effect of airflow and cage-structure interactions on FI was assessed at 30 and 60 min across two experimental conditions under varying airflow: (i) single/dual cage (mesh-5), and (ii) the MMC cage with meshes 1–5 (Fig. 1). Interactions were estimated using binomial GLMs, while the magnitude of variation was quantified as odds ratios.

In the single- and dual-cage systems, airflow (the presence or absence of a baffle) was the primary factor influencing FI (Fig. 4a, Table S4). GLM results indicated that the baffle significantly decreased FI at both 30 minutes ($P = 0.004$) and 60 minutes ($P < 0.001$). Pairwise comparisons showed 5.9 - and 10.78-fold increases in the odds of FI without the baffle at the two timepoints (Fig. 4a). The absence of a baffle

produced approximately 20–30% higher FI at 30 minutes and 50–70% higher inhibition at 60 minutes compared with the baffle setup (Fig. 4B). While fan speed did not influence FI at 30 minutes ($P = 0.62$), it became significant at 60 minutes ($P = 0.0006$), with a 2.44-fold increase in FI (Fig. 4a, Table S4). For cage configuration (dual vs. single), no significant interaction was observed at either time point ($P \geq 0.16$), indicating comparable responses between the two configurations. Consequently, single- and dual-cage data were pooled for subsequent analyses of ventilation effects

In the MMC cage system, the airflow had a stronger effect on FI, with GLM results showing significant effects at both 30 and 60 minutes (Table S4). Pairwise comparisons revealed a 6-fold increase in FI at 30 min and a 19-fold increase at 60 min in the absence of the baffle (Fig. 4b). The absence of a baffle increased FI by approximately 30–50% at 30 minutes, and at 60 minutes, inhibition was nearly 100% across all mesh types. By contrast, with a baffle, the FI at 60 min ranges from 50% to 80% depending on the mesh structure. Mesh effects were significant at 30 minutes ($P \leq 0.049$) but less consistent at 60 minutes ($P \geq 0.059$) (Table S4). No significant interactions between mesh type and airflow were found ($P > 0.05$), indicating that airflow was consistent across cage setups.

Effect of Airflow on Volatile Insecticide Efficacy

Clear separation in response time was observed between airflow treatments for susceptible and resistant mosquitoes, with susceptible mosquitoes consistently reaching inhibition thresholds earlier than resistant mosquitoes, although the magnitude of this difference varied across conditions (Fig. S6). Airflow configuration influenced the temporal dynamics of volatile insecticide effectiveness. Δ time values were expressed relative to a baffle baseline, and time to reach 25%, 50%, and 80% FI differed consistently between baffle and no-baffle conditions across all assays (Fig. 5a).

In resistant populations, airflow configuration (baffle vs no baffle) strongly altered FI dynamics: the absence of a baffle reduced the time to 50% inhibition by approximately 15–25 min and enabled attainment of 80% inhibition, whereas under baffle conditions this threshold was often not reached (Fig. 5b). In susceptible mosquitoes, inhibition thresholds were reached under both airflow conditions, with comparatively small differences (<10 min). Consistent with these patterns, airflow significantly increased the rate of reaching inhibition thresholds (Cox models; HR ≈ 5–12 for No baffle vs Baffle, $P < 0.05$ across thresholds). This effect was stronger in resistant mosquitoes, whereas in susceptible mosquitoes it was smaller but remained significant at FI_{25} and FI50 ($P < 0.05$), with no difference detected at FI_{80} ($P > 0.05$) (Table S6).

Consistent with the observed effects on FI dynamics, 24-h mortality varied significantly with airflow configuration (binomial GLM, $P < 0.05$) and showed a significant strain × airflow interaction ($P < 0.05$), indicating that the effect of airflow differed between strains (Fig. 5c; Table S6). Overall, mortality was substantially higher in susceptible mosquitoes (65–100%) than in resistant mosquitoes (approximately 5–15%) across all conditions. Strain-specific analyses showed that airflow significantly affected mortality in susceptible mosquitoes across all conditions ($P < 0.05$), whereas in resistant mosquitoes,

mortality was largely unaffected by airflow, with only the assay with low fan speed and no baffle differing significantly from the reference low–baffle condition ($P < 0.05$).

