

Hatch Plasticity in Response to Varied Inundation Frequency in *Aedes albopictus*

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J. Med. Entomol. 46(4): 766–771 (2009)

ABSTRACT Eggs of container-breeding mosquitoes are able to withstand drought conditions as an egg and hatch when submerged. Frequent rainfall can be simulated by frequent submersion, and drought conditions can be simulated by infrequent submersion. We examined the hatch response of *Aedes albopictus* (Skuse) eggs to simulated drought conditions. *Ae. albopictus* eggs from a strain originating outside Kobe, Japan, were subjected to one of three treatments; high-frequency hatch stimulation consisting of submerging the eggs in a nutrient broth mixture every 3 d, low-frequency hatch stimulation consisting of submerging the eggs every 7 d, and delayed high-frequency hatch stimulation. Eggs that were subjected to lower-frequency stimulation showed a significant decrease in hatch delay, which was the opposite of the predicted response. This decrease in hatch delay may be an example of hatch plasticity in response to drought conditions. This response could not be explained as a result of the difference in the ages of the eggs on any given stimulus. A decreased hatch delay response to potential drought conditions combined with rapid larval development may enable *Ae. albopictus*, whose eggs are not as desiccation resistant as some other container-breeding mosquitoes, to survive extended drought.

KEY WORDS *Aedes albopictus*, hatch rate, phenotypic plasticity, hatch delay, drought

Environmental stress, or any environmental condition that is less than optimal for an organism, is a strong force in natural selection. Organisms may use a variety of responses to cope with the stress. One of these mechanisms is phenotypic plasticity, or the expression of different phenotypes by the same genotype under varying environmental conditions (Bradshaw and Hardwick 1989, Harvell 1998, Schlichting and Pigliucci 1998, Pfennig and Murphy 2002). Phenotypic plasticity is likely to evolve in conditions of frequent environmental change preceded by an environmental cue (Van Tienderen 1991; Schlichting and Pigliucci 1995, 1998) as well as in populations that may exhibit moderate migration between subpopulations (Kingsolver et al. 2002). One common example of phenotypic plasticity is entering a diapause state to avoid adverse environmental conditions, a trait observed in many mosquito species (Cohen 1970, Taylor 1980).

One such environmental change that can pose a risk to mosquito larvae is the desiccation of aquatic habitats. Although mosquito larvae face the threat of habitat desiccation, the eggs of many container-breeding mosquitoes are able to resist desiccation for extended periods of time. Desiccation resistance of the eggs varies from species to species and is influenced by a

number of factors including egg size, the state of the embryo (diapause or nondiapause), and the natural habitat (urban or forest settings) (Sota and Mogi 1992a, b). If an environmental cue preceding habitat desiccation can be used to predict the possibility of desiccation, a change in the hatch response of the egg may minimize the threat of habitat desiccation. Container-breeding mosquitoes are able to detect some environmental cues during the egg stage of development. In addition to detecting and responding to a drop in ambient oxygen concentration in the liquid medium (the trigger for hatching), some container-breeding mosquito species can detect changes in photoperiod, which are used as cues predicting the onset or end of winter (Mori et al. 1981, Shroyer and Craig 1983, Focks et al. 1994). If potential desiccation can be detected, the mosquito may be able to survive a potential drought by avoiding hatching. We predict that during drought-like conditions, *Aedes albopictus* eggs will avoid hatching even when presented with suitable opportunities.

Under drought conditions, rainfall is less frequent, and the eggs are stimulated to hatch less often. Frequency of inundation (or hatch stimulation) may be a potential cue that mosquitoes use to assess their habitat and environment before hatching. Like many mosquito species, *Ae. albopictus* (Skuse) mosquitoes exhibit installment hatching (Gillett 1955, Mogi 1976). During a rainfall event, the eggs of container-breeding

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mosquitoes are submerged beneath the water, but only a fraction of the eggs may hatch. Results from a preliminary experiment using an established colony (>25 generations) of *Ae. albopictus* indicated a possible plasticity response to variable frequency of submersion (Vitek and Livdahl 2006). These results indicated that the mosquitoes seemed to decrease their hatch delay in response to a decrease in the frequency of stimulation. However, populations reared in the laboratory for extended periods of time may undergo intensive artificial selection. Hatch delay is generally selected against in laboratory colonies, because non-hatching eggs are discarded and not subjected to continued stimulation until they hatch. Previous work with *Ae. albopictus* has shown that two wild strains with differing hatch responses rapidly become more similar to each other when kept in the laboratory for successive generations (Mogi 1976). Limited oviposition opportunity, adaptation to artificial diurnal cycles, and genetic bottlenecks may also influence the behavior of colonized mosquitoes.

