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MOSQUTIO TRACKING

Report

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Disclaimer

The views and ideas expressed herein are those of the author(s) and do not necessarily imply or reflect the opinion of the Institute.

ABBREVIATIONS

3D	Three dimensional
95% CI	95% confidence interval
Ae.	*Aedes*
An.	*Anopheles*
BG-Cage	Biogents Cage
CO_2	Carbon dioxide
DEET	Diethyltoluamide
EBAAP	Ethyl butylacetylaminopropionate
FHF	Finite helix fit
LED	Light emitting diode
NIR	Near-infrared
PMD	*para*-Menthane
Swiss TPH	Swiss Tropical and Public Health Institute
WHO	World Health Organization

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SUMMARY

Mosquitoes are vectors of infectious diseases that can be fatal such as malaria or dengue. For many mosquito-borne diseases, we have neither a vaccine nor a specific treatment and, therefore, travellers to disease endemic countries are advised to avoid mosquito bites by applying of topical repellents. Topical repellents are biocides and have to be registered while efficacy tests are key for their evaluation. Repellent efficacy is typically assessed under laboratory conditions in the arm-in-cage test in which study participants expose their repellent-treated forearm at regular intervals into a cage containing a large number of host-seeking female mosquitoes until the repellent fails. However, the arm-in-cage test confines the mosquitoes to a very small space and mosquitoes may not behave in the same way they would under a normal use situation. Indeed, previous studies have shown that the arm-in-cage test may considerably underestimate the repellents' efficacy under natural conditions. To gauge the value of the arm-in-cage test and to make evidence-based recommendations for improving efficacy testing, it is essential to understand how mosquitoes perceive and interact with topical repellents in different contexts. Here, we measured the behaviour of host seeking mosquitoes in the arm-in-cage test using a 3D infrared video camera system. We tracked mosquito flight paths of two key disease vectors, *Aedes aegypti* and *Anopheles stephensi*, as they interact with a repellent-treated forearm in the arm-in-cage test. The tested repellents included 20% ethanolic solutions of *N,N*-diethyl-meta-toluamide, *p*-menthane-3,8-diol, icaridin and ethyl butylacetylaminopropionate. We found no clear evidence that volatile chemicals, rather gustatory excito-repellency upon contact with the treated skin, repelled the mosquitoes. Our observations cast doubts on the validity of the arm-in-cage test as the only basis for making label claim recommendations of topical mosquito repellents.

1. INTRODUCTION

Biting mosquitoes (order Diptera; family Culicidae) are vectors of medically important infectious diseases caused by parasites and viruses, including the two most prevalent vector-borne diseases, malaria and dengue. In 2019, malaria caused an estimated 229 million cases globally and resulted in more than 400,000 deaths while most cases occur in sub-Saharan Africa in children under 5 years (WHO, 2020). The disease is caused by parasites of the genus *Plasmodium* that are transmitted from human to human through the bite of an *Anopheles* mosquito. Dengue is the most prevalent arthropod-borne viral infection globally and is primarily transmitted by two key vectors, *Aedes aegypti* and *Ae. albopictus*. More than 3.97 billion people in over 128 countries are at risk of contracting dengue (Brady *et al.*, 2012), with an estimated 96 million symptomatic cases and an estimated 40,000 deaths every year (Bhatt *et al.*, 2013). In addition to dengue, mosquitoes also transmit other important viral diseases such as chikungunya fever, Zika virus infections, yellow fever, West Nile fever and Japanese encephalitis (Service, 2012).

Protective measures, including community-based vector control such as indoor residual spraying, insecticide-treated nets or larviciding, as well as personal protection such as mosquito nets and repellents, are key for disease prevention - even more so as for most of the mosquito-borne diseases neither specific treatments nor vaccines are available.

Personal protection is of particularly relevance for Swiss citizens if travelling to disease endemic countries. Effective personal protection to avoid mosquito bites may be achieved by a combination of long-sleeved light clothing and application of topical repellents on the bare skin. In Europe, repellents are classified as biocides and must be registered before coming to the market (EC, 2012a). The registration process also includes demonstration of the product's efficacy. Efficacy of topical repellents is usually assessed under laboratory conditions in the so-called arm-in-cage test (EC, 2012b; EPA, 2010; WHO, 2009).

In the arm-in-cage test, the repellent is applied to the bare forearm between the wrist and the elbow. Upon application, the repellent-treated arm is exposed at regular intervals (*e.g.* every 30 min) for a few minutes (*e.g.* 3 min) while the number of bites is recorded. The procedure is repeated until the repellent fails to provide complete protection. Complete protection is usually defined as the time between application of the repellent and the exposure at which the first confirmed bite occurs. A confirmed bite is a bite followed by a second bite within the same or two consecutive exposures.

A great advantage of the arm-in-cage test is that the protocol is relatively simple to follow, has a clear measurable endpoint and can easily be standardised under laboratory conditions. However, results from arm-in-cage laboratory studies may markedly differ from field studies measuring the same endpoints and repellent formulations (Frances, Mackenzie, Klun et al., 2019; Obermayr, Rose & Geier, 2010), even if the methodology and endpoints are carefully standardised allowing for such comparisons (Colucci & Müller, 2018). Moreover, a laboratory study has shown that repellents last also much longer when they are tested in a walk-in chamber, an alternative setup for testing repellents, even if the experimental conditions such as mosquito species, temperature, humidity etc. are comparable (Trongtokit *et al.*, 2005). A plausible explanation for these discrepancies are differences in biting rates, which may simply be related to mosquito densities. As a remedy, Moreno-Gómez et al. (2021) suggested calibrating biting rates in laboratory assays to rates reported from the field by adjusting the number of mosquitoes released into the experimental space. Intriguingly, even if the mosquito density is kept constant in an arm-in-cage test, biting rates are still lower in larger cages (Barnard *et al.*, 1998).

While part of the observed discrepancy between field and laboratory are certainly due to differences in mosquito densities, the arm-in-cage test confines the test mosquitoes within an extremely small space next to the repellent-treated arm, a context that is different in many respects from the conditions host-seeking mosquitoes would encounter in nature. In nature, mosquitoes seek hosts from much longer distances, up to tens of metres away from the host. At a longer distance from the host, carbon dioxide (CO_2) initiates upwind flight in the mosquito towards the source while mosquitoes integrate both olfactory and visual cues. Closer to the host, other cues, including host skin volatiles, visual features, heat and moisture are integrated by the mosquito leading eventually the host-seeking female to its target (van Breugel, Riffell, Fairhall et al., 2015; Figure 1). While recent work has shed some light into how female mosquitoes locate a human host by integrating different cues that is context-dependent (Cardé, 2015), it remains largely unknown how topical repellents affect this multimodal sensory integration of cues and how this would affect outcomes of efficacy assays with varying setups.

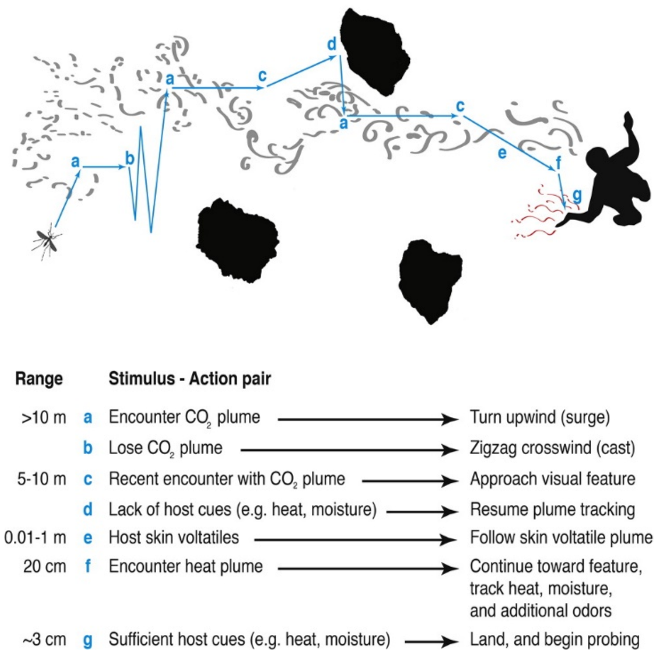

Figure 1: Host-seeking behaviour in mosquitoes. *The figure shows the sequences of stimuli used by female mosquitoes and elicited behavioural modes when seeking a human host for a blood meal. Source: van Breugel et al. (2015).*

Neurological evidence suggests that synthetic repellents like diethyltoluamide (DEET), butylacetylaminopropionate (EBAAP) and icaridin are masking human odours, while natural repellents like lemon eucalyptus oil would actually repel mosquitoes by activating odour receptors (Afify *et al.*, 2019). Yet, the behavioural mode of action of mosquito repellents remains largely unknown because measuring simple endpoints such as the number of bites or landings does not provide any information on how mosquitoes perceive and interact with repellents. Measuring mosquito behaviour beyond simple endpoints such as biting rates has been technically extremely challenging. Thanks to the latest developments, affordable camera systems can now be built that are versatile, and can record and evaluate the behaviour of free-flying insects - including mosquitoes (Hawkes & Gibson, 2016; McMeniman *et al.*, 2014). This opens up new ways to better understand the effect of repellents and ultimately design bioassays that mirror the natural host-seeking behaviour of mosquitoes in a more realistic way. Such bioassays would provide stronger predictive evidence of efficacy under a real use scenario, minimising the risk of falsely failing a product that would actually work under a real-use scenario – or even worse – exposing consumers to unnecessarily high levels of potentially toxic compounds.

The aim of the present study was to configure a three-dimensional (3D) infrared video tracking system for recording mosquito flight paths and measure the behaviour of mosquitoes as they interact with topical repellents.

2. MATERIALS AND METHODS

2.1 Treatments

For the laboratory experiments, we tested four different active ingredients (Table 1) commonly present in commercially available topical mosquito repellent formulations, including diethyltoluamide (DEET), *para*-menthan (PMD), icaridin and ethyl butylacetylaminopropionate (EBAAP). The formulations were prepared by Labor Hartmann GmbH (Uznach, Switzerland) as 20% (m/m) ethanolic solutions specifically for the current study and were stored in the dark at room temperature until use.

Table 1: Active ingredients topically applied in the experiments as 20% (m/m) ethanolic solutions.

Active ingredient	Other names	Formula
Diethyltoluamide (DEET)	*N,N*-Diethyl-*m*-toluamide *N,N*-Diethyl-3-methylbenzamide	$C_{12}H_{17}NO$
Icaridin	1-(1-Methylpropoxycarbonyl)-2-(2-hydroxyethyl)piperidin Bayrepel Picaridin KBR3023	$C_{12}H_{23}NO_3$
para-Menthan (PMD)	1-Methyl-4-isopropylcyclohexanee 4-Isopropyl-1-methyl-cyclohexan	$C_{10}H_{20}$
Ethyl butylacetylaminopropionate (EBAAP)	IR3535® Ethyl *N*-acetyl-*N*-butyl-*β*-alaninate	$C_{11}H_{21}NO_3$

We applied the active ingredients topically on the bare forearm between the wrist and the elbow at an application rate of 1 ml per 600 cm^2 as recommended in the WHO guidelines for testing topical repellents (2009). We adjusted the application volume by estimating the surface area of the forearm, calculated as the distance between the elbow and the wrist, multiplied by the average of the circumferences of the elbow, the wrist and between the elbow and the wrist.

DEET is the oldest active ingredient and had been developed by the US Army in 1946 before it came on the market in 1956 and is, therefore, widely regarded as the "gold standard" for mosquito repellents (Roberts & Reigart, 2004). DEET is widely used in commercially available products today. Icaridin is an alternative synthetic active ingredient that has been developed by molecular modelling techniques in Germany 50 years after DEET. It demonstrates repellent properties comparable to those of the DEET (Boeckh *et al.*, 1996; WHO, 2001). PMD is naturally derived compound that is effective against a range of mosquito species (Carroll & Loye, 2006; Colucci & Müller, 2018; Trigg, 1996). PMD originates from the residuum of the hydrodistillation of the leaves of the lemon eucalyptus tree (*Corymbia citriodora*) but may also be produced synthetically. EBAAP, available under the commercial trademark IR3535® (Merck KGaA, Darmstadt, Germany), is a synthetic molecule derived from the amino acid beta-alanine. It was designed in the early 1970s. Its efficacy against *Ae. aegypti* and *Culex quiquefasciatus* is similar to that of DEET (Cilek *et al.*, 2004), however, *An. gambiae* mosquitoes seem more tolerant to EBAAP (Costantini *et al.*, 2004).