Overall, the mortality pattern contrasted with the airflow-driven variation observed in FI dynamics (Fig. 5a,b). In resistant mosquitoes, mortality did not differ from the strain-specific mean ($P > 0.05$; Table S7), except for a significant reduction under low-speed no-baffle conditions ($P < 0.05$). In susceptible mosquitoes, mortality differed from the strain-specific mean under most conditions ($P < 0.05$): lower under low-speed baffle conditions and higher under medium-speed conditions.

Discussion

Passive emanators, as a new class of vector-control interventions, lack specific guidelines for evaluating product performance, monitoring resistance, and comparing results across studies. To date, Peet–Grady (PG) chamber methods provide the best available framework for testing volatile compounds under laboratory conditions.[7,15] While these methods provide an important foundation, some assay parameters relevant to passive emanators have not been systematically examined. Here, we show that ventilation configuration (fan speed and the presence or absence of a baffle), cage geometry, and cage mesh structure influence mosquito responses to passive transfluthrin emanators, measured as flight inhibition (FI) and mortality.

The high level of reproducibility (similarity between replicates and between chambers at the same conditions) observed across the experimental conditions evaluated in this study supports the applicability of the previously-described standardised PG-chamber methodology for passive emanator testing[15]. Despite the wide range of airflow and cage configurations examined, assay responses remained highly reproducible across experiments (Fig. 2a,b; Fig. S1). This consistency likely reflects the use of standardised fan alignment, which minimises airflow heterogeneity within the chamber. The few instances of reduced reproducibility occurred under low-speed baffled airflow conditions and were most evident in cages with the smallest mesh openings (single- and dual-cage).

Cage mesh structure influenced both mosquito responses and assay reproducibility. Consistent with the reduced reproducibility observed under some airflow conditions, the greatest variation occurred in cages with the smallest mesh openings and mesh characteristics indicative of greater resistance to airflow exchange, whereas more permeable mesh structures generally produced more consistent responses (Fig. 2c; Fig. 3a). Previous studies using aerosolised insecticides have reported similar effects of cage porosity on mosquito exposure and bioassay outcomes. Mesh aperture is an important determinant of airflow and insecticide penetration into mosquito holding cages, with substantial variation in aperture dimensions and airflow properties among the cage meshes evaluated here (Table S1). Smaller mesh apertures have been shown to substantially reduce airflow penetration into mosquito cages and have also been associated with reduced mosquito mortality during space-spray exposure.[16,24]

The observed effects of mesh structure are particularly relevant because WHO guidelines for evaluating household insecticide products recommend mosquito cages with approximately 0.5 mm mesh openings for PG-chamber testing of volatile insecticides, such as mosquito coils, vapouriser mats, and liquid vapourisers.[7] In the present study, the single- and dual-cage configurations (BugDorm cages) used mesh openings closest to this recommendation. Relative to these WHO-like cages, the MMC cage designs, which used mesh configurations with openings approximately 2.5- to 5.5-fold larger (Fig. 1), frequently produced substantially stronger FI responses, with some comparisons associated with approximately 10- to 20-fold increases in the odds of inhibition (Fig. 4b). Taken together, these findings demonstrate that relatively modest differences in mesh structure can translate into large differences in measured mosquito responses, highlighting the importance of cage design when developing and standardising passive emanator bioassays.

The strongest mesh effects were observed under baffled airflow conditions, suggesting that mesh structure and ventilation interact to influence mosquito exposure. One possible explanation is that meshes with smaller openings restrict airflow exchange between the chamber and cage interior, thereby altering volatile insecticide transport and generating greater heterogeneity in mosquito exposure. Because airborne transfluthrin concentrations were not measured in this study, it was not possible to disentangle the relative contributions of differences in volatile transport, local concentration gradients, and other airflow-mediated processes to the observed effects.

