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Behavioral Responses of Nymph and Adult *Cimex lectularius* (Hemiptera: Cimicidae) to Colored Harborages

Corraine A. McNeill,[1,2] Roberto M. Pereira,[3] Philip G. Koehler,[3] Seth A. McNeill,[1] and Rebecca W. Baldwin[3]

[1]Department of Science and Mathematics, Union College, 3800 S. 48th St., Lincoln, NE 68506-0050 (comcneil@ucollege.edu; semcneil@ucollege.edu), [2]Corresponding author, e-mail: comcneil@ucollege.edu, and [3]Department of Entomology and Nematology, University of Florida, Steinmetz Hall, Natural Area Drive, Gainesville, FL 32611-0620 (rpereira@ufl.edu; pgk@ufl.edu; baldwinr@ufl.edu)

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Abstract

Behavioral bioassays were conducted to determine whether bed bug adults and nymphs prefer specific colored harborages. Two-choice and seven-choice behavioral color assays indicate that red (28.5%) and black (23.4%) harborages are optimal harborage choices for bed bugs. Yellow and green harborages appear to repel bed bugs. Harborage color preferences change according to gender, nutritional status, aggregation, and life stage. Female bed bugs prefer harborages with shorter wavelengths (lilac—14.5% and violet—11.5%) compared to males, whereas males prefer harborages with longer wavelengths (red—37.5% and black—32%) compared with females. The preference for orange and violet harborages is stronger when bed bugs are fed as opposed to when they are starved. Lone bed bugs (30%) prefer to be in black harborages while red harborages appear to be the optimum harborage color for bed bugs in more natural mixed aggregations (35.5%). Bed bug nymphs preferred different colored harborages at each stage of development, which is indicative of their developing eye structures and pigments. First instars showed no significant preference for any colored harborage soon after hatching. However, by the fifth instar, 27.5% of nymphs significantly preferred red and black harborages (which was a similar preference to adult bed bugs). The proportion of oviposited eggs was significantly greater under blue, red, and black harborages compared to other colored harborages tested. The use of visual cues such as specific colors offers great potential for improving bed bug monitoring tools by increasing trap captures.

Key words: bed bug, *Cimex lectularius*, color, vision, harborage

Insects use visual cues for a wide variety of behaviors including, but not limited to, recognizing hosts, finding mates, ovipositing, and seeking shelter. These visual cues are often based on the perception of color. The ability to distinguish between different wavelengths of light, as opposed to different light intensities, is termed color vision (Menzel and Backhaus 1991, Cuthill 2006). Last accessed: 3 March 2016. Many insects exhibit color vision and color preferences. Color preference, which is a receptor-neural strategy (Menzel and Backhaus 1991), is an example of how the images that an insect sees can produce a biologically significant behavioral response. In addition to abiotic factors, such as temperature and moisture, and biological factors, such as the size and development of the compound eyes, responses to light are important for understanding how insects respond to visual stimuli (Weiss 1943).

The common bed bug, *Cimex lectularius* (L.), is a nest parasite, which implies that, when it is not feeding, it is searching for a crack or crevice to hide in, and spends the majority of time within that harborage. Older bed bug monitoring tools that use a combination of heat, kairomones, and CO_2 are expensive, inaccessible to the general public, and can be considered impractical (Weeks et al. 2010). However, semiochemical pheromones, which have been found to attract males, virgin females, and bed bug nymphs during harborage studies, have great potential for monitoring bed bugs via semiochemical-baited traps. Ten semiochemical pheromonal compounds (nonanal, decanal, (*E*)-2-hexanal, (*E*)-2-octanal, (2E, 4E)-octadienal, benzaldehyde, (RS)-limonene, 6-methyl-5-hepten-2one, and benzyl alcohol) which attract bed bugs to harborages are crucial for improving bed bug monitoring tools (Siljander et al. 2008, Weeks et al. 2010). These semiochemical traps can act as temporary or permanent refuge (harborage) monitors when they are carefully placed (for example under bed legs) to intercept bed bugs that tend to aggregate in dark, enclosed spaces following a bloodmeal. These pheromone traps can also be used to lure bed bugs from their harborages into a better trap (Weeks et al. 2010).

In addition to pheromones, color is also an important clue that hematophagous insects use to seek shelter (Green and Cosens 1983, Steverding and Troscianko 2004). These authors showed that traps with a blue exterior and black and red interior were very effective at attracting and optimizing tsetse fly trap captures.

Early studies by Aboul-Nasr and Erakey (1969) have documented that the common bed bug is able to distinguish between different wavelengths of light. Short-wavelength colors such as violet (hematoxylin 0.005% solution) and bluish-green (fast green 0.001%) were preferred compared with the other colors tested. Red (eosin 5%) had attractive qualities, while yellow (Bouin's solution) appeared to be the least attractive. More recent studies by Singh et al. (2015) have also shown that aggregations of specifically adult male bed bugs and third–fifth-instar bed bugs prefer black and red harborages compared with other tested colors.

The goal of this research sought to broaden the scope of work carried out by Aboul-Nasr and Erakey (1969) as well as Singh et al. (2015) by 1) examining the effect of gender, nutritional status, aggregation, and bed bug life stage on harborage color choice, and 2) determining whether color influences where female bed bugs oviposit. Results from these objectives will be fundamental to understanding the bed bug behavior to improve monitoring devices and traps used in bed bug management.

Materials and Methods

Bed Bug Rearing and Maintenance

The bed bugs used in these experiments were the Harlan strain of the common bed bug (Harold Harlan, Armed Forces Pest Management Board, U.S. Department of Defense, Washington, D.C.). These bed bugs were reared at the Urban Entomology Laboratory at the University of Florida (Gainesville, FL) at 23–24°C, ~50% RH, and under a photoperiod of 12:12 (L:D) h using methods similar to Pfiester et al. (2009). This particular strain of Harlan bed bugs has been in culture since 1973 in Fort Dix, NJ (Bartley and Harlan, 1974). Cultured bed bugs were maintained in 240-ml plastic containers that were lined with 9-cm filter paper circles and had several harborages made from folding strips of white-colored Whatman no. 1 filter paper (Whatman International Ltd, Maidstone, England) in a fan-like manner. To avoid conditioning influences, the white-colored harborages used in the color harborage experiments were a different type of white-colored cardstock (Michael Stores Inc, Irving, TX) and were not the same white filter paper harborages used for culturing. To prevent escape and facilitate feeding, the top of the plastic holding containers was covered with 90-μm mesh screw-top lids. Each week, bed bugs were fed on live chickens until they were fully engorged. All colony maintenance was in accordance with the University of Florida Institutional Animal Care and Use Committee (UF/IACUC) protocol E876.

Bed bugs in two nutritional stages were used: fed and starved. Starved bed bugs were those that had not been fed within their normal once a week feeding regimen. Fed bed bugs were those that had been blood fed 1–2 d before experimentation.

