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<th>Winter Activity and Diapause of Aedes albopictus (Diptera: Culicidae) in Hanoi, Northern Vietnam</th>
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<td>Author(s)</td>
<td>Tsunoda, Takashi; Chaves, Luis Fernando; Nguyen, Giang Thi Tra; Nguyen, Yen Thi; Takagi, Masahiro</td>
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Tsunoda et al.: Winter activity of *Aedes albopictus*

Development, Life history

**Winter activity and diapause of *Aedes albopictus* (Diptera: Culicidae) in Hanoi, northern Vietnam**

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ABSTRACT We studied the winter activity of *Aedes albopictus* (Skuse) from November 2008 to April 2009 in Bat Trang village of Hanoi, Vietnam. We selected 12 houses and collected: (i) adults with BG sentinel traps, (Fontes et al.) pupae from household water containers, and eggs (iii) with ovitraps. *Aedes albopictus* adults, pupae and eggs were not collected from early January to early February. Though the egg hatching probability tended to be initially high at longer day-length, the maximum probability gradually shifted to shorter day-length, as the observation period elapsed. When females were reared under long day-length and their eggs were immersed 1 or 5 weeks after oviposition, >50% of eggs hatched within 20 days. However, when females were reared under short-day length and their eggs were immersed after 1 week, hatching was suppressed for 60 days. When females were reared under short day-length, the median hatching day occurred earlier in eggs kept dry for 5 and 10 weeks after oviposition than in those dried for only 1 week. This indicates that the extended dry periods accelerate egg hatching. Our results showed that hatchability gradually changed with day-length, suggesting that selection for overwintering is not as strong relative to *Ae. albopictus* living in the temperate zone, where winter conditions are less favorable than in tropical and subtropical areas.

KEY WORDS bet-hedging, photoperiod, sub-tropics, hatchability
Many major insect vectors of human pathogens have become “domesticated,” breeding in close proximity to humans and seeking human blood-meals (Powell and Tabachnick 2013). The Asian tiger mosquito, *Aedes albopictus* (Skuse), is a major “domesticated” vector of Dengue and Chikungunya viruses (Reiter et al. 2006). *Aedes albopictus* colonizes artificial containers, such as used tires and bamboo stalks (Medlock et al. 2012, Bonizzoni et al. 2013), and this species is now cosmopolitan throughout tropical and temperate zones.

The occurrence of *Ae. albopictus* across temperate (Mori et al. 1981) and tropical (Suwonkerd et al. 1996) latitudes likely implies a diverse set of strategies to deal with changing environments (Levins 1968). For example, *Ae. albopictus* females from temperate latitudes, where winter temperatures limit insect development and activity, lay diapausing eggs when pupae and/or adults are exposed to short day-length at 25ºC (Mori and Wada 1978, Mori et al. 1981). Moreover, *Ae. albopictus* diapausing eggs have an increased stress resistance that might enhance survival during long-distance transport, and could partially explain the remarkable success of this species as a successful an invasive species (Denlinger and Armbruster 2014). In contrast, *Ae. albopictus* populations from subtropical environments lay eggs during the winter, with a small proportion of eggs hatching without undergoing any diapause (Higa et al. 2007). Thus, the study of *Ae. albopictus* overwintering in the transition area between subtropical and temperate environments is critical to understand how life history strategies could have shaped its invasion of new habitats worldwide (Lounibos 2002).

Hanoi is located in northern Vietnam, where minimum winter temperatures sometimes fall below 10ºC (Weatherbase 2013). Temperatures below 10ºC imply that *Ae. albopictus* here could often be below its developmental zero point (Chen and Huang 1988). *Aedes albopictus* is believed to have been originally restricted to Southeast Asian forests (Smith 1956). Wing morphometrics suggest that Hanoi *Ae. albopictus* populations are more closely related to those of Japan and Korea than to southern Vietnam (Morales et al. 2013). Given this, we asked whether *Ae. albopictus* in Hanoi enters diapause during winter. Specifically, we investigated the field activity of *Ae. albopictus* immatures and adults during the winter and also examined the effect of photoperiod on egg diapause in the laboratory.

