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DEVELOPMENT AND SURVIVAL OF *ANOPHELES GAMBIAE* EGGS IN DRYING SOIL: INFLUENCE OF THE RATE OF DRYING, EGG AGE, AND SOIL TYPE

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ABSTRACT. Little is known about the contribution made by the egg stage of African malaria vectors to the rapid rise in adult populations following the onset of seasonal rains. To examine this issue, we evaluated the viability of *Anopheles gambiae* eggs in drying soil in the laboratory. Survival data were collected from field-caught mosquitoes kept in sandy loam soil and laboratory-reared colonies kept in sandy loam soil and black cotton soil. Under high, medium, and low soil-moisture regimes, egg viability declined sharply with increased duration of drying. Eggs remained viable in drying sandy loam soil for 1, 5, and 10 days, but not after 15 or 20 days. The most dramatic decline in hatching success occurred between drying days 1 (78–83% hatch) and 5 (20–23% hatch). In contrast, eggs reared in high-moisture black cotton soil remained viable for up to 15 days. Furthermore, after 5 drying days, high-, medium-, and low-moisture soils averaged 59, 47, and 31% hatching success, respectively. We recovered unhatched eggs from sandy loam soils to examine the developmental status of the embryos. A majority of the unhatched eggs that were recovered from days 15 and 20 in sandy loam soils contained fully developed late-stage embryos. Thus, unhatched eggs completed embryonic development but probably died before receiving an appropriate hatching stimulus. Our results suggest that the absolute moisture content of the soil does not alone determine hatching success of anopheline eggs. Rather, soil moisture, together with the rate of drying, physiological factors associated with the age of the egg, and the type of soil in which the egg rests likely influence survival.

KEYWORDS *Anopheles gambiae,* egg stage, desiccation, soil type, malaria

INTRODUCTION

Anopheles gambiae, Anopheles arabiensis, and *Anopheles funestus,* the main malaria vectors in Sub-Saharan Africa, are confronted with highly variable and challenging climatic conditions in the tropics during the dry season (Mattingly 1971). The abundance of the malaria vector species falls dramatically with the onset of the dry season, and this can depress the incidence of severe malaria (Wilkinson et al. 1978, Snow et al. 1993). The onset of the rains brings a rapid explosion in mosquito numbers and a concomitant increase in malaria (Omer and Cloudsley-Thomson 1970, Mbogo et al. 1993, Snow et al. 1993). The mechanisms by which anopheline mosquitoes survive the long periods of dry and hot conditions are not understood, although such knowledge is important for the design of effective vector-control strategies (Minakawa et al. 2001).

Contributions to long-term survival during the dry season could be made during the egg, larval, and/or adult (including pupal) stages. Anopheline mosquito eggs do not survive prolonged desiccation (Deane and Causey 1943), which could prevent the egg stage from making a significant contribution to long-term survival during the dry season. However, *Anopheles* larvae have been observed immediately after the flooding of dry larval development sites (Beier et al. 1990). Parallel studies in the laboratory demonstrated that egg stages survive and hatch after 12 days of storage in moist conditions (Beier et al. 1990). These observations suggested that the egg stage could be important for short-term survival, as larvae typically cannot survive more than 2 days without water (Muirhead-Thomson 1945). Adult stages could also make an important contribution to dry-season population dynamics. Studies conducted in Burkina Faso and Sudan indicate that *An. gambiae* adults enter a state of dormancy at the onset of the dry season (Omer and Cloudsley-Thomson 1970). The mechanisms underlying this physiological adaptation are poorly understood. Thus, data on the Anophelinae are not conclusive with respect to how each life stage contributes to long-term survival during the dry season and the rapid population rise that occurs at the onset of the rains.

In this study, we further examined the tolerance of *An. gambiae* egg stage to desiccation stress. We compared desiccation resistance in eggs collected from field-caught mosquitoes and from laboratory populations. Further, to study whether the soil in which the egg rests contributes to desiccation tolerance, we raised eggs in two different soil types: a sandy soil from the Kenyan coast and a black cotton soil from Suba District in western Kenya.

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Finally, we examined the developmental status of eggs that did not hatch upon exposure to a standard hatching stimulus.