Visual Arena

The visual bioassays were conducted in a Lab Tek, extra deep, Petri dish (150 by 20 mm; ThermoFisher Scientific, Pittsburgh, PA). The base of each Petri dish was minimally scourged with 60 grit Aluminum Oxide sandpaper (Gator Finishing Products, Fairborn, OH) to allow bed bugs to walk more easily within the arena without influencing the edge-following behavior of bed bugs. To prevent positional biases, within each experiment, a clean Petri dish arena was randomly selected and placed within a large Pyrex dish (25 by 33 cm; Anchor Hocking, Co. Lancaster, OH), to further prevent bed bugs from escaping.

Each Petri-dish arena contained two or seven color choices (depending on the type of experiment) that were arranged as small tent-like harborages. The colored tent-harborages (2 cm long by 1 cm wide) were made from various colored cardstock paper (Michaels Stores Inc., Irving, TX). The wavelength of each harborage color was measured using an Ocean Optics USB 2000 Spectrometer with a Sony ILX 511 linear silicon CCD array detector (Ocean Optics, Dunedin, FL). The same spectrometer was also used to measure the reflectance from the integument of 20 unfed bed bugs (10 males and 10 females). The peak width for each reflectance spectrum was calculated by using the full width at half maximum (FWHM) algorithm (Barua et al. 2014, Saint-Gobain 2014).

Two 32-watt 4-Pin CFL daylight bulbs (2040 lumens each; Phillips Lighting Company, Somerset, NJ), and six 15-watt F15T8-D fluorescent daylight bulbs (700 lumens each; General Electric, Indonesia) were suspended above the test areas and provided ample light for the experiments. All harborages were under the same light conditions. The experimental room was maintained at 26–27°C and average RH of 60.5%. All bed bugs were placed in the experimental room for acclimatization 24 h prior to the bioassays. As the bed bug harborage color choice experiments were conducted, the doors were closed, and no human hosts were present inside the experimental room. Gloves were used in all situations to keep human odors off all harborages and arenas.

Adult Visual Bioassays

Two-Choice Assays

Bioassays were performed to determine whether bed bugs would show significant gender and nutritional status differences in response to various colors when selecting a suitable harborage. Bed bugs were given the choice of each of eight color harborages: lilac, violet, blue, green, yellow, orange, red, and black against white (standard). Both harborage choices were in the arena 3 cm apart and 2.5 cm from the perimeter of the Petri dish to prevent edge effects (Fig. 1A). A single bed bug was then placed in the middle of the Petri dish arena and was given 10 min to make a choice of going under a particular colored harborage. This was considered to be one replication. After the end of the 10-min period, the harborage under which the bed bug was found was recorded. After each replicate, new colored harborages were placed in the arena and the positions of the control and colored tents were randomized to prevent positional biases. Each colored harborage and each bed bug was used only once. Arenas were washed with soapy water and dried before used for another bioassay. This experiment was replicated 40 times with males and females starved for 7–10 d, and males and females fed within 1–2 d. Data from the two-choice assay determined the harborage colors that were used in the seven-choice assay.

Seven-Choice Assays

Bioassays were performed to determine whether bed bugs would show significant gender, nutritional status, and aggregation differences in response to various wavelengths of reflected light when selecting a suitable harborage. The harborage colors chosen for this experiment were dependent on their attractiveness in relation to the white standard as determined in previous two choice bioassays. Any color that was marginally significant or significantly less preferred than white was not included in the choice of seven colors. Therefore, the following seven harborage color choices tested simultaneously were: lilac, violet, blue, green, orange, red, and black. The colored harborages were arranged in a semicircular manner in the

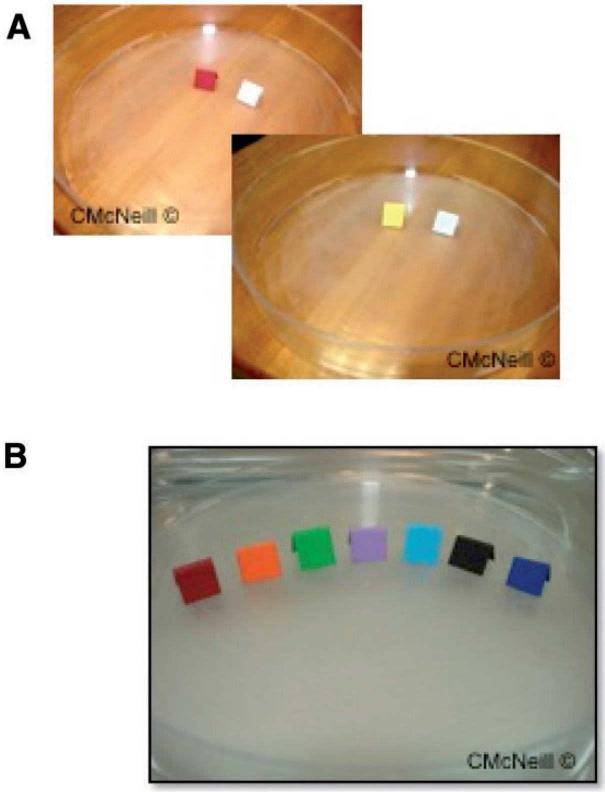

Fig. 1. Various sized petri dish arenas were used for color harborage experiments. (**A**) Smaller petri dishes were used for the two-choice assays. (**B**) Larger petri dishes were used for the seven-choice assays.

arena with each end harborage ~2.5 cm from the edge of the Petri dish to control for edge effects (Fig. 1B). Bed bugs were tested either individually (one at a time) or aggregated (groups of 10 at a time). Aggregated bed bugs were either groups of all males, all females, or a 1:1 ratio of males and females. One replicate consisted of all seven color choices randomly arranged (based on an Excel randomization algorithm) with either one or 10 bed bugs in the center of the arena. Bed bugs were given 10 min to make a choice for a particular colored harborage. At the end of the 10-min period, the number of bed bugs under each harborage was counted and recorded. At the end of the experiment, all harborages were discarded. No harborages were re-used. Arenas were washed with soapy water and dried before used for another bioassay. There were 80 replicates of individually tested bed bugs for each group (7–10-d starved males and females, 1–2-d fed males and females). Also, there were 10 replicates of aggregated bed bugs (100 insects in total) for each group (7–10-d starved males and females, 1–2-d fed males and females). No bed bug or colored harborage was used more than once.

Nymph Visual Bioassays

Seven-Choice Bioassay

These bioassays setups were similar to the seven-choice bioassays for the adults. Individual nymphs were tested. All five nymphal stages were tested with all seven harborage colors (lilac, violet, blue, green, orange, red, and black) included as in the previous adult experiment, and the bioassay was replicated 80 times for each nymphal stage.