Compared with ventilation and mesh structure, cage geometry had relatively limited effects on mosquito responses, with the previously described single- and dual-cage configurations producing comparable outcomes across all test conditions.[15] This suggests that dual-cage assays remain a feasible approach for high-throughput testing of passive spatial emanators, as they enable simultaneous testing of multiple mosquito strains without compromising assay efficiency. The shallower MMC mesh-5 (BugDorm mesh) cage produced higher FI responses than the standard-depth single BugDorm cage in resistant mosquitoes under some airflow conditions (Fig. 2d; Fig. S3C). Since the single- and dual-cage setups yielded similar results, the differences are probably not solely due to cage volume. Instead, changes in airflow around and within the cage, especially with reduced depth, may explain the differences. Earlier studies with aerosol insecticides also found that cage shape can affect airflow and insecticide delivery, especially when comparing flat and round cages.[24] However, because airflow inside the cages was not measured directly in that study, it was not possible to pinpoint the exact reasons for these differences. These results show that cage features such as mesh structure and cage geometry can affect mosquito exposure and should be considered when improving PG-chamber methods for passive emanators.

Although cage design seemed to influence mosquito exposure, ventilation configuration produced the largest and most consistent effects observed in this study. The presence or absence of the baffle exerted a substantially greater influence on mosquito responses than changes in fan speed alone (Fig. 4; Table S4). The absence of significant airflow × cage interactions suggests that ventilation exerted broad effects across assay configurations rather than acting primarily through specific cage designs. These findings are particularly noteworthy given that current WHO guidelines for evaluating household

insecticide products recommend using both a baffle and fan speed of approximately 4–5 m/s during PG-chamber testing of volatile insecticides.[7] However, our results suggest that these two parameters do not contribute equally to airflow conditions within the chamber. While increasing fan speed produced only a modest (~1.3-fold) increase in air velocity, the presence of the baffle reduced airflow by approximately 1.6-fold at the fan and nearly 3-fold at the ceiling, where the passive emanator was positioned (Fig. S4). Consistent with these airflow measurements, mosquito responses were more strongly associated with the presence or absence of the baffle than with differences in fan speed (Fig. 4), suggesting that the baffle exerted a substantially greater influence on chamber airflow dynamics as related to mosquito exposure under the conditions tested.

These observations may help explain why previous studies have adopted contrasting approaches to airflow management during PG-chamber testing. In aerosol bioassays, baffles have been shown to reduce aerosol dispersion and increase insecticide deposition on chamber surfaces, while fan orientation can generate heterogeneous insecticide distribution and location-dependent mosquito responses.[15] By contrast, passive emanator studies have adjusted fan orientation to minimise direct airflow towards the emanator, recognising the potential for direct airflow to increase the release of volatile insecticides from the treated substrate.[13]

Whether the ventilation-driven heterogeneity reported in aerosol testing also occurs during passive emanator testing remains to be determined, but our findings suggest that airflow configuration can substantially alter exposure conditions in mosquito cage-based bioassays for volatile insecticides. Similar observations have been reported in evaluations of household ambient insecticides, where air circulation improved agreement between cage-based and free-flying mosquito assays, further emphasising the importance of airflow dynamics when assessing volatile insecticides.[25]

Passive emanators, therefore, can pose a distinct methodological challenge for mosquito cage-based bioassays compared with aerosols, mosquito coils and heated vapourisers. In these active deployment systems, ventilation primarily affects insecticide distribution after release. By contrast, passive emanators rely on evaporation from a treated substrate, so airflow may influence both insecticide transport and release. Consistent with this, the highest levels of flight inhibition in the present study occurred under unrestricted airflow, whereas baffled configurations reduced airflow, particularly at the emanator location, resulting in slower or lower FI responses (Figs. 3, 4, and S5). These differences may reflect variations in transfluthrin vapour dispersion within the chamber, airflow-mediated effects on transfluthrin release from the emanator, or a combination of both.

Resistance status in this study was defined according to the WHO's conventional susceptibility criteria for contact insecticides, as there is currently no widely accepted operational definition of resistance to passive emanators or other spatial-acting interventions. The influence of ventilation on mosquito responses was particularly evident when examining FI dynamics in resistant mosquitoes (Fig. 5a,b). Compared with susceptible mosquitoes, which reached high levels of flight inhibition rapidly under most test conditions, resistant mosquitoes exhibited more distinct response patterns across airflow

configurations over time. This increased sensitivity improved the ability to distinguish between assay conditions. These findings suggest that pyrethroid resistant strains may provide a more informative model for assay optimisation and for evaluating new active ingredients and passive emanator products, since methodological or product-related differences may be masked when highly susceptible mosquitoes are used.

