



Behavioural Responses of Tropical Bed Bug *Cimex hemipterus* (F.) (Hemiptera: Cimicidae) to Coloured Harborage

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Highlights

- Two-choice and three choice colour assays were conducted to determine whether bed bugs will choose black, red or white coloured harborage.
- Both fed and starved male, female and nymph strongly preferred red and black coloured harborage compared to white coloured harborage.
- Oviposition assays showed that female bed bugs preferred to laid their eggs on red coloured harborage compared to black coloured harborage.

Behavioural Responses of Tropical Bed Bug *Cimex hemipterus* (F.) (Hemiptera: Cimicidae) to Colored Harborage

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Abstract: Population of the tropical bed bug *Cimex hemipterus* (F.) (Hemiptera: Cimicidae), a temporary ectoparasite on both humans and animals, have surged in many tropical countries. Tropical bed bugs preferences when selecting a suitable harborage and oviposition site were investigated. Two-choice and three choice colour assays were conducted to determine whether bed bugs will choose black, red or white coloured harborages. Then, fifty 1st instar were reared in containers containing black, red and white (control) paper served as the harborages and observed for 12 weeks. Both fed and starve male, female and nymph strongly preferred red and black coloured harborage compared to white coloured harborage. Oviposition assays showed that female bed bugs preferred to laid their eggs on red coloured harborages compared to black coloured harborages. Rearing experiment showed that there was no significant difference ($p > 0.05$) between final population size of tropical bed bug. However, tropical bed bugs reared in container with red paper (600 ± 89.238) have the highest number of individuals followed by black (473 ± 133.841) and white (485 ± 84.234) paper. Bed bug preference towards coloured harborage provide useful information for those developing new bed bug control method or improving bed bug infestation monitoring devices.

Keywords: Tropical Bed Bug, Reproduction, Harborage Colour, Rearing Behavioural

INTRODUCTION

Global resurgence of the bed bugs has sparked interest among scientist and pest control companies in developing effective and efficient control and management strategies (Abd Rahim *et al.* 2014, Ab Majid & Zahran 2015; Zahran *et al.* 2016; Lim & Ab Majid 2020). Knowledge of biology and ecology of the bed bug, *Cimex lectularius* or *Cimex hemipterus* under various environmental conditions is crucial to improve the management techniques. Bed bugs colonies

are required to be tested and this is proved to be a problem since bed bug is an obligatory blood feeder which mean they are required to be fed on a human or animal host for nutritional and reproduction purposes (Abd Rahim *et al.* 2016; Miller *et al.* 2013; Zorrilla-Vaca *et al.* 2015).

Visual cues are used by insects for a wide variety of behaviours including, but not limited to, finding mates, recognising hosts, seeking shelter and ovipositing. These visual cues are often based on the perception of colour. The ability to distinguish between different wavelengths of light, as opposed to different light intensities, is termed colour vision (Menzel & Backhaus 1991; Cuthill 2006). Many insects exhibit colour vision and colour preferences. Colour preference, which is a receptor-neural strategy (Menzel & Backhaus 1991), is an example of how the images that an insect sees can produce a biologically significant behavioural response. In addition to abiotic factors, such as temperature and moisture (How & Lee 2014), 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).

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 colours such as violet and bluish-green were preferred compared with the other colours tested. Red had attractive qualities, while yellow 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 colours. Additionally, McNeil *et al.* (2016) reported that harborage colour preferences change according to gender, nutritional status, aggregation and life stage. Female bed bugs prefer harborages with shorter wavelengths (lilac and violet) compared to males, whereas males prefer harborages with longer wavelengths (red and black) compared with females. The preference for orange and violet harborages is stronger when bed bugs are fed as opposed to when they are starved. Bed bug nymphs preferred different coloured harborages at each stage of development, which is indicative of their developing eye structures and pigments.