Oviposition Bioassays

Bioassays were conducted to test if female bed bugs prefer to oviposit eggs in harborages of specific colors. Seven color harborages were tested simultaneously (lilac, violet, blue, green, orange, red, and black). Immediately following the usual feeding regime, female bed bugs were allowed to mate for 1 h in the testing room (24–26°C, 50–51% RH). Following mating, 10 female bed bugs were placed in a Petri dish arena with seven colored harborages listed above and left for 72 h to oviposit. After 72 h eggs under each colored harborage were counted. This oviposition bioassay experiment was replicated 10 times (100 females in total).

Statistical Analysis

Nominal logistic regression was used to determine whether gender, nutritional status, and aggregation influenced harborage color choice. Two-choice and seven-choice preference data were analyzed using the χ^2 analysis (JMP 9.0.2 SAS Institute Inc. 2010). Mean separation was determined by comparing upper and lower 95% confidence interval (CI) limits for overlap. Means with confidence interval limits that did not overlap were considered significantly different. Also, adult bed bug data were pooled to determine overall bed bug color preference and these data were also analyzed using χ^2 analysis and comparison of 95% confidence limits. One-way analysis of variance (ANOVA; JMP 9.0.2 SAS Institute Inc. 2010) was used to determine the proportion of eggs that was deposited under each colored harborage within each replicate and also the proportion of females present under each colored harborage. For the egg analysis, we did not include the replicates that had fewer than 10 eggs because in these replicates, not all harborages would have had a chance to have one egg. Means of eggs under each colored harborage were separated using Student's *t*-test. The Bonferroni adjustment for α given multiple tests was $\alpha_{new} = [1 - (1 - \alpha)^n]/n = [1 - (1 - 0.05)^{21}]/21 = 0.031$ in the seven-choice experiments (Keppel and Wickens 2004). The α significance level of 0.05 was used for all other tests.

Results

Spectrometer Readings

The colored cardstock used for bed bug visual harborage cues represented the full breadth of the visual spectrum (~400–700 nm). All colored harborages had a single broad spectral peak, except the lilac harborage which had two broad peaks—violet and red. The primary peaks for the colored harborages, based on their reflectance curves, are as follows (Fig. 2A): lilac (two primary peaks: violet $\lambda_{max} = 437$ nm [peak width: 135 nm] and red $\lambda_{max} = 664$ nm [peak width: 74 nm]), violet $\lambda_{max} = 457$ nm (peak width: 70 nm), blue $\lambda_{max} = 470$ nm (peak width: 129 nm), green $\lambda_{max} = 520$ nm (peak width: 64 nm), orange $\lambda_{max} = 608$ nm (peak width: 98 nm), red $\lambda_{max} = 639$ nm (peak width: 68 nm), and yellow $\lambda_{max} = 578$ nm (peak width: 174 nm). The peak wavelength at which there was reflectance from the bed bug integuments was in the red region of the spectrum: males $\lambda_{max} = 685$ nm (peak width: 150 nm), females $\lambda_{max} = 679$ nm (peak width: 145 nm; Fig. 2B).

Behavioral Bioassays

Based on nominal logistic regression, bed bug harborage color choice was influenced by all factors tested: gender ($\chi^2 = 49.9$, df = 6, $P < 0.0001$), nutritional status ($\chi^2 = 15.8$, df = 6, $P = 0.0151$), aggregation ($\chi^2 = 50.6$, df = 6, $P < 0.0001$), and interactions of gender

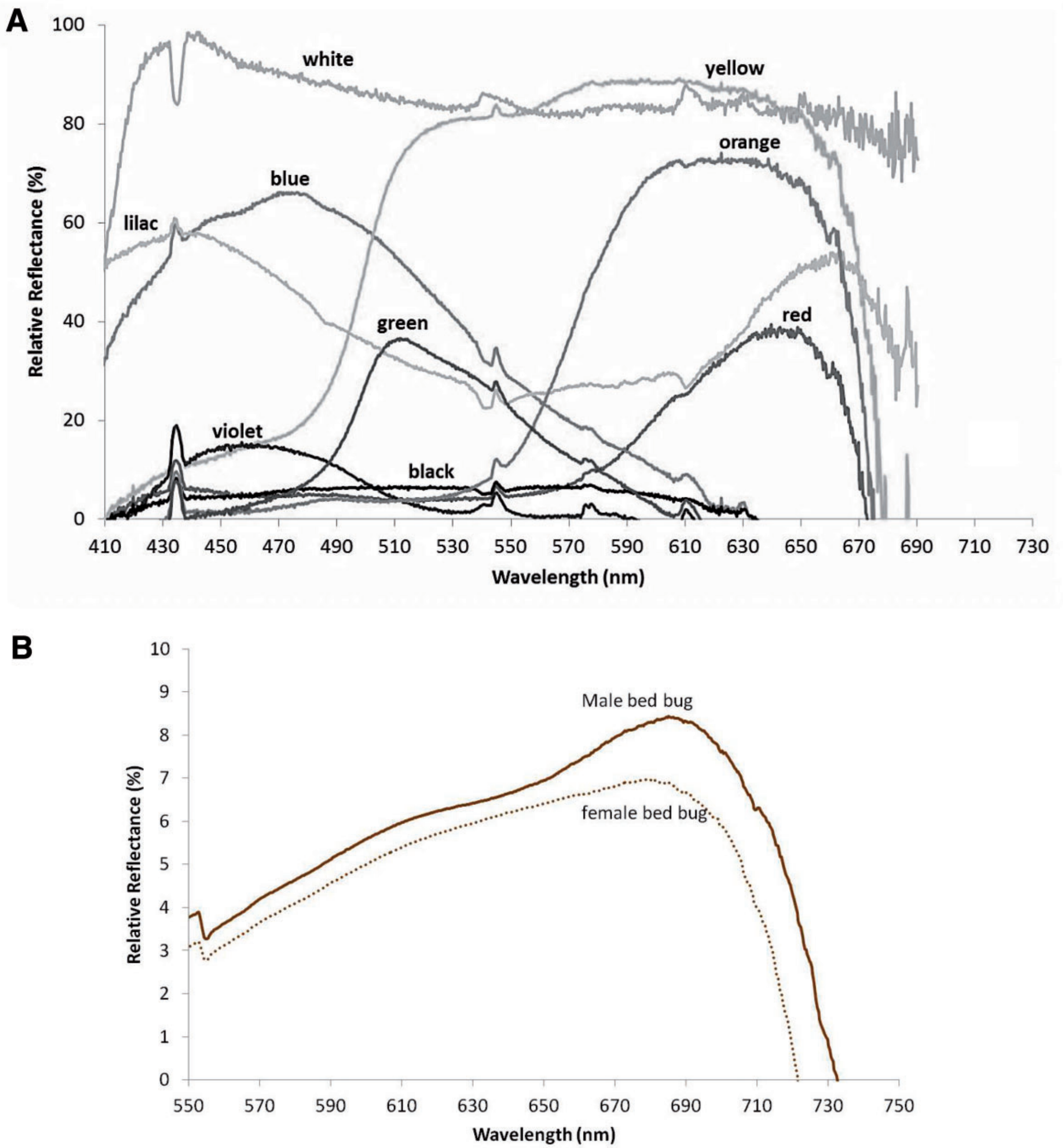

Fig. 2. Reflectance spectra were determined by an Ocean Optics Spectrometer (Dunedin, FL). (**A**) Reflectance spectra were obtained from colored cardstock. (**B**) Reflectance spectra were obtained from male and female adult bed bugs. (Online figure in color.)

and aggregation ($\chi^2 = 14.28$, df = 6, $P = 0.0266$) and gender and nutritional status ($\chi^2 = 69.68$, df = 18, $P < 0.0001$).