In contrast to the pronounced effects observed for FI, mortality responses were less sensitive to airflow configuration, particularly in resistant mosquitoes (Fig. 5c; Table S6). The recognised mode of action of volatile pyrethroids, which can modify mosquito behaviour through non-lethal mechanisms, including deterrence, disrupted host-seeking and reduced feeding at lower concentrations, rather than acting solely through acute toxicity which occurs at higher concentrations.[8,26] FI may be a more sensitive endpoint for detecting differences in exposure conditions and product performance, whereas mortality reflects responses at higher exposure thresholds. FI and mortality should therefore be viewed as complementary rather than interchangeable measures of passive emanator efficacy.

Beyond their implications for laboratory assay optimisation, these findings underscore the broader importance of ventilation in defining optimal conditions for passive emanator deployment. Passive emanators are intended for use in occupied spaces, where airflow patterns are inherently heterogeneous. Airflow can vary considerably between houses, rooms and environmental conditions. If ventilation can alter mosquito responses within the controlled environment of the PG-chamber, it is plausible that similar processes contribute to variation in passive emanator performance under operational conditions. While laboratory findings might not directly translate to field performance, these results emphasise the importance of understanding how airflow influences volatile insecticide transport and exposure heterogeneity in real-world settings.

Conclusion

Taken together, the findings of this study, which comprehensively explored the effect of key test parameters, demonstrate that both cage design and ventilation are key determinants of mosquito response in PG-chamber assays with passive spatial emanators. Mesh structure, cage geometry and ventilation likely act in concert to influence mosquito exposure, potentially by affecting transfluthrin vapour transport within the chamber and into cage interiors. In particular, the use of larger-aperture cage mesh, within the range evaluated (approximately 1.2–2.7 mm aperture diameter), may improve mosquito exposure and assay sensitivity while maintaining apertures sufficiently small to prevent mosquito escape. The limited effect of cage geometry also supports the use of higher-throughput dual-cage configurations. Together with standardised ventilation conditions, these parameters provide a basis for refining current WHO recommendations, although further validation across products and laboratories would be required before defining specific conditions as optimal.

Declarations

Contributors

WFSM conceptualised the manuscript, wrote the original draft, designed the study, conducted data collection and curation, performed data analysis and visualisation, and secured funding. TH conducted data collection and curation. RSL secured funding and reviewed and edited the manuscript. All authors read, revised, and approved the manuscript.

Declaration of interests

The authors declare that they have no competing interests.