2.2 Human bait

Mosquitoes were attracted to one of the forearms of the person conducting the experiments. This allowed for consistency between experiments as humans may show variable attractiveness to mosquitoes, while, here, we focused on the mosquito rather than variability between human hosts.

Since the study did not involve study participants; and hence does not fall under the Swiss Federal Human Research Act (HRA 810.30), the study protocol was only submitted to the institutional review board but not to Ethikkommission Nordwest- und Zentralschweiz EKNZ.

2.3 Test mosquitoes

For the laboratory experiments, we used two highly anthropophilic mosquito species, the yellow fever mosquito, *Aedes aegypti*, and the Asian malaria vector, *Anopheles stephensi*. Both species are recommended model organisms for testing topical repellents (WHO, 2009).

Aedes aegypti (Figure 2a) is the key vector of several arboviruses (arthropod-borne viruses), including dengue, yellow fever, chikungunya and Zika, and is present throughout the tropics and subtropics. It is a particularly good disease vector as it is competent for many arboviruses, well adapted to breed in small, abundant breeding sites in the urban environment and extremely anthropophilic. Moreover, *Ae. aegypti* bites during the day when people are active outdoors and equally enters human dwellings (Christophers, 1960).

Anopheles stephensi (Figure 2b) is an important urban vector of the malaria parasites, *Plasmodium falciparum* and *P. vivax*, and is widespread from the Middle East to the Indian subcontinent and China. Over the last decade, *An. stephensi* has invaded the Horn of Africa and continues to spread (Carter *et al.*, 2018). Similar to *Ae. aegypti*, the mosquito may use small water containers to breed that are abundant in urban settings. In contrast to *Ae. aegypti*, *An. stephensi* bites at night.

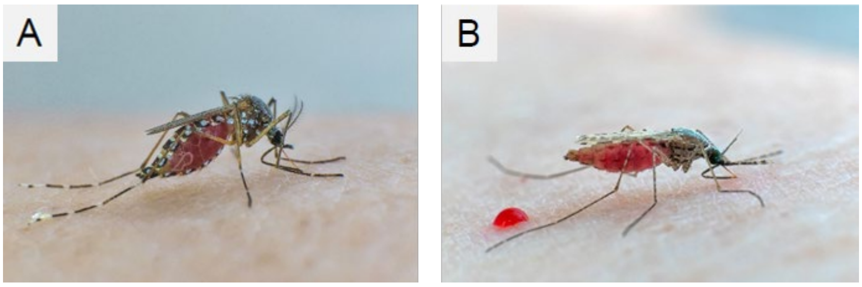

Figure 2: Mosquito species used in the laboratory experiments. A. *Female* Aedes aegypti. ***B.*** *Female* Anopheles stephensi*. Photo credit: Joachim Pelikan, Swiss TPH.*

We maintained the colonies in our specialized facilities at a room temperature of 27±2 °C, a relative humidity of 65±5% and a day-night-cycle of 12 hours. Further details on the origin and maintenance of the *Ae. aegypti* and *An. stephensi* colonies are provided in Owusu *et al.* (2015). In the experiment, we used 5- to 10-day-old females from our laboratory colonies. We removed the female mosquitoes from a stock cage in which females and males were allowed to mate, and mosquitoes had access to water and a 10% sucrose solution *ad libitum*. Before the experiments, we starved the mosquitoes for 14 hours during which they had only access to water but not to the sucrose solution.

2.4 Video tracking system

In order to record the mosquito flight trajectories, we used the software Trackit 3D Fly suite (Scitracks, Bertschikon, Switzerland), installed on a desktop computer in combination with two acA2040-90umNIR USB 3 digital cameras (Basler AG, Ahrensburg, Germany) and three near-infrared (NIR) light emitting diode (LED) lamps that were custom-made by Ingenieurbüro Warnke (Wietzendorf, Germany). Trackit 3D estimates the position of an object based on its contrast against the background; either as a dark object against a bright background or vice versa (Fry *et al.*, 2000). The cameras have a resolution of 4 megapixels and acquire images at a rate of 90 frames per second. The cameras were equipped with Fujinon DV3.4x3.8SA-SA1 lenses (Fujifilm Holdings K.K., Tokio, Japan) and Midopt BP850 NIR band-pass filters (Midwest Optical Systems, Palatine, IL, USA) that only pass light at a wavelength above 850 nm. This combination allows tracking mosquitoes under varying light conditions (*i.e.* bright light or in darkness) in accordance to their dial activity pattern as mosquitoes are not sensitive to light in the NIR range (Gibson, 1995).

The cameras were positioned to suit the experimental needs best and to maximise the cameras' overlapping field of view. Once we had positioned the cameras, we calibrated the system with the Trackit 3D in-built calibration function by moving a board with a defined chequered pattern through the space of the experimental arena. Once the system was calibrated, we tracked a rotating pendulum to validate if the tracking system would produce the trajectories expected from a moving pendulum (Figure 3). The positional data of the mosquitoes, or pendulum, were recorded at a frequency of 50 Hz (*i.e.* the positon was measured every 20 ms) and immediately stored as *x*-, *y*- and *z*-coordinates along the actual time onto the hard drive of the desktop computer.

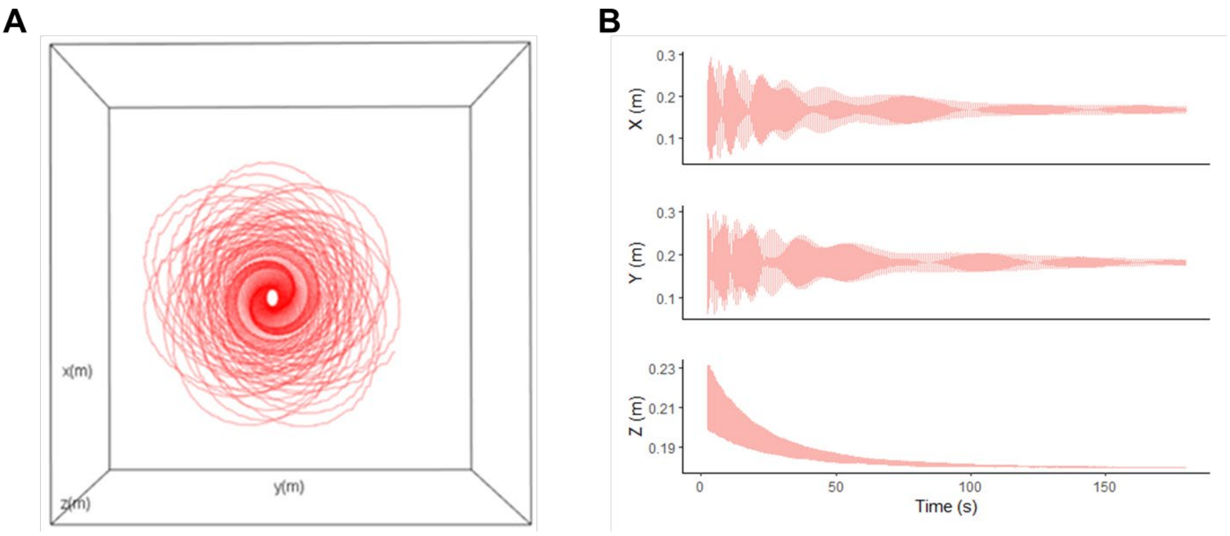

Figure 3: Validation of the calibration of the video tracking system using a pendulum. A. *The pendulum produces a typical rose-shaped pattern when projected to the* xy*-plane.* ***B.*** *Amplitudes as a function of time. As expected they decrease over time until reaching zero.*

2.5 Arm-in-cage experiments

We performed the arm-in-cage experiments in a specialised, climate-controlled walk-in room in the basement of Swiss TPH in Basel.

The design and dimensions of the test cage applied in the present study was based on our standard cage we use for routine evaluation of topical repellents (Colucci & Müller, 2018) with slight modifications allowing for the infrared video tracking system to follow mosquitoes inside a test cage (Figure 4). The experimental cage measured 40 cm x 40 cm x 40 cm while the bottom, side and top panels were made of acrylic glass with two panels being opaque white to produce a homogenously illuminated background against which the mosquitoes could be tracked as a dark object. The front panel, also made of acrylic glass, had a round opening and a sleeve made of netting fabric through which the arm can be inserted and exposed to the mosquitoes. The back panel consisted of mesh allowing some airflow. In addition, the panel pointing away from the cameras was fitted with a cylindrical handle bar that allowed stabilising the arm during the exposure by holding to it and keeping the arm at the same position between assays. In addition to physically stabilise the arm, the software had been updated from the already commercially available version in order to avoid tracking the arm as a mosquito.

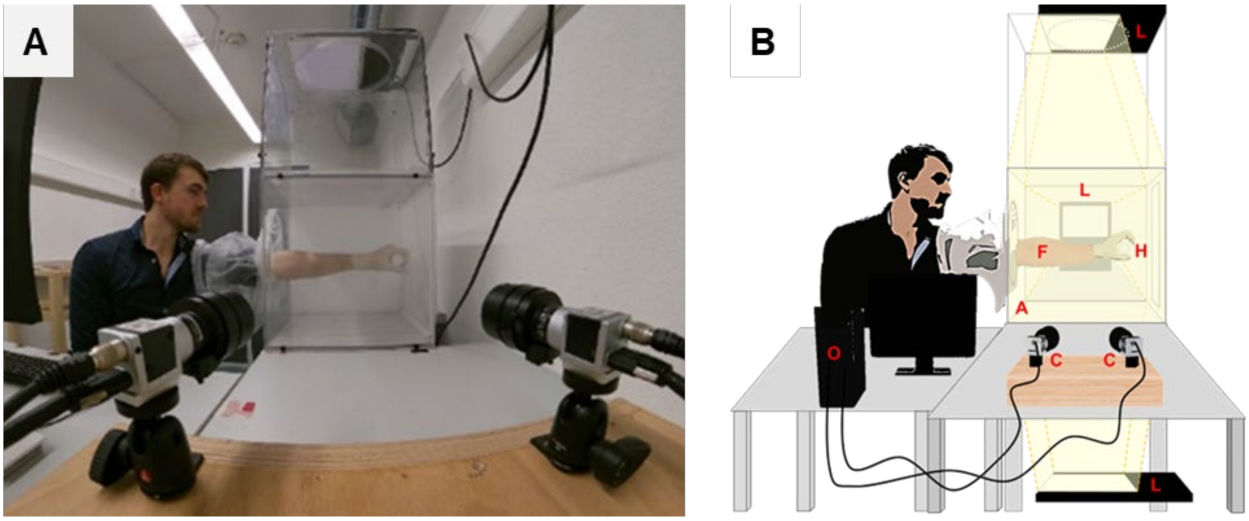

Figure 4: Arm-in-cage test setup with the 3D infrared video tracking system. *The experimental cage measured 40 cm x 40 cm x 40 cm. The bottom, side and top panels are made of acrylic glass with two panels being white opaque to create a homogenously illuminated background against which the mosquitoes could be tracked as a dark object. The front panel, also made of acrylic glass, had a round opening and a sleeve through which the arm can be exposed to the mosquitoes. The back panel (to the right in the image) consisted of a mesh allowing some airflow.* ***A.*** *Original view from the cameras' position.* ***B.*** *Sketch of the setup showing the different elements, including the opening to insert the forearm (F). Inside the cage was a cylindrical handle made of acrylic glass (H) allowing for stabilising the arm and keeping the arm at the same position between experiments. The two cameras (C) are pointing towards the tracking area while three NIR lamps (L) illuminate the cage. The cameras are connected to a desktop computer and screen (O) placed near the experimental cage allowing for simultaneous in controlling the system and exposing the arm to the mosquitoes inside the cage.*

In the standard protocol of the arm-in-cage test, the repellent is applied to the bare forearm between the wrist and the elbow. The repellent-treated arm is then exposed at regular intervals (*e.g.* every 30 or 60 min) for a few minutes during which the number of landings and bites is recorded (EC, 2012b; EPA, 2010; WHO, 2009). The procedure is then repeated until the repellent fails to provide complete protection. Complete protection is defined as the time between application of the topical repellent and the interval with the first confirmed bite. A confirmed bite is a bite followed by a second bite either within the same or during two consecutive exposures.