Adult Two Color-Choice Bioassays

All bed bug groups (starved and fed, males and females) strongly preferred red and black harborages to white harborages (Fig. 3A–D). (RED: starved males $\chi^2 = 22.5$, df = 1, $P < 0.0001$, starved females $\chi^2 = 22.5$, df = 1, $P < 0.0001$, fed males $\chi^2 = 16.9$, df = 1, $P < 0.0001$, fed females $\chi^2 = 16.9$, df = 1, $P < 0.0001$; BLACK: starved males $\chi^2 = 14.4$, df = 1, $P = 0.0001$, starved females $\chi^2 = 10.0$, df = 1, $P = 0.0016$, fed males $\chi^2 = 19.6$, df = 1, $P < 0.0001$, fed females $\chi^2 = 16.9$, df = 1, $P < 0.0001$). Fed males ($\chi^2 = 14.4$, df = 1, $P = 0.0001$), fed females ($\chi^2 = 19.6$, df = 1, $P < 0.0001$), and starved males ($\chi^2 = 8.1$, df = 1, $P = 0.0044$) significantly preferred orange harborages over white harborages (Fig. 3A, B, and D). Starved males and females showed a preference for lilac harborages over white harborages (males: $\chi^2 = 12.1$, df = 1, $P = 0.0005$; females: $\chi^2 = 6.4$, df = 1, $P = 0.0114$; Fig. 3C and D), whereas fed males and fed females showed no preference between these two colored harborages ($\chi^2 = 3.6$, df = 1,

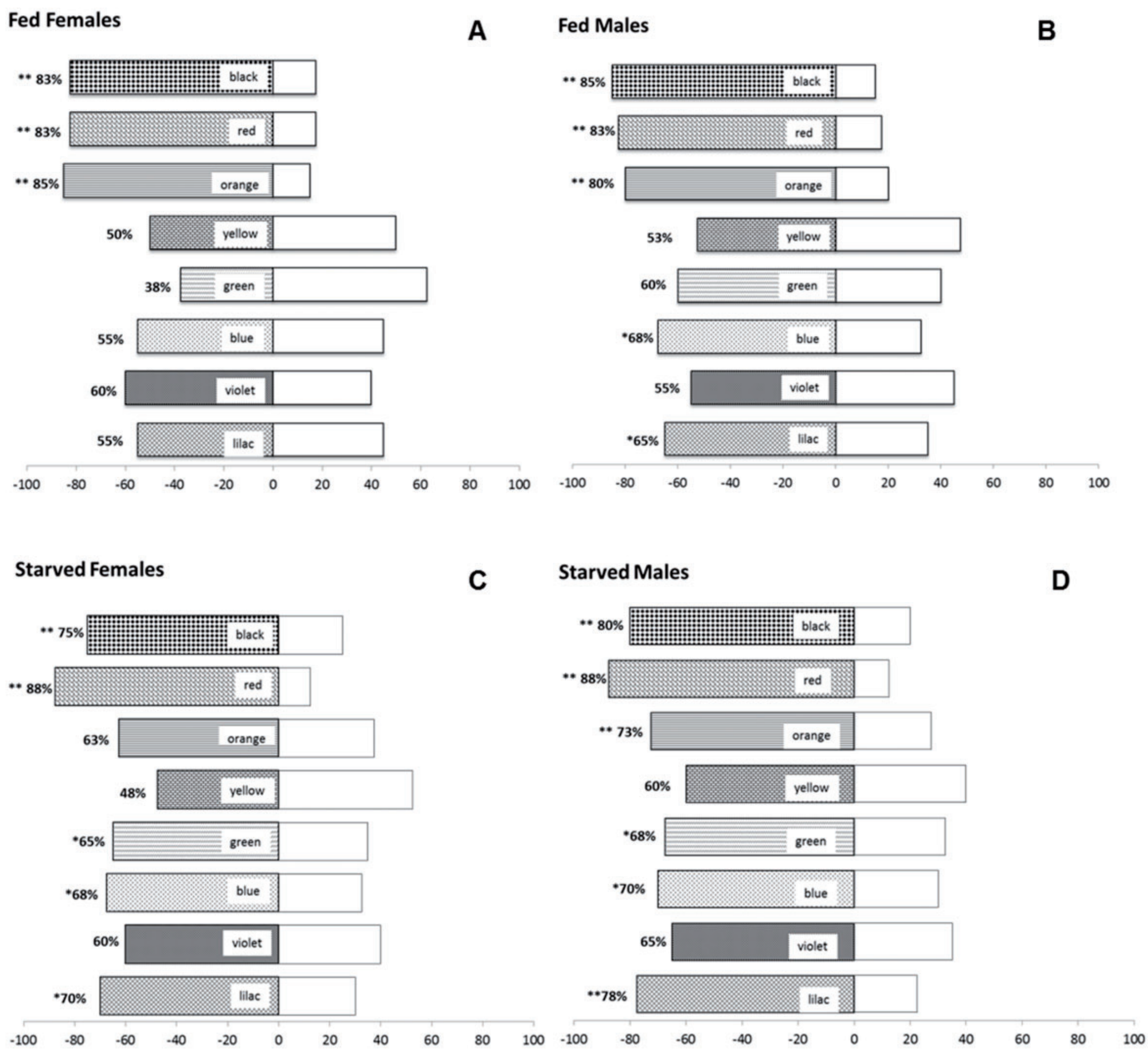

Fig. 3. The response of adult bed bugs to each colored harborage versus a white standard harborage was tested. (**A**) Responses of 40 fed females are shown. (**B**) Responses of 40 fed males are shown. (**C**) Responses of 40 starved females are shown. (**D**) Responses of 40 starved males are shown. * indicates significant differences in χ^2 tests at $P < 0.05$, 95% confidence intervals; ** indicates significant differences in χ^2 tests at $P < 0.01$, 99% confidence intervals.