The objectives of this study were:

1. to determine whether tropical bed bugs would show significant gender and nutritional status differences in response to different colours when selecting a suitable harborage,
2. to determine whether female bed bugs would prefer to oviposit eggs on harborage of specific colours, and
3. to determine the effect of coloured harborages to bed bug's population.

MATERIALS AND METHODS

Bed Bugs Culture and Rearing

The tropical bed bugs, *C. hemipterus*, were reared in Household and Structural Urban Entomology Laboratory, Universiti Sains Malaysia. The samples were originated from specimens collected at Kuala Lumpur International Airport (KLIA) back in 2014. Bed bugs were reared in plastic containers (8 cm in height, 8 cm in diameter) containing folded white A4 paper strips (Double A, Chachoengsao, Thailand) as their harborage. The paper strips were placed

perpendicular to the bottom of the container to provide a surface for the bed bugs to walk and deposit eggs. Plastic containers were covered with a piece of fine net cloth (13 cm × 13 cm) and a rubber band to hold it in place (Abd Rahim *et al.* 2015). The bed bugs were fed weekly on a human volunteer's arm. Bed bugs were carefully transferred into sample vials which then covered with a fine net cloth and a rubber band to hold it in place. After net cloth was secured, sample vials were placed on the forearm of the volunteer and left for 20 min so that the bed bugs can feed and sucked the blood (Abd Rahim *et al.* 2016). The feeding of bed bugs on human host followed the protocol approved by the Human Research Ethics Committee USM (HREC) with code USM/JEPeM/19120868. The tropical bed bugs colonies were cultured in an incubator with temperature set at $26 \pm 1^\circ\text{C}$, $65 \pm 5\%$ relative humidity (RH) and a photoperiod of 12 h: 12 h (light: dark).

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 (blood fed 7 d before experimentation). Fed bed bugs were those that had been blood fed 1–2 d before experimentation. Three stages of bed bug were used in this study; male, female and the fifth instar. Every replicate use a new individual which was randomly selected from the rearing containers.

Visual Arena

The visual bioassays were conducted in a Lab Tek, extra deep, Petri dish (90 by 20 mm; ThermoFisher Scientific, Pittsburgh, PA). The base of each Petri dish was minimally scoured with 60 grit sandpaper to allow bed bugs to walk more easily within the arena without influencing the edge-following behaviour of bed bugs. To prevent positional biases, within each experiment, a clean Petri dish arena was randomly selected and placed within a large metal tray (30 by 30 cm), to further prevent bed bugs from escaping.

Each Petri-dish arena contained two or three colour choices (depending on the type of experiment) that were arranged as small L-shaped harborages and placed perpendicular to the petri dish floor (Fig. 1). The L-shaped coloured harborages (2 cm long by 1 cm wide) were made from various coloured A4 paper. All harborages were under the same light conditions. The experimental room was maintained at 26°C – 27°C and average RH of 60.5%. All bed bugs were placed in the experimental room for acclimatisation 24 h prior to the bioassays. As the bed bug haborage colour 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 odours off all harborages and arenas.