**Materials and Methods**

**Meteorological Data.** Daily temperature and rainfall records for Hanoi, from November 2008 to April 2009 were obtained from the national Center for Hydro Meteorological Forecasting, Vietnam.
Monitoring of *Ae. albopictus*. From November 2008 to April 2009 we sampled *Ae. albopictus* in Bat Trang, a suburb of Hanoi. We monitored 12 houses, which were sampled biweekly. During each sampling session: (1) All the water-filled containers in each house were examined for the presence/absence of larvae and pupae. “Container index” was defined as (the number of positive container × 100) / the number of total containers. Small containers such as flower vases were emptied into a cup containing clean water to collect the immature mosquitoes. Jars and concrete tanks were collected with the quantitative sampling method that estimates the number of immatures (Knox et al. 2007). Though Bat Trang is famous for ceramics, we defined pottery left outside over 2 weeks as discarded. Pupae were collected with pipettes and reared to adults, which were identified using standard mosquito identification keys (Stojanovich and Scott 1966). (2) One BG sentinel trap (BioGents, Regensburg, Germany) was placed in a storeroom of each house for 22h (from 1000 to 0800) to collect adults. (3) An ovitrap was placed within a 3 m radius of each house. The trap consisted of a plastic bucket (20 cm diameter, 17 cm height) filled with 3 l of water for 1 week. The number of eggs from eight paper strips (30 cm × 7 cm) placed inside each ovitrap was counted under a dissecting microscope.

**Experiment 1: Egg Hatching from *Ae. albopictus* Grown under Different Photoperiods.** *Aedes albopictus* larvae were collected from Bat Trang in August 2009 and kept at room temperature. Second-instar larvae were divided into six incubators at 25°C (treatments) with different photoperiods: (1) 9h light: 15h dark, (2) 10h light: 14h dark, (3) 11h light: 13h dark, (4) 12h light: 12h dark, (5) 13h light: 11h dark, (6) 14h light: 10h dark to examine the threshold photoperiod of diapause. All adults used in this experiment were confirmed as *Ae. albopictus* before oviposition started. Eggs were collected from females reared in each incubator. Eggs were also dried for two days, and kept in each incubator for five days prior to the hatching experiment. For the experiment, we prepared 8 cups that contained 20 eggs each per treatment, eggs were submerged in deionized water, and repeated twice. During the 100 d observation period for egg hatching, water was changed every day and eggs were kept at 25°C. Unhatched eggs were dissected under a microscope to examine embryonation.

**Experiment 2: Egg Hatching from *Ae. albopictus* Grown at Different Photoperiods, with Different Post-oviposition Age and Hatching Photoperiod.** Eggs from *Ae. albopictus* raised at 25°C in the following two photoperiods: (1) 10 h light: 14h dark (i.e., short-day) and (2) 14h light: 10h dark (i.e., long-day) conditions were removed and dried. They were submerged in plain water 1, 5, and 10 weeks after oviposition, and hatched under the two photoperiods defined previously, i.e., short-day
and long-day. The resulting treatments, i.e., the combination of *Ae. albopictus* development photoperiod until oviposition and post-oviposition age, were conducted using 20 eggs per cup. Hatching was observed for 100 d in all the treatments, each treatment comprising 12 cups. After 100 d immersion, unhatched eggs were then dissected under a microscope to examine embryonation. As in the previous experiment, all adults used in the experiment were confirmed as *Ae. albopictus* prior to oviposition.

**Statistical Analysis.** To study the critical photoperiod of *Ae albopictus*, we fitted a negative binomial generalized linear model (Venables and Ripley 2002) to the number of eggs that hatched in 25 day intervals during the 100 d of experiment 1, as a function of both day-length and the day when unhatched eggs were counted. We chose a negative binomial model to account for the over-dispersion in the number of hatched eggs (Mangel 2006). The model and parameter estimates are presented in Table S1.