$P = 0.057$; Fig. 3A and B). A comparison of blue with white harborages showed that starved males ($\chi^2 = 6.4$, df = 1, $P = 0.0114$), starved females ($\chi^2 = 4.9$, df = 1, $P = 0.0269$), and fed males ($\chi^2 = 4.9$, df = 1, $P = 0.0269$) significantly preferred blue to white colored harborages (Fig. 3B–D). Green harborages were only preferred to white by starved males ($\chi^2 = 4.9$, df = 1, $P = 0.0269$; Fig. 3D). Also, no bed bug group showed any preference for yellow over white harborages (starved males: $\chi^2 = 1.6$, df = 1, $P = 0.2059$, starved females $\chi^2 = 0.10$, df = 1, $P = 0.7518$, fed males: $\chi^2 = 0.10$, df = 1, $P = 0.7518$, fed females $\chi^2 = 0.00$, df = 1, $P = 1.00$; Fig. 3A–D), or for violet over white harborages (starved males: $\chi^2 = 3.6$, df = 1, $P = 0.058$, starved females $\chi^2 = 1.6$, df = 1, $P = 0.2059$, fed males: $\chi^2 = 0.40$, df = 1, $P = 0.5271$, fed females $\chi^2 = 1.6$, df = 1, $P = 0.2059$; Fig. 3A–D).

Adult Seven-Choice Bioassays

When all data were pooled to compare all seven colors for bed bug harborage preference, red harborages were significantly preferred compared with all other colored harborages ($F = 10.23$, df = 6, $P < 0.0001$) except black (Fig. 4), whereas black and orange harborages were not significantly different from each other. The order of preference was: red (28.5%), black (23.4%) > orange harborages (17.9%) > blue (10.9%), lilac (9.3%) > violet (6.8%) > green (2.8%). Yellow harborages were not included in the choice of multiple colors to be tested because yellow was not significantly different compared with the white standard for any of the bed bug groups tested.

Gender significantly influenced bed bug color harborage choice ($\chi^2 = 49.9$, df = 6, $P < 0.0001$). Male bed bugs significantly preferred black and red harborages compared to female bed bugs, whereas female bed bugs significantly preferred lilac and violet compared to male bed bugs. Nutritional status also influenced bed bug color choice. Bed bugs preferred orange and violet harborages significantly more when they were starved ($\chi^2 = 15.8$, df = 6, $P = 0.0151$) as opposed to when they were fed.

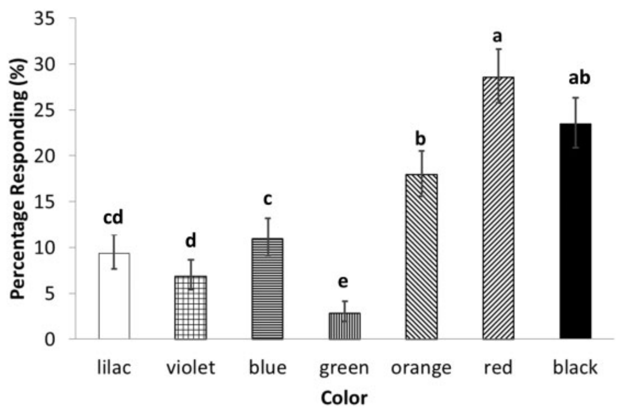

Fig. 4. Overall color preferences of adult bed bugs to various harborage colors. Different letters are significantly different means ($P < 0.031$; 96.9% confidence intervals based on Bonferroni corrections).

There was also a significant interaction between gender and nutritional status, which significantly influenced bed bug color harborage choice ($\chi^2 = 69.68$, df = 18, $P < 0.0001$; Fig. 5). Fed females significantly preferred violet harborages in comparison to fed and starved males. On the other hand, starved males significantly preferred red and black harborages in comparison to fed females.

Harborage color preferences changed depending on how bed bugs were grouped ($\chi^2 = 67.94$, df = 12, $P < 0.0001$). Bed bugs preferred green significantly more when they were alone than when they were in same gender groups (Fig. 6). A mixed group of males and females significantly prefer red and violet harborages than when they are alone. Also lone bed bugs and bed bugs groups of the same gender significantly preferred black harborages over groups of bed bugs of mixed gender. Interestingly, bed bugs showed no difference in preference for blue, orange and lilac colored harborages whether bed bugs are alone, in same-gender aggregations or mixed-gender aggregations.

Nymph Seven-Choice Bioassays

When first-instar bed bug nymphs that had never been fed were exposed to all seven harborage color choices, none of these harborage colors were significantly preferred over any other (Fig. 7A). However, after these first-instar nymphs were allowed to feed, some color preferences were observed ($\chi^2 = 14.15$, df = 6, $P = 0.028$; Fig. 7B). Significantly fewer bed bug nymphs were found under the lilac harborage compared with orange and black harborages. Color harborage preferences were also observed for second instars ($\chi^2 = 25.18$, df = 6, $P = 0.0003$), third instars ($\chi^2 = 29.02$, df = 6, $P < 0.0001$), fourth instars ($\chi^2 = 29.02$, df = 6, $P < 0.0001$), and fifth instars ($\chi^2 = 43.2$, df = 6, $P < 0.0001$). Second-instar nymphs significantly preferred black, green, orange, red, and violet harborages over blue harborages (Fig. 7C). Third-instar nymphs significantly preferred green and red harborages over blue, lilac, and violet harborages (Fig. 7D). Fourth-instar nymphs significantly prefer red and blue harborages over lilac, violet, and green harborages (Fig. 7E). For fifth-instar nymphs, black, blue, orange, red, and lilac harborages were all significantly preferred to green and violet harborages (Fig. 7F).

Oviposition Bioassay

Significantly more eggs were found in red, blue, orange, and black harborages compared with green harborages ($F = 4.49$, df = 6, $P = 0.0008$; Fig 8). Red, blue, and black harborages were also the harborages that had significantly more females during the oviposition bioassay ($F = 2.70$, df = 6, $P = 0.0214$). Although females were found under green harborages, no eggs were laid under green harborages during this bioassay.

Discussion

Although bed bugs are mostly active during the night, a peak of activity has been observed at the time the lights are turned on (Mellanby 1939). This flurry of activity has been explained by harborage-seeking behavior (Romero et al. 2010). Therefore, understanding the physiological color preferences of bed bugs under lighted conditions is important, especially for infestations in human habitations where the light–dark cycles are neither necessarily dictated by natural night–day cycles, nor occur gradually.