When an egg is submerged after a rainfall event, it may respond by either hatching or not hatching. Three possible responses may be observed after exposing eggs to changes in frequency of hatch stimulation: eggs may either show an increased hatch rate (faster hatching or less hatch delay), a decreased hatch rate (slower hatching or increased hatch delay), or no change in the hatch rates. We hypothesized that mosquito eggs will respond to potential drought-like conditions by altering their hatching behavior. We predict that eggs exposed to simulated drought conditions would show a decreased hatch rate or increased hatch delay. This would enable desiccation-resistant eggs to survive during periods of infrequent rainfall and potential drought and to hatch when rainfall frequency increases.

Materials and Methods

Eggs from *Ae. albopictus* were collected outside of Kobe, Japan, in the summer of 2000 using standard cup style egg traps. Kobe, Japan, experiences a mean of 97 d of ≥ 1 mm rain each year based on a 30-yr average from 1961 to 1990 or slightly more than one wetting event every 4 d (Hong Kong Observatory 2003). The National Climatic Data Center (NCDC) indicates that Kobe, Japan, experienced an average of 132 precipitation days with ≥ 0.01 in during this same time period, or a wetting event approximately once every 3 d (National Oceanic and Atmospheric Administration 2008).

Eggs were stored on damp cardboard in a mosquito incubator that was maintained at constant high humidity ($\geq 75\%$), photoperiod (16-h:8-h light:dark cycle), and temperature (23°C). The eggs were hatched using standard nutrient broth, and the strain was reared in the laboratory to an F₄ generation. Eggs from the F₄ generation were collected within 24 h of being laid on pine tomato stakes. These eggs were divided into batches of ≈ 50 eggs and stored damp on the pine stake in plastic vials. The experimental began when a

Table 1. Schedule for experimental treatments

Treatment	Inundation interval (d)	First inundation	Last inundation
Low frequency	7	Day 1	Day 64
High frequency	3	Day 1	Day 28
Delayed high frequency	3	Day 37	Day 64

Day 1 is the day of the first inundation.

sufficient number of eggs were collected, 29 d after the initial eggs from the F₄ generation were oviposited. Eggs used for this experiment were oviposited throughout the entire 29-d period. This resulted in a different starting age for the various egg batches, even within the same treatment. The difference in initial age of the eggs was included in the statistical analysis.

Eggs were subjected to two differing treatments. Eggs were inundated in a nutrient broth media every 3 d (high frequency [HF]) or every 7 d (low frequency [LF]; Table 1). Inundation every 3 d is similar to the observed rate in the field, whereas inundation every 7 d is less frequent than observed under natural conditions in Kobe and simulates possible drought conditions. During drought conditions, there may be many periods when rainfall is less frequent than every 7 d. However, previous research on hatch rates had indicated no statistical difference between random hatch stimulation based on a probability of rainfall and inundation at regular intervals with the same overall probability (Vitek and Livdahl 2006). The 7-d interval used in this experiment simply represents a long-term decrease in the frequency of rainfall relative to the normal yearly frequency.

Submerging the HF eggs in the nutrient broth at a faster rate resulted in an age discrepancy toward the end of the experiment. On any given wetting event after the first inundation, the ages of eggs subjected to LF treatment were older than eggs subjected to the HF treatment. A second level of treatment called "delay" was added to adjust for this. The first wetting event of the delay treatment was postponed for 36 d and was carried out on day 37 of the experiment. After this holding period, the repeated inundation began. This delay was designed so that the eggs subjected to delayed HF stimulation (DHF) would reach their 10th stimulus at the same time as the eggs subjected to LF treatment (Table 1). This resulted in the DHF eggs being the same age at the time of the 10th stimulus as the LF eggs.