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Figures

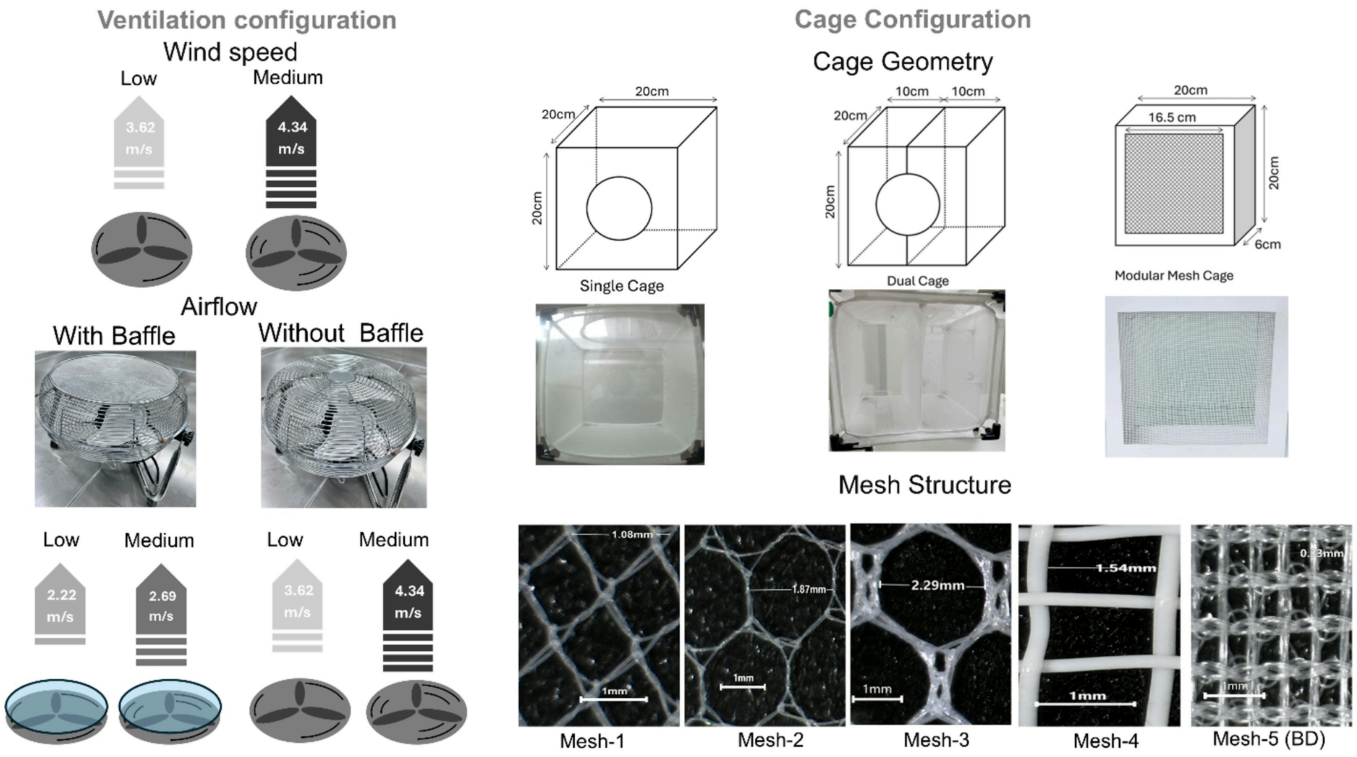


Figure 1

Schematic overview of the experimental variables evaluated in the PG-chamber spatial emanator bioassay.

Ventilation conditions, cage configurations, and mesh structures were systematically evaluated for their effects on mosquito flight inhibition (FI) and 24-h mortality following exposure to transfluthrin passive emanator.

