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Principles of Semi-Field Study Design to Obtain Realistic Data about the Effects of Anthropogenic Climate Change on the Biological and Ecological Traits of *Culex pipiens (L.)*

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Review Article

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ABSTRACT

Mosquitoes, representing an ectothermic metazoan parasite, are hypothesized to be highly affected by concurrent climate change. It is also particularly emphasized that field-based data from the studies conducted under realistically fluctuating natural conditions are needed to perform accurate measurements of the impact of such climate effects. For this purpose, a specific greenhouse unit was established in a highly endemic natural habitat of *Culex pipiens* located on the Tekirdag Namik Kemal University campus. This protocol description-based study was performed in order to reveal a detailed application process of a semi field study unit as well as to underline particular advantages and drawbacks of such units, which were designed for the purpose of obtaining field data, and finally to prompt certain related suggestions. In the preparation of the study documentation, protocols and studies on a sample unit were taken into account in order to base the principles on realistic data.

1. Introduction

More than 3500 species of mosquitoes, which constitute the most important arthropod group for human beings as well as animals, have been reported worldwide so far [1]. This importance is due to both the number of agents that they can transmit to the different host groups and the fact that these agents, which can be seen in humans and animals around the world, may cause serious life-threatening diseases [2]. Mosquitoes are common throughout the world; however, species diversity and/or density varies from region to region, and humid tropical/subtropical regions contain ¾ of the known species [3].

Although there is a plethora of studies on the effects of temperature on arthropod life history traits, these have mainly been carried out under laboratory conditions, often applying constant temperature regimes on laboratory colonies. However, it is well known that the laboratory colony or natural population status of the mosquitoes used in the studies, or the territories of the study, can significantly affect the thermal response of insects to varying degrees [4-9]. Furthermore, insects routinely experience variable climatic parameters on a daily, monthly, or seasonal basis in their natural habitats. Various biological and population traits [10, 11] and vector capacities [12-14] are characterized under the constantly variable drivers under the natural circumstances. Therefore, in order to obtain more reliable data on such parameters, it is much more convenient to carry out the relevant studies directly in the natural environment of insects or in a realistic simulation of their natural habitat [15, 16]. Particular emphasis is placed on the requirement for studies conducted under realistic fluctuating field conditions to accurately measure the impact of climate change on living systems, including vectors, as climate change is predicted to be characterized by variations in temperature values and increased frequency of periodic thermal extremes [14, 17-21].

Although the importance of field data is well known, there are no detailed protocols for obtaining detailed field data on mosquitoes. In particular, the establishment of field simulations is a method that is rarely applied in the world. This is of course also due to the particular challenges of obtaining field data on mosquitoes compared to many other parasites. However, it is now a fundamental necessity to put in place some initiatives in order to reach realistic field data in any case.

In this study, the characteristics of a field simulation complex, which can be used in mosquito studies and the principles of its used protocol, are discussed in some basic aspects. In order to base the analysis and inferences on realistic data, the operation of an existing and actively used sample facility was taken as basis. The sample facility is established in an area containing rather high *C. pipiens* natural population endemically, together with some other mosquito species [22]. In addition to having a population density at the level required for a detailed study in the area, the fact that this species is an ideal model for some ecological studies such as evolutional

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adaptation [23] also played a role in its selection. In this review, study designs that can be conducted directly under natural conditions in order to obtain field-based data on the life history traits and ecological features of *C. pipiens* in and/or around the greenhouse system, which is designed specifically and established in Tekirdag Namik Kemal University campus are addressed from various aspects.

2. Materials and Methods

2.1 Principles of the study area selection

The study area is placed in the agricultural research area of the Tekirdag Namik Kemal University campus which is located in the central district of Tekirdag province, in the coastal region of the Marmara Sea, and on the southern part of Eastern Thrace. The site is located in a peri-urban environment and consists of woodlands, fields, greenhouses, and buildings. Trials are conducted in and/or around a greenhouse system (40º59'32.12"N, 27º34'43.93"E) designed as a simulation of natural condition for arthropod research (Fig. 1). The region, which is located in the subtropical zone, is under the influence of "summer arid and hot temperate climate type (Csa)" according to the Köppen-Geiger climate classification [24]. The study area was preferred based on the following features: i) the natural prevalence of *C. pipiens*, which is the most common mosquito species in Tekirdag and almost all regions of Turkey and constitutes the main subject of our study, ii) since the aim of the study was to determine how the natural process proceeds, the mosquitoes to be examined should not have been exposed to special conditions or passed through some special process, and iii) the absence of a clinically important disease outbreak due to active vector dynamic related to the mosquito species belonging to the natural fauna of the region.

2.2. Establishment of a purposive greenhouse

The greenhouse system (5 m x 7 m x 2,5 m), which does not have any thermal insulation, artificial lighting, or air conditioning, is established in a wooded part of the study area. The greenhouse is covered with wire mesh and the ceiling is covered with white nylon tarpaulin to prevent the entry of wild animals, direct sunlight, and rain (Fig. 1A).