While in the regular arm-in-cage test, 100 to 200 female mosquitoes are introduced into the cage, we only tracked one mosquito at the time. Tracking only one mosquito allows to unambiguously assigning all behavioural measurements to the same individual, which may not be the case if several mosquitoes are tracked simultaneously even though as such, the system would be capable to do so. Yet, individual mosquitoes could leave the field of view, for example, when flying behind the arm from the cameras' view. Another modification to the procedures of the standard protocol was that we recorded the host seeking behaviour at 30 minutes, 3.5 hours and 6.5 hours post application of the topical repellent solution. However, we kept the 3 minutes exposure time as stated in the WHO guidelines (WHO, 2009).

We performed the experiments under artificial light at an intensity of 640 Lux, a temperature of 27±2 °C, a relative humidity of 65±5% and a CO_2 concentration ranging between 450 ppm and 750 ppm. Ambient temperature, humidity and CO_2 were monitored with Tinytag data loggers (Gemini Data Loggers Ltd, Chichester, UK).

In preparation of an experiment, we shaved the forearms of the human bait because the video tracking system may incidentally track the skin hairs as moving mosquitoes. Before the experiment, we washed both forearms with odourless soap and dried them with a towel. Once dried we swabbed the forearms with ethanol and a paper tissue, and let it air dry.

In the experiment, we randomly treated one forearm with one of the four repellents in Table 1 at an application rate of 1 ml per 600 cm^2 between the wrist and elbow while the other forearm was left untreated. Which arm was treated and the allocation of a repellent on a given experimental day was randomised using the R function "sample" (R Core Team, 2019). The treatment of the forearm took place at 08:30 hours and each arm was exposed for 3 min at 30 min, 3.5 h and 6.5 h after application of the repellent while we randomly selected which of the two arms was exposed first.

Using a mouth aspirator, we released one mosquito into the cage at a time, started the video tracking and exposed the forearm for 3 min. Once the arm was inside the cage, we blew twice air from the mouth into the cage to provide a CO_2 stimulus initiating the mosquito to start host seeking (McMeniman *et al.*, 2014). At the end of the 3-minute exposure, we stopped the video tracking and removed the mosquito from the cage before repeating the procedure with the other forearm.

2.6 Alternative experimental setups

2.6.1 Biogents Cage test

In the arm-in-cage test, the forearm of a person is inserted into the cage containing the host-seeking mosquitoes. This situation is quite challenging for Trackit 3D software because the system tracks mosquitoes as a moving contrast against the background and, therefore, not only mosquitoes but also any object generating a contrast will be incidentally tracked. To avoid unwanted interference of the exposed forearm with the tracking system, we initially set out to work with a simplified set-up in which the arm is not visible to the camera lenses. At the time, an elegant solution seemed to be the Biogents Cage (BG-Cage) test (Figure 5), an alternative laboratory bioassay for measuring the efficacy of topical repellents (Obermayr *et al.*, 2010).

In the BG-Cage test, the repellent-treated forearm is placed underneath the cage and a defined area of the skin is exposed to the mosquitoes through a screened window at the bottom, measuring 3.8 cm x 14.8 cm (Figure 5). In contrast to the cage used in the arm-in-cage test, the BG-Cage measures about half the volume (*i.e.* 41 cm x 41 cm x 16 cm). In the actual test, the BG-Cage is only filled with 30 mosquitoes instead of 100 to 200 individuals as in the arm-in-cage test, and after each single exposure, the air can be replaced by a ventilation system with fresh air to prevent saturation of volatiles inside the cage.

Figure 5: Biogents Cage test. *In contrast to the arm-in-cage test, the arm is placed underneath the cage and mosquitoes can only bite through a screened hole at the bottom. Source: https://eu.biogents.com/wp-content/uploads/slideshow-bg-cage-test-en.jpg*

Unfortunately, the frame and the floor of the BG-Cage consist of metal that is highly reflective in infrared light. Therefore, we painted the frame with black paint that is not visible in infrared light (Figure 6). However, while having solved the issue with the light reflections, the BG-Cage still turned out as not suitable because the frame was partially obstructing the field of view of the cameras.

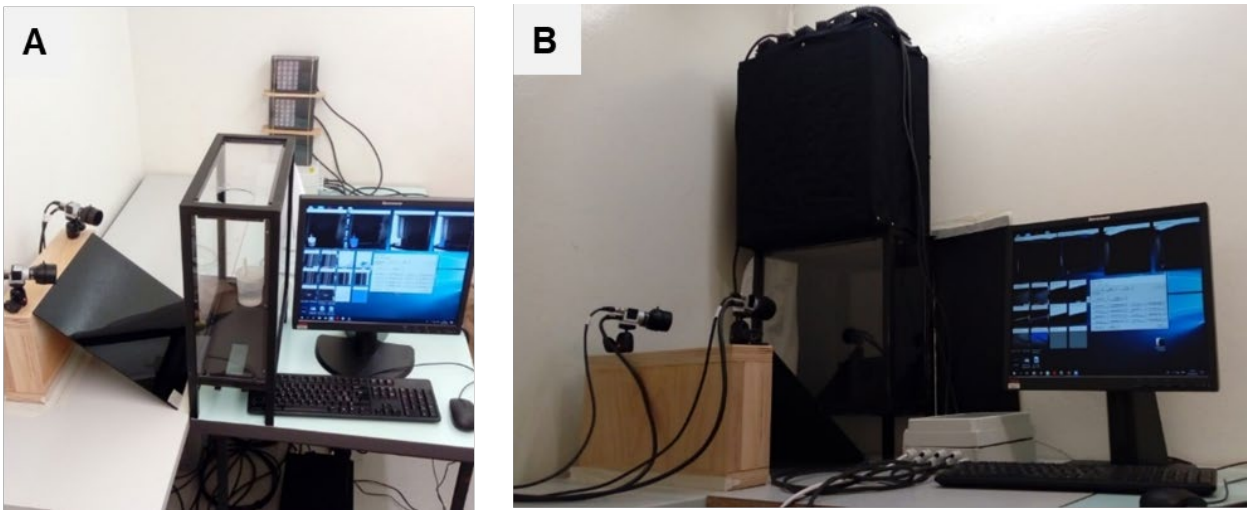

Figure 6: Biogents Cage test in combination with the video tracking system. *In order to track mosquitoes inside the cage of the Biogents test cage different settings were evaluated in an attempt to minimise light reflections on the metal frame of the original cage (Figure 5). A key issue to overcome was the reflections by the metal frame that confused the camera system.* ***A.*** *Biogents cage with three NIR lamps placed on the side of the cage on the wall of the room and* ***B.*** *with one lamp mounted on top of the cage.*

2.6.2 Field setup

One of the original aims of the project was to track mosquitoes also in nature and compare the host seeking behaviour against the artificial setting in the arm-in-cage test. Unfortunately, setting up the tracking system in the laboratory already proofed very challenging and led to significant time delays. One of the key issues in the laboratory we did not anticipate was the difficulty in finding the right light conditions that would provide a homogeneous illumination and good contrast of the tracked mosquito against the background across the entire experimental arena. Another issue was the system could initially not cope with a moving background (*i.e.* the arm inside the cage). For these reasons, we had decided to focus on the laboratory setup first and assure we can reliably track mosquitoes in that setting.

Despite the technical challenges in the laboratory, we were able to develop a setup that would allow tracking mosquitoes under field conditions (Figure 7). However, the system still needs to be fully validated as by the time, the system was up and running under field conditions, no mosquito was landing on the human bait during the field tests. Yet, albeit for less than 1 s we were able to track another insects and we believe the system would be a suitable way forward to equally track mosquitoes in the field.

Figure 7: Video tracking system setup in the field. *Wild mosquitoes approaching the volunteer are tracked by two cameras placed on tripods and tracked against the dark background. As the handle in the arm-in-cage test, the pole serves to stabilise the exposed arm of the experimenter. Except for the forearm, the person was protected from mosquito bites with a bee keeper's hat, an overall and latex gloves through which mosquitoes cannot bite.*

We set up the tracking system at the University of Basel research station in the nature reserve Petite Camargue Alsacienne, France (47°37'18.5" N, 7°32'05.7" E). In contrast to the setup for the arm-in-cage test in the laboratory, here, the mosquitoes are being tracked as bright objects against a dark background. The dark background is provided by a grey woollen blanket (POLARVIDE, IKEA AG, Switzerland). We mounted the blanket, measuring 170 cm x 130 cm, at 180 cm above the ground suspended from a string tied across two tent poles. Similarly to the arm-in-cage video tracking setup in the laboratory, we used a metal pole placed at 110 cm in front of the blanket, serving as a handle for stabilizing the hand of the treated forearm. We mounted the two cameras on tripods at 65 cm above the ground and 220 cm in front of the blanket while the two cameras were separated by 100 cm. The experimental arena was illuminated with four Raytec LED lamps, facing the blanket from a distance of 320 cm. The setup allows for tracking insects within a 3D space of about 1 m^3, while in theory it should be possible to increase the trackable space even further depending on the available light conditions.

2.7 Data analysis

2.7.1 Data processing

Trackit 3D produces Cartesian *x*-, *y*- and *z*-coordinates as a function of time with at a time resolution of 50 Hz, that is the position is measured every 20 ms. The tracking software stores the data as CSV files on the hard drive of the desktop computer. In the CSV file, the positional data are already filtered for outliers and missing observations. We then imported the data into the open source statistical software package R version 4.0.2 (R Core Team, 2019). Before analyzing the data, we plotted the data and visually inspected them for consistency.

2.7.2 Estimating velocity, curvature and torsion

A 3D trajectory may completely be described by its translational velocity $\boldsymbol{V}$ (with a speed *s*), curvature $\boldsymbol{\kappa}$ and torsion $\boldsymbol{\tau}$ as a function of time (Figure 8). As the data points corresponded to a discrete time series with one measurement every 20 ms, we applied the finite helix fit (FHF) approach of Crenshaw et al. (2000) which has previously also been applied to describe flight trajectories for other dipterans (Müller & Robert, 2001).

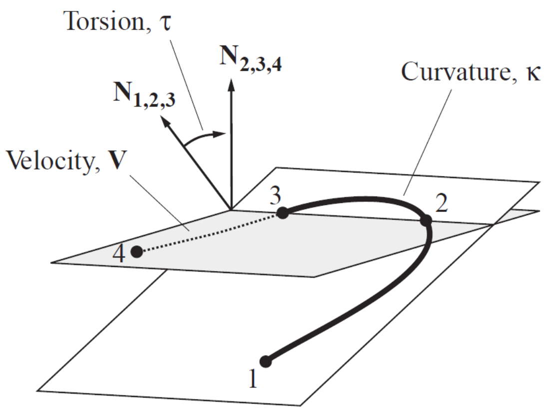

Figure 8: Finite helix fit approach to parametrize 3D flight trajectories. *Velocity* **V** *is defined as the distance covered and is measured in m* s^{-1}*. Curvature* **κ** *is the angular change between the vector given by point 1 to 2 and the vector defined by the positions 2 to 3 and is measured in rad* s^{-1}*. Torsion* **τ** *is defined as the angular change between the subsequent normal vectors* $\mathbf{N}_{1,2,3}$ *and* $\mathbf{N}_{2,3,4}$ *of the planes defined by the points 1–3 and 2–4, respectively. Figure adapted from Müller & Robert (2001).*

2.7.3 Analysis of spatial activity

For the analysis of the spatial activity, we only kept data points when mosquitoes were in mid-air (*i.e.* with a speed above 0.015 ms^{-1}). We grouped and superimposed the trajectories by mosquito species and treatment (*n* = 30 for each condition), including video tracking of mosquitoes flying in an empty cage. For the presentation of the data, we either plotted the superimposed trajectories or counted the number of positional data points within squares of 2.5 cm by 2.5 cm from the superimposed trajectories onto the *xy*-, *xz*- and *yz*-planes.