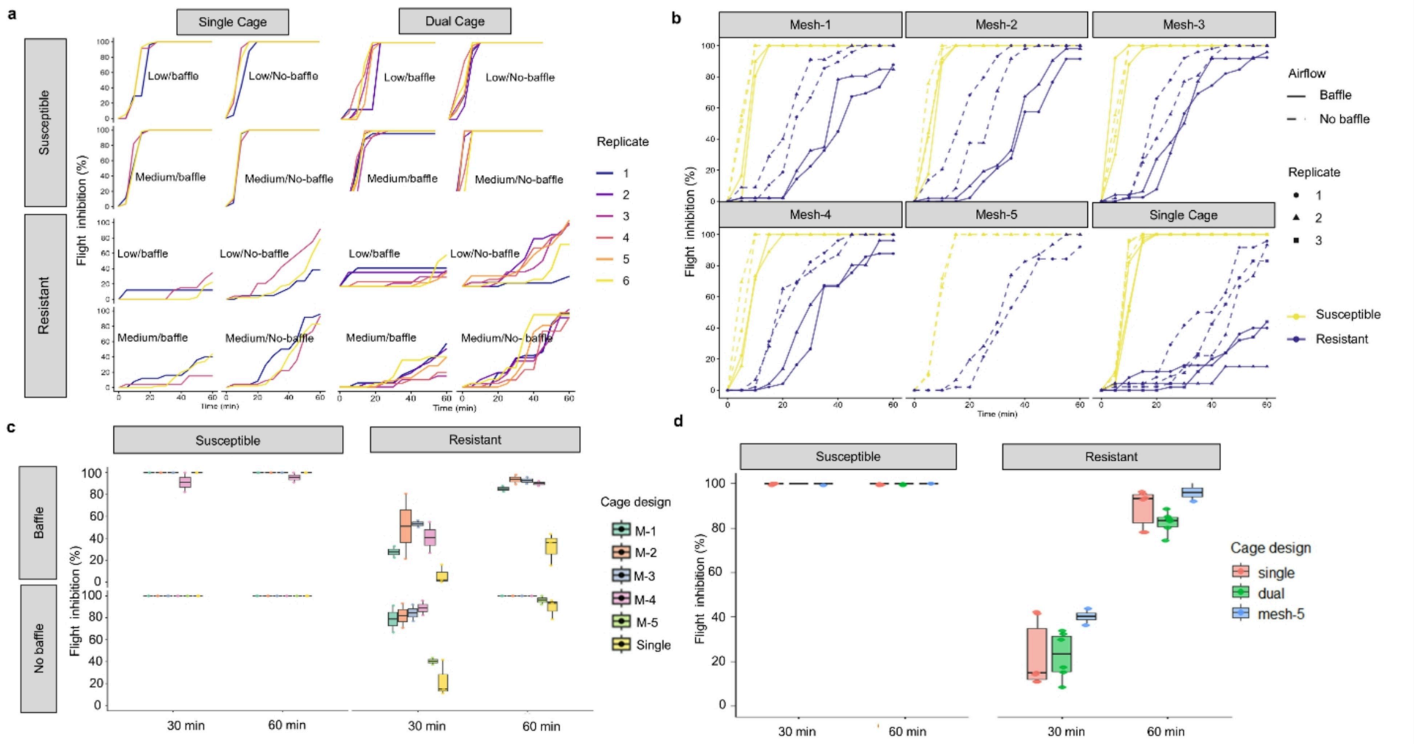


Figure 2

Reproducibility and effects of cage design on mosquito flight inhibition (FI).

(a) Reproducibility across experimental replicates, assessed using FI time-response trajectories in single- and dual-cage configurations under varying ventilation conditions. (b) Corresponding reproducibility analysis for MMC-cage configurations incorporating different mesh types. (c) Effects of mesh structure on FI at 30 and 60 min in baffled and unbaffled ventilation configurations. (d) Effects of cage geometry on FI at 30 and 60 min in single-, dual- and MMC-cage configurations using the same mesh type.

