Effects of Water Color and Chemical Compounds on the Oviposition Behavior of Gravid *Culex pipiens pallens* Females under Laboratory Conditions

Jing Li, Tianfu Deng, Hongjie Li, Li Chen, and Jianchu Mo

**ABSTRACT** Oviposition behavior is a plastic trait that can be affected by a variety of physical factors and chemical cues. The effects of water color and three chemical compounds on the oviposition behavior of *Culex pipiens pallens* (Diptera: Culicidae) females were studied under laboratory conditions. Solutions of methylene blue (50 mg/L) and brilliant blue (50 mg/L and 200 mg/L) attracted significantly more gravid *C. pipiens pallens* females than did the distilled water control. Among the three chemical compounds tested, only 0.1 mg/L acetic acid solution showed significantly greater oviposition attraction activity than the control solutions of distilled water or acetone (25 mg/L) in water. A mixture of 25 mg/L methylene blue plus 0.05 mg/L acetic acid attracted significantly more gravid females to lay eggs than did distilled water, methylene blue solutions alone, or acetic acid solutions alone. A solution of 25 mg/L methylene blue and 0.05 mg/L acetic acid might have potential for use in traps for monitoring population densities of *C. pipiens pallens*.

**KEY WORDS** *Culex pipiens pallens*, gravid female, oviposition, dye, compound

The selection of an oviposition site is an important component of the behavioral repertoire of mosquitoes (Diptera: Culicidae), and the correct choice is crucial for the survival of progeny. The attractiveness of these sites to ovipositing mosquitoes is dependent upon a number of physical, chemical, and biological cues presented under natural conditions (Millar et al. 1994, Dhileepan 1997, Olagbemiro et al. 1999, Geetha et al. 2003).

Water color has an important influence on the oviposition activity of mosquitoes. Studies have shown that dyed water is more attractive to ovipositing mosquitoes than un-dyed water under both laboratory and field conditions (Williams 1962, Beehler & DeFoliart 1990, Beehler et al. 1993, Iseoe et al. 1995). Black water was the most preferred oviposition site for the gravid *Culex quinquefasciatus* Say (Diptera: Culicidae) females (Frank 1985). Dhileepan (1997) demonstrated that black and red water were the most preferred
oviposition sites for gravid *C. annulirostris* Skuse and *C. molestus* Ficalbi females, respectively.

Natural chemical cues also influence mosquito oviposition behavior (Geetha et al. 2003), and 3-methylindole was shown to be the most attractive chemical to gravid *Culex* females under both laboratory and field conditions (Millar et al. 1992, Mordue (Luntz) et al. 1992, Blackwell et al. 1993, Beehler et al. 1994). In addition, the influence of some fatty acids on the oviposition behaviors of *C. quinquefasciatus* and *C. tarsalis* Coquillett has also been evaluated under laboratory condition (Hwang et al. 1980, 1982, 1984). However, chemical cues and water color do not act independently in natural situations, as they typically interact with other factors at the oviposition site for mosquito species (Dhileepan 1997, Mboera et al. 1999, Olagbemiro et al. 2004).

*Culex pipiens pallens* Coquillett is an important disease vector widely distributed in northern and central China. This species transmits lymphatic filariasis, Japanese B encephalitis, and other viral diseases (Li et al. 2002, Tao et al. 2006). Since 2005, the Chinese government has monitored population densities of *C. pipiens pallens* by counting the average number of egg rafts in the water containers of ovitraps (Service 1993). Thus, the “Mosq-ovitrap” (Lin et al. 2005) has become an important tool for monitoring mosquito densities in China. However, because of lack of information on the oviposition site preference of female *C. pipiens pallens*, the monitoring of population dynamics of this mosquito has been unsatisfactory. The objective of this study was to study the effects of water color and certain chemical compounds on the oviposition behavior of gravid *C. pipiens pallens* females.

**Methods and Materials**

**Mosquito rearing.** *Culex pipiens pallens* was maintained in a rearing room at 26°C, 65% RH, and L:D 14:10 h photoperiod. Larvae were reared in plastic basins filled with dechlorinated tap water. Commercial rat food formulated with wheat flour, wheat bran, corn powder, soybean powder, fish meat powder, yeast, salt, and vitamins was provided as nutrient for mosquito larvae. Pupae were collected and allowed to emerge in a gauze cage (35 × 35 × 35 cm). A diet of 5% sucrose solution was provided for the adults. Females were allowed to feed on the blood of a live mouse at the fifth day after emergence. Twenty-four hours after blood feeding, the gravid females were used for experiments.

**Chemical compounds.** Methylene blue, acetic acid, and ethyl acetate were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Carmine, brilliant blue, fruit green, and tartrazine dye were purchased from Shanghai Dye Research Institute Co., Ltd. (Shanghai, China). Dimethyl disulfide were purchased from Sigma-Aldrich Co., Ltd. (Shanghai, China).

