Topic Introduction

Using Videography to Study the Biomechanics and Behavior of Freely Moving Mosquitoes

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Female mosquitoes of most species require a blood meal for egg development. When biting a human host to collect this blood meal, they can spread dangerous diseases such as malaria, yellow fever, or dengue. Researchers use videography to study many aspects of mosquito behavior, including in-flight host-seeking, takeoff, and landing behaviors, as well as probing and blood feeding, and more. Here, we introduce protocols on how to use videography to capture and analyze mosquito movements at high spatial and temporal resolution, in two and three dimensions.

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STUDYING MOSQUITO BEHAVIOR USING VIDEOGRAPHY

Mosquitoes are dipteran insects with a slender body, long legs, and relatively high-aspect ratio wings. When flying, mosquitoes beat their wings back and forth at exceptionally high frequencies and low amplitudes. Female mosquitoes of most species need protein found in vertebrate blood meal for egg development. To acquire this blood meal, female mosquitoes bite a blood host, such as a human. Not only are mosquito bites annoying, but they can result in the contraction of several dangerous infectious diseases, including malaria, yellow fever, dengue, chikungunya, and Zika virus (Lee et al. 2018). As a result, the behavior of this deadly disease-carrying insect is studied extensively using videography systems (Dickerson et al. 2012, 2015a,b, 2018; Dickerson and Hu 2014; Bomphrey et al. 2017; Sutcliffe et al. 2017).

Using video analysis, one can capture and quantify mosquito behavior at temporal resolutions that cannot be captured by other means. By using multiple synchronized cameras in a single video system, one can reconstruct the full three-dimensional (3D) kinematics of moving mosquitoes. Videography system complexity can be tuned to study a large range of mosquito behaviors, including feeding, egg laying, biting of blood hosts, and landing on and taking off from a range of complex surfaces, including H_2O and human hosts (Muijres et al. 2017; Smith et al. 2018, 2020). Videography also makes it possible to capture various in-flight behaviors, such as searching for food, blood hosts, and mates, as well as in-flight escape maneuvers (Cribellier et al. 2022). Such mosquito behavior studies often aim to contribute to the development and improvement of vector control methods and techniques (Cribellier et al. 2018, 2020; Batista et al. 2019). For example, based on knowledge of the in-flight search and escape dynamics of host-seeking mosquitoes, one can improve repellents and mosquito traps and create pathways for new control tactic development.

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A mosquito's minute stature and uncommonly high wingbeat frequencies present a considerable challenge for in vivo biomechanical measurements. Therefore, dedicated high-speed cameras are needed for high-fidelity visualization. Recent developments in high-speed camera technology and computing power have made the required hardware more available and affordable. For all videography-based studies on moving mosquitoes, the choice of video system depends directly on the goal of the current study (see Fig. 1). Therefore, to effectively design a videography experiment, researchers should first clearly define their research question, and then determine hardware requirements. With the research question and hardware in mind, researchers can then select an appropriate software tool for video analysis. However, the process of designing a videography-based mosquito behavior study is an ongoing process based on continual feedback and is thus nonlinear.

In the accompanying protocols, we present the videography experiment design process in detail, and provide best practices, guidelines, and alternatives for experimental and analysis tools for studying mosquito behavior using videography. For guidelines and points of consideration for designing a generic videography experiment for studying mosquito behavior, see Protocol: **Designing a Generic Videography Experiment for Studying Mosquito Behavior** (Muijres et al. 2022). For a discussion on how to use a real-time tracking system, called Braid, to study moving mosquitoes, see Protocol: **Real-Time Tracking of Multiple Moving Mosquitoes** (Straw et al. 2022). The Braid tracking system facilitates fully automated location tracking of multiple mosquitoes moving freely about an arena without the need of storing video data. Moreover, how to use the software tool DLTdv to track the body, wing, and leg kinematics of moving mosquitoes in 3D and at high spatial and temporal resolution is detailed in Protocol: **Tracking the Body, Wing, and Leg Kinematics of Moving Mosquitoes** (Hedrick et al. 2022). DLTdv can be used to track mosquito movements both manually and automatically. Finally, how to quantify the body, leg, and wingbeat kinematics of moving mosquitoes post–data collection is discussed in Protocol: **Quantifying and Analyzing Mosquito Movement from Video Tracking Results** (Muijres 2022).