2.7.4 Definition of mosquito behavioural categories

To quantify and compare mosquito behaviour between the different treatments in the arm-in-cage test we classified the observed behaviour according to six measurable distinct behavioural categories (Figure 9):

Touch and go: Flight trajectories that show a brief contact with the arm (≤1 s) immediately followed by a trajectory pointing away from the arm and rapid acceleration in flight speed.

Bouncing: Flight trajectories that indicate a contact with the arm or glove immediately followed (≤1 s) by another contact

Landing: Flight trajectories with a single contact with the arm with a duration >1 s

Probing: Trajectories that show a single contact with the arm with a duration >1 s followed by probing, blood-feeding or both

Swooping: Tracks that show no contact of the mosquito with the arm while the mosquito was in mid-air

Resting: Tracks when the mosquito was immobile or walking on the walls or the sleeve of the cage and had no contact with the inserted arm (not shown in Figure 9).

The different behavioural categories were analysed in terms of frequency and duration as a function of treatment, exposure interval and mosquito species.

2.7.5 Statistical analysis

We did all statistical analysis in the open source software R (R Core Team, 2019) and created the graphs with the "rgl" (Adler *et al.*, 2020) and "ggplot" (Wickham, 2016) packages.

To estimate average speed, curvature and torsion and to test for differences between species and treatments we fitted generalised linear models. While for speed we could assume Gaussian distributed errors, we transformed the individual values for curvature and torsion before including them in the statistical models. For curvature and torsion, we transformed the data using the Box-Cox method implemented in the function "boxcox" of the R package MASS (Venables & Ripley, 2002). To transform the data we picked the λ value that maximized the log-likelihood based on the plot produced by the boxcoc function. We then back-transformed the data for presentation in the report.

For statistical testing we set the level of significance at $\alpha = 0.05$.

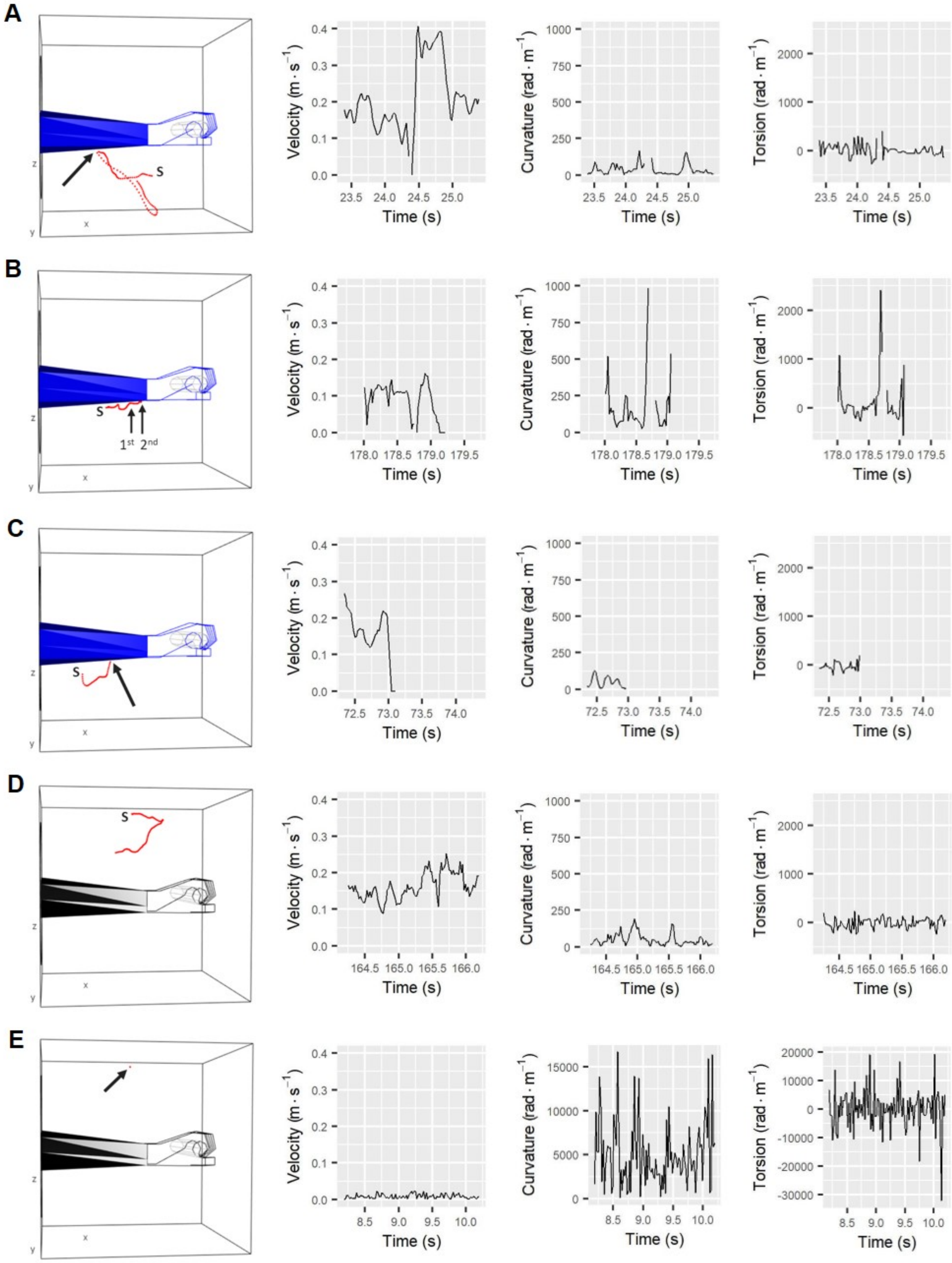

Figure 9: Examples of the different behavioural categories. *The panels on the left show a 3D projection of the trajectories while the panels on the right show the flight FHF parameters, including velocity, curvature and torsion.* ***A.*** *Example of a flight trajectory showing "touch and go".* ***B.*** *Trajectory representing "bouncing".* ***C.*** *Flight trajectory showing "landing".* ***D.*** *Trajectory showing "swooping".* ***E.*** *Trajectory showing "resting". Typical features of the behaviours are indicated by black arrows. Arm colour; blue: right arm, black: left arm. "S" indicates the start of the flight path.*

3. RESULTS AND DISCUSSION

We recorded 480 mosquito flight trajectories, including 240 trajectories of mosquitoes interacting with a repellent-treated arm (*i.e.* 10 mosquito trajectories per combination of repellent, mosquito species and exposure interval post application) and 240 trajectories in combination with an untreated arm. In addition, we recorded 30 trajectories each for *An. stephensi* and *Ae. aegypti* when released into an empty cage. In theory, each trajectory consists of 180 s of *xyz* positional data points at a frequency of 50 Hz, corresponding to 9,000 data points per trajectory and 4,850,000 data points in total. However, the tracking system could not always track the position of the mosquito because mosquitoes could leave the field of view, for example, when flying behind the arm. In addition, we removed the first 8 seconds as this was the time lag to allow for inserting the arm and for the system to learn the background before initiating the tracking of the mosquitoes.

3.1 Location of spatial activity

While the spatial activity between the treatments was fairly consistent, there was a distinct difference in the spatial activity pattern between *Ae. aegypti* and *An. stephensi*. When an arm was inserted, *Ae. aegypti* spent most of the time flying in the upper part of the cage above the arm. In contrast, *An. stephensi* about evenly occupied the entire space of the cage while host seeking (Figure 10).

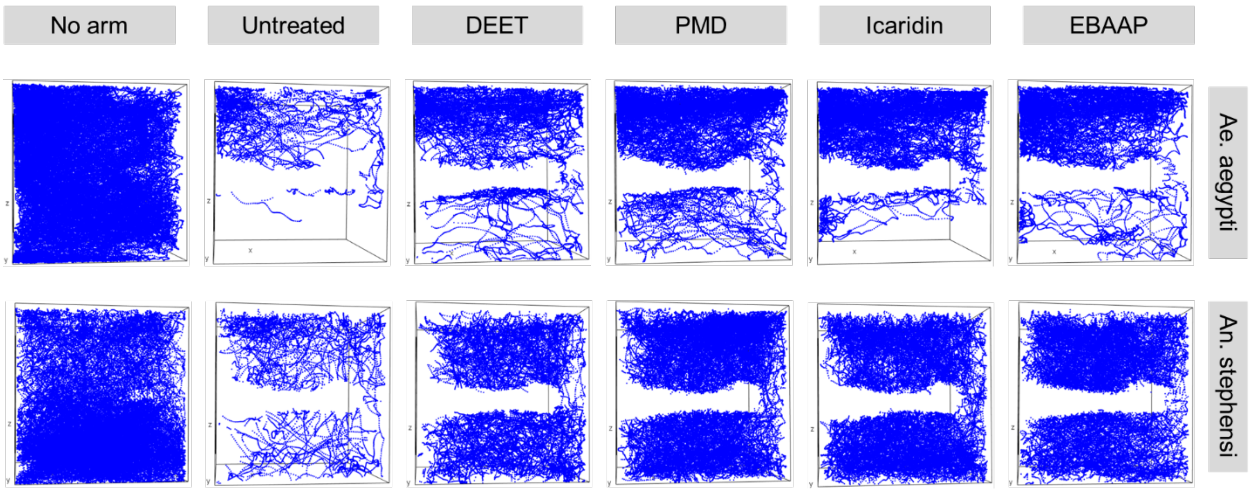

Figure 10: Summary of spatial activity in the arm-in-cage test cage. *Each panel shows the summary of all trajectories recorded by treatment and mosquito species across all tests.*

A possible explanation for the difference in the spatial activity between the two species could be that they are differentially attracted to human odours. *Aedes aegypti* is an extremely anthropophilic species (Scott *et al.*, 1993), while *An. stephensi* is more opportunistic and shows a variable preference for humans and animals depending on host species availability (Sinka *et al.*, 2011). Moreover, olfaction, vision and heat trigger independent host-seeking behavioural modules (van Breugel *et al.*, 2015) and might be used in a different way by the two test species, which could be reflected in their flight trajectories. In this context, it is noteworthy that *Ae. aegypti* is a day active mosquito (Christophers, 1960) and, therefore, the mosquito likely relies even more on visual cues than *An. stephensi* albeit it has been shown that vision also plays a role in nocturnal *Anopheles* mosquitoes (Gibson, 1995; Hawkes & Gibson, 2016). Van Breugel et al. (2015) have also shown for *Ae. aegypti* that CO_2 – which was blown into the cage at the onset of the experiments – initiates not only host-seeking behaviour as previously described by McMeniman *et al.* (2014) but also elicits a strong attraction to visual features.

However, even without a stimuli and no arm in the cage the two mosquito species still show a different pattern in using the space of the cage, which becomes apparent from Figure 10 and even more so from the projections onto the *xz*- and *yz*-planes in Figure 11 and Figure 12. While *Ae. aegypti* spent most of the time flying near the top of the cage and towards the sleeve for the arm, *An. stephensi* was mainly flying in the lower far corner of the cage. When presented to a repellent-treated arm, *Ae. aegypti* was flying mainly above the arm (Figure 11), while *An. stephensi* used the space of the cage more evenly - with the exception of PMD where they also flew close to the top panel of the cage (Figure 12).