**Experiments.** All laboratory experiments were carried out in a 190 cm × 150 cm × 140 cm mosquito net in the laboratory (26 ± 1°C, 65 ± 5% RH, and L:D 14:10 photoperiod). Seventy-five gravid females were used for each experiment. The number of egg rafts laid by females in each beaker was recorded four days after the start of each experiment.

Experiment 1 was designed to assess the attractiveness of different colored water to gravid female mosquitoes. The dyes tested in this experiment were methylene blue, carmine, brilliant blue, fruit green, and tartrazine. The
concentrations of dyes in distilled water were 200, 50, 12.5, and 3.1 mg/L. For each of the three replicates, 21 beakers (100 ml) with 50 ml distilled water or dye solution were placed equidistantly in a random pattern around the edge of the mosquito net.

Experiment 2 was designed to assess the attractiveness of acetic acid, ethyl acetate, and dimethyl disulfide to gravid female mosquitoes. The concentrations of these compounds in distilled water were 100, 10, 1, 0.1, and 0.01 mg/L. During formulation, acetic acid was dissolved directly in distilled water, whereas ethyl acetate and dimethyl disulfide were first dissolved in pure acetone and then diluted with distilled water to the experimental concentrations. The final concentration of acetone in the formulated solutions was 25 mg/L, and a water:acetone solution of this concentration was used as an additional control for this experiment. For each of the eight replicates, 17 beakers (100 ml) with 50 ml distilled water (Control 1), 25 mg/L acetone solution (Control 2), or one of the compound solutions were placed equidistantly in a random pattern around the edge of the mosquito net.

Experiment 3 was designed to assess the combined attractiveness of blue water color and the acetic acid to gravid female mosquitoes. Methylene blue was used as the colorant in distilled water at concentrations of 25 mg/L, 50 mg/L, and 75 mg/L. Acetic acid in distilled water was used at concentrations of 0.05 mg/L, 0.10 mg/L, and 0.50 mg/L. For each of the five replicates, 16 beakers with 50 ml of a treatment solution were placed equidistantly in a random pattern around the edge of the mosquito net.

Statistical analyses. For all experiments, the number of egg rafts in each beaker was recorded and used for analysis. The data were analyzed with ANOVA and LSD by the DPS® software (Tang & Feng 2002) to detect any significant differences between treatments.

Results

Effect of water color on the oviposition behavior of gravid females. The water-color experiment showed that 50 mg/L of methylene blue solution and 50 mg/L and 200 mg/L of brilliant blue solution were significantly preferred by the gravid females of C. pipiens pallens compared with distilled water (ANOVA: $F = 3.462$, df = 20, 42; $P = 0.0003$) (Table 1). In this experiment, $10.00 \pm 2.52$ egg rafts were laid in 50 mg/L methylene blue solution, $8.67 \pm 2.33$ egg rafts in 200 mg/L brilliant blue solution, and $6.33 \pm 4.10$ egg rafts in 50 mg/L brilliant blue solution (Table 1). No significant differences were observed between the other dye solutions and distilled water ($P > 0.05$, Table 1).

Effect of chemical compounds on the oviposition behavior of gravid females. Among the three compounds tested, only 0.1 mg/L of acetic acid solution significantly attracted more gravid C. pipiens pallens females to oviposit than did the controls of distilled water or 25 mg/L acetone solution ($F = 3.715$; df = 16, 119; $P = 0.0001$). There were no significant differences among the other compounds and control solutions ($P > 0.05$, Table 2).

Combined effect of color and compound on the oviposition behavior of gravid females. Results indicated that combinations of methylene blue with acetic acid had a significant effect on the oviposition behavior of C. pipiens pallens females ($F = 4.595$; df = 15, 64; $P = 0.0001$). The mixture of 25 mg/L
Table 1. Number of egg rafts laid by *C. pipiens pallens* females in water colored by different dyes.

