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Responses of *Anopheles gambiae, Anopheles stephensi, Aedes aegypti,* and *Culex pipiens* mosquitoes (Diptera: Culicidae) to cool and humid refugium conditions

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ABSTRACT: Like all terrestrial arthropods, mosquitoes must cope with the threat of desiccation. To gain insight into their survival strategies, we recorded the behavioral responses of *Anopheles gambiae, Anopheles stephensi, Aedes aegypti,* and *Culex pipiens* offered zones of different microclimatic conditions in laboratory cages. The cooled refugium was at 25.6° C, 86% RH and the control was at 28.5° C, 75% RH, i.e., a difference in saturation deficit of 3.9 mm Hg between the two zones. We show that newly-emerged adults, with no access to water or sugar, prefer the cooler and more humid refugium with a saturation deficit half that in the control and where the mosquitoes could reduce their metabolic rate. This response is delayed in *Ae. aegypti,* perhaps because the energy reserves accumulated as larvae are higher in this species. This study shows that mosquitoes under stress can use their thermohygroreceptor cells to guide them to locations that facilitate survival.

Keyword Index: Microclimatic preferences, *Anopheles gambiae, An. stephensi, Aedes aegypti, Culex pipiens*.

INTRODUCTION

Anopheline, Culicine, and Aedine mosquitoes are major vectors of disease agents. The *Anopheles gambiae* Giles complex is responsible for approximately 80% of the global malaria morbidity and mortality that occurs in sub-Saharan Africa, and *Anopheles stephensi* Liston transmits the disease in Asia. *Culex pipiens* (L.) is a vector of West Nile virus. Activity of these three species of mosquito is maximal during the night, whereas *Aedes aegypti* (L.), a major vector of yellow fever, is diurnally active.

The metabolism of mosquitoes is influenced by environmental parameters, such as temperature and humidity. Like all terrestrial arthropods, mosquitoes must cope with the threat of desiccation, such that preferences for suitable microclimatic conditions could be expected depending on their physiological state. Moreover, microclimatic preferences have a particular bearing on disease transmission as it pertains to pathogen incubation and sporogonic duration in the case of malaria transmission. All mosquito species do not show the same resistance to desiccation: when reared under identical conditions, female *An. arabiensis* were shown to possess significantly higher desiccation resistance than those of *An. gambiae* of the same age, a characteristic that has been ascribed to the higher body water content of *An. arabiensis* (Gray and Bradley 2005). In contrast to this, diapausing *Cx. pipiens* females, which contain less water than nondiapausing individuals, combat desiccation by their larger size (lower surface to volume ratio), higher levels of insulating cuticular lipids, and a lower metabolic rate (Benoit and Denlinger 2007). *Drosophila* selected for enhanced desiccation resistance show increased levels of hemolymph solutes for homeostasis (Folk et al. 2001), but to our knowledge, no such phenomenon has been described to date for mosquitoes.

To gain insight into the survival strategies of *An. gambiae, An. stephensi, Cx. pipiens,* and *Ae. aegypti*, we recorded the behavioral responses of these disease vectors when deprived of access to water and sugar but offered zones at different microclimatic conditions in laboratory cages. We show that newly-emerged adults search for a cooler and more humid refugium, but this response is delayed when the energy reserves of a species are higher.