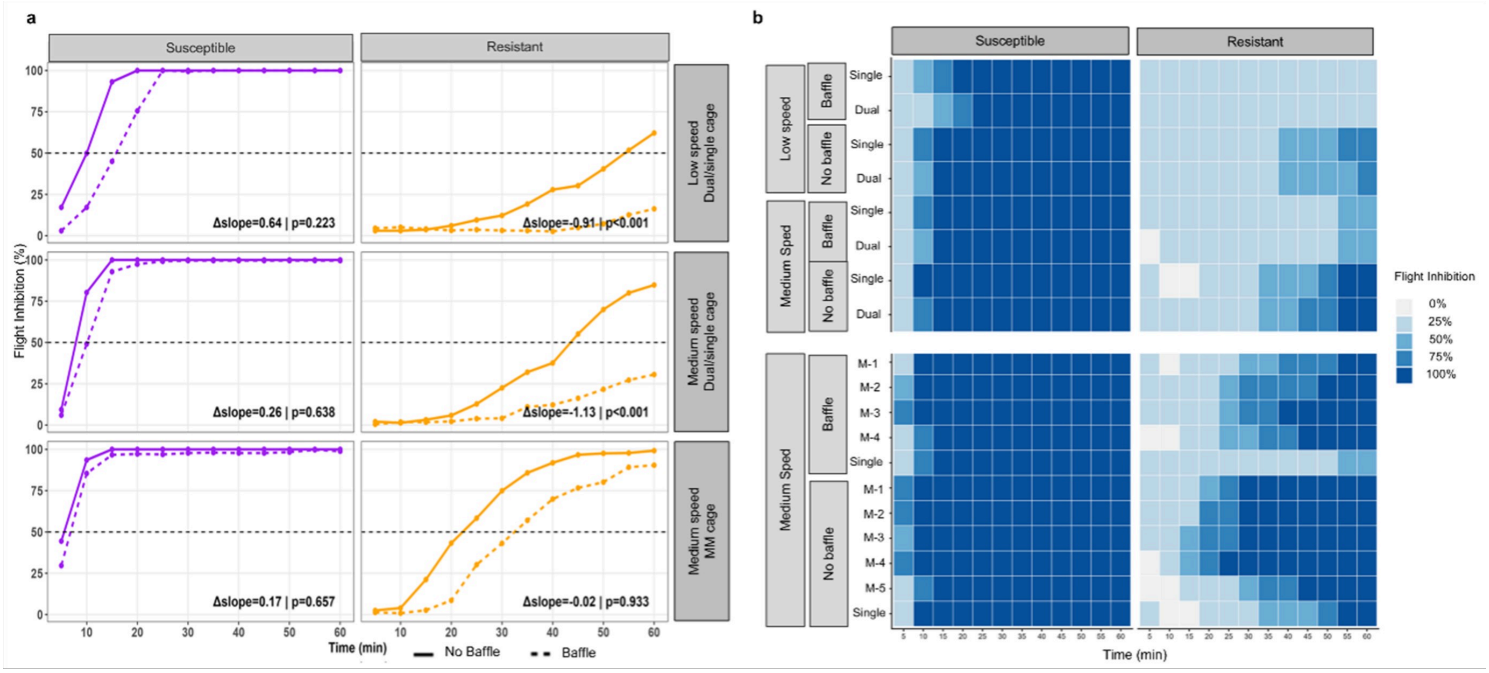


Figure 3

Effects of ventilation configuration on mosquito flight inhibition.

(a) FI time-response trajectories under different ventilation configurations in susceptible and resistant mosquitoes. Δslope values represent differences in FI response rates between ventilation configurations. (b) Heatmap showing the temporal progression of FI across ventilation configurations in susceptible and resistant mosquitoes.

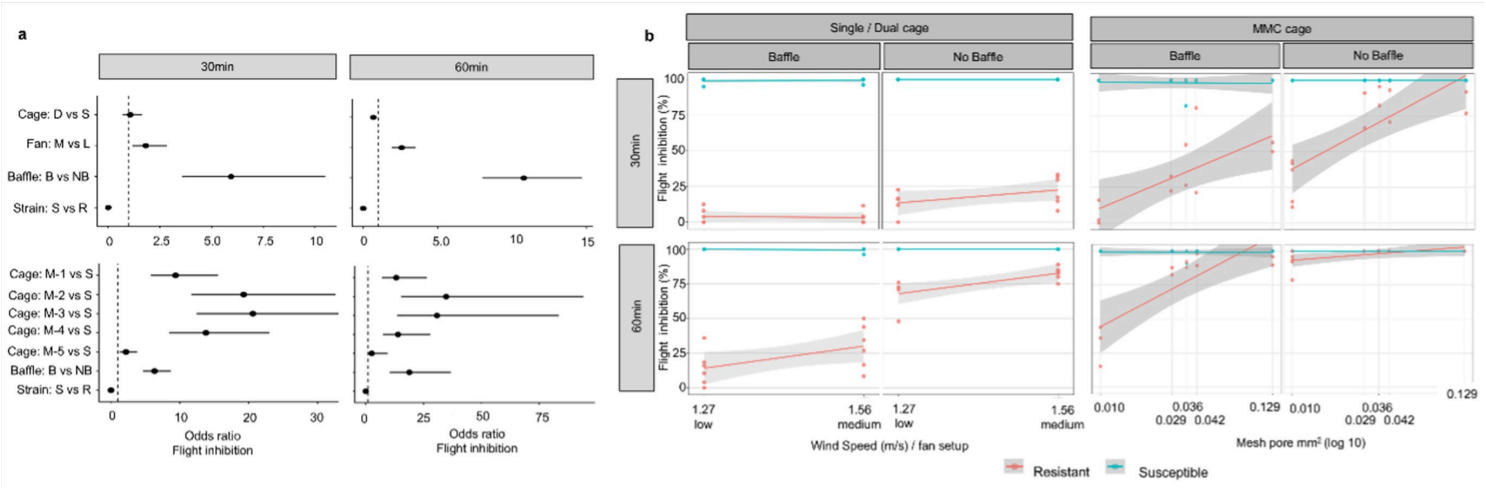


Figure 4

Effects of airflow and cage structure on mosquito flight inhibition (FI).

(a) Forest plots showing odds ratios (OR ± 95% CI) from binomial GLMs evaluating the effects of airflow, fan-speed setting, cage configuration and mesh structure on FI at 30 and 60 min. Dashed vertical lines

indicate the null effect (OR = 1). (b) FI responses across airflow conditions in single/dual-cage and MMC-cage systems at 30 and 60 min. The x-axis represents measured airflow intensity (m/s) under baffled and unbaffled ventilation configurations. Points represent individual replicates, coloured by mosquito strain. Solid lines show fitted trends, and shaded areas represent 95% confidence intervals.