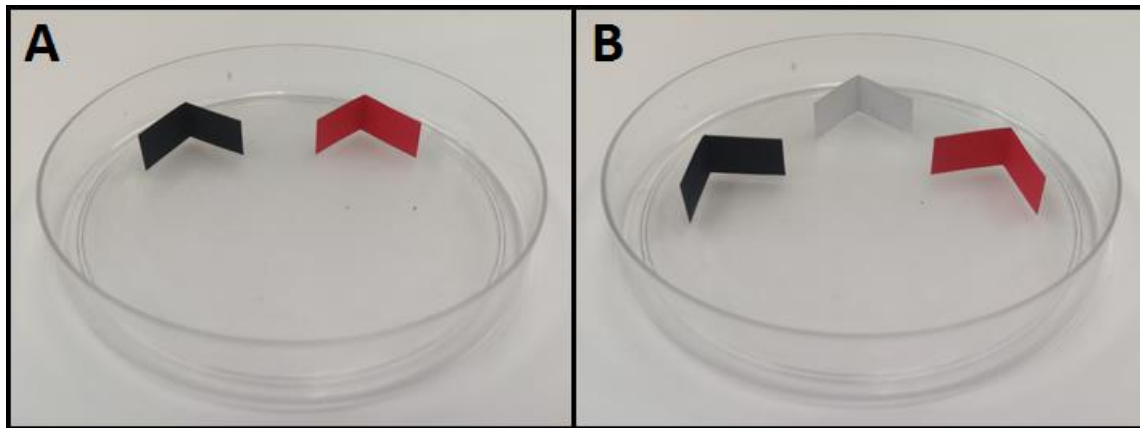


Figure 1. Two colours (A) and three colors (B) choice assays arena.

Color Choice Assays

Bioassays were performed to determine whether tropical bed bugs would show significant differences in response to various colours when selecting a suitable harborage. Bed bugs were given the choice of three colour harborages: red, black and white (control). Two types of experiment were conducted:

1. Two choice harborage colours (black vs. white, red vs. white and black vs. red).
2. Three choice harborage colours (black vs. red vs. white).

Order of the colour harborages was randomised within each Petri dish. Paper harborages were placed in the arena 2 cm apart and 1 cm from the perimeter of the Petri dish to prevent edge effects.

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 climbing to a particular coloured harborage. This was considered to be one replication. After the end of the 10-min period, the harborage which the bed bug was found was recorded. After each replicate, new coloured harborages were placed in the arena and the positions of the control and coloured harborages were randomised to prevent positional biases. Each coloured harborage and each bed bug was used only once. This experiment was replicated 40 times with males, females and 5th instar.

Oviposition Experiment

Experiments were conducted to test if female bed bugs prefer to oviposit eggs on harborages of specific colours. Three colour harborages were tested simultaneously (black, red and white). Immediately following the usual feeding regime, female bed bugs were allowed to mate for 4 h. Following mating, 5 female bed bugs were placed in a Petri dish arena with three coloured harborages listed above and left for 7 days to oviposit. After 7 days, eggs on each coloured harborage were counted. This oviposition experiments were replicated 10 times (50 females in total) simultaneously.

Rearing Using Colored Harborages

Ten males and 10 females that had been fed 7 days ago were collected from the colonies and fed through artificial feeding system until they reached engorged weight. All fed adults were placed in a new container and after 7 days, harborage paper containing eggs were observed until 50 first instars were obtained. The first instars were then transferred to containers with respective coloured harborage (white as control, red and black), three replicates for each colour (Fig. 2). The physical condition of the container (transparent) and the net covering it (polkadot muslin net) were not changed since the same kind were used for the rearing of the bed bug colonies in Household and Structural Urban Entomology Laboratory since 2014. The only condition that changed was the colour of the harborages, red and black was added as well as white (as control – white paper has been used as the harborage since 2014). Colour papers (Benchmark Paper Products Sdn. Bhd., Penang, Malaysia), black, red and white, were cut to 15 cm × 6 cm rectangular shape and circular shape with 5 cm diameter. Circular paper was placed at the bottom of the container and the rectangular paper was folded 8 times so it can fit into the container and placed perpendicular to the circular one at the bottom.

The colonies were fed on a human volunteer for every 7 days. Live individuals were counted before each feeding session. Live individual counted based on their posture and movement in the container. The ones that were in an upside-down position with no signs of movement at all after 10 sec of observation were counted as dead (Ab Majid & Zahran 2017). The number of molted bed bugs were also counted based on how many exoskeleton were presented in the container. In addition, number of adults, male and female were counted when they were emerged.



Figure 2. Bed bugs were reared in containers with coloured harborages.

DATA ANALYSIS

Nominal logistic regression was used to determine whether gender (male, female and nymph) and nutritional status influenced harborage colour choice. Two-choice and three-choice preference data were analysed using the chi-square analysis. The statistical analysis was performed by using SPSS version 26 (IBM Corp., Armonk, NY, USA).