MATERIALS AND METHODS

Mosquitoes

An. gambiae (16CSS strain, derived in 1974 from wild-caught adults originating from Lagos, Nigeria, West Africa) and *An. stephensi mysorensis* (originating from the Swiss Tropical Institute, Basel) colonies were maintained in a walk-in climate chamber under a 14:10 light-dark cycle with 2 h simulated sunset and sunrise at 28° +/- 1° C and 80 +/- 1% relative humidity (RH). The scotophase lasted from 20:00 to 06:00. The *Ae. aegypti* colony (UGAL strain from Georgia, U.S.A.) was maintained in a second walk-in climate chamber under a 12:12 h light-dark cycle at 26° +/- 2° C and 65 +/- 2% RH during the day and 22° +/- 2° C and 90 +/- 2% RH during the night. The *Cx. pipiens* colony (from Oberwil, Switzerland) was maintained on a bench at laboratory conditions. Adults of the anautogenous mosquito species were fed on guinea pigs every two weeks and *Cx. pipiens* on 10% sucrose. Eggs were collected on moist filter paper and 400 larvae per species were reared in pans with 400 ml of distilled water and fed pulverized Tetramin® (Timmermann and Briegel 1993). This density provided optimal nutrition for larvae, synchronous eclosion, and adults of homogeneous size. One experiment was made with each species of a defined age: 36 one-day-old *An. gambiae* (17 females and 19 males), 100 one-day-old *An. stephensi* (not sexed), 100 one-day-old *Cx. pipiens* (72 females and 28 males), 110 one-day-old *Ae. aegypti* (63 females and 47 males) and 105 two-day-old *Ae. aegypti* (63 females and 42 males). The two groups of *Ae. aegypti* were from the same generation. Experiments with *An. gambiae*, *An. stephensi,* and *Cx. pipiens* were performed during the scotophase under the Anopheles rearing conditions. Experiments with *Ae. aegypti* were made during the photophase in the Anopheles rearing chamber. Light was provided by fluorescent tubes (Philips TLD, 32 W at 36 KHz) providing 1110 lux at the base of the cage for the *Ae. aegypti* observations. The experimental mosquitoes had no access to either sucrose or water from emergence.

Experimental refugia

Mosquitoes were held in the Plexiglas® rearing cages (35x35x55 cm high) where they had emerged. Cages have an opening (15 cm diameter) on one side, closed by nylon netting (1 mm mesh size) to provide access. In addition, a circular (10 cm diameter) opening on the top of the cage closed by plastic netting (1 mm mesh size) permitted circulation of the surrounding conditions within the cage. Two identical Peltier elements attached to aluminum discs (90 mm diameter, 10 mm thick) were placed on opposite sides (245 mm apart) on the Plexiglas® roof of the cage. One of the Peltier elements served to cool a zone underneath it at the top of the cage and the other served as control (environmental cabinet conditions). Heat was eliminated from the Peltier element by passing water through it from a water bath (Heger et al. 2006). The control element was also connected to the pump to ensure that any vibrations caused by passage of water were similar in the two zones. The positions of the cooled and control Peltier elements alternated between experiments. A filter paper disk (90 mm diameter, Whatman No. 1001 090) was attached to the inside roof of the cage under each element. Temperature and humidity was measured at 5 mm below the center of the filter paper disk using a thermo-hygro probe (Vaisala HMP50, +/- 0.6° C, +/- 3% RH). RH levels under the filter paper disks were recorded every 30 s using Scopview (version 1.03, AGA Associates, U..SA.) for 2 h to measure the humidity conditions established with time under the cooled and control Peltier elements.

Recording and analysis of mosquito behavior

Images of the numbers of mosquitoes on the cooled and control Peltier elements at the top of the cage were acquired with an IR sensitive camera (Panasonic wvBP310) from 80 cm outside the cage. Images were stored every 0.5 s using a time-lapse recorder (Panasonic AG-7350). Constant near-infrared illumination (950 nm) from a lamp (WFN-II, Model LED 30, Videor Technical E. Hartig GmbH, Germany) enabled video observations for the nocturnally active species. The lamp was positioned at 90 cm from the center of the top of the cage and 20° lower. Recordings of mosquito landings at night started 1 h into the scotophase. Behaviors of *Ae. aegypti* were recorded between 14:00 and 17:30 for one-day-old mosquitoes, and between 08:30 and 11:30 for two-day-old mosquitoes. In each experiment, the number of mosquitoes present on the filter paper disks on the cooled and control zones was counted at 15 min intervals. The duration of pauses made by a sample of the *An. gambiae* that landed on the cooled zone was measured from the video records made during the first (21:00 to 00:00) and second part (00:00 to 06:00) of the night.