MATERIALS AND METHODS

Experimental animals

Experiments were performed with eggs from *An. gambiae* adults that were maintained as a laboratory colony in Nairobi or collected in July and August 2000 by aspiration methods from human dwellings in the Jaribuni area along the Kenyan coast. Blood-fed and gravid females were kept in cages at room temperature and about 70% relative humidity under a natural photoperiod. The cages were supplied with Whatman filter paper no. 2 (Whatman, Kent, UK) moistened with distilled H_2O as a substrate for egg laying and a 6% (w/v) sucrose solution for feeding. Although we did not perform polymerase chain reaction on the mosquitoes, previous studies of this area of Jaribuni indicate that more than 95% are *An. gambiae* s.s. (Mbogo et al., unpublished data)

Egg survival assays

Black cotton soil with high water-retention capacity and high organic content was collected from mosquito larval habitats in Suba district, and sandy loam soil was collected from Kilifi district. After drying for several days at room temperature, the soil was homogenized and measured into 100-g portions. Known quantities of distilled water (dH_2O) were added to each soil portion to produce high- (35 ml dH_20; 26% [w/w]), medium- (25 ml; 20% [w/w]), and low- (15 ml; 13% [w/w]) moisture soils (the residual moisture content of these portions was estimated immediately prior to the experiments by measuring the weight loss after complete oven drying of a representative soil sample). One hundred eggs laid overnight were distributed to each tray of soil. The soils with eggs were allowed to dry for 1, 5, 10, 15, and 20 days at room temperature. The weights of the soil trays were measured daily to determine the amount of water lost. Preliminary experiments indicated that soils dried rapidly if left uncovered, so we extended the time of drying by covering each soil sample with similarly perforated plastic trays (10–12 holes per tray, each approximately 0.5 cm diameter). At the end of the drying period, the soil and eggs were submerged in dH_2O as a hatching stimulus. The trays with submerged eggs were covered securely with mosquito netting. The 1st instars were removed and counted each day thereafter until no new larvae were seen for 2 consecutive days. For field-caught mosquitoes, the 1st instar from each tray were raised to the 4th instar and verified as *An. gambiae* by morphological criteria. For hatching controls, 100 eggs were submerged in dH_2O at day 0.

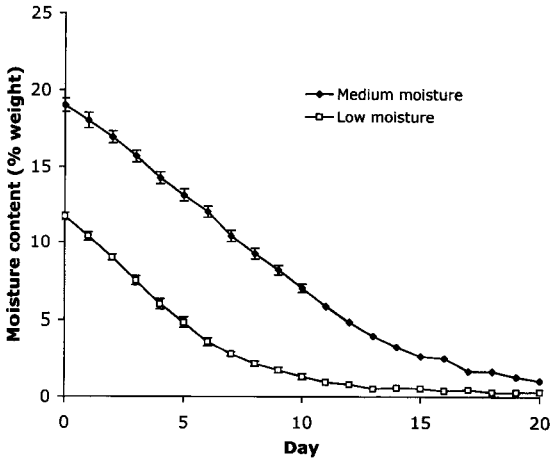

Fig. 1. Average daily rates of water loss for medium- and low-moisture sandy loam soils. Shown are mean + SE (days 1–10) or mean (days 11–20) for each day of the 20 days of the drying regime ($n = 4$). No means are calculated for days 11–20. Closed symbols represent medium- (M) moisture samples; open symbols represent low- (L) moisture samples.

Embryonic development of *An. gambiae* under drying conditions

To determine the developmental status of embryos that did not hatch after drying, floating eggs were manually recovered from the trays and placed in a 2.5% solution of commercial bleach (Ace, Nairobi) for approximately 3 h or until the chorion was transparent. Once visible, the embryos were either observed within the intact egg or dissected from the egg to identify the presence or absence of visible developmental landmarks of late embryos, including eye pigmentation, segmentation, and cuticle deposition (Trpis et al. 1973).

RESULTS

Effect of soil moisture on egg hatching success: Field-caught populations

We assessed the effect of soil moisture content and rate of drying on egg viability by allowing soils containing batches of eggs to dry for a variable number of days and then submersing the soils in water to stimulate hatching. For experiments on field-caught mosquitoes, we investigated medium-moisture (M) and low-moisture (L) soils. The average water content of medium soils declined steadily by 1.2% day^{-1} (w/w) until day 11 and progressively less until day 20 (Fig. 1). Low-moisture soils lost water by 1.4% day^{-1} until day 6 and progressively less until day 20 (Fig. 1).