To simulate a natural wetting event, a 0.5-g/liter nutrient broth mixture was created and aerated for 30 min using a fish tank aerator. This concentration nutrient broth solution has been shown to result in hatch rates that are not significantly different from field hatch rates (Vitek and Livdahl 2006). New nutrient broth was created for each hatching stimulus. The hatch stimulus consisted of submerging the wooden stake in this nutrient broth mixture for 24 h. After 24 h, the stake with the eggs was removed from the liquid and stored in a sealed plastic vial in an incubator. Any hatched larvae were removed and counted. The eggs

Table 2. ANOVA for the effects of frequency of stimulation (treatment), no. of eggs (eggs), and a possible age effect (age) on the hatch fraction during the first hatch stimulus

Source	df	SS	F	P
Treatment	1	7.60×10^{-3}	1.93	0.17
Age	1	3.00×10^{-4}	0.08	0.78
Eggs	1	6.80×10^{-1}	172.08	<0.0001
Error	43	8.87×10^{-1}		

were subjected to 10 sequential wetting events at the predetermined stimulation frequency according to the experimental treatment. After 10 inundations, all remaining unhatched eggs were dissected to determine viability as indicated by the presence of unhatched larvae. All hatch fractions and rates were calculated by excluding nonviable eggs.

The hatch fractions of the first wetting event were compared using a multiple analysis of variance (ANOVA), using number of eggs, treatment, and age influence as independent variables. For subsequent analyses, the hatch fractions of the first wetting event were included as a covariate. Age of the eggs caused by the initial delay in starting the experiment was included in all analyses.

Of the delayed hatching eggs (eggs that did not hatch on the first wetting event), two different statistical analyses were conducted. The first analysis examined the difference between responding and nonresponding egg batches using a Wald χ^2 analysis. A responding egg batch is one that exhibited any hatching after the first wetting event, whereas a nonresponding egg batch is an egg batch that had no eggs hatch after the first wetting event. For the second analysis, a logistic regression model of the hatch rates was used to estimate the wetting event at which 50% of the delayed-hatch eggs (eggs that are unhatched after the first wetting event) would hatch. This estimate of hatch delay was called the median stimulus and was calculated for all egg batches and constituted the observations for the comparisons of hatch delay. The median stimuli values were analyzed with a multiple ANOVA. The hatch fraction on the first wetting event, the age of the eggs, the hatch treatment, and the number of eggs in the egg batch were included as independent variables. On all multiple ANOVA models, nonsignificant interactions were removed from the model. All statistical analysis was conducted using JMP v4.0 software (SAS Institute 2001).

Results and Discussion

All experimental batches had eggs hatch in response to the initial wetting event. An initial analysis of the hatch fractions on the first inundation showed that there was no difference between the treatments (Table 2) caused by either age of the eggs or frequency of stimulation. The dependent variable is the number of larvae hatched on the first inundation for each egg batch, divided by the total number of viable eggs in the egg batch. There were no significant interactions. Age was calculated from the date the eggs were oviposited

Table 3. ANOVA for the effects of frequency of stimulation (frequency), age effect (age), no. of eggs (eggs), and the hatch fractions (first hatch) during the first stimulus on the median stimulus value

Source	df	SS	F	P
Age	1	1.83	0.35	0.56
Frequency	1	62.43	12.03	0.001
First hatch	1	1.35	0.26	0.61
Eggs	1	2.44	0.47	0.50
First hatch \times frequency	1	31.83	6.14	0.02
Error	49	254.19		

for each egg back to the first hatch stimulus (used to include age as a possible variable) including the initial age difference in the DHF treatment. Frequency of stimulation was either high or low. The number of eggs in the egg batch did have a significant influence on that hatch rates ($F = 172.08$, $P < 0.0001$). This has been observed in previous experiments and may be a form of negative feedback. Bacterial populations are responsible for the drop in the oxygen concentration in the hatch medium, which acts as the actual hatch stimulus (Gjullin et al. 1941, Judson 1960, Fallis and Snow 1983). As more larvae hatch, they feed on the bacteria, limiting any further drop in oxygen concentration in the hatch medium, which inhibits further hatching during the 24-h period that eggs are submerged (Livdahl and Edgerly 1987).

One response to varied frequency of wetting events was to simply avoid hatching (either for the LH or the HF stimulation). In all three treatments combined, only one egg batch showed no hatch response after the first treatment. This egg batch was a replicate in the HF treatment. The Wald χ^2 analysis indicated that a hatch or no hatch response was not significant (χ^2 value = 0.0025, $P = 0.9988$, $df = 1$) and is not a response to frequency of stimulation treatment.