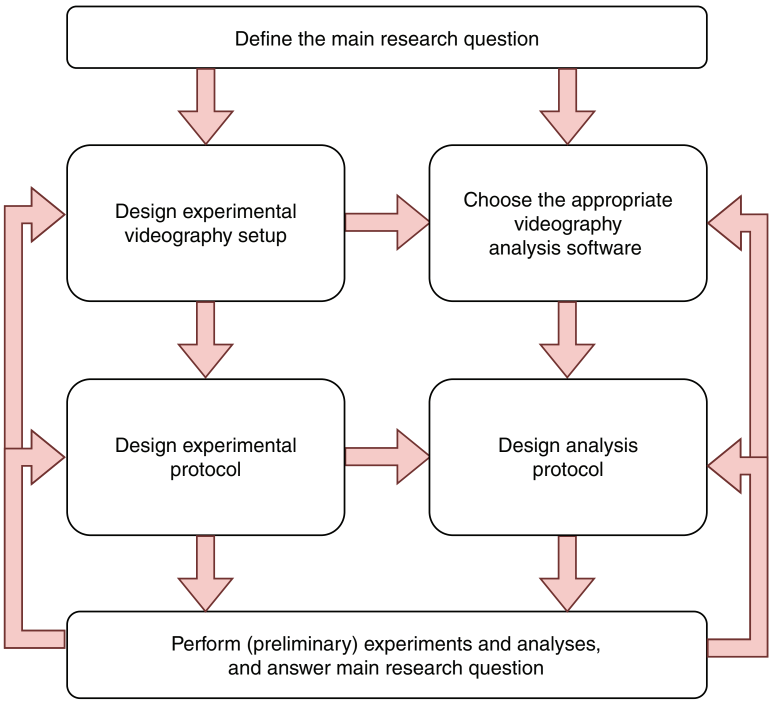

FIGURE 1. The workflow for designing a videography-based mosquito behavior study.

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DESIGNING YOUR VIDEOGRAPHY EXPERIMENT

As with any research project, it is crucial to align the experimental design and procedure with the main research question. The workflow for experimental videography design targeting moving mosquitoes is summarized in Figure 1. This plan consists of four steps.

1. Define your main research question.
2. Based on the main research question, determine the hardware requirements for the experimental setup. The most important questions to answer are thus what size the experimental arena should be, how many cameras are needed, and what camera lighting conditions are required. For details, see the section on hardware requirements below and Protocol: **Designing a Generic Videography Experiment for Studying Mosquito Behavior** (Muijres et al. 2022).
3. Based on the research question and experimental design, choose the appropriate analysis software and tools for processing and analyzing the video data. Here, make certain the chosen software permits the extraction of the type(s) of data produced by the videography system, and that the analysis output can be used to answer the main research question. For details, see the section on analysis software requirements below, and Protocol: **Real-Time Tracking of Multiple Moving Mosquitoes** (Straw et al. 2022) and Protocol: **Tracking the Body, Wing, and Leg Kinematics of Moving Mosquitoes** (Hedrick et al. 2022).
4. Following videography design and software selection, you should design your experimental and analysis methods, such that they are aligned with the research question and the used hardware and software. For details, see the "analysis software requirements" section below and Protocol: **Quantifying and Analyzing Mosquito Movement from Video Tracking Results** (Muijres 2022).
5. Finally, build the experimental setup, perform your research, and answer your research question.

Note that here we describe this plan as a single set of steps, but the experimental setup may often need to be redesigned and optimized based on preliminary experiments and analyses, as indicated by the return arrows in Figure 1.

Hardware Requirements

As stated above, the nature of the desired data determines the number of cameras needed in a particular experiment. The most important consideration here is whether 3D movements need to be measured. A single-camera setup can only track movements accurately within the viewing plane of the camera. With a multicamera setup, whereby multiple (synchronized) cameras simultaneously film an event, 3D movements can be reconstructed. As a general rule, using more cameras increases precision measurement and mitigates view obstruction. However, multicamera systems are more complex to set up, calibrate, and operate, and data analysis is more labor-intensive. Therefore, a multicamera setup should only be used if needed. Consult Protocol: **Designing a Generic Videography Experiment for Studying Mosquito Behavior** (Muijres et al. 2022) to determine the level of system complexity appropriate for your experiments.