The hatching success of eggs from field-caught and laboratory populations did not differ significantly when provided with the same hatching stimulus ($P > 0.10$; Kruskal–Wallis test; Fig. 2). Hatch-

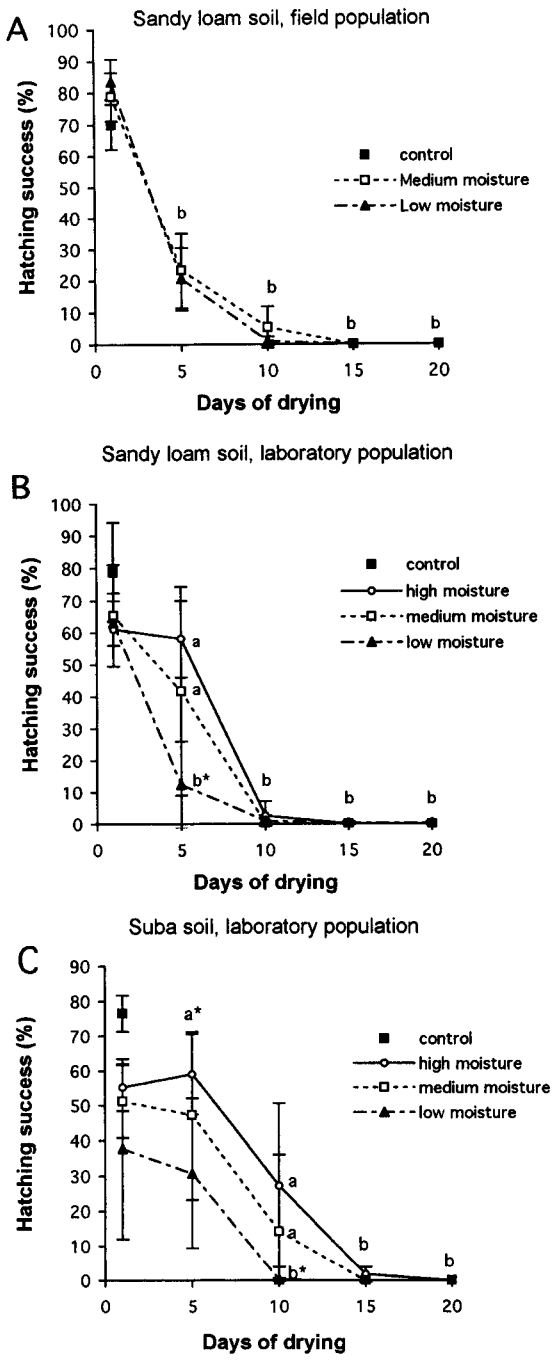

Fig. 2. Percent hatch (mean + SD) of eggs from field-caught and laboratory mosquitoes versus the number of days of drying in (A) field-caught mosquitoes maintained in sandy loam soil, (B) laboratory mosquitoes maintained in sandy loam soil, and (C) laboratory mosquitoes maintained in cotton soil from Suba district. * = significant difference between values obtained in different moisture regimes (high versus medium versus low moisture). [a] $P > 0.05$ (not significantly different from day 1). [b] $P < 0.05$ (significantly different from day 1).

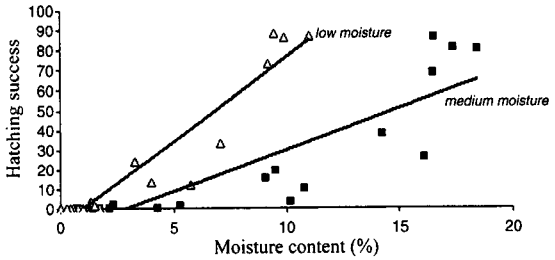

Fig. 3. Effect of soil moisture content on hatching success for field-caught mosquitoes in sandy loam soil. Percent soil moisture content (w/w) was plotted against the number of larvae recovered from each tray. Open triangles indicate data points for low-moisture soils, black squares indicate data points for medium-moisture soils.