A Kruskal-Wallis rank sum test was used to compare the hatching day of eggs with different post-oviposition ages in the four different light treatments resulting from the combination of growing photoperiod and hatching photoperiod of Experiment 2. For Experiment 2, we also developed an egg hatching hazard model. *Aedes albopictus* egg hatching (eh$_X$) was estimated daily using the equation eh$_X$ = Eh$_X$/eh$_0$, where Eh$_X$ indicates the cumulative number of eggs that remained without hatching up to day X, and eh$_0$ the initial number of eggs in each treatment, i.e., 120. Since eh$_X$ can be seen as analogous to a survival schedule, daily egg hatching was analyzed using a Cox proportional hazard model. The Cox hazard model considered the additive effects of light treatment and the number of weeks eggs were dormant (i.e., post-oviposition age) as covariates driving the hatching hazards. In the Cox hazard model egg hatching was modeled using a baseline hazard function $h_0$, so that the hazard function $h(t)=h_0\exp(f(covariates(t)))$ measured the proportional increase in egg hatching. When implementing the Cox hazard model, we only counted the eggs that were still viable (alive) at day 100 when the experiment finished. We compared whether unhatched eggs across the range of post-oviposition times and light treatments had similar dead to alive odds ratios at the end of the experiment (day 100), using a binomial generalized linear model (Faraway 2006).

**Results**

**Field Survey of *Ae. albopictus*.** Mean temperature dropped below 17°C from late December to early March, with the lowest in late January (Fig. 1A). Precipitation decreased suddenly from mid-November (Fig. 1B). Though precipitation was above
80mm per day in early November, there was little rain from mid-November to mid-March. Discarded containers were the most abundant container in most households (Table 1). One household (No. 12) had the highest number of flower vases, Bonsai, and discarded containers. The mean number of containers with water was 3.0 to 10.6 from November to February and it was above 10 from March to April (Fig. 2A). There was no correlation between mean number of containers with water and the precipitation from the previous survey date to the current survey (P>0.05). The container index was highest in early November and then decreased gradually, reaching a minimum in late February (Fig. 2B). The container index gradually increased from March onward. Density of pupae per container was low from November to February and increased from March onward (Fig. 2C).

We collected 3,244 Culex pipiens quinquefasciatus Say, 102 Ae. albopictus, 38 Armigeres subalbaltus (Coquillett), 21 Cx. vishuni Theobald, 14 Anopheles sinensis Wiedemann, 11 Cx. tritaeniorhynchus Giles, and 6 An. tessellatus Theobald from November to April by BG sentinel trap. The number of Ae. albopictus adults per trap decreased from November, reaching a minimum in January, and increased from February onward (Fig. 3). Ovitraps were positive at a rate of over 0.5 until mid-December, then less than 0.4 from late December to early March (Fig. 4A). The number of eggs decreased from November onward, nearly reaching zero from late December to early March, except for late February (Fig. 4B). Both positive rate and the mean number of eggs increased after late March, though there were fewer eggs in spring than in autumn.

**Laboratory Diapause Experiment.** The egg hatching probability tended to be high at longer day-length for the 25 d observation period (Fig. 5). However, as the observation period was extended, the day-length at the maximum hatching probability was reduced. The hatching probability was highest at an 11 hour day during 75 days of the observation period in the Experiment 1.

When females were reared under long day-length (Long-Long, Long-Short) and their eggs immersed 1 and 5 weeks post-oviposition, >50% of eggs hatched within 20 d (Fig. 6A, B). Hatchability of eggs from females reared under long-day conditions and immersed 10 weeks post-oviposition was between 0.6 and 0.7 after 100 d. When females were reared under short-day (Short-Long, Short-Short) and their eggs immersed after 1 week, hatching were suppressed for about 60 days (Fig. 6C, D). When eggs of short-day females were immersed 5 or 10 weeks post-oviposition, hatchability was higher than that of 1-week post-oviposition eggs. The Cox Proportional Hazards model showed that both time post-oviposition and light treatment had an effect on egg
hatching (Table 2). When we examined unhatched eggs for embryonation at the end of the 100 d observation period, both time post-oviposition and light treatment had an effect on embryonation status (Table 3).