RESULTS

The median temperature was 25.55° C (+/- 1° C) in the cooled zone at the top of the cage and 28.50° C (+/- 0.2° C) in the control zone. After applying current, the RH steadily

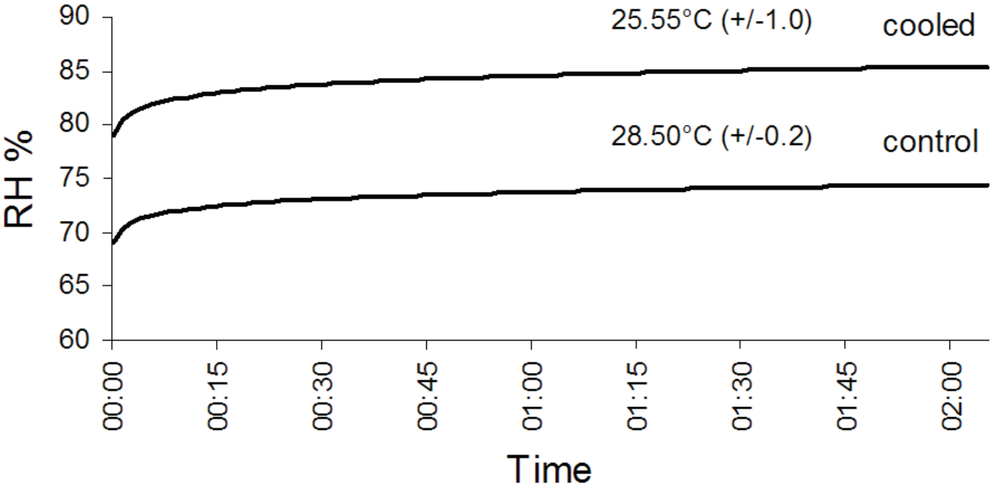

Figure 1. Establishment of a higher humidity over 2 h under the cooled Peltier element after applying current, compared to the control. The RH showed an increase under both the cooled and control zones due to absorption of humidity by the filter paper disk with time. Median values for the temperature under each zone are indicated.

increased under the cooled Peltier to stabilize approximately 2.5 h later at 85.75%, that is 10.84% higher than under the control, i.e., saturation deficits of 3.71 mmHg under the cooled Peltier element and 7.61 mm Hg under the control (Figure 1).

Successively more *An. gambiae* were recorded on the cooler zone as the night progressed, and at 9.5 h into the scotophase half of the mosquitoes had landed on the cooler zone (Figure 2A). The response of *An. stephensi* was similar to *An. gambiae*, but not as strong, with 14% of the mosquitoes on the cooler zone after 6.25 h into the scotophase compared to only 2% on the control (Figure 2B). *Cx. pipiens* showed a very clear response with 32% of the mosquitoes on the cooler zone at 7.25 h into the experiment (Figure 2C). No such attraction to the cooler zone was recorded for one-day-old *Ae. aegypti* (Figure 2D). However, by the morning of day two, *Ae. aegypti* started to show a preference for the colder zone, with a constant increase in this preference over 3 h, by which time 34% of the mosquitoes were on the cooler zone (Figure 2E).

There was a tendency for *An. gambiae* mosquitoes to remain longer on the cooler zone as the night progressed. However, the median of the resting periods on the cooler, more humid zone were not significantly different between the first and the second part of the night (134 s for the period 21:00-00:00 compared to 191.5 s for the period 00:00-06:00, Mann-Whitney U test, $P>0.05$). This is due to the high variation in the pauses, as individuals already on the cooler, more humid zone were disturbed by the successively increasing numbers of mosquitoes arriving there as the night progressed. To provide a measure of this disturbance, the duration of pauses that fell between the 10th and the 90th percentiles varied between 20.4 s and 812.2 s during the first part of the night, whereas these values varied between 22.9 s and 2373.9 s during the second part of the night.