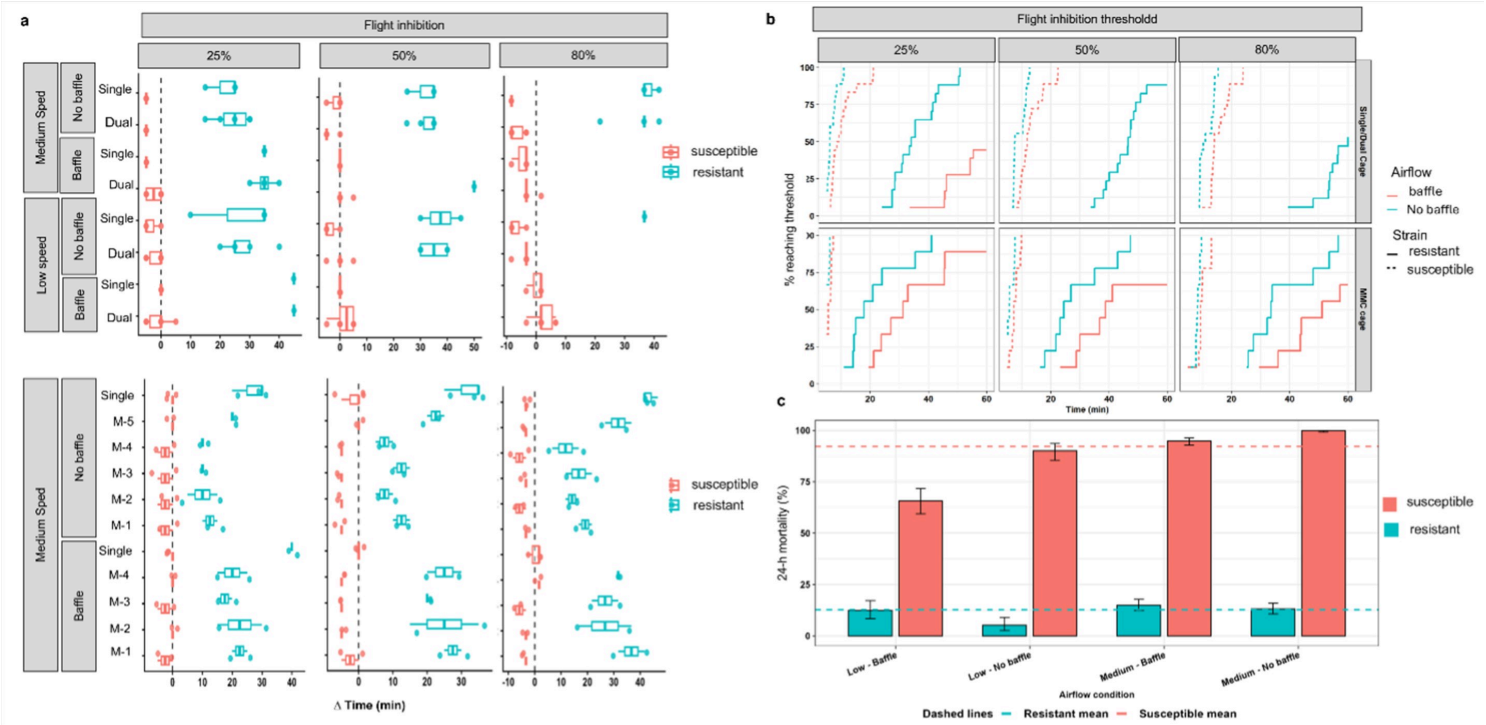


Figure 5

Airflow-dependent variation in flight inhibition (FI) and mortality responses.
(a) Differences in time required to reach FI thresholds (FI_{25}, FI_{50} and FI_{90}) under different airflow conditions in susceptible and resistant mosquitoes, expressed relative to the corresponding baffled condition. (b) Relative rates of attaining FI thresholds under different airflow conditions, estimated using Cox regression models (HR ± 95% CI). (c) 24-hour mortality across airflow conditions in susceptible and resistant mosquitoes. Dashed horizontal lines indicate strain-specific mean mortality.

Supplementary Files

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