2.3. Recording of climatic parameters

Temperature and humidity values of the study area were recorded hourly by means of dataloggers (TFA Dostmann Klimalogg Pro 30.3039, Germany) placed inside the greenhouse. Dataloggers are positioned in such a way that they do not receive direct sunlight. Rainfall data are recorded daily by rain gauges (TFA Dostmann 47.1008, Germany) placed around the greenhouse. Wind data is obtained from the General Directorate of Meteorology.

2.4. Lure of mosquitoes to the vicinity of the study area

It has been reported that CO2 and various odors emitted by the host are attractive features in mosquitoes' host selection [25]. It has also been emphasized that the addition of bird uropygial gland odorants to CO2 supplied traps will increase the collection of Culex species [26]. Besides, *C. quinquefasciatus* has a specific interest in fecal odorants of poultry [27]. To lure mosquitoes in the vicinity of the study area, ten domestic chickens (*Gallus domesticus*) were constantly maintained in a coop complex (4 m x 5 m x 4 m) covered with wire mesh on three sides, a wall on one side, and a tarpaulin on the ceiling. Feces obtained from chickens were collected in certain areas around the greenhouse. In this way, it is aimed to accelerate the orientation of the mosquitoes existing

Fig 1. Study area (A: greenhouse system, B: containers used for the larvae rearing in the greenhouse system).

in the vicinity to the greenhouse environment and to stimulate their feedings in their natural process. Mosquitoes that head to the study area immediately enter the resting mode on the walls or ceilings of the reinforced concrete area in the coop after feeding on the chickens, and these gravid females wait in this mode until they are ready to lay eggs. Chickens are both natural hosts that mosquitoes can feed on, and the presence of chickens, birds, other animals, or humans in an area play as an effective attractant for *C. pipiens*, whose host selectivity is quite flexible [28].

2.5. Obtainment of mosquito egg rafts for any trials on larvae

Obtaining egg rafts is a basic requirement for conducting life history traits and population dynamics in the *C. pipiens* mosquito. Accordingly, breeding/laying containers were placed around the greenhouse system for the supply of egg rafts. The egg rafts laid in the containers were collected daily and used for relevant studies performed to determine the larval development process under natural circumstances.

2.6. Design of the study targeted to examine the details of life history traits

The main purpose of the experiments, which will investigate the life history characteristics of *C. pipiens*, is to monitor the larval development of the local population from the egg laying to the adult emergence under the local temperature regime. As long as the reproductive dynamics of the natural *C. pipiens* population in the study area continues, it should be aimed to establish trial groups on a monthly basis. The duration of the study should be planned so that at least a one-year cycle is completed. Egg rafts laid directly by mosquitoes obtained from the breeding/laying containers placed in the vicinity of the greenhouse system for the establishment of the trials. Trials should be set up on the day the first instar larvae emerge from these egg rafts. Egg rafts obtained from breeding/laying containers should be taken into egg incubation containers that contain water supplied with fry food. Incubation containers should be followed in the greenhouse until the first stage larvae emerge. A trial group belonging to the relevant month should be established from the larvae emerged in the relevant month. First instar larvae hatching from at least six egg rafts on the same day should be collected in a single pool for each trial group. In total 288 larvae randomly selected from this pool can be divided into 36 clear glass cups (bottom diameter: 6 cm; top diameter: 8 cm; height: 8 cm; water depth 5.5 cm) containing 150 ml of commercial water and eight larvae in each glass. The day on which eight larvae are distributed to the glasses should be marked as the day 0 (starting day). Here, we recommend that the number of larvae to be used should not fall below 200 for each trial to obtain accurate data for the statistical analysis and relevant modellings. Again, it is recommended that the number of larvae to be placed in each glass cup described above should not exceed eight. Because, in a study in which the intraspecies crowding effect was investigated in the larvae of *C. pipiens* mosquitoes, the success of adult emergence in the container containing eight larvae seems ideal among the experimental groups in containers prepared in the same way [29]. Exceeding this number will trigger the larval crowding effect, negatively affecting the main objectives of the experiment. Each cup should be placed one by one in transparent plastic containers (h: 20 cm; diameter: 20 cm) to prevent the escape of emerging adults (Fig. 1B).

The upper opening of the containers should be covered with gauze and a cotton-plugged hole should be created in the middle of the gauze to collect the emergent adult mosquitoes with a mouth aspirator or any other aspirator types. Water loss due to evaporation during daily checks on glasses should be supplied by adding equal amount of commercial water. Larvae should be fed according to the protocol related to juvenile fish food and feeding should be discontinued from the pupal stage [30]. In order to be able to directly observe the effect of meteorological parameters, special attention should be paid to prevent the larvae from being exposed to nutritional deficiency and crowding in the experimental groups, since it is essential to eliminate other factors.