The median day of hatching was also significantly different when females were reared under long-day (Long-Long, $\chi^2=91.00$, d. f. =1, $P<0.001$; Long-Short, $\chi^2=133.21$, d. f. =1, $P<0.001$) (Fig. 7A, B). When females were reared under short-day, the median day of hatching was earlier in eggs dry for 5 and 10 weeks than those dry 1 week (Short-Long, $\chi^2=165.67$, d. f. =1, $P<0.001$; Short-Short, $\chi^2=318.02$, d. f. =1, $P<0.001$) (Fig. 7C, D). This indicates that extended time until immersion accelerates hatching of eggs in diapause.

**Discussion**

Our results indicate that reproduction of *Aedes albopictus* was markedly suppressed during winter in Hanoi, although there was some oviposition. The primary reason for low winter reproduction is that flight activity is reduced, as seen *Ae. aegypti* (Christophers 1960), as the average air temperature is 17°C and the minimum average temperature is 12°C in January (Weatherbase 2013). Unlike *Ae. aegypti* which prefers concrete tanks underground, *Ae. albopictus* prefers containers put outside (e.g. garbage and flowerpots) (Tsunoda et al. 2014), exposing them to colder temperatures. Data indicate that *Ae. aegypti* is sluggish below 17°C and flies with difficulty at 12-14°C (Christophers 1960). Thus, flight activity would seem problematic for *Ae. albopictus* in January.

A second consideration is egg diapause in winter. Geographic variation of the photoperiodic response is known for North American and East Asian populations of *Ae. albopictus*, and populations collected from Taiwan and Hong Kong do not exhibit diapause (Hawley et al. 1987, Philippi and Seger 1989). However, though Hanoi is located at a lower latitude than Taiwan and Hong Kong, our data show that hatchability of *Ae. albopictus* eggs collected here were affected by day-length, as eggs laid under short-day delayed hatching. Since the hatching rate of *Ae. albopictus* eggs is low during winter in Okinawa Island despite a high embryonation rate, they are considered in “light dormancy” (Higa et al. 2007). Our results suggest the same situation in Hanoi.

Bet-hedging is defined as a strategy, where unpredictably variable environments favor genotypes with lower variance in fitness at the cost of lower mean fitness (Cohen 1966, Philippi and Seger 1989, Hopper 1999, Ripa et al. 2010). When species do not have reliable cues for the start of unfavorable environmental conditions, natural
selection will favor either genotypes with an obligate diapause commencing before 
conditions become unfavorable, or genotypes that produce both diapausing and 
non-diapausing phenotypes with or without modification of diapause frequency by 
environmental cues (Seger and Brockmann 1987, Hopper 1999). Diapause theory 
suggests that in environments with unpredictable lengths of favorable seasons, the 
proportion of individuals diapausing should increase during the favorable season as the 
likelihood of completing another generation declines. This leads to a gradual 
increase in the proportion diapausing as the season advances, suggesting risk-spreading 
as long as the variation in diapause frequency is not genetic (Seger and Brockmann 

Egg-hatching in Aedes mosquitoes is regarded as bet-hedging, since emergence is 
often staggered even if environmental conditions are favorable and development is 
prepared (Evans and Dennehy 2005). Since a bet-hedging strategy implies an 
evolutionary tradeoff between risk aversion and early reproduction, many organisms 
may be affected by competing selective pressures for both immediate and delayed 
hatch (Khatchikian et al. 2010). Selection for risk-spreading might explain variation 
in frequency of diapauses in species with facultative diapause (Walker 1980, Bradford 
genetic variation in the duration of egg dormancy in populations occupying larval 
habitats that occasionally become entirely unsuitable, the model shows that a more 
catastrophic environment will favor the late-hatching allele, presenting the possibility 
for a decline in the intrinsic rate of increase (Denlinger and Armbruster) with an 
increase in environmental uncertainty (Livdahl 1979). In Aedes triseriatus, both low 
precipitation and high variability in precipitation directly increase the delaying pattern, 
which is an adaptive bet-hedging strategy that allows the species to manage desiccation 
risks (Khatchikian et al. 2010).