<table>
<thead>
<tr>
<th>Dye</th>
<th>Dye concentration in distilled water (mg/L)</th>
<th>Number of egg rafts (Mean ± SE)¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylene blue</td>
<td>200</td>
<td>1.33 ± 0.88 de</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>10.00 ± 2.52 a</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>1.33 ± 0.67 de</td>
</tr>
<tr>
<td></td>
<td>3.1</td>
<td>1.67 ± 0.33 cde</td>
</tr>
<tr>
<td>Brilliant blue</td>
<td>200</td>
<td>8.67 ± 2.33 ab</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>6.33 ± 4.10 abc</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>0.67 ± 0.33 de</td>
</tr>
<tr>
<td></td>
<td>3.1</td>
<td>2.00 ± 1.15 cde</td>
</tr>
<tr>
<td>Carmine</td>
<td>200</td>
<td>5.33 ± 2.19 bcd</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>3.33 ± 0.33 cde</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>3.00 ± 1.00 cde</td>
</tr>
<tr>
<td></td>
<td>3.1</td>
<td>2.67 ± 1.20 cde</td>
</tr>
<tr>
<td>Fruit green</td>
<td>200</td>
<td>3.33 ± 0.88 cde</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>2.00 ± 1.15 cde</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>1.33 ± 0.33 de</td>
</tr>
<tr>
<td></td>
<td>3.1</td>
<td>1.00 ± 0.58 de</td>
</tr>
<tr>
<td>Tartrazine</td>
<td>200</td>
<td>0.67 ± 0.33 de</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>1.00 ± 0.58 de</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>1.33 ± 0.88 de</td>
</tr>
<tr>
<td></td>
<td>3.1</td>
<td>0.00 ± 0.00 e</td>
</tr>
<tr>
<td>Distilled water</td>
<td>0</td>
<td>1.33 ± 0.88 de</td>
</tr>
</tbody>
</table>

¹Means in the same column followed by different letters are significantly different (*P* < 0.05, ANOVA).

Methylene blue and 0.05 mg/L acetic acid induced the most *C. pipiens pallens* females to lay eggs. Significantly more egg rafts were laid by females in this solution than in distilled water, in methylene blue-water alone, or in acetic acid solution alone (Table 3). However, there were no significant differences in oviposition between this mixture and six of the eight other mixtures of methylene blue and acetic acid, and only two mixtures of these materials (75 mg/L methylene blue + 0.10 mg/L acetic acid and 50 mg/L methylene blue + 0.50 mg/L acetic acid) attracted significantly fewer egg rafts. Also for this experiment, there were no significant differences between distilled water, methylene blue solution alone, or acetic acid solution alone (*P* > 0.05) (Table 3).

**Discussion**

Water color is an important factors influencing oviposition behavior of female mosquitoes. Our study showed that *Culex pipiens pallens* females laid significantly more eggs in dark blue water (50 mg/L of methylene blue solution, or 50 mg/L and 200 mg/L of brilliant blue solution) than in distilled water. This behavior has also been shown for *C. tarsalis*, *C. quinquefasciatus*, *C. annulirostris*, and *C. molestus* (Beehler & DeFoliart 1990, Yap et al. 1995,
Dhileepan 1997). Beehler et al. (1993) suggested that dark colors attract more gravid C. quinquefasciatus because females respond to the increased optical density of the dyed water rather than to the volatile components of the dye.

Hwang et al. (1980, 1982, 1984) reported that small chain aliphatic acids (e.g., acetic, propionic, isobutyric, butyric acids), homologues of C\textsubscript{5} to C\textsubscript{13} and C\textsubscript{16} to C\textsubscript{22} fatty acids, elicited negative oviposition activity when tested for C. quinquefasciatus, C. tarsalis, and Aedes aegypti (L.). However, a recent study showed that larger chain fatty acids of C\textsubscript{12} to C\textsubscript{18} isolated from the egg cuticle of A. aegypti exhibited oviposition activity at one of three concentrations (1, 10 and 100 mg/L) tested (Ganesan et al. 2006). In our study, acetic acid exhibited significantly stronger attractiveness to the gravid C. pipiens pallens females at 0.1 mg/L concentration compared with distilled water.

There have been few studies on the effects of esters of fatty acids on the oviposition behavior of mosquitoes (Perry & Fay 1967, Ganesan et al. 2006). However, the current study showed that 10 mg/L and 0.1 mg/L of ethyl acetate water solution had significantly higher attractive activity to gravid C. pipiens pallens females compared with distilled water. Torres-Estrada et al. (2007) suggested that ethyl acetate in the extract of Spirogyra majuscula Kützing algae (Zygemataceae) might also be involved in attraction of gravid Anopheles pseudopunctipennis Theobald (Diptera: Culicidae) females to oviposition sites.

<table>
<thead>
<tr>
<th>Chemical Compound</th>
<th>Compound concentration in distilled water (mg/L)</th>
<th>Number of egg rafts (Mean ± SE)\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>100</td>
<td>0.25 ± 0.16 c</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.25 ± 0.37 bc</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1.37 ± 0.46 bc</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>7.12 ± 1.94 a</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>1.87 ± 0.69 bc</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>100</td>
<td>3.00 ± 0.82 b</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.12 ± 0.23 bc</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1.75 ± 0.65 bc</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>2.62 ± 1.25 bc</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>1.50 ± 0.38 bc</td>
</tr>
<tr>
<td>Dimethyl disulfide</td>
<td>100</td>
<td>2.25 ± 0.73 bc</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>3.62 ± 0.75 bc</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>3.37 ± 0.86 b</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>2.00 ± 0.98 bc</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>1.00 ± 0.27 bc</td>
</tr>
<tr>
<td>Control 1\textsuperscript{b}</td>
<td>0</td>
<td>1.25 ± 0.31 bc</td>
</tr>
<tr>
<td>Control 2\textsuperscript{c}</td>
<td>0</td>
<td>1.75 ± 0.73 bc</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Means in the same column followed by different letters are significantly different (\textit{P} < 0.05, ANOVA).