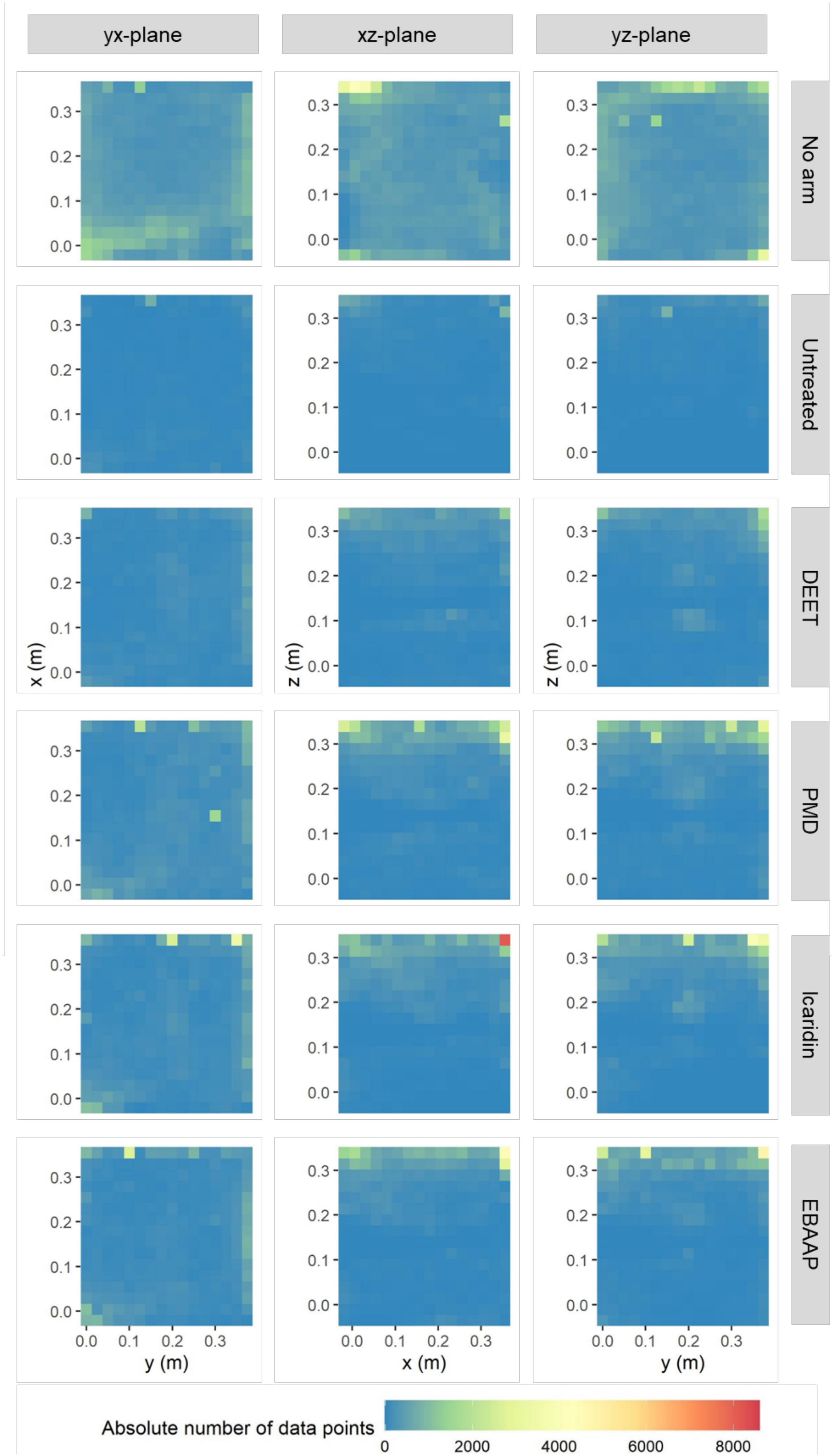

Figure 11: Two dimensional projection of spatial activity in* Aedes aegypti *in the arm-in-cage test cage. *Each panel shows the summary of all trajectories recorded by treatment and mosquito species across all tests projected onto the yx-, xz- and yz-plane.*

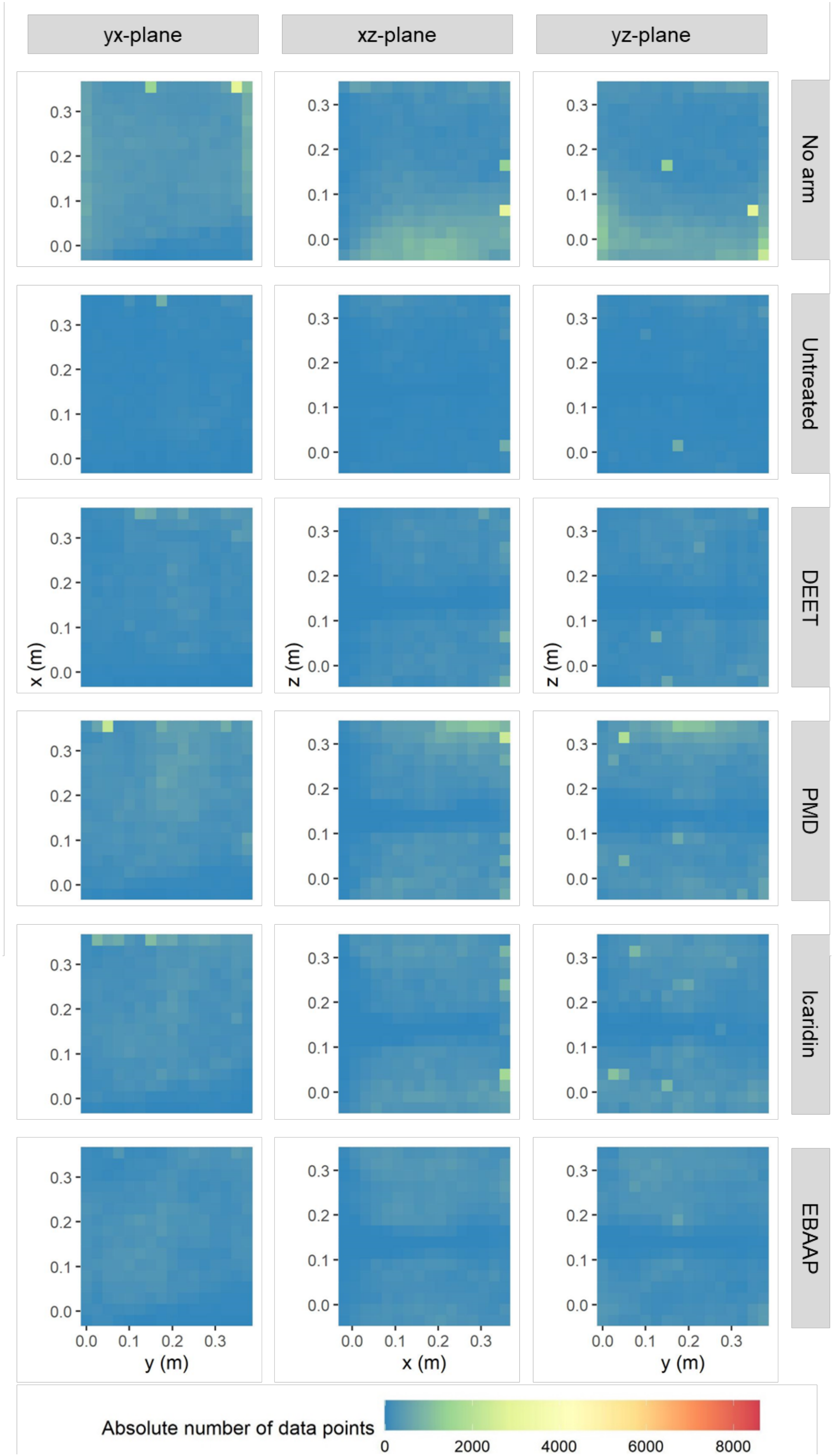

Figure 12: Two dimensional projection of spatial activity in* Anopheles stephensi *in the arm-in-cage test cage. *Each panel shows the summary of all trajectories recorded by treatment and mosquito species across all tests projected onto the yx-, xz- and yz-plane.*

3.2 Analysis of velocity, curvature and torsion

The average velocity (*i.e.* instantaneous flight speed) depended on the mosquito species and whether the arm was treated with a repellent or not, while we neither saw a statistically significant difference between the repellents nor the exposure intervals (Figure 13). In *Ae. aegypti* we also saw differences in the flight speed in the empty cage.

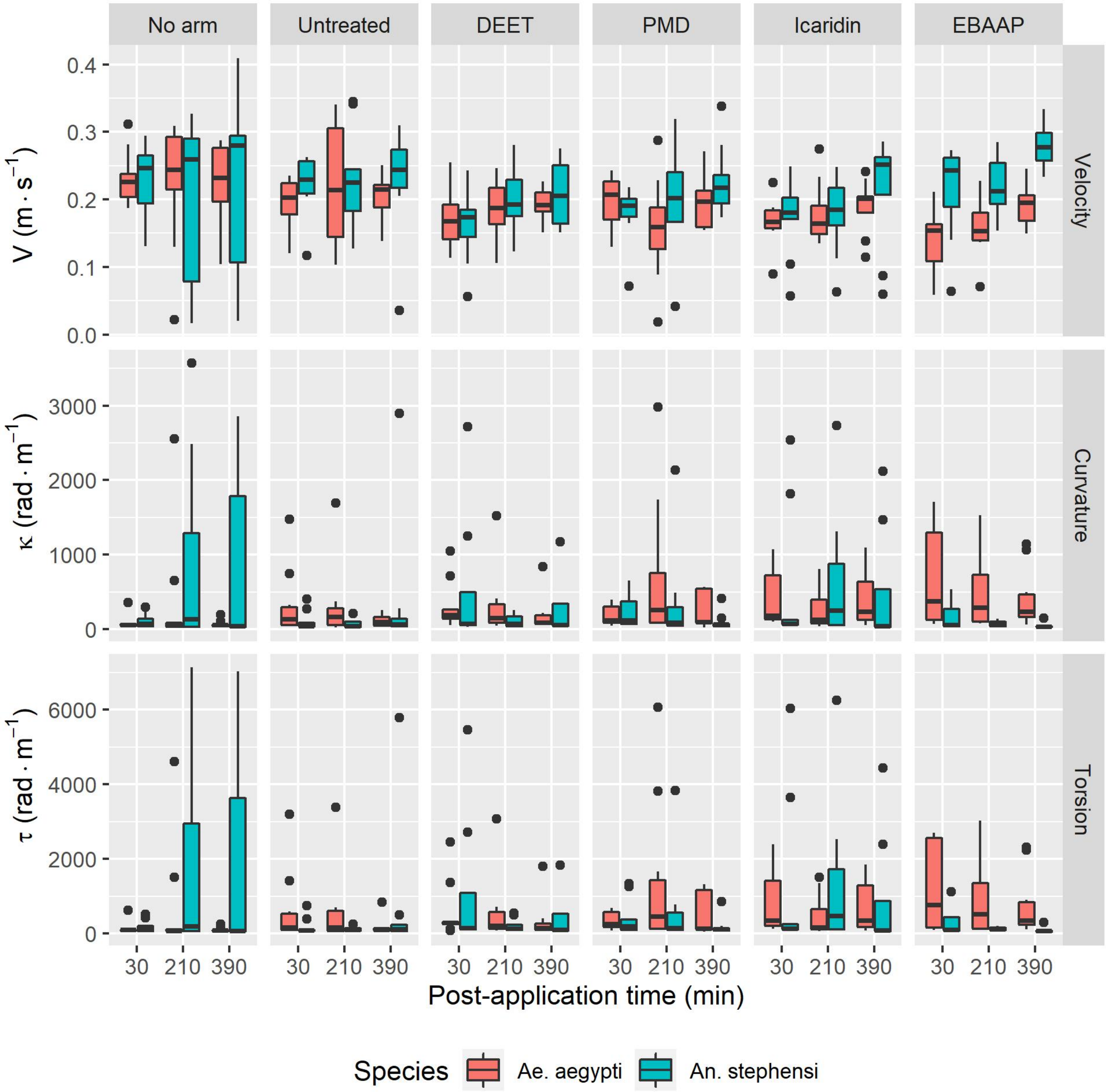

Figure 13: Summary statistics of the FHF estimates for velocity, curvature and torsion. *The centre line through each box represents the median while the boxes represent the interquartile distance (IQD). The whiskers extend to the extreme values of the data (*i.e. *±1.5×IQD from the median) and dots indicate possible outliers. Since positive and negative values would average to zero, torsion is presented in absolute values.*