One-way analysis of variance was used to determine the proportion of eggs that was deposited on each coloured harborage within each replicate. Means of eggs on each coloured harborage were separated using student's t-test.

Two-way analysis of variance (ANOVA) was performed to determine the significant difference between live individuals for each week for the rearing population. One-way ANOVA was performed to determine the significance difference between the population after 12 weeks.

RESULTS

Colour Choice Assays

A chi-square test was performed to evaluate the relationship between bed bug's nutritional status and preferred harborage colour. There was no statistically significant relationship between fed bed bugs and choice of red or white coloured harborage $\chi^2 = 0.752$, $df = 2$, $p = 0.686$. Fed male (75%), female (70%) and nymph (75%) strongly preferred red over white coloured harborage (Fig. 3). There was also no statistically significant relationship between fed bed bugs and choice of black or white colored harborage $\chi^2 = 5.236$, $df = 2$, $p = 0.073$. Fed male (65%), female (73%) and nymph (60%) preferred black over white coloured harborage (Fig. 4). On the other hand, the chi-square test yielded no statistically significant relationship between fed bed bugs and choice of black or red coloured harborage $\chi^2 = 2.500$, $df = 2$, $p = 0.287$. Fed male (65%) and female (68%) showed strong preference towards black coloured harborage compared to red coloured harborage while fed nymph showed similar (50%) preference towards both red and black harborages (Fig. 5).

Similarly, starved male (72.5%), female (72.5) and nymph (80%) also showed strong preference towards red over white coloured harborage (Fig. 6), although there was no statistically significant relationship between starved bed bugs and choice of red or white coloured harborage $\chi^2 = 0.800$, $df = 2$, $p = 0.670$. Interestingly, there was a statistically significant relationship between starved bed bugs and choice of black or white coloured harborage $\chi^2 = 7.081$, $df = 2$, $p = 0.029$. starved male (75%), female (65%) and nymph (90%) showed strong preference towards black over white coloured harborage (Fig. 7). There was no statistically significant relationship between starved bed bugs and choice of black or red coloured harborage $\chi^2 = 2.887$, $df = 2$, $p = 0.236$. Starved nymph showed a preference for black (52.5%) over red coloured harborage while starved female showed similar (50%) preference towards black and red coloured harborages, and starved male bed bug preferred red (65%) over black coloured harborage (Fig. 8).

Three choice colour assays showed that white coloured harborage is the least preferred harborages. However, there was no statistically significant relationship between fed bed bugs and choice of coloured harborage $\chi^2 = 7.832$, $df = 4$, $p = 0.098$. Fed female (50%) and nymph (58%) strongly preferred black over red and white (Fig. 9). On the contrary, only fed male preferred red (43%) over black (30%) and white (27%) coloured harborage. There was also no statistically significant relationship between starved bed bugs and choice of coloured harborage $\chi^2 = 2.173$,

df = 4, $p = 0.704$. Starved male preferred black (42.5%) over red (37.5%) and white (20%) coloured harborage (Fig. 10). Starved female (47.5%) and nymph (47.5%) showed a preference towards red over black and white coloured harborage.