ing success in M and L soils at day 1 were 78.8 + 7.6% and 83.5 + 7.0%, respectively, but fell to significantly lower levels after 5 days (23.3 + 11.8% and 20.1 + 9.9%, respectively; $P < 0.05$; Wilcoxon rank sum; Fig. 2A). After 10 days, M soils yielded a 5.3 + 6.5% hatch, while L soils gave a 1.0 + 1.4% hatch (Fig. 2A). Larvae were not observed from either moisture treatment after 15 and 20 days of drying (Fig. 2A). In no case was hatching success significantly different for medium- and low-moisture soils that were dried for identical periods of time (Fig. 2A). However, M and L soils with comparable moisture contents (attained after different lengths of drying; see Fig. 1) yielded different numbers of larvae (Fig. 3). Under such conditions, a higher hatchability was seen in L soils that dried to a given moisture more quickly. These results suggest that the age of the egg in the soil and/or the length of time that the egg has been exposed to desiccating conditions is a more reliable indicator of egg viability than is absolute soil moisture content.

Effect of soil moisture on egg hatching success: Laboratory populations

The mean hatching success of laboratory embryos kept in high-moisture sandy loam soils was 60% through 5 days of drying, but dropped to 2.6% after 10 days of drying (Fig. 2B). Eggs kept in medium-moisture soil had approximately 60% hatching success after 1 day of drying and 40% after 5 days in drying soil. After 10 days of drying, the hatching success of laboratory mosquitoes in medium-moisture soils fell to near 0%. Eggs in low-moisture soils showed no significant differences in hatching success relative to high- and medium-moisture soil after 1 day of drying (Wilcoxon rank sum). Hatching success declined significantly after 5 days of drying in low-moisture soils (12% hatching success; $P < 0.05$; Wilcoxon rank sum), and differed significantly from hatching success in high-moisture soils (Fig. 2B)

We examined the effect of soil type on egg re-

sistance to desiccation. Cotton soil from Suba district supported higher sustained hatching success of laboratory eggs than did sandy loam soil. The hatching success from high-moisture cotton soils, e.g., was 59 + 11.6% (mean + SD) after 5 days of drying and declined to 27 + 23% after 10 days (Fig. 2C). Some hatchlings were observed after 15 days of drying in high-moisture soils. Low-moisture cotton soils, by contrast, only produced larvae after 1 and 5 days of drying, suggesting that soil moisture content is an important determinant of hatching success regardless of soil type. Indeed, significant differences were observed between hatching success in high- and low-moisture soils at days 5 ($P = 0.03$; Wilcoxon rank sum) and 10 ($P = 0.03$; Fig. 2C).

Developmental status of embryos

Batches of eggs from field-caught mosquitoes were bleached prior to providing a hatching stimulus to assess the proportion containing fully developed embryos. Out of 74 bleached eggs, 73 (98.6%) contained late-stage embryos showing pronounced pigmentation of the eyes and hatching spine (data not shown). Desiccated eggs that fail to hatch when provided an appropriate stimulus might do so either because they are dead or because they are arrested in a state of diapause of quiescence. To assess whether embryos that failed to hatch after several days of drying in soil were fully developed or arrested, eggs were recovered from soils and their chorions bleached to reveal the embryo inside. Two hundred thirty-three unhatched eggs were recovered from 19 different submerged soils, with the majority (211) coming from day 15 and 20 soils. Eighty of these eggs were successfully bleached and/or dissected. Of these, 50 (63%) contained fully developed embryos but none of these became active upon removal from the egg. The remainder of the eggs either contained no embryos or embryos that were destroyed during the bleaching process.

We normally observed a tendency for eggs containing fully developed embryos to hatch upon exposure to bleach. In one trial, 75% (24/32) of eggs hatched within 2 h of exposure to bleach, while 10.8% (5/46) of eggs from the same batch hatched if exposed to water only. Thus, either a particular physical property of bleach acts as an effective hatching stimulus, or an effect of bleach, such as the removal of floats and sinking of the egg, enhances the stimulus for hatching. In no case, however, did eggs recovered from desiccation experiments hatch upon exposure to bleach. Together, these findings suggest that desiccation does not affect a particular phase of embryonic development but rather causes the death and degradation of eggs.