In contrast to *Ae. aegypti*, *An. stephensi* flew with an overall mean speed of 0.207 $m \cdot s^{-1}$ (95% confidence interval, 95% CI: 0.197 – 0.22 $m \cdot s^{-1}$). On average, that is 0.019 $m \cdot s^{-1}$ (95% CI: 0.005 – 0.033 $m \cdot s^{-1}$) faster than *Ae. aegypti* (t = 2.736, $p < 0.01$), while the differences between the treatments were statistically not significant. To our knowledge, there are no data available from the literature for flight speed in *An. stephensi*. Measurements in the related species, *An. gambiae* s.l., have shown slightly higher values. For example, based on two dimensional flight trajectories, Parker et al. (2017) estimated the average instantaneous flight speed of *An. arabiensis* released into an experimental hut in Tanzania at 0.327 $m \cdot s^{-1}$ while under laboratory conditions, *An. gambiae* s.s. flew with an average speed of 0.346 $m \cdot s^{-1}$ (Parker *et al.*, 2015). Similar values have been reported for *An. coluzzii* for tortuous flight tracks with a speed of 0.29 $m \cdot s^{-1}$ from mosquitoes in a flight tunnel while also using a Trackit 3D system (Hawkes & Gibson, 2016).

Aedes aegypti flew with an average speed of 0.216 $m \cdot s^{-1}$ (95% CI: 0.189 – 0.244 $m \cdot s^{-1}$) when the cage was empty which is similar to flight speed measurements for wild *Ae. aegypti* mosquitoes tethered to a flight mill (*i.e.* about 0.19 $m \cdot s^{-1}$; Bargielowski et al., 2012). However, in contrast to *An. stephensi*, flight speed was lower in *Ae. aegypti* when mosquitoes were exposed to a treated arm ($t = -4.700$, $p < 0.001$). In that case, the average flight speed was reduced by 0.05 $m \cdot s^{-1}$ (95% CI: 0.062 – 0.039 $m \cdot s^{-1}$).

The curvature in *An. stephensi* was on average 76.5 $rad \cdot m^{-1}$ (95% CI: 66.6 – 88.7 $rad \cdot m^{-1}$), while *Ae. aegypti* made turns that were about twice as sharp ($t = -4.453$, $p < 0.001$). In *Ae. aegypti*, the average curvature was 137.2 $rad \cdot m^{-1}$ (95% CI: 118.3 – 160.3 $rad \cdot m^{-1}$). Although there was a slight statistical tendency for a reduced curvature when the cage was empty, average curvature was not statistically significantly different between the treatments for *An. stephensi*. In contrast, there was a statistical significantly increase in the curvature for EBAAP ($t = 3.184$, $p < 0.01$), icaridin ($t = 2.242$, $p < 0.05$) and when no arm was in the cage ($t = -2.909$, $p < 0.01$). However, the differences are minute and its biological relevance questionable as they were 1.2 $rad \cdot m^{-1}$ (95% CI: 1.1 – 1.3 $rad \cdot m^{-1}$), 1.1 $rad \cdot m^{-1}$ (95% CI: 1.0 – 1.2 $rad \cdot m^{-1}$) and 0.9 $rad \cdot m^{-1}$ (95% CI: 0.8 – 1.0 $rad \cdot m^{-1}$) for EBAAP, icaridin and no arm, respectively. In conclusion, we saw marked differences in curvature between the two mosquito species but not between treatments.

The pattern in average torsion is very similar to curvature (Figure 14). Indeed, the two parameters were highly correlated with each other ($r = 0.99$, $p < 0.001$). A possible explanation is that, on average, the mosquitoes' flight trajectory would follow a helical flight path while sharper turns would also lead to curvature. From Figure 14 it becomes also apparent that curvature and torsion depend on velocity. The data may be interpreted as mosquitoes of both test species flew in a straighter manner at higher speed as we would intuitively expect.

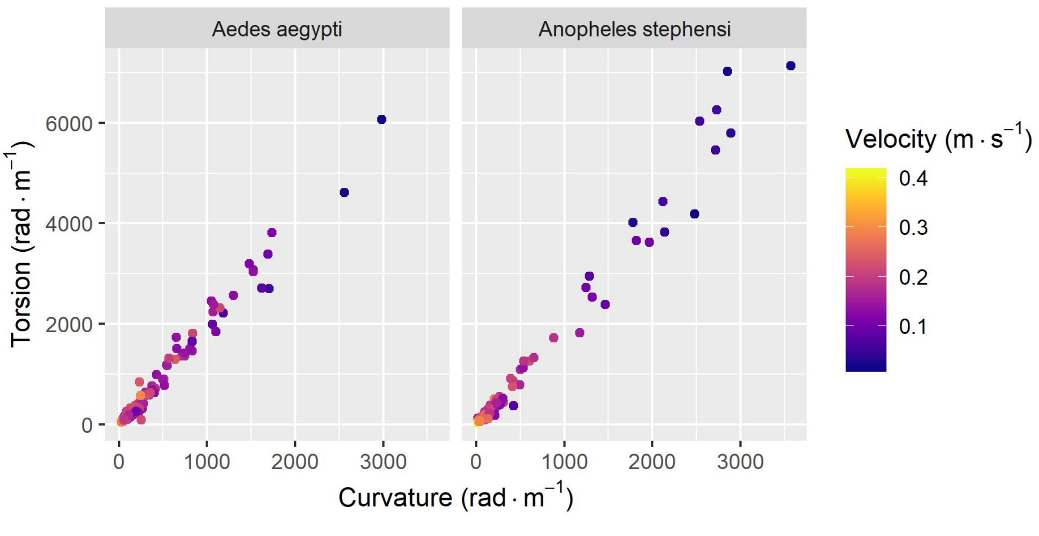

Figure 14: Relationship between velocity, curvature and torsion. *Average curvature and torsion are highly correlated in both mosquito test species (r = 0.99, p < 0.001). Both curvature and torsion depend on velocity; the faster the mosquitoes flew, the straighter were their trajectories.*

3.3 Analysis of behavioural categories

3.3.1 Mosquito interactions with the exposed arm inside the cage

All four repellents reduced the number of bites substantially while simultaneously increasing the number of contacts (Table 2). Most of the time, the mosquitoes were either bouncing or showing a "touch and go" behaviour (Figure 14 and Figure 15), suggesting that they were attracted to the arm but irritated upon contact with the repellent. This may be interpreted as "taste and avoid". Intriguingly, it has been previously shown for mutant *Ae. aegypti* that they are not repelled by volatile DEET but still repelled upon contact with DEET, suggesting repellents may have multi-modal actions (DeGennaro *et al.*, 2013).

Table 2: Total number of mosquitoes that probed or landed on the exposed arm across all tests

Treatment	Species	Total	Contact with arm	Bites
Untreated	*Aedes aegypti*	30	3	26
	Anopheles stephensi	30	5	26
DEET	*Aedes aegypti*	30	10 $Z = 5.467$; $p < 0.0001$	6 $Z = -3.082$; $p < 0.01$
	Anopheles stephensi	30	15 $Z = 2.517$; $p < 0.05$	1 $Z = -3.197$; $p < 0.01$
PMD	*Aedes aegypti*	30	20 $Z = 5.835$; $p < 0.0001$	7 $Z = -3.082$; $p < 0.01$
	Anopheles stephensi	30	19 $Z = 5.236$; $p < 0.0001$	6 $Z = -3.238$; $p < 0.01$
Icaridin	*Aedes aegypti*	30	19 $Z = 4.621$; $p < 0.0001$	6 -3.238; $p < 0.01$
	Anopheles stephensi	30	17 $Z = 5.379$; $p < 0.0001$	1 $Z = -3.197$; $p < 0.01$
EBAAP	*Aedes aegypti*	30	15 $Z = 3.976$; $p < 0.0001$	6 -3.238; $p < 0.01$
	Anopheles stephensi	30	19 $Z = 5.728$; $p < 0.0001$	11 $Z = -2.215$; $p < 0.05$

The number of contacts with the arm and the number of probing were counted mutually exclusively so that a probing was not counted as a contact and vice versa. The values in small print are the test statistics from a generalised linear model with the number of probing or contacts as the outcome and the repellent as the fixed term with a Poisson distribution and a log link function. Total: number of mosquitoes. Contact with arm: the number of how many times the mosquitoes had contact with the arm. Bites: the number of mosquitoes probing (i.e. biting).

Afify *et al.* (2019) found that natural repellents, including lemon grass oil and eugenol, elicit a strong response of the olfactory neurons in *An. coluzii*, while DEET, EBAAP and icaridin did not have the same effect, presumably because synthetic repellents reduce the amount of odorants reaching the mosquito's antenna. Since in our experiments the mosquitoes still reached the arm, our results seem not to corroborate these findings. However, apart from using other mosquito species, an explanation would be that in the current setup mosquitoes might rely on different or additional, non-olfactory cues provided by the exposed arm. Indeed, mosquitoes integrate multiple cues when seeking a human host, including vision, temperature and humidity and - depending on the context of stimulus presentation - this may determine behavioural outcomes (Cardé, 2015). While the mosquitoes may rely on close-range host cues other than odorants, they are still repelled by the repellents. Either the repellents still have an effect on olfactory mechanisms mediated through the mosquito's antenna or the effect is rather contact-mediated, a hypothesis that seems to be supported by the observations made in this study. If this is indeed the case, relying on arm-in-cage tests for product evaluation and registration could potentially lead to false conclusions failing repellents that would actually work in a more realistic use scenario such as a field trial even in a room test.