When using a single-camera setup, ensure that movements occur mostly within the camera viewing plane. By doing so, single-camera setups have been used to determine, in high temporal and spatial resolution, the center of mass of mosquitoes impacted by falling H_2O drops (Dickerson et al. 2012), mosquitoes falling through dense gas (Dickerson et al. 2015b), taking off (Smith et al. 2018), in short flight sequences (Ortega-Jiménez and Combes 2018), and probing for holes in a net (Dickerson et al. 2018). Single-camera setups have also been used to track moving body parts such as tarsi (Smith et al. 2018), wings (Dickerson and Hu 2014), and halteres (Dickerson et al. 2015b).

Many movements of mosquitoes are inherently 3D, and when 3D motion is of interest a multicamera system is needed. Depending on the spatial and temporal resolution of the multicamera system, one can study the 3D kinematics of moving mosquitoes at varying levels of detail. A low-resolution system allows for location tracking of mosquitoes within a relatively large area, up to meters in size. Tracking over a relatively large volume allows, for example, the study of flying mosquito interaction with large objects such as mosquito traps (Cribellier et al. 2018, 2020; Batista et al. 2019). Multicamera systems with both high spatial and temporal resolutions can be used to track the body and wingbeat kinematics of mosquitoes in detail. This facilitates the observation of the bio(fluid) mechanics of mosquito flight (Bomphrey et al. 2017; Nakata et al. 2020) during takeoff (Muijres et al. 2017) and landing (Smith et al. 2020).

Analysis Software Requirements

There are several software tools available for analyzing videography data of moving animals. These include both open-source and commercial software. The choice of analysis tool is driven by the research question, the resulting required measurement complexity and analysis volume, and the researcher's experience. Below, we list seven analysis packages ranging from basic to complex, but this is by no means a comprehensive list.

1. Tracker Video Analysis and Modeling Tool is likely the simplest and most accessible two-dimensional tracking software available. It runs in Java and was developed by Douglas Brown at Cabrillo College (Brown 2007). The software allows for digitizing single-camera video data and is user-friendly but has limited options.
2. Direct Linear Transformation data viewer (DLTdv) is a MATLAB package for analyzing video data of moving animals (Hedrick 2008), packaged for use in MATLAB or the freely available MATLAB runtime environment. It is developed and maintained by Tyson Hedrick at the University of North Carolina at Chapel Hill. It is user-friendly, has extensive tutorials and support, and has many features. It can be used to digitize animal movements in a single camera or simultaneously in multiple cameras. It includes sophisticated camera calibration routines, including a DLT calibration for 3D reconstruction, and lens distortion corrections (using the accompanying pose and position-based "Wand calibration tools"). The analysis tool allows for both manual and automatic tracking of movements. The automatic tracking can be done using classic machine vision techniques and using a deep learning algorithm (Breslav et al. 2017). We discuss this system in detail in Protocol: **Tracking the Body, Wing, and Leg Kinematics of Moving Mosquitoes** (Hedrick et al. 2022).
3. Braid is an open-source real-time tracking software for animal movement research developed by Andrew Straw at Freiburg University (https://strawlab.org/braid) and is a rewrite of a similar software called Flydra (Straw et al. 2011). Because it tracks moving animals in real time, it is fully integrated in the experimental videography system and thus sets specific requirements for the hardware. This software can track multiple mosquitoes in real time based on video streams, and therefore does not require saving and postprocessing of video data. We discuss the Braid system in detail in Protocol: **Real-Time Tracking of Multiple Moving Mosquitoes** (Straw et al. 2022).
4. Track3D is a commercial 3D videography-based tracking software developed by the company Noldus Information Technology in collaboration with professor Willem Takken (Laboratory of Entomology) and professor Johan van Leeuwen (Experimental Zoology Group) of Wageningen University (Noldus et al. 2011; Spitzen et al. 2013). It is a user-friendly software package that allows automatic 3D tracking of the position of a moving animal such as a mosquito and has good postprocessing and visualization tools.
5. Hull Reconstruction Motion Tracking (HRMT) is an automated insect tracking algorithm developed by the Itai Cohen Group (Ristroph et al. 2009). HRMT allows for automatic tracking of detailed wing and body kinematics of moving mosquitoes and is therefore particularly useful for studying mosquito flight in detail. That said, HRMT requires the use of several high-speed cameras and expert knowledge of machine vision algorithms (Ristroph et al. 2009; Walker et al. 2012; Bomphrey et al. 2017).