Both adult and immature bed bugs are able to differentiate between different colors, and preferentially select harborages based on color-specific visual cues. Typically, bed bugs tend to feed and find harborages during dark periods, so color preferences may not be important in those cases. Studies by Singh et al. (2015) documented that bed bugs cannot differentiate between different colored harborages under completely dark conditions. However, these harborage color bioassays indicate the important role that light plays for bed bugs as they locate a suitable hiding or nesting area. Harborages (crack and crevices) are very important to bed bugs because they spend 90% of their time in harborages, and when not in a harborage, they are either actively searching for a host or looking for new harborage sites (Pinto et al. 2007). It has been speculated that a bed bug would go to any harborage in an attempt to hide. However, these color experiments show that bed bugs do not hide in just any harborage; rather they will select a harborage based on its color when moving in the light. Specific colors may represent 1) an opportune oviposition site or mating arena or 2) safety due to the presence of other bed bugs and/or a site that limits visibility by predators.

When the seven most attractive colors were tested simultaneously, bed bugs preferred to hide in red and black harborages more than any other color harborage. These findings are similar to those reported previously (Singh et al. 2015). Sand flies such as *Phlebotomus papatasi* (Hoel et al. 2007) and *Lutzomyia shannoni* (Mann et al. 2009), whose crepuscular or nocturnal feeding behavior is similar to bed bugs, have also been shown to be attracted to red wavelengths. Red and black colored cloths were also shown to be most attractive to *Aedes aegypti* mosquitoes (Brett 1938). Singh et al. (2015) stated that it is unclear whether bed bugs can differentiate between red and black harborages, but rather that both colors possibly appear dark to bed bugs and are therefore those colors are chosen based on their strong photonegative attractions. Another reason for the strong preference for red harborages could be that bed bugs themselves appear red as observed by the integument reflectance. Red harborages may represent the presence of other bed bugs, and so bed bugs would be attracted to red harborages for aggregation purposes.

Nutritional status and population density are important factors influencing the search for new habitats in hematophagous triatomines (Minoli and Lazzari 2006). It appears that, once bed bugs are fed, the color of a harborage becomes more important, perhaps because the bed bugs shift from an interest in food, to an interest in suitable harborages where the bed bugs would be less disturbed. In this case, orange and violet colors appear to represent preferred wavelengths. The reason for this is unclear, but could be related to

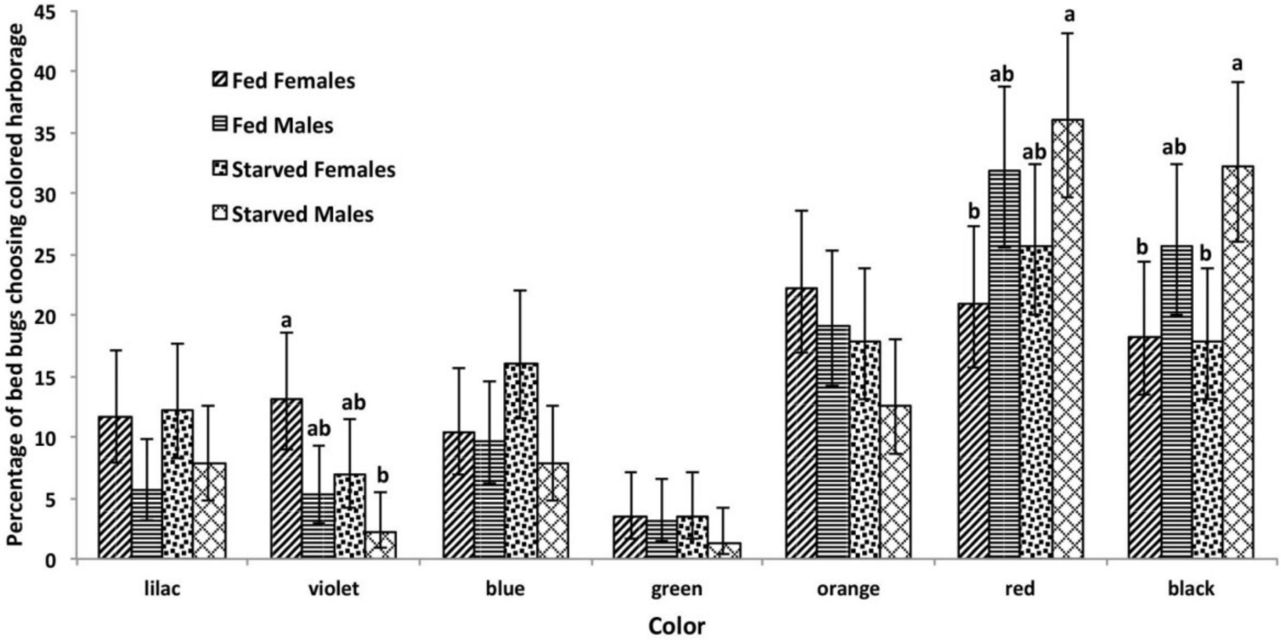

Fig. 5. Percentage of fed and unfed male and female bed bugs that chose different colored harborages. Ten individual bed bugs for each treatment group (fed males, starved males, fed females, and starved females) were used in each replication, and each group was replicated eight times. Within each color group, different letters are significantly different means ($P < 0.031$; 96.9% confidence intervals based on Bonferroni corrections).

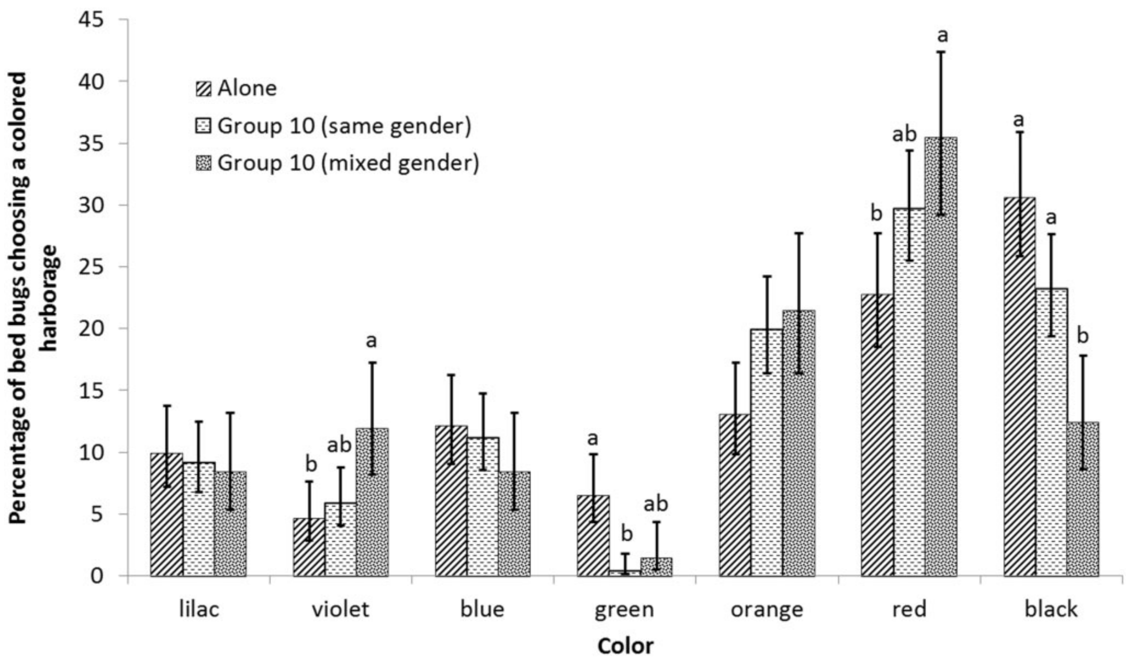

Fig. 6. Percentage of bed bugs that chose different colored harborages when making selections alone or in groups of 10 individuals of same or mixed gender. Ten individual bed bugs were used for each replication and each lone group was replicated eight times. A group of 10 bed bugs was used for each replication in both aggregation treatments. There were with ten replicates of both aggregated treatments. Within each color group, different letters are significantly different means ($P < 0.031$; 96.9% confidence intervals based on Bonferroni corrections).