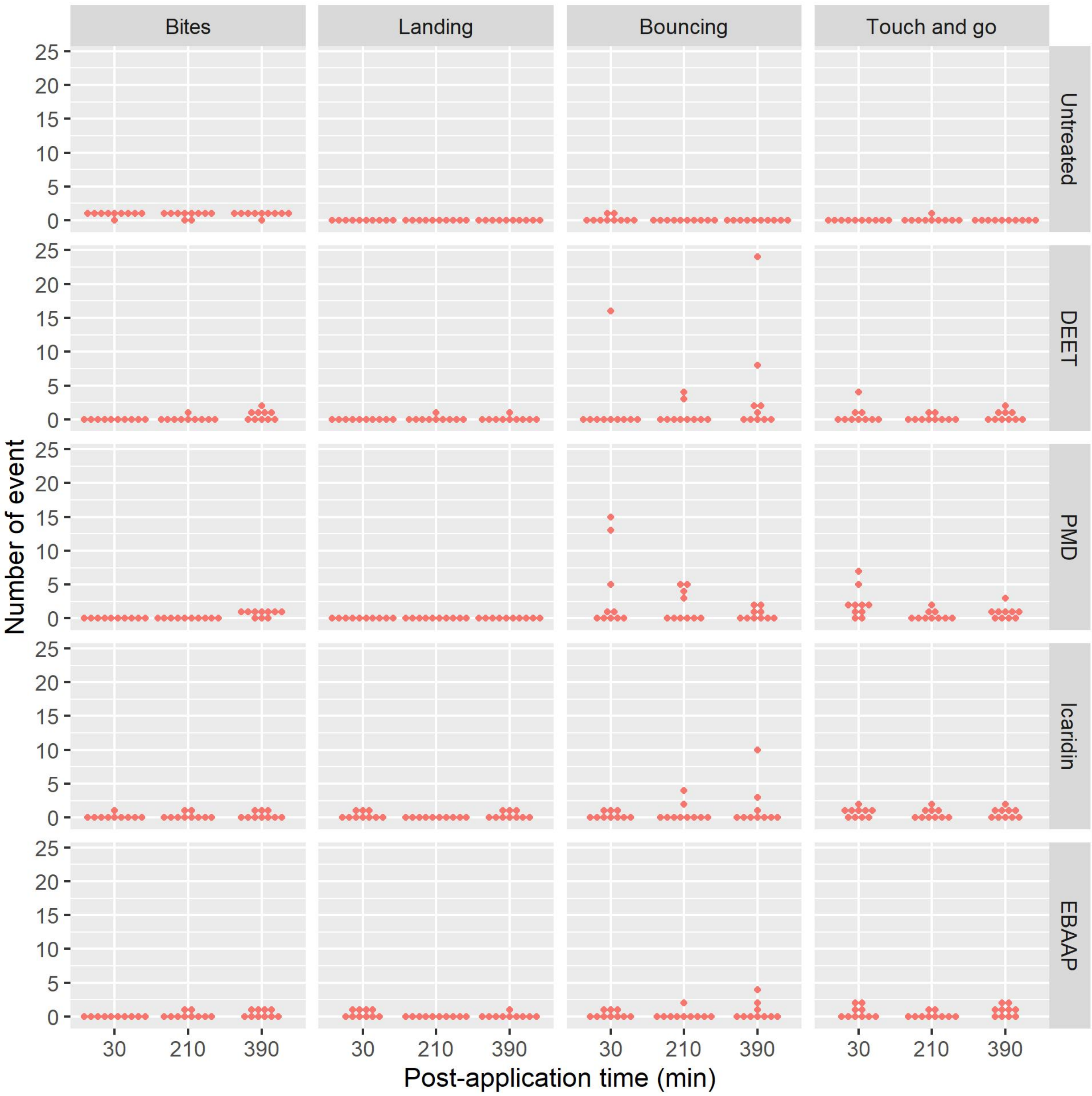

***Figure 15: Summary of different types of interactions with the arm of the human bait in* Aedes aegypti.** *Each dot represents a single mosquito (n = 30 per treatment). Landings and bites were counted mutually exclusively.*

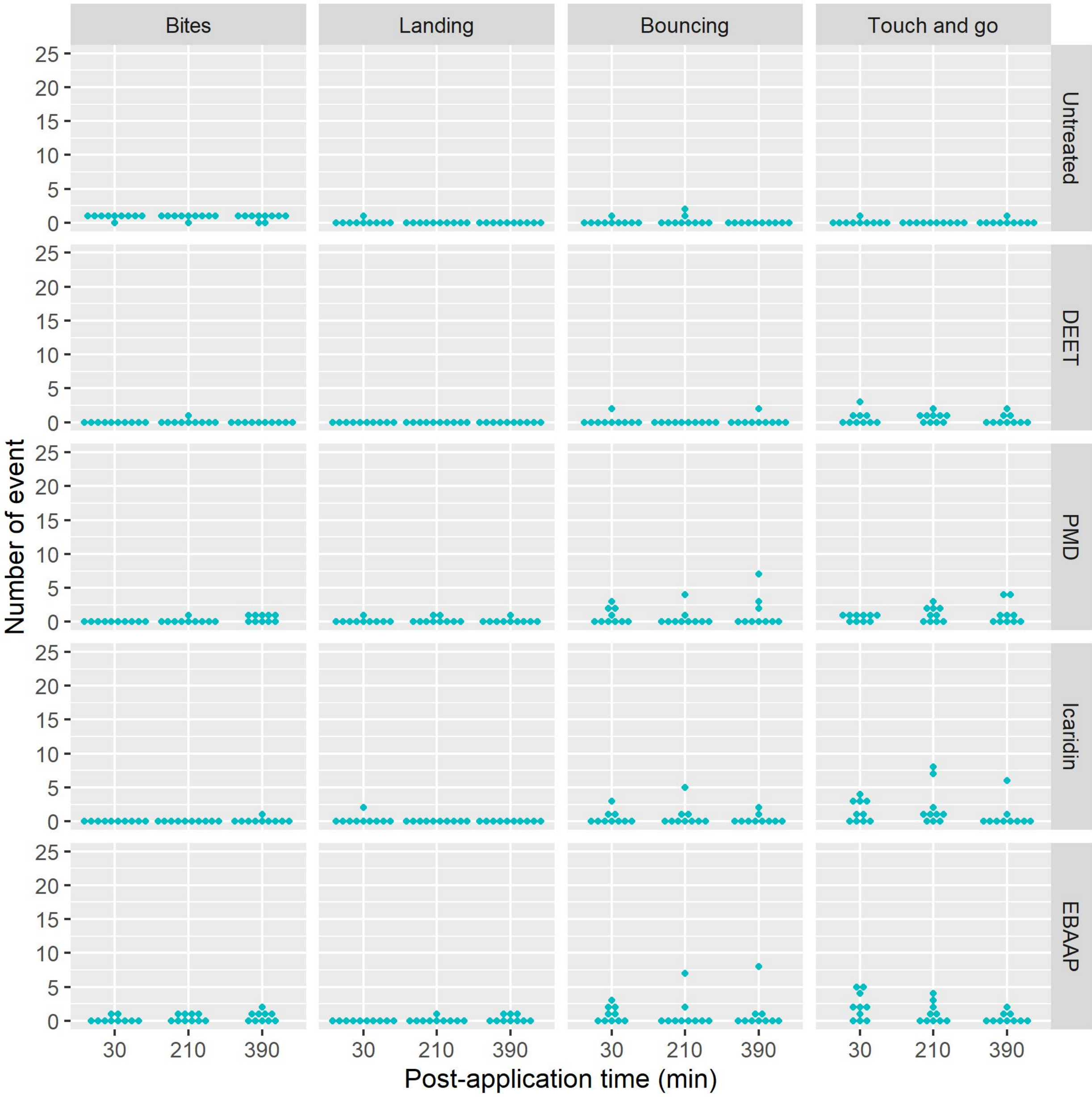

Figure 16: Summary of different types of interactions with the arm of the human bait in* Anopheles stephensi*. *Each dot represents a single mosquito (*n *= 30 per treatment). Landings and bites were counted mutually exclusively.*

3.3.2 Mosquito activity over time within a single exposure

While a single exposure of the arm was 180 s, we removed the first 8 s from the individual data sets since they correspond to the time needed to insert the arm and grab the handle. This initial movement of inserting the arm creates a lot of background noise, taking a short while for the camera system to adapt.

During a single exposure, both *An. stephensi* (Table 3) and *Ae. aegypti* (Table 4) spent most of the time resting, except when the arm was untreated and the mosquitoes only landed shortly before starting to probe and taking a blood meal. The second longest behavioural mode after resting was swooping. Again, in the case of the untreated arm, swooping was a lot shorter as the mosquitoes almost immediately located the arm and spent little time in mid-air.

Table 3: Mean total activity time (seconds) of female* Anopheles stephensi *spent in different behavioural categories over the 3 min exposures in the arm-in-cage tests

Treatment	Bouncing	Swooping	Touch'n'go	Landing	Probing	Resting
DEET	0.1 (0.0 – 2.0)	18.2 (0.0 – 64.5)	0.6 (0.0 – 3.0)	0.0 (0.0 – 0.0)	2.5 (0.0 – 75.0)	150.6 (57.5 – 172.0)
EBAAP	0.9 (0.0 – 8.0)	24.3 (0.0 – 79.8)	1.2 (0.0 – 5.0)	2.0 (0.0 – 39.0)	22.0 (0.0 – 139.0)	122.4 (27.9 – 172.0)
Icaridin	0.5 (0.0 – 5.0)	30.8 (0.0 – 83.0)	1.4 (0.0 – 8.0)	0.5 (0.0 – 15.0)	0.6 (0.0 – 18.0)	138.1 (87.0 – 172.0)
PMD	0.8 (0.0 – 7.0)	37.1 (0.0 – 104.1)	0.9 (0.0 – 4.0)	1.5 (0.0 – 21.0)	12.8 (0.0 – 159.0)	118.8 (10.5 – 172.0)
Untreated	0.1 (0.0 – 2.0)	6.8 (0.0 – 24.3)	0.1 (0.0 – 1.0)	0.5 (0.0 – 14.0)	112.2 (0.0 – 170.9)	52.4 (0.0 – 172.0)

The first 8 seconds during which the arm was introduced into the cage were removed from the data set. All categories were mutually exclusively measured. The numbers in brackets show the minimum and maximum for each behavioural category.

Table 4: Mean total activity time (seconds) of female* Aedes aegypti *spent in different behavioural categories over 3 min exposures in the arm-in-cage tests

Treatment	Bouncing	Swooping	Touch'n'go	Landing	Probing	Resting
DEET	2.0 (0.0 – 24.0)	16.4 (0.0 – 60.1)	0.4 (0.0 – 4.0)	0.4 (0.0 – 7.0)	15.1 (0.0 – 163.0)	137.7 (1.2 – 172.0)
EBAAP	0.4 (0.0 – 4.0)	28.1 (0.0 – 62.5)	0.5 (0.0 – 2.0)	0.5 (0.0 – 6.0)	4.8 (0.0 – 58.0)	137.6 (104.6 – 172.0)
Icaridin	0.8 (0.0 – 10.0)	30.1 (0.0 – 97.6)	0.6 (0.0 – 2.0)	0.9 (0.0 – 9.0)	9.6 (0.0 – 102.0)	130.0 (54.1 – 172.0)
PMD	1.9 (0.0 – 15.0)	31.1 (0.0 – 99.1)	1.1 (0.0 – 7.0)	0.0 (0.0 – 0.0)	7.8 (0.0 – 87.0)	130.1 (67.9 – 172.0)
Untreated	0.1 (0.0 – 1.0)	6.2 (0.0 – 30.5)	0.0 (0.0 – 1.0)	0.0 (0.0 – 0.0)	117.7 (0.0 – 171.9)	48.1 (0.0 – 172.0)

The first 8 seconds during which the arm was introduced into the cage were removed from the data set. All categories were mutually exclusively measured. The numbers in brackets show the minimum and maximum for each behavioural category.

The time allocation to different behaviours within a single exposure is shown in Figure 16. Here, we grouped the durations of mosquito activity throughout a single exposure into eight intervals of 21.5 s.

When the arm was untreated, the average time a mosquito required locating the arm and to land on it was about 1 min (*i.e.* within the 3rd interval in Figure 16). The time spent probing on the untreated forearm is considerably longer than with any of the topical repellents tested, even if the repellent failed to provide complete protection. When mosquitoes were exposed to a treated forearm, their flight activity decreased towards the end of the exposure period even if they did neither land nor probe. Mosquitoes becoming irritated, and perhaps even intoxicated, upon physical contact with the repellent, or were they were giving up, may explain this observation.