DISCUSSION

The mosquito species tested all showed a clear preference for the cooled and more humid zone on the roof of the cage, where the saturation deficit was approximately half that of the control zone. The climatic conditions under the latter were comparable to those in the environmental cabinet. We did not observe a preference by one-day-old *Ae. aegypti* for the cooler, more humid zone, but *Ae. aegypti* deprived of access to water and sugar responded to cooler, more humid conditions on day two of life. In contrast to the other mosquito species studied here, larval food supply in *Ae. aegypti* is of primary importance in determining imaginal body size and energy reserves in this species (Briegel 1990), such that newly-emerged adult *Ae. aegypti* survive on reserves accumulated as larvae. However, as these reserves were being depleted in our unfed *Ae. aegypti* with time, we interpret our finding regarding this species as a switch to a microclimatic preference that reduces metabolic rate and risk of desiccation by day two of life. It has been shown that the attraction of yellow fever mosquitoes to a wet surface and the moist air associated with it is dependent on their dehydration state (Bar-Zeef 1960).

We have observed that the numbers of mosquitoes alighting on the cooler, more humid zone on the roof of the cage increased throughout the test period, i.e., during the night for *An. gambiae*, *An. stephensi,* and *Cx. pipiens* and during the day for the diurnally active *Ae. aegypti*. The number of arriving mosquitoes and interindividual disturbance was certainly too high on the colder, more humid zone in the second half of the night to properly evaluate an increase in the duration of pauses. In the experiment with *An. gambiae*, with only 36 mosquitoes present in the cage, the disturbance generated was manifested in the higher variance associated with the duration of pauses as the night progressed. Despite this, a progressive increase in the number of mosquitoes on the cooler, more humid zone was recorded for each species, a manifestation of acceptance by the mosquitoes of increasing density with time on the cooled zone. The strong preference by all four mosquito species used in this study for the cooler zone at a lower saturation deficit is interpreted as an adaptation to maintain water balance (Edney 1977, Hadley 1994) as well as to limit energy loss through a lower metabolic rate.

The comparable body sizes of *Ae. aegypti* and the *Anopheles* species (the latter within 7% of the body size of *Ae. aegypti*) used in our experiments (Timmermann and Briegel 1993) excludes an allometric factor as accounting for the differences in behavior we have recorded for these two genera in this study. This lends credence to the hypothesis that the behavioral discrepancy stems from a fundamental difference in the levels of caloric content to explain the delayed response of one-day-old *Ae. aegypti*. In this species, the caloric content is composed of lipids that are exponentially related to body size and higher in males, and of proteins and carbohydrates that are linearly related to body size in both sexes (Briegel 1990). The latter author has also shown that newly-eclosed and starving females mobilize carbohydrates nearly completely followed, respectively, by lipids and proteins. Our *Ae. aegypti* were starving on the morning of day two of life, so their preference for the cooler zone would serve to abate caloric depletion through a lowering of metabolic rate. In the case of *Cx. pipiens*, in addition to the insulating effects of cuticular hydrocarbons and lower metabolic rate that protect diapausing females (Benoit and Denlinger 2007), they can further succeed in reducing water loss and increase longetivity through the fine-tuning of their thermohygroreceptors (Kellog 1970, Davis and Sokolove 1975) that serve to guide them to zones with a cooler microclimate with air at lower saturation deficit levels. Hygroreceptive neurons assist desiccated larvae of the tobacco hornworm, *Manduca sexta*, in finding water (Rowley and Hanson 2007).

