Journal of Insect Physiology 71 (2014) 114–121

Contents lists available at ScienceDirect

Journal of Insect Physiology

journal homepage: www.elsevier.com/locate/jinsphys

Straightforward multi-object video tracking for quantification of mosquito flight activity

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ARTICLE INFO

Article history:
Received 27 May 2014
Received in revised form 7 October 2014
Accepted 10 October 2014
Available online 23 October 2014

Keywords:
Mosquito tracking
Mosquito activity
Mosquito flight
Aedes albopictus

ABSTRACT

Mosquito flight activity has been studied using a variety of different methodologies, and largely concentrates on female mosquito activity as vectors of disease. Video recording using standard commercially available hardware has limited accuracy for the measurement of flight activity due to the lack of depth-perception in two-dimensional images, but multi-camera observation for three dimensional trajectory reconstructions remain challenging and inaccessible to the majority of researchers. Here, *in silico* simulations were used to quantify the limitations of two-dimensional flight observation. We observed that, under the simulated conditions, two dimensional observation of flight was more than 90% accurate for the determination of population flight speeds and thus that two dimensional imaging can be used to provide accurate estimates of mosquito population flight speeds, and to measure flight activity over long periods of time. We optimized single camera video imaging to study male *Aedes albopictus* mosquitoes over a 30 h time period, and tested two different multi-object tracking algorithms for their efficiency in flight tracking. *A. Albopictus* males were observed to be most active at the start of the day period (06h00–08h00) with the longest period of activity in the evening (15h00–18h00) and that a single mosquito will fly more than 600 m over the course of 24 h. No activity was observed during the night period (18h00–06h00). Simplistic tracking methodologies, executable on standard computational hardware, are sufficient to produce reliable data when video imaging is optimized under laboratory conditions. As this methodology does not require overly-expensive equipment, complex calibration of equipment or extensive knowledge of computer programming, the technology should be accessible to the majority of computer-literate researchers.

1. Introduction

Reunion Island suffered a severe outbreak of chikungunya in 2006, when en epidemic spread across several countries of the southwest of the Indian Ocean (Benedict et al., 2007). The main vector of Chikungunya, the mosquito *Aedes albopictus*, is geographically widespread in Reunion occurring both in the vicinity of human dwellings and in remote habitats (Delatte et al., 2009, 2010). While the activity of *A. albopictus* males includes relatively inconspicuous behaviors such as sugar feeding or mating, it is the act of moving and particularly flight that brings them into contact with sugar sources and mates. Knowledge of the daily patterns of flight activities which contribute to physiological fitness is of crucial importance. Despite the increasing importance of *A. albopictus* as a global disease vector, little is known about activity and strategies for mating, and males are considered to be occasionally swarm-forming, but predominantly opportunistic in mating (Clements, 1999; Hawley, 1988; Oliva et al., 2013). Surprisingly, flight behaviors of *A. albopictus* males have been overlooked as the majority of field and laboratory studies on this species have been directed at understanding the flight activity pattern of female mosquitoes and how environmental cues or other stimuli (e.g. host-related olfactory or visual stimuli) interfere with the flight paths to their hosts (Cardé and Gibson, 2010; Takken et al., 2001). *A. Albopictus* males may fly great distances over a 24 h

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http://dx.doi.org/10.1016/j.jinsphys.2014.10.005
0022-1910/

period and are difficult to track. Quantitative measurements of their flight activity patterns may provide a method for directing and measuring the effectiveness of the Sterile Insect Technique (SIT), in addition to enabling detailed spatiotemporal modelling of mosquito dispersion in the field.

Measuring patterns in mosquito flight activity often consists in the manual scoring of behavioral attributes (Noldus, 1991), or video recording of behavioral sequences, using a video camera and computer assisted analyses (Cummins et al., 2012; Jones, 1964; Reiser, 2009), or has previously been achieved by the use of flight mills (Nakamori and Simizu, 1983), break-beam technology (Gentile et al., 2009; Rund et al., 2012) or acoustic measurements in specialized sound-proof flight cages (Nayar and Sauerman, 1971). However, none of the widely used assays is universally adapted or accessible for the quantitative investigations of flight activity of male *A. albopictus*, because they are either subjective, invasive, require specialized equipment or give results that are not reproducible. Furthermore, many of the assays are performed over short periods of time. Manual scoring of mosquito behaviors limits the number of individuals that can be simultaneously monitored, and in the time devoted to visually recording subtle and composite behaviors of individual flies. Automated video tracking systems have revolutionized the study of mosquito behavioral patterns. Video visualization facilitates the analysis of a single or multiple insects engaged in a wide range of complex behaviors, while overcoming the experimental bottlenecks associated with manual recording. Most available literature concerning mosquito flight patterns in the laboratory use visual observations, or single camera footage of the flight activity of individual mosquitoes.