As precipitation is low during winter in Hanoi, it is reasonable to conclude that 
delaying egg hatching under short-day would develop as risk aversion for low and 
unpredictable precipitation in winter. Since season and precipitation are closely 
related in Hanoi, the delaying pattern would be influenced by day-length. The 
sharpness of the photoperiodic response will be greater the larger the standard 
development from the mean maturation date of a particular generation, which may explain 
why mosquitoes, which usually have short generation times, have diapausing fractions 
which increase only gradually as the season advances (Cohen 1970). Insects that 
were under greater pressure from natural selection for timing of the induction of the 
overwintering exhibit a steeper curve through the critical photoperiod (Lees 1968).
The strains of *Ae. albopictus* from northern Asia and North America showed higher overwintering survival rates than the strains from tropical Asia, Hawaii, and Brazil in field experiments (Hawley et al. 1989). Our results show that hatchability gradually changed with day-length, suggesting that natural selection for overwintering is not as strong as in temperate zone *Ae. albopictus*.

Our study also showed that *Ae. albopictus* eggs hatched at higher rates as the period before or after immersion in water was prolonged, suggesting that hatching is dependent on the energy content of the eggs themselves. Diapausing insects with low energy reserves have higher mortality during diapause than those with enough energy reserves (Hahn and Denlinger 2007). Metabolism is proportional to temperature in diapausing insects (Irwin and Lee 2003). The low temperatures during winter greatly favor conservation of energy reserves to maintain high survival (Irwin and Lee 2000). Though diapause is not uncommon phenomenon among tropical insects, metabolic depression is still important in diapausing insects living here (Denlinger 1986). When winter temperatures are mild, as in Hanoi, it may be hard for eggs to maintain low metabolic levels, even if they enter diapause.

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and Associates LLC.
Table 1. The mean number ± S. E. of water containers in the houses surveyed from November 2008 to April 2009

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</tbody>
</table>

1) House hold, 2) Round concrete tank, 3) Other concrete tank, 4) Plastic drum, 5) Flower vase, 6) Pig through, 7) Toilet concrete tank, 8) Discarded.
Table 2. Cox Proportional Hazards for *Aedes albopictus* egg hatching as a function of day-length (Light Treatment) and time since oviposition (Time)

| Factor          | Hatching Hazard | Estimate | S.E. | Z     | Pr(>|z|) |
|-----------------|-----------------|----------|------|-------|---------|
| Time (Weeks)    |                 |          |      |       |         |
| 1               | 1               | -        | -    | -     | -       |
| 5               | 1.478           | 0.391    | 0.073| 5.332 | <0.0005*|
| 10              | 1.165           | 0.153    | 0.082| 1.861 | 0.0627  |
| Light Treatment |                 |          |      |       |         |
| Long-Long       | 1               | -        | -    | -     | -       |
| Long-Short      | 0.519           | -0.656   | 0.082| -8.021| <0.0005*|
| Short-Long      | 0.388           | -0.948   | 0.103| -9.227| <0.0005*|
| Short-Short     | 1.388           | 0.328    | 0.082| 4.017 | <0.0005*|

*Statistically significant, P<0.05.
Table 3. Odds for unembryonated vs embryonated eggs at the end of the experiment (day 100) as a function of time since oviposition and light treatment

| Factor          | Odds Ratio | Estimate | S.E. | Z   | Pr(>|z|)  |
|-----------------|------------|----------|------|-----|-----------|
| Time (Weeks)    |            |          |      |     |           |
| 1               | 1          | -        | -    | -   | -         |
| 5               | 9.764      | 2.279    | 0.247| 9.221| <2e-16*   |
| 10              | 4.273      | 1.452    | 0.237| 6.139| 8.33E-10* |
| Light Treatment |            |          |      |     |           |
| Long-Long       | 1          | -        | -    | -   | -         |
| Long-Short      | 0.182      | -1.704   | 0.213| -7.997| 1.28E-15* |
| Short-Long      | 0.386      | -0.951   | 0.213| -4.455| 8.38E-06* |
| Short-Short     | 0.186      | -1.679   | 0.240| -7.009| 2.39E-12* |

*Statistically significant, P<0.05.
Table S1 Negative Binomial generalized linear model parameter estimates employed to generate the surface of Fig. 5.