Frequency of stimulation had a significant influence on the median stimulus as shown on Table 3. LF stimulation resulted in less hatch delay than HF stimulation. The LF treatment resulted in an average median hatch stimulus of 4.207 hatch stimuli, whereas the HF treatment resulted in an average median hatch stimulus of ≈ 6.397 hatch stimuli. These values were significantly different from each other ($F = 12.03$, $P = 0.001$). These results indicate that eggs subjected to less frequent hatch stimulation require fewer inundation events to hatch. Despite this increased hatch rate, the larvae will actually hatch at a later date because of the less frequent inundation. Age, initial hatch rate, and number of eggs were all nonsignificant factors influencing the hatch rate of the eggs.

There was a significant interaction between the initial hatch rates and the frequency of stimulation, shown in Fig. 1 ($F = 6.14$, $P = 0.02$). HF stimulation had an expected negative slope (a slope of -6.00), although a regression analysis indicates it was nonsignificant ($P = 0.167$). For HF stimulation, high initial hatch rates resulted in a lower median stimulus. LF stimulation had the opposite effect; as initial hatch rates increased, the median stimulus value also in-

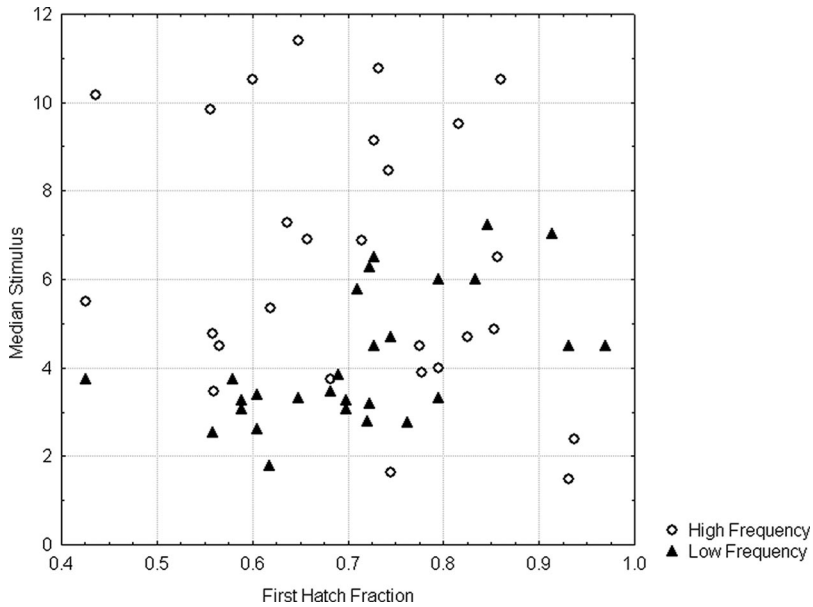


Fig. 1. The relationship between first hatch fraction and the median hatch stimulus. Although there was no significant difference in the hatch fractions on the first stimulus because of frequency of stimulation ($F = 1.93$, $P = 0.17$), there was a significant interaction between the treatment (frequency of stimulation) and the first hatch fraction on the median hatch stimulus ($F = 6.14$, $P = 0.02$). Eggs subjected to a low frequency of stimulation shows a increasing median hatch stimulus as the first hatch fraction increased. The opposite effect was seen in eggs subjected to a high frequency of stimulation.

creased. A regression analysis for the LF data were significant ($P = 0.002$), resulting in a regression line with a positive slope (6.78), although the r^2 value was not extremely high ($r^2 = 0.304$). This significant result indicates that infrequent stimulation results in a decrease in hatch rates or increased hatch delay (despite the fact that LF stimulation overall had less hatch delay than HF stimulation). A positive slope is only possible if the hatch rates changed after the initial hatch stimulus. This interaction between treatment and initial hatch rate was unexpected and does not lend itself to an easy interpretation. Re-examining this interaction with a larger sample size should help determine whether this interaction is accurate or whether it is caused by a small sample size. One explanation is that a change in response under LF stimulation may be the result of a change in the microbial fauna in the hatching medium. Aspbury and Juliano (1998) indicated that wetting and drying of detritus in the larval habitat has a negative influence on larval development and fitness. They hypothesized that this was caused by a change in microbial fauna as a result of the drying. Extended drying of the eggs may also influence the microbial population in the hatching medium caused by changes in the microbial fauna found on the egg itself. An increase in the microbial population results in a decrease in the oxygen concentration in the hatch medium, which acts as the hatch stimulus. If the microbial fauna on the egg changes because of repeated or extended drying (as seen in the LF treatment), the rate or degree of the drop in oxygen concentration in the hatch media may also change, potentially decreasing the hatch rate of