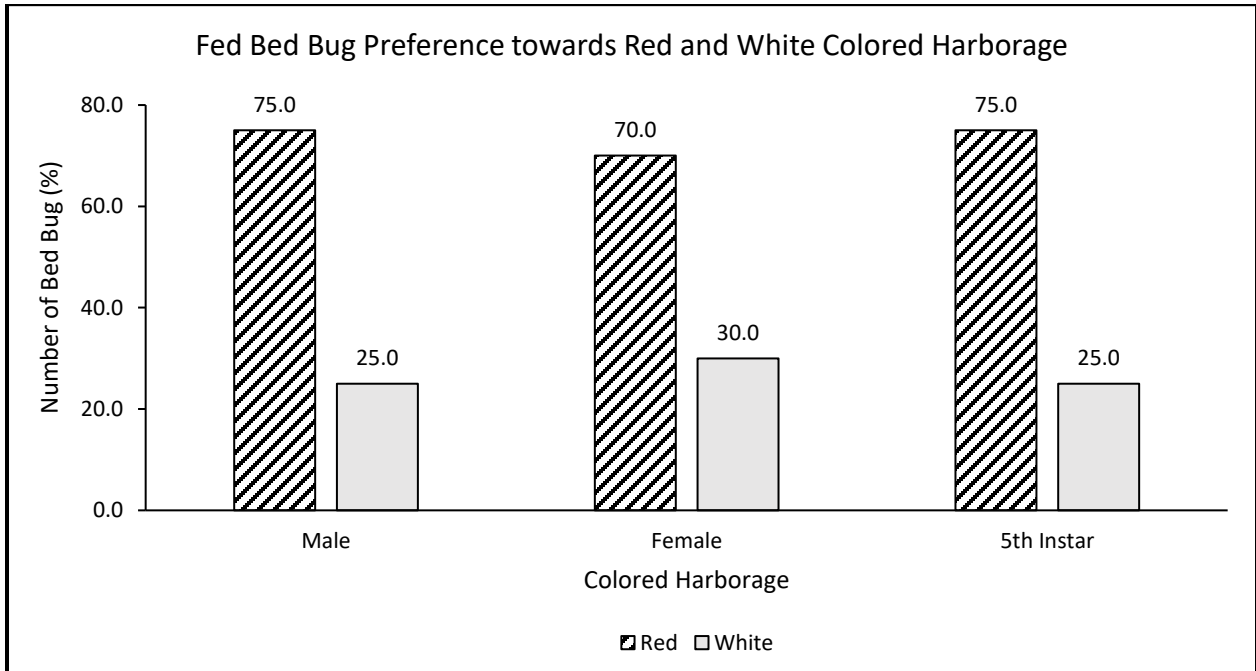


Figure 3. The responses of fed bed bugs to red or white coloured harborage.