Single object tracking systems can provide a considerable amount of information about each mosquito that is recorded, but since statistically-significant characterization of any individual mosquito requires the analysis of multiple mosquitoes, collecting data on one mosquito at a time is time-consuming. The automation of single object tracking is relatively straightforward, whilst multi-object tracking remains a complex problem that can be addressed using a variety of different bioinformatic analysis pipelines (Ouellette et al., 2006; Wood et al., 2012).

An adequate tracking system or algorithm must find a balance between the robustness and reliability necessary for high-throughput, long-duration experiments and the flexibility to handle large numbers of interacting insects under given conditions. Single camera setups are a suitable solution, however they are unable to record the depth of observed objects, and therefore tracks are recorded in two dimensions (2D). For this reason dual or multi-camera setups are increasingly employed for the observation of insect flight, which permits the reconstruction of 3D tracks from multiple views of the insects (Beeuwkes et al., 2008; Butail et al., 2012; Spitzen et al., 2013). Although multi-camera techniques remain essential for a complete description and analysis of complex individual interactions (El-Sayed et al., 2000; Khan et al., 2005; Wu et al., 2009), they have a number of drawbacks: they are prohibitively expensive for many laboratories, require precise imaging calibration for their correct installation, and use relatively complex and computationally demanding techniques for 3D position reconstruction. At present, no standard software packages exist to perform 3D reconstruction. Multi-camera techniques are therefore inaccessible to the majority of experimentalists.

In this article, we have three primary aims: (i) to determine the limitations of 2D observation of flying objects by computer simulation, (ii) to generate a reliable and accessible laboratory protocol for tracking male mosquitoes, and (iii) to quantify the nycthemeral patterns of flight activity for groups of *A. albopictus* males using a simple single-camera setup.

2. Materials and methods

2.1. Mosquitoes

The main mosquito vectors in Reunion Island (Latitude/Longitude: 21° 06′ S, 55° 36′ E) include the dominant species *A. albopictus*, but also *Aedes aegypti* and the malaria vector *Anopheles arabiensis*. From a previous assessment environmental and climatic factors appear to provide favorable conditions for *A. albopictus* in the Island (Boyer et al., 2014). In the present study, all adult males used have been from the F27 and 28 generations of laboratory-reared *A. albopictus* strain originating from the urban area located 2 km north-east of Saint-Denis (20°55′13.80″S; 55°30′52.87″E), the capital of Reunion Island. Immature and adult mosquitoes were reared in a climate-controlled room, maintained at a temperature of 27 ± 2 °C and 75 ± 2% relative humidity (RH) with a 12:12 h light/dark (LD) cycle. Newly emerged females were blood-fed on parafilm® membrane using 2.5 ml of sheep blood (Prolab, Saint-Pierre, Reunion Island), maintained at 37 °C using a Hemotek® membrane feeding system. Eggs laid by females were kept at room temperature after a three-day period of maturation and egg hatching was triggered using a highly dehydrated rabbit food left overnight in the rearing water (hay pellet, *Compagnie des Grains du Capricorne*, Le Port, Reunion Island). Larvae were reared at a density of approximately 200 first instar larvae in a white plastic tray (30 × 40 cm with a depth of 6 cm) containing 1 L of distilled water. The growing larvae were fed a powdered rabbit-food and fish-food (Tetra GmbH, TetraMin, Heinsberg, Germany) every 24 h. Upon pupation, male and female pupae were separated manually under a binocular microscope based on terminalia. There were separately put in small plastic cups and placed into insect holding cages (30 × 30 × 30 cm) where emergence occurred. Newly emerged adults were maintained under strict environmental conditions kept constant throughout the experimental period, being continuously supplied with 10% sucrose solution on cotton wicks. Adults males used for experiments were less than 48 h old.

2.2. Monitoring mosquito flight

Each flight arena consisted of a 30 × 40 × 10 cm acrylic-sided box with rounded corners and a transparent Perspex base. The box was sealed using a mosquito-netting lined lid, allowing light into the chamber and mosquitoes to feed on sugar solution across the netting. The scene was constantly backlit using two angled infrared LED lamps (MicroLight IR-56), and the 12:12 LD cycle with abrupt transitions was achieved using timer-interrupted (lights on from 06h00 to 18h00) standard overhead white lights. A white Perspex sheet was used as a backdrop to achieve even illumination across the whole scene, improving video contrast and definition by minimizing shadows and reflections. The flight arena and the camera were kept within a controlled environment room (temperature of 27 ± 2 °C and 75 ± 2% relative humidity) to ensure equilibration of temperature and humidity within the flight chamber.

On each experimental occasion, random batches of young male mosquitoes were placed in the flight arena, 24 h before the start of filming to allow an adjustment time to the new conditions. To minimize variation between tests and all replicates the following experimental procedure was employed throughout: a rectangular cotton pad soaked with 10% sugar solution was placed on the mosquito netting, covered with a small plastic tray to reduce evaporation, and left in position as a food source from the moment the mosquitoes were placed in the flight arena and throughout the duration of filming. Filming encompassed both dark and light cycles and began at 12h00 on the first day, and was stopped at