hormonal changes that occur after feeding because almost every area of postembryonic development in insects is under hormonal control (Nijhout, 1999). For starved bed bugs, harborage color appeared to be less important perhaps because hungry bed bugs are more likely to be found searching for a host in order to obtain a bloodmeal rather than looking for a harborage.

Specific colors along the visible color spectrum appear to be biologically significant for bed bugs depending on their gender and nutrient status. Overall, bed bugs prefer red and black harborages, and for starved males red and black harborages were preferred compared with starved or fed females. However violet harborages became more attractive to females when they were fed compared to

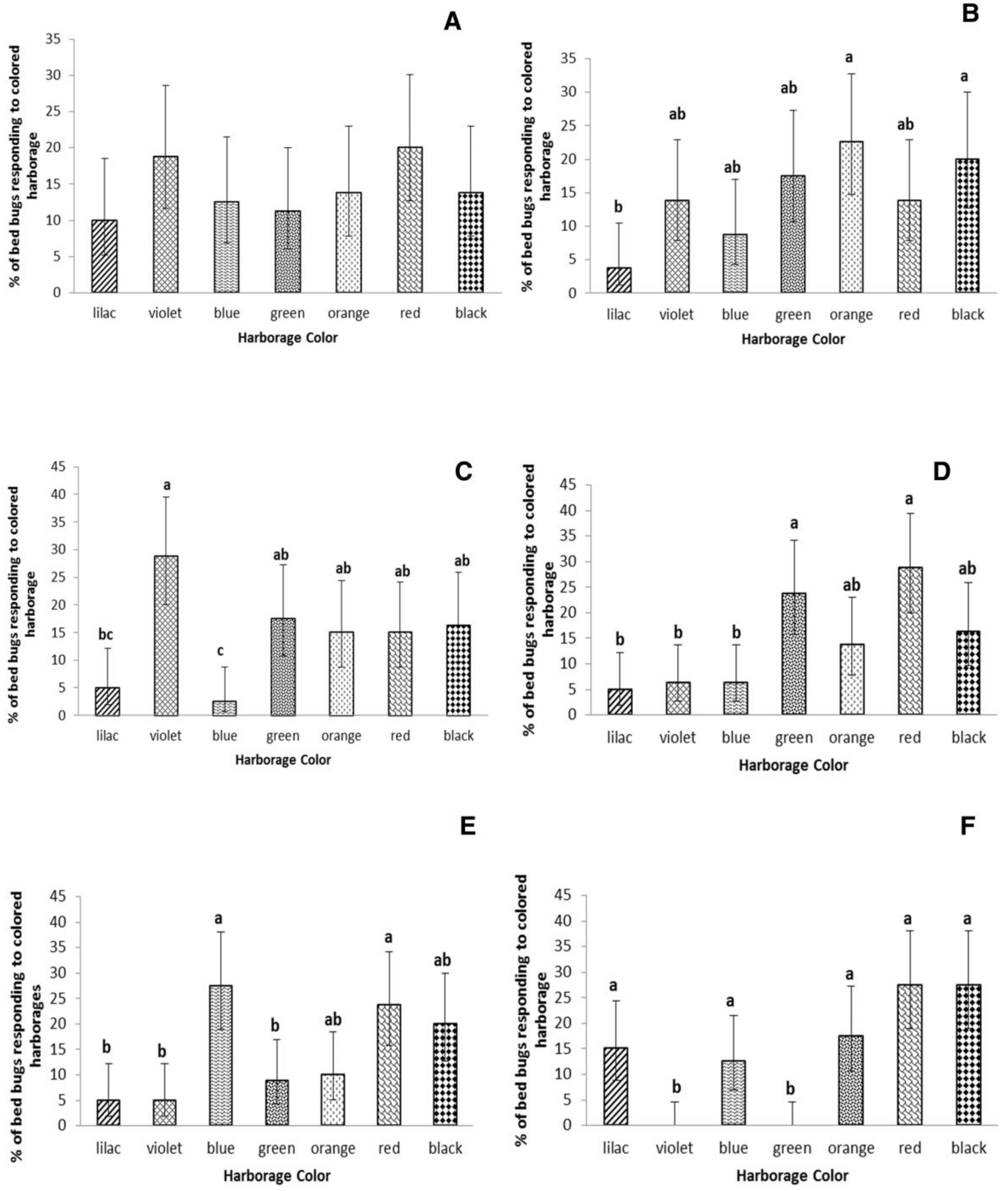

Fig. 7. Data show percentage of bed bug nymphs responding to various colored harborages. (**A**) Responses of first instars (never fed). (**B**) Responses of first instars fed but not yet molted. (**C**) Responses of second-instar nymphs. (**D**) Responses of third-instar nymphs. (**E**) Responses of fourth-instar nymphs. (**F**) Responses of fifth-instar nymphs. One bed bug nymph (for each nymphal stage) was used for each replication and each nymphal stage was replicated eight times. Within each graph, different letters are significantly different means ($P < 0.031$; 96.9% confidence intervals based on Bonferroni corrections).

males that were starved. Perhaps the violet wavelength is desensitized when a female bed bug is hungry, but becomes important for harborage finding after a bloodmeal is consumed. To our knowledge, no studies have empirically investigated whether nutritional status affects harborage color choice for hematophagous insects, and so this may be the first study to highlight this phenomenon. In nature, the influence of nutritional constraints is common and can affect several areas of insect behavior such as sexual selection, as