Observations similar to those reported for the four mosquito species here have also been shown for the hematophagus triatomine, *Rhodnius prolixus*, that manifests a preference for zones at a lower temperature and higher humidity (Heger et al. 2006). Partially dehydrated nymphs of the European tick, *Ixodes ricinus*, will walk along a humidity gradient towards the wet end (Crooks and

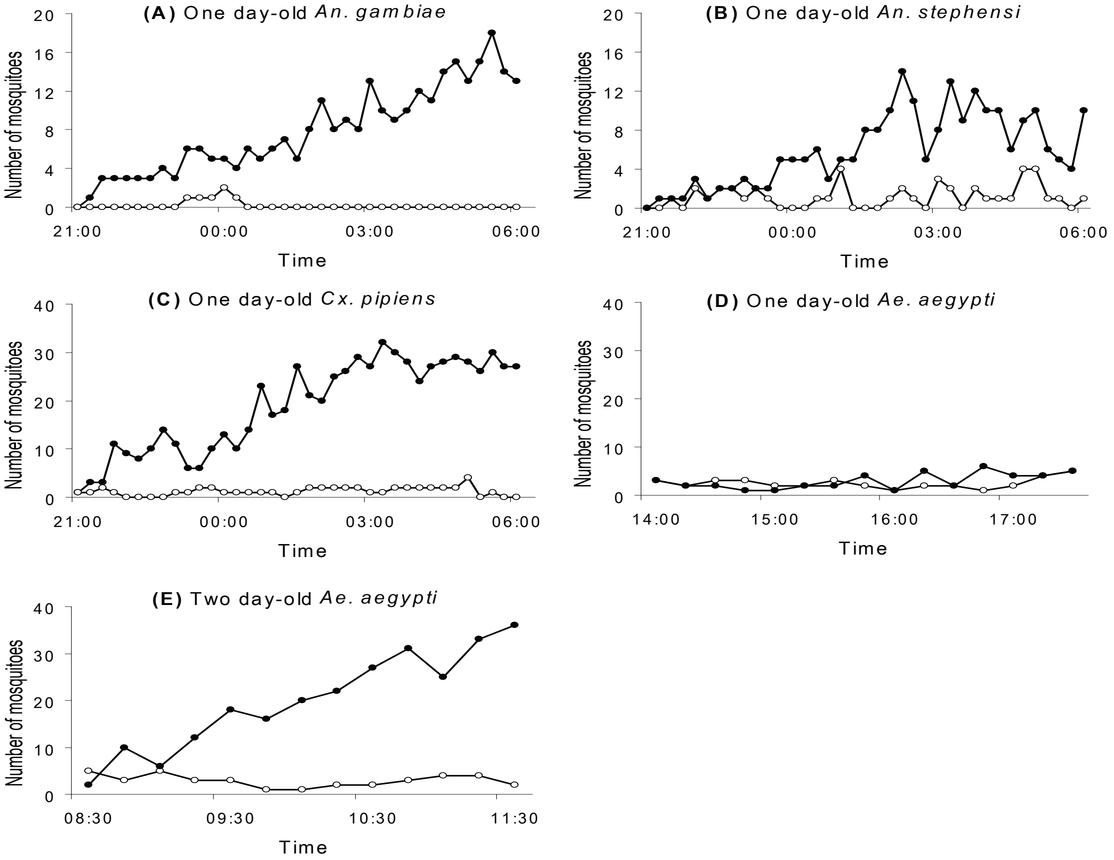

Figure 2. Cumulative numbers of one day-old *An. gambiae* (A), *An. stephensi* (B), *Cx. pipiens* (C), and of one and two day-old *Ae. aegypti* (D and E) on the cooled and more humid zone (solid circles) compared to the control zone (open circles) on the roof of the cage.

Randolph 2006). Furthermore, temperature and saturation deficit are both critical in determining the survival of tsetse flies, as has been reported for *Glossina morsitans morsitans* and *Glossina pallidipes*, where these environmental factors have been shown to differentially affect flies of various ages (Hargrove 2001). Moreover, according to the latter author, the effect of an increase in these two microclimatic parameters is accentuated in tsetse with low fat levels. Under these conditions, the exclusively hematophagous *Glossina* tend to seek food in high-risk situations in order to survive (Hargrove and Packer 1993). Such a constraint is not overbearing in mosquitoes whose survival is not dependent on the blood meal alone.

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