\textsuperscript{b} Distilled water.

\textsuperscript{c} Distilled water plus 25 mg/L acetone.
However, in our study, ethyl acetate water solution was not attractive to gravid *C. pipiens pallens* females. Hog waste lagoons and other animal waste sites are common places in which *Culex* mosquitoes are produced (O’Meara & Evans 1983). Dimethyl disulfide was demonstrated to be a component in hog lagoon odors (Zahn et al. 2001). Trexler et al. (2003) reported that dimethyl disulfide did not elicit the oviposition response of *Aedes albopictus* (Skuse) (Diptera: Culicidae) in laboratory bioassay and in field trial. Similarly, in our study, *C. pipiens pallens* females also did not show a significant preference to dimethyl disulfide solution compared with the control solutions. Interactions between chemical attractants and physical cues have been documented as affecting the ovipositional behavior of some mosquito species (Beehler et al. 1993, Mboera et al. 1999, Olagbemiro et al. 2004). Beehler et al. (1993) reported that gravid *C. quinquefasciatus* females used both visual and chemical cues in the selection of an oviposition site, and water dyed with ink acted synergistically with a five-component chemical attractant mixture (3-methylindole, indole, 4-methylphenol, 4-ethylphenol, and phenol) under laboratory conditions. In our study, most of the mixtures of methylene blue and acetic acid induced a significant ovipositional response by *C. pipiens pallens* females. The solution of 25 mg/L methylene blue and 0.05 mg/L acetic acid was especially attractive and induced the most females to lay eggs. This solution could be of potential use in traps for monitoring population densities of *C. pipiens pallens* in the field.

### Table 3. Number of egg rafts laid by *C. pipiens pallens* females in mixed water solutions of methylene blue and acetic acid.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Number of egg rafts (Mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>75 mg/L methylene blue</td>
<td>0.40 ± 0.24 c</td>
</tr>
<tr>
<td>50 mg/L methylene blue</td>
<td>2.00 ± 0.63 c</td>
</tr>
<tr>
<td>25 mg/L methylene blue</td>
<td>1.20 ± 0.37 c</td>
</tr>
<tr>
<td>0.50 mg/L acetic acid</td>
<td>0.40 ± 0.24 c</td>
</tr>
<tr>
<td>0.10 mg/L acetic acid</td>
<td>3.00 ± 1.41 bc</td>
</tr>
<tr>
<td>0.05 mg/L acetic acid</td>
<td>1.40 ± 0.60 c</td>
</tr>
<tr>
<td>75 mg/L methylene blue + 0.50 mg/L acetic acid</td>
<td>4.60 ± 1.08 abc</td>
</tr>
<tr>
<td>75 mg/L methylene blue + 0.10 mg/L acetic acid</td>
<td>1.40 ± 0.93 c</td>
</tr>
<tr>
<td>75 mg/L methylene blue + 0.05 mg/L acetic acid</td>
<td>3.40 ± 0.98 abc</td>
</tr>
<tr>
<td>50 mg/L methylene blue + 0.50 mg/L acetic acid</td>
<td>2.40 ± 1.17 bc</td>
</tr>
<tr>
<td>50 mg/L methylene blue + 0.10 mg/L acetic acid</td>
<td>5.20 ± 2.54 abc</td>
</tr>
<tr>
<td>50 mg/L methylene blue + 0.05 mg/L acetic acid</td>
<td>6.20 ± 1.50 abc</td>
</tr>
<tr>
<td>25 mg/L methylene blue + 0.50 mg/L acetic acid</td>
<td>3.60 ± 0.93 abc</td>
</tr>
<tr>
<td>25 mg/L methylene blue + 0.10 mg/L acetic acid</td>
<td>9.40 ± 3.53 ab</td>
</tr>
<tr>
<td>25 mg/L methylene blue + 0.05 mg/L acetic acid</td>
<td>10.20 ± 1.32 a</td>
</tr>
<tr>
<td>Distilled water</td>
<td>0.80 ± 0.59 c</td>
</tr>
</tbody>
</table>

*Means in the same column followed by different letters are significantly different (P < 0.05, ANOVA).*
References Cited