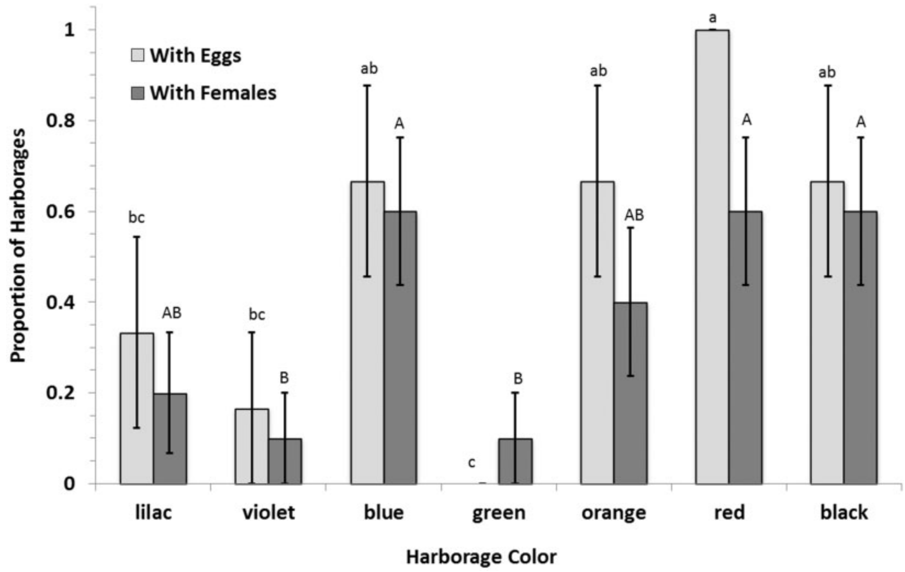

Fig. 8. Data show bed bug oviposition preference and post oviposition harborage choice. A group of 10 bed bugs was used for each replication in oviposition bioassays and the experiment was replicated 10 times. Different letters are significantly different means ($P < 0.031$; 96.9% confidence intervals based on Bonferroni corrections). Lower case letters indicate comparison of means among proportion of females found in each colored harborage. Upper case letters indicate comparison of means among proportion of eggs laid in each colored harborage.

well as reproductive investments, life span, and gender physiology (Tigreros et al. 2014). Given the influence of nutritional constraints in insects for various aspects of insect behavior, it is not surprising that nutritional status can influence color harborage choice. In fact, color preference shifts with regards to nutritional status has also been observed in other animals such as pigeons (Delius 1968) and toads (Fal'tsman and Bastakov 1999).

Apparently bed bugs behave differently when they are in a mixed group of males and females as opposed to being alone or in a same-gender group. In mixed aggregations, bed bugs prefer to be in red harborages. Surprisingly only 12.5% of bed bugs from a mixed aggregation chose a black harborage despite cracks and crevices being black (dark) areas. It appears that red may be the optimum harborage color for bed bugs in their naturally occurring mixed aggregations, although lone bed bugs or bed bugs in a same-gender aggregations prefer to be in black harborages.

In addition to nutritional status and aggregation, harborage color preferences were also influenced by gender. Male bed bugs preferred to hide under red and black (longer wavelength) harborages, whereas female bed bugs preferred to hide under lilac and violet (shorter wavelength) harborages. A similar trend was observed when males and females were in groups of 10 according to their gender.

This information is particularly useful when considering behavioral and physiological mechanisms that lead bed bugs seek to out and colonize new harborages. Although most bed bugs are found heavily aggregated in harborages within most infestations, isolated bed bugs have been observed away from the primary harborage sites (Pfiester et al. 2009). For a wandering bed bug, red harborages may signify highly populated areas where competition for space may be a problem. In that case, lone bed bugs may seek out other black harborages, which may signify a minimally populated area suitable for colonization and egg laying. Also, female bed bugs tend to prefer female–female aggregations over female–male aggregations for fear of excessive mating (Pfiester et al. 2009). This may explain why black harborages were more attractive to female bed bugs because this may represent a safer harborage away from the males.

Yellow and green were least preferred or somewhat repellent to bed bugs. These findings further support early studies by Aboul-Nasr and Erakey (1969) that similarly document the unattractiveness of yellow colors to bed bugs. It appears that yellow harborages are not seen as suitable by bed bugs because they may be closely related to brightly lit areas, which are contrary to the nocturnal habits of these insects. Unattractive visual colors such as yellow and green should be taken into consideration in the development of monitoring tools or traps for bed bugs. These colors have been considered unattractive to other blood-sucking insects such as mosquitoes, sand flies, triatomines, and other insects such as houseflies and weevils (Brett 1938, Hausmann et al. 2004, Reisenman and Lazzari 2006, Hoel et al. 2007, Diclaro et al. 2012).

Immature bed bugs do exhibit harborage color preferences but these preferences change between successive instars and are most likely to be under hormonal control as development progresses. In insects, many developmental processes and events, as well as alternate developmental pathway choices are very heavily influenced by hormones (Nijhout, 1999). First-instar nymphs that have never taken a bloodmeal could not differentiate between colors and should therefore be considered color-blind. However, once those first instars were fed but had not yet molted to the next instar, some color preference began to occur. Color preferences in the second instar were most different compared to all other instars. From the third instar to the fifth instar, color preferences appeared to be more similar to the adult. This shifting of color preferences may be due to developmental differences in structure, shape, and number of ommatidia in the eye in each instar as well as neurological processes in the brain.

It appears that colors also influence where female bed bugs will oviposit. Larger proportions of eggs were found under red, black, and blue harborages, whereas fewer proportions of eggs were found in green harborages. Black and blue colors were also found to be the preferred oviposition sites for other insect species such as the

Mediterranean fruit fly *Ceratitis capitata* and the Asian tiger mosquito *Aedes albopictus* (Katsoyannos et al. 1986, Hoel et al. 2011). Although red and black harborages are strongly preferred for various stages of bed bugs, females will oviposit under a wider range of colored harborages similar to that shown for *Ae. aegypti* females (Panigrahi et al. 2014), but will avoid ovipositing under green harborages. Green or yellow-green colors are usually associated with an outdoor environment having plants and bright light, which is not where bed bugs are normally found. Bed bug avoidance of green colors for harborages and for oviposition sites may be strong to ensure avoidance of unsuitable habitats. This type of behavior was also implied by Aboul-Nasr and Erakey (1969) who documented that strong avoidance of bright harborages supported their cryptic, nocturnal behavior.

Results from these studies indicate that bed bugs have different color preferences for their harborage and oviposition sites. Harborage color preferences are influenced by gender, nutritional status, and aggregation, and will change with life stage. Red and black harborages (no statistically significant difference was observed between these colors) appear to be the most attractive harborages for a wide range of bed bug life stages, whereas yellow and green harborages appear to be the least attractive for most bed bug life stages. Singh et al. (2015) have speculated that red and black colors may only appear as general dark colors to bed bugs based on their photonegative reactions, and it is not known whether bed bugs can really differentiate between red or black colors. This study has given further support for bed bug preferences that may indicate that a mechanism exists for color discrimination in bed bugs. Our findings should be useful in bed bug trap design as an attempt to enhance trap captures.

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