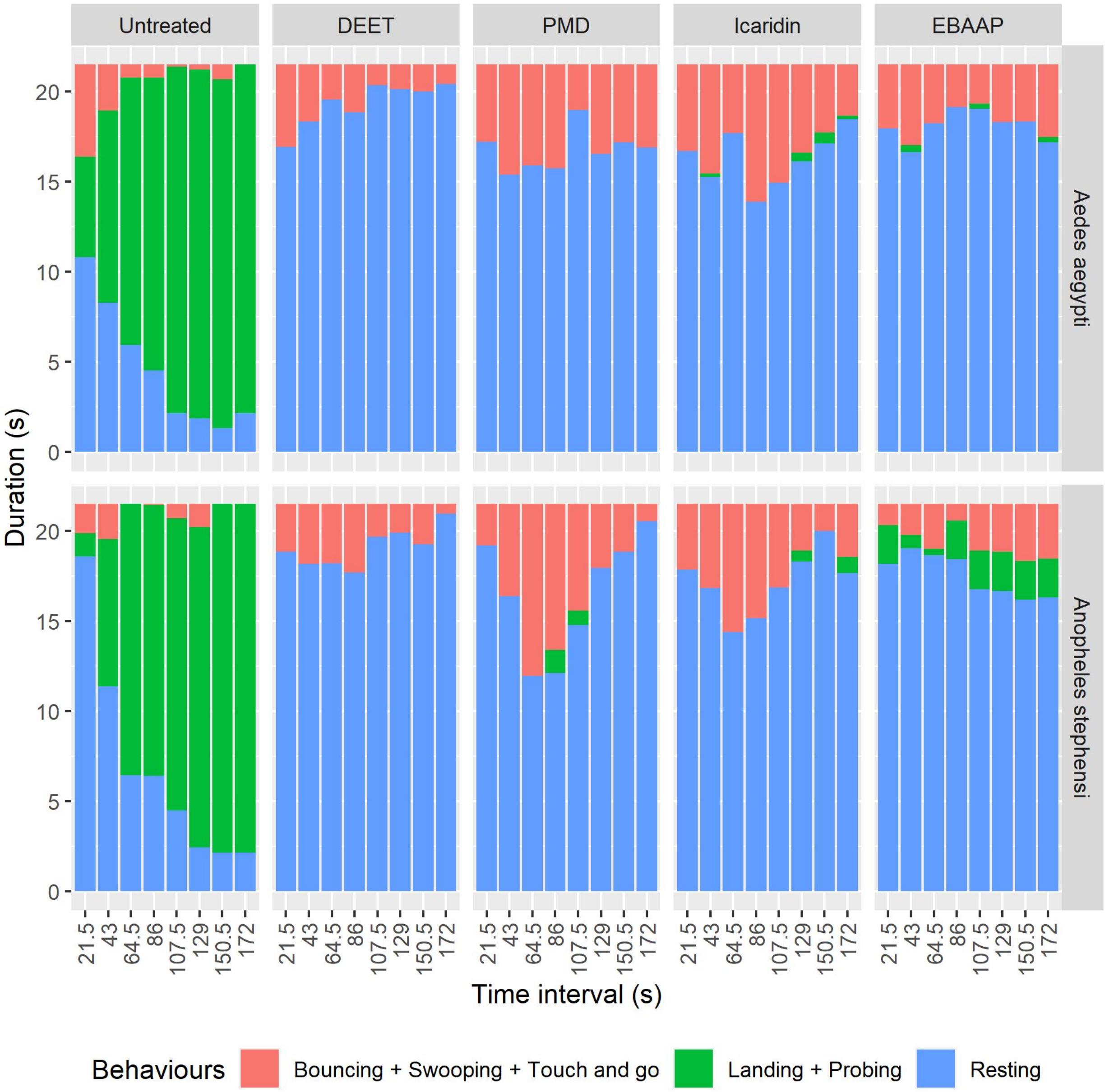

Figure 17: Mean total activity time during the first exposure spent on different behavioural categories. *The colours correspond to behavioural categories grouped by "active and flying" (red), "active but stationary" (green), and "stationary while inactive" (blue).*

Among the repellents, DEET seems to affect behaviour most in that the mosquitoes spent less time flying but more time resting while landing and probing was very brief, if at all. We observed most landing and probing activity in *An. stephensi* in combination with EBAAP. EBAAP had a weaker effect on *An. stephensi* than *Ae. aegypti* as shown by the increased proportion of landing and probing and shortened time spent on flying.

3.3.3 Mosquito activity across the three exposure intervals

In the control, the mosquitoes spent a similar amount of time between the different activities across the three exposure periods (*i.e.* 30, 210 and 390 min) with most time spent on landing and probing (Figure 17). There is a tendency that *Ae. aegypti* spends more time flying at the beginning of the experiment, while *An. stephensi* shows more activity towards the end of the experiment. This could be an effect of diurnal activity patterns and merits further investigation.

Time spent on landing and probing was markedly reduced when the mosquitoes interacted with a repellent-treated arm (Figure 17). Here the host-seeking mosquitoes spent more time in mid-air. While flying activity was increased for all repellents, activity showed to be lowest in mosquitoes when they were exposed to DEET. In contrast, mosquitoes were more active when interacting with PMD while PMD showed the least effect in *An. stephensi*.

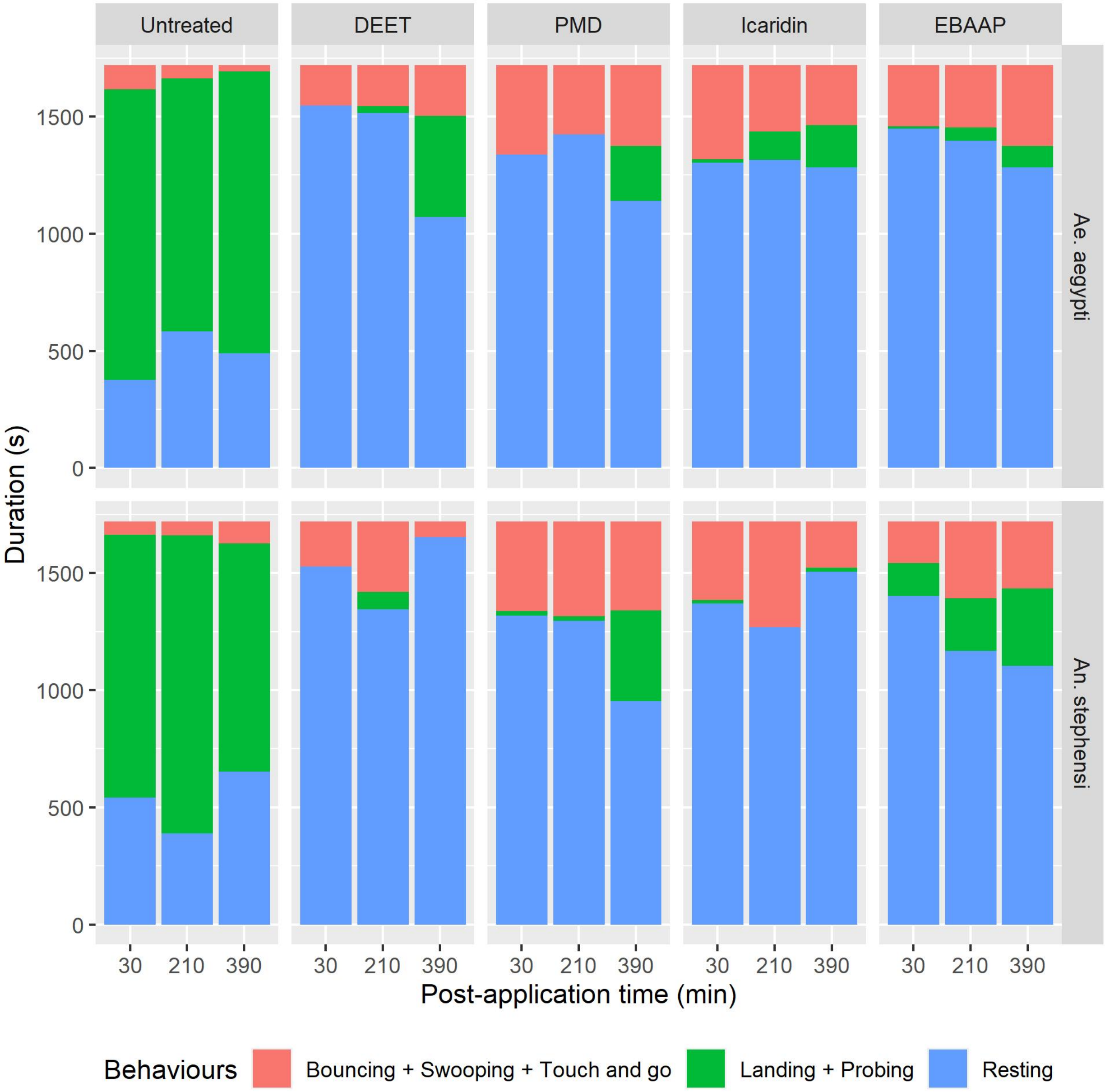

Figure 18: Total activity time during the different exposure intervals in the different behavioural categories. *The colours correspond to behavioural categories grouped by "active and flying" (red), "active but stationary" (green), and "stationary while inactive" (blue).*

4. CONCLUSIONS AND RECOMMENDATIONS

To gauge the value of the arm-in-cage test and to make evidence-based recommendations to improve efficacy testing, it is essential to understand how mosquitoes perceive and interact with topical repellents in different settings and its context. Here, we measured the behaviour of laboratory-reared *Ae. aegypti* and *An. stephensi* females as they interact with a repellent-treated forearm in the arm-in-cage test using a 3D infrared video camera system to track their flight paths. In this setting, we found no clear evidence for mosquitoes being repelled by volatile chemicals. We hypothesis that gustatory excito-repellency upon contact with the treated skin is the topical repellent's key mode of action in the context of the arm-in-cage test. Indeed, the context in which stimuli are presented and how they are perceived and integrated by the mosquito's sensory system may lead to different behavioural outcomes (Cardé, 2015). This raises the question as to how representative the arm-in-cage assay is for a real use scenario where mosquitoes seek humans from larger distances and have neither contact with the treated arm nor do they rely on the same host cues for its location.

Our initial plan was to do measurements both in the arm-in-cage test and in the field to compare how well the laboratory setup reflects the situation under the real use scenario. Yet, we have not achieved measuring behaviour in nature due to technical challenges at the beginning with the laboratory set-up. We also realise that before going into the field, observations under more controlled conditions, such as a walk-in chamber or a semi-field setup, would allow for better comparison between different situations while maintaining factors such as mosquito species, ambient temperature and humidity constant.

As a next step, we, therefore, suggest to repeat similar experiments in which we record again the flight paths but in a larger space such as the walk-in chamber shown (Figure 18a). The experiment could even be expanded to a larger room even set up in a semi-field environment (Figure 18b).

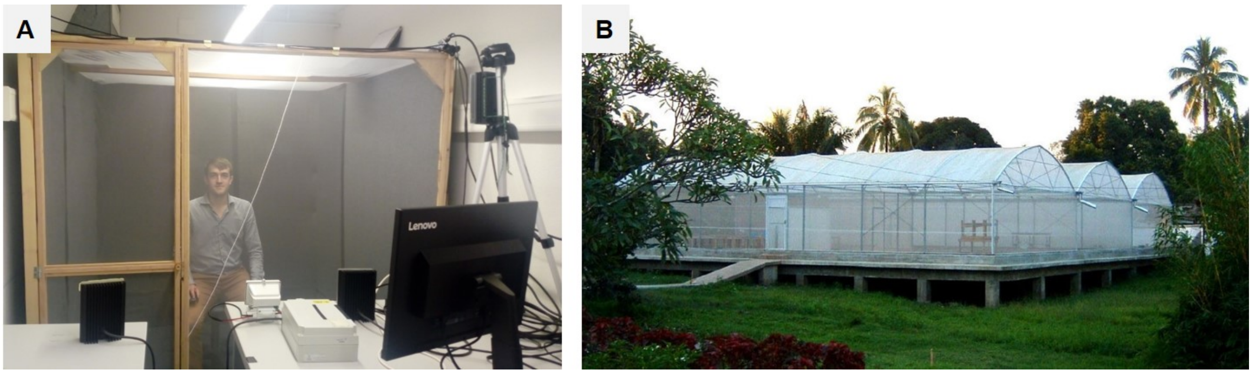

Figure 19: Experimental cages for tracking free-flying mosquitoes in a larger spaces. A. *A screened cage available at Swiss TPH with a volume of 6.9 m^3 (i.e. 1.9 m x 1.9 m x 1.9 m) which is 107 times larger than the space in the arm-in-cage test (i.e. 0.4 m x 0.4 m x 0.4 m = 0.64 m^3). The cameras are mounted on the top of the two corners facing the front allowing tracking the whole space inside the cage.* ***B.*** *Semi-field system set-up in Ifakara, Tanzania. Semi-field systems have been shown to be useful to study efficacy of topical repellents in larger space while still controlling the release of mosquitoes (Ferguson et al., 2008; Sangoro et al., 2014).*

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