In the experimental groups, the number of living larvae in the glasses, the number of dead larvae, the number of daily formed pupae, the number of living pupae, the number of dead pupae, the number of emergent males and females and the temperature level in the greenhouse system should be recorded daily. The developmental process of the larvae in the cups should be monitored daily from the first instar larval stage to adult emergence and/or death of the last aquatic form. Relevant examinations must be performed before 18:00 in the evening. All records of larvae, pupae and adult emergence and temperature taken in a 24-hour period from 18:00 to 18:00 for consecutive days should be used as single-day data.

Wing measurements can be taken from randomly selected 30 male and 30 female individuals from the emergent adult collected in a pool. It has been stated that wing length is an indicator of body size in adult mosquitoes [17, 31]. By measuring the wing lengths of the adults, it can be determined to what extent they are affected by the periodic temperature regime exposed during the development. Measurements should be made from the anal lobe to the wing tip, using the same wing (left wing in this study) for each individual [32]. Species identification of mosquitoes used throughout the study was performed under a stereomicroscope using specific identification keys based on the morphological characteristics of different developmental stages [33-35].

In the study designed such a way described above, the development time of the larva until the pupae, the development time of the emergent males and females, the survival rate and wing length, the number of deaths in the larvae and pupae, the daily ambient temperature values experienced during development (daily maximum, minimum and average temperatures, and daily temperature) were taken as indicators to measure the effects of natural circumstances to the development process of *C. pipiens* aquatic forms.

2.7. Design of experiments to monitor propagation characteristics and population dynamics

In this study, it has been aimed to determine the monthly reproductive characteristics and population dynamics of *C. pipiens* under the fluctuating natural conditions. In this direction, breeding containers were placed at the different distances in the vicinity of the greenhouse system, some of

Fig 2. The localities of breeding containers placed in the study area

which were outdoors and some were indoors. The number of containers and their site may vary according to the conditions of the area where the study is performed [36]. The containers were placed in different foci with different characteristics at some extent; one plastic and one tire container were placed side by side in each focus (Fig. 2). It has been aimed to reveal four facts in the selection of the type and location of the containers, namely the following: i) how far from the feeding focus (chicken house) do mosquitoes lay eggs? ii) is there a difference of interest in tire and plastic containers in the selection of the laying site? iii) do indoor and outdoor preferences change in egg laying? and iv) how does the monthly course change in respect of all these preferences?

It is recommended that this study be conducted for at least one year to clearly demonstrate monthly and seasonal propagation characteristics and population dynamics. Egg rafts in all containers should be counted every day and collected by recording the container codes and other relevant records. Photographs of the rafts should be taken under a stereomicroscope to determine egg numbers per raft. Then, the rafts should be taken into glass cups supplied with fry food one by one and kept until the larvae hatch. The hatching times should be noted by counting the larvae when they emerge.

A wooden board is mounted on top of the breeding containers to prevent them from overflowing by rain and being exposed to direct sunlight. In addition, each container is protected with wire to prevent animals from getting inside and drinking water. Six liters of distilled water up to six cm high was put into rectangular plastic containers (25 cm x 38 cm; h: 27 cm). Six liters of distilled water was poured into the tire (outer diameter: 47 cm, inner diameter: 38 cm, length: 47 cm, width: 25 cm) with a depth of six cm. In order to lure mosquitoes to the water bodies placed in the containers, the attraction technique with straw infusion was used as described in the related study [37].

3. Conclusion

Considering the evaluations made on the unit used as a model, it is understood that the following parameters should be considered in fieldwork simulations: i) since *C. pipiens* prefers to lay eggs in containers at the shaded foci for most of the year, rearing containers used in the studies should be kept out of direct sunlight, ii) The instructions for the use of the devices used in the measurement of meteorological parameters should be fully complied with, e.g., at least three rain gauges should be used considering possible local differences, and the average value should be used as ambient data, iii) thermometers must be placed in such a way that they do not receive direct sunlight; this will result in highly erroneous temperature readings being recorded and the related error will manifest itself especially with abnormal increases in temperature difference between day and night, iv) natural mosquito breeding areas in the selected locality should be determined and whether they will affect the study should be evaluated, v) it should definitely be taken into account that larval crowding and feeding deficiencies can result in very crucial problems in larval development, and vi) it should be known that the presence of animal hosts, which are kept in accordance with natural conditions, is one of the most important factors in attracting mosquitoes to a certain area.

When the designs of field research described in this study were evaluated together, the following aspects have been determined: i) the number of egg rafts laid by *C. pipiens* on a daily basis under the circumstances of realistic natural conditions in a year; ii) whether the number of eggs per egg raft changed on a monthly basis; iii) the embryonal development time and the performance of larva hatching from egg rafts conceived on a monthly basis; and iv) the development time and performance from first instar larvae to adult emergence revealed successfully on a monthly basis. For this purpose, field-based data on *C. pipiens* life history traits and ecology were accurately obtained using the described study area design and the performed study protocols in this document.

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