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6. Model-based 3D insect tracker was originally developed by Ebraheem Fontaine at the California Institute of Technology (Fontaine et al. 2009). It has been used to track the movements of swimming fish, moving worms, and flying insects, including mosquitoes (Fontaine et al. 2009; Muijres et al. 2014, 2017). Similar to the HRMT method, these algorithms require expert knowledge of machine vision. However, model-based tracking tends to require fewer cameras (typically three) than HRMT (four or more).
7. Deep Lab Cut (DLC) is a Python-based program that uses sophisticated deep-learning algorithms to track moving animals (Mathis et al. 2018). It was developed by Alexander and Mackenzie Mathis at École Polytechnique Fédérale de Lausanne and shows great potential for behavioral research on mosquitoes. It has an active community of developers and good tutorials and tech support. To effectively use deep-learning algorithms, a dedicated workstation with a high-end graphics card is needed, although simple artificial neural networks can be developed even on a laptop. DLC supports single-camera analysis and two-camera stereoscopic setups for 3D reconstruction.

In the accompanying protocols, we present high-level instructions for tracking moving mosquitoes in real time and in detail. For the real-time tracking of multiple moving mosquitoes, we present Braid (see Protocol: **Real-Time Tracking of Multiple Moving Mosquitoes** [Straw et al. 2022]). For detailed tracking of body, wing, and leg movements of mosquitoes, we highlight DLTdv because of its long history of use, number of users, available documentation, ease of use, and overall capability (see Protocol: **Tracking the Body, Wing, and Leg Kinematics of Moving Mosquitoes** [Hedrick et al. 2022]). DLTdv can also perform both manual and automatic tracking, whereby automatic tracking can be done using a classic machine vision algorithm and a deep-learning algorithm.

Within particular dimensionality constraints, each software tool listed above can ultimately provide the user with the same types of data. For example, Tracker, the simplest package, can produce the same types of tracks as DLC, but perhaps at a higher manual labor cost. Thus, the choice which software is best for your project may not be clear. There is in fact no correct or incorrect choice. Instead, consider the experience of the user, your short- and long-term goals, the video quality, and available computational power when approaching the various software packages. Tracker requires the least initial investment but lacks high-throughput abilities. In contrast, DLC requires much greater computing capabilities, familiarity with Python, and considerable algorithm training before any data can be realized. However, the payoff is the ability to track points in multiple videos without much user input. If videos are inconsistent one to the next, or otherwise demand a high degree of human subjectivity, a manual tracker with the ability to switch to (semi)automatic tracking such as DLTdv will be the natural choice. Finally, if one is only interested in the position of the mosquito, Braid might be the best option. When set up and well-calibrated, Braid provides location data of mosquitoes without any postprocessing, allowing you to track a large number of mosquitoes at low labor costs.

Regardless of the analysis software, every analysis must include a calibration routine to transform video data (pixel-based coordinates) to a measurement with physical units. For single-camera systems, a scale reference such as an object of known dimension often suffices. Once a pixels/length metric is determined, multiple videos can be captured in sequence without providing a new scale, so long as no camera adjustments are made. Multicamera systems often use more sophisticated calibration routines, where an object with known dimensions is placed in the camera's field of view. Depending on the calibration routine, this can be a 3D object with many reference points, a checkerboard placed at different locations, or even a moving stick or set of light-emitting diodes. These calibration routines are often part of the tracking software. Camera calibrations are specific to the precise relative position of the cameras, lens settings, and pixel resolution. Changes to any of these parameters, even a small inadvertent nudge given to one of the cameras, will reduce calibration accuracy. Because the accuracy and quality of the videography experiments depends directly on the quality of the calibration, calibration routines need to be performed very carefully and regularly.

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