DISCUSSION

Previous studies have suggested that the egg stage of *An. gambiae* may contribute to the short-term survival of these mosquitoes during the dry season. Studies conducted in western Kenya, e.g., have shown that *An. gambiae* eggs can survive for limited periods in dry soil and hatch on exposure to water (Beier et al. 1990). In the laboratory, *An. gambiae* eggs can remain viable for up to 18 days (Deane and Causey 1943). However, the limits of this desiccation tolerance and the underlying physiological mechanisms of embryo longevity have yet to be elucidated.

Our data demonstrated that only a small fraction of *An. gambiae* eggs can survive for 15 days in drying cotton soil from the Suba district in western Kenya and that coastal sandy soils and cotton soils yield different hatching successes after desiccation. The limits to desiccation tolerance are likely determined by a combination of factors, including soil moisture content, rate of soil drying, egg age, and the type of soil in which the egg rests. These results provide a basis for future studies on the mechanisms and importance of egg stage survival in *An. gambiae* during drying conditions.

Eggs of *An. gambiae* can survive in moist cotton soil for up to 15 days but only 10 days in sandy loam soil from coastal regions (Fig. 2). Our experimental conditions were designed to partially mimic the natural conditions experienced by eggs, which become embedded in soil that dries slowly. Under this treatment, several factors might account for the trend toward longer egg survival in black cotton soil. First, because of its fine particles, the cotton soil may retain water better than the sandy soil of the coast. Second, with time, eggs may become more embedded by soil particles, so that the sandy soil may be more abrasive to the chorion of the eggs. Perhaps, in these conditions, abrasion of the chorion could be significant and in cotton soil, the chorion might provide a more effective barrier to water loss than in sandy soil.

Large differences in soil moisture content, such as between high- and low-moisture soils after 10 or 15 days of drying, can yield significantly different hatching rates (Fig. 3). Generally, however, a strong correlation between hatching success and soil moisture content held only for cohorts of eggs that were of similar age and exposed to similar moisture conditions. For example, on some days, medium- and low-moisture treatments with different moisture contents gave statistically similar hatch rates in sandy loam soil (Fig. 3). Furthermore, medium- and low-moisture soils with comparable moisture contents (obtained after drying for different amounts of time) gave statistically different hatch rates. In such cases, the medium-moisture soils, which had been drying for several days longer than the low-moisture soils, yielded a lower hatching success. These results suggest a complex relationship be-

tween egg age and drying status in determining hatching success. Key factors determining hatching success in these cases appear to be either 1) the age of the egg in soil or 2) the absolute difference between the starting moisture content and the moisture content at the time of soil submersion (because medium-moisture soils would have lost more water over this period).

What physiological parameters contribute to the decrease in survivorship of anopheline eggs after prolonged drying? One likely factor is a limited resistance to desiccation of the egg shell (arising from the physical composition of the chorion and extraembryonic membranes). In addition, however, aging of the egg could contribute to decreased hatchability independently of drying. If egg age were an important limit to hatching success, we would expect that egg survivorship would decline with time even if soil moisture is constant. Data from previous studies (Beier et al. 1990) have shown that eggs kept on moist filter paper only survive up to 12 days. Similarly, our preliminary data on eggs kept in cotton soil indicate that daily water replacement only partially rescues the decline in hatchability at days 5, 10, and 15 (57, 32, and 5% hatching, respectively versus 23, 5, and 0% hatching without water replacement; Fig. 2A). These limited periods of viability, despite apparently sufficient moisture, suggest that no true diapause or aestivation with its associated reduced metabolism is occurring. Therefore, the embryos are likely continuing to metabolize yolk reserves prior to receiving a hatching stimulus. Viability would be expected to decrease sharply as these reserves are depleted. The nutritional status of embryos, measured by how much yolk is present through successive days of drying, is therefore worth closer investigation.

By comparison with other mosquito eggs, most notably those in the genera *Aedes* and *Ochlerotatus*, *An. gambiae* eggs show little resistance to desiccation (Clement 1992). Our data show that eggs of *An. gambiae* can survive for up to 15 days under drying conditions and that their susceptibility to desiccation depends on soil type. Thus, the limited desiccation tolerance of *An. gambiae* eggs may contribute only to short-term survival in moist or drying soil. Even limited desiccation tolerance could, however, act to maintain low levels of anopheline mosquitoes during times of unpredictably intermittent or low rainfall, and thus contribute to the regulation of anopheline mosquito populations through wet and dry seasons.

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