the eggs. This potential decrease in hatch rate was not observed in the LF treatment (which had an overall increase in hatch rate relative to the HF treatment), but a decreased hatch response may be more evident in egg batches that had a high initial hatch rate, resulting in a significant interaction term.

There was no influence from the age of the eggs. Delay did not have any significant influence on the median stimulus, indicating that age of the eggs was not a factor influencing the hatch response. This contradicts previous work with a colony strain of *Ae. albopictus*, showing that egg age does influence hatch rates (Vitek and Livdahl 2006). It is possible that the frequency of stimulation treatment may alter or overshadow any influence that age has on the hatch rates of eggs. Alternatively, colony strains that are hatched at regular intervals may be more sensitive to age factors, because the colony has undergone artificial selection to encourage regular oviposition and hatching.

The mosquito eggs clearly responded to varied frequency of hatch stimulation. Although the hatch rates on the first stimulus were not significantly different from each other, the calculated values for the median hatch stimulus were significantly different for the two treatments. However, the observed response of hatch rate to frequency of stimulation was the opposite effect than was predicted—eggs hatched earlier under simulated drought conditions, whereas under simulated frequent rainfall, eggs exhibited greater hatch delay. Eggs were expected to delay hatching until more favorable conditions exist under simulated drought conditions. By hatching faster, the larvae are seemingly putting themselves at risk in a potentially

unstable habitat. However, hatch rate may be influenced by desiccation resistance in the eggs. *Ae. albopictus* eggs are more desiccation resistant than some forest-dwelling species (Sota and Mogi 1992a) but not as desiccation resistant as some other urban species such as *Aedes aegypti* L. (Juliano et al. 2001). Mosquito species with eggs that are less desiccation resistant may have a better chance of surviving drought if the larvae hatch. Mosquito species with desiccation resistant eggs such as *Ae. aegypti* may show greater hatch delay in response to infrequent stimulation.

Development rate may also show a degree of plasticity in response to hatch stimulation frequency as well, which could explain these results. Development rates in mosquito larvae have been shown to be influenced by habitat desiccation (Juliano and Stoffregen 1994, Schäfer and Lundström 2006). Aspbury and Juliano (1998) determined the prior wet/dry cycles on detritus can also influence larval development, indicating a potential series of complex relationships between wet/dry cycles, larval development, and food resources. Mosquito eggs may hatch and larvae may develop more rapidly in response to drought conditions.

These preliminary data indicate that mosquito embryos may be able to detect cues regarding habitat stability and risk and may be able to respond to potentially limit that risk. Although organisms can and do respond to direct cues associated with habitat desiccation (Chodorowski 1979, Juliano and Stoffregen 1994, Schäfer and Lundström 2006), this study indicates that mosquitoes may detect and respond to indirect cues from their environment as well. In this experiment, we used two frequencies of stimulation, inundating eggs once every 3 or 7 d. Testing a greater range of frequencies, including frequencies of inundation less than once every 7 d, would yield a more accurate estimate of hatching behavior during long-term or extreme drought conditions. In addition to the frequency of stimulation, other indirect cues of drought such as humidity, quantity of rain during a rainfall, and duration of inundation may also induce a plasticity response and influence hatching behavior of *Ae. albopictus* or other container-breeding mosquitoes. These additional cues should be examined both in isolation and in conjunction with each other and frequency of stimulation. The combined influences may result in a greater change in hatching behavior. By developing a greater understanding of what influence mosquito hatching and development behavior, it may be possible to refine our ability to predict changes in mosquito populations, leading to an enhanced ability to control mosquito populations.

Acknowledgments

We thank S. Prager and W. Green for technical assistance in this research. We also thank J. Fung for providing appreciated comments on the manuscript. This research was funded by NIH Grant R15AI41191.

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Received 1 August 2007; accepted 11 February 2009.