| Parameter          | Estimate | S. E. | z value | Pr(>|z|) |
|--------------------|----------|-------|---------|----------|
| Intercept (μ)      | -21.87   | 4.96  | -4.408  | <0.0001* |
| Time (α)           | 0.304    | 0.030 | 10.02   | <0.0001* |
| Light (β)          | 2.70     | 0.81  | 3.326   | <0.0008* |
| Time² (γ)          | -0.0008  | 0.0001| -6.267  | <0.0001* |
| Light² (δ)         | -0.0675  | 0.0339| -1.988  | <0.046*  |
| Time*Light (θ)     | -0.0161  | 0.0019| -8.398  | <0.0001* |
| Dispersion (κ)     | 3.50     | 0.78  |         |          |

*Statistically significant (P<0.05)

Model fitted had the following structure:

\[ E_{hi}(\text{Time, Light}) = \omega \]

\[ \omega = \exp(\mu + \alpha \times \text{Time} + \beta \times \text{Light} + \gamma \times \text{Time}^2 + \delta \times \text{Light}^2 + \theta \times \text{Time} \times \text{Light} + \text{error}) \]

Where \( E_{hi} \) is the number of hatched eggs at a given time interval (Time) and kept under different day lengths (Light), and has a mean equal to \( \omega \), and a variance equal to \( \omega + \omega^2/\kappa \), where \( \kappa \) is the dispersion parameter of the negative binomial distribution. The Akaike Information Criterion (AIC) of this model was 423, and was minimum when compared with simpler, and more complex, versions of the model presented in the above equation (differences were greater than 10 AIC units, also supporting that the model was not over-parameterized). Finally, to generate the hatching probability the recorded number of eggs and the surface fitted by the model were divided by 240, the number of eggs in each treatment at the beginning of the experiment.
Figure legend

Fig. 1. Meteorological data from November 1, 2008, to April 30, 2009, in Hanoi. (A) Daily mean temperature. (B) Daily precipitation.

Fig. 2. (A) Mean number of containers with water in a household of Bat Trang from early November 2008 to late April 2009. (B) Container index of *Aedes* mosquitoes in Bat Trang from early November 2008 to late April 2009. Number in parenthesis is total number of containers holding water. (C) Mean number of *Ae. albopictus* pupae per container of Bat Trang from late November 2008 to late April 2009. Bars indicate SE.

Fig. 3. Mean number of *Ae. albopictus* adults collected by BG Sentinel trap in Bat Trang from early November 2008 to late April 2009. Bars indicate SE.

Fig. 4. (A) Ovitrap positive rate of ovitrap in Bat Trang from late November 2008 to late April 2009. Numbers under x-axis are the collection date. (B) Density of *Aedes albopictus* eggs collected from ovitraps in Bat Trang from late November 2008 to late April 2009. Bars indicate SE.

Fig. 5. Critical photoperiod for *Aedes albopictus* egg hatching. The x axis is the egg day-length during Experiment 1, the y axis the day cumulative hatching was recorded, colors indicate egg predicted hatching probability for different times and day lengths (a color graded scale is at the right of the main plot). Circles represent the observed data, and circle size is proportional to the observed hatching probability, and a reference scale is provided to the right of the main plot. For reference, the black dot in the bottom-left corner of the plot corresponds to a hatching probability of 0.1. Parameters employed for the probability surface construction are presented online only in Table S1.

Fig. 6. *Aedes albopictus* egg hatching trajectories under different light treatments and post-oviposition times (A) Long-Long light treatment (lt), (B) Long-Short lt (C) Short-Long lt and (D) Short-Short lt. For the post-oviposition times, refer to the inset legend in panel D.

Fig. 7. Median day of *Aedes albopictus* egg hatching after immersion in water under
different day-length. (A) Long-Long, (B) Long-Short, (C) Short-Long, (D) Short-Short. ‘Long’ means 14 h light and 10 h dark condition. ‘Short’ means 10 h light and 14 h dark condition. Combination of day-length (e.g. Long-Long) indicates the day-length condition of eggs before and during the observation.
Fig. 1

A

Daily mean temperature (°C)

B

Precipitation / day (mm)

November 2008 to April
Fig. 2

A) No. of container/house

B) Container Index

C) No. of pupa/container
Fig. 4

A) Positive rate of ovitraps

B) No. of Aedes eggs / ovitraps
Fig. 6
Fig. 7