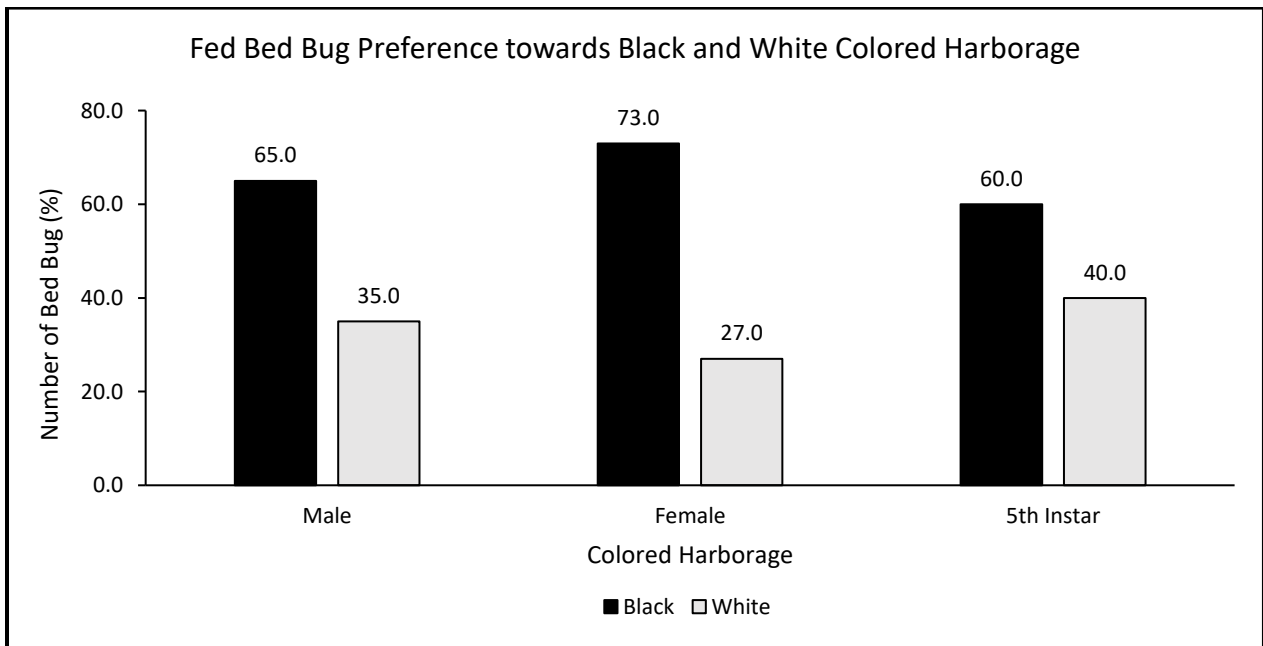


Figure 4. The responses of fed bed bugs to black or white coloured harborage.

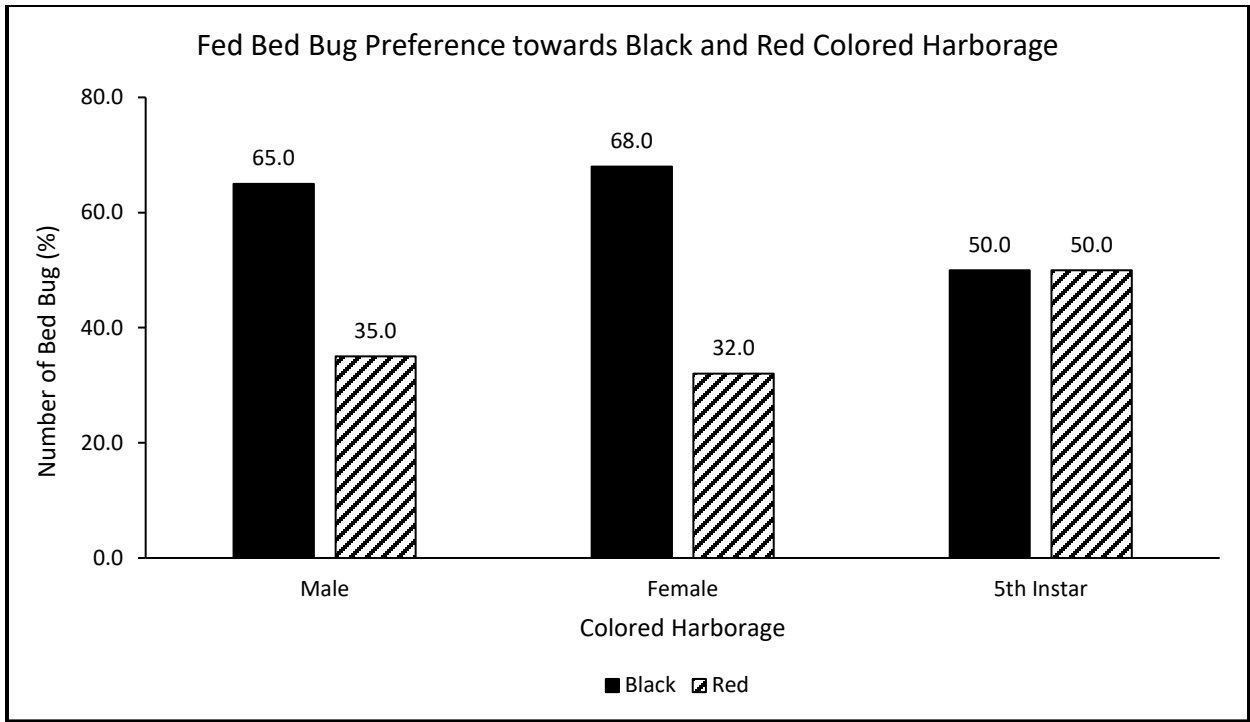


Figure 5. The responses of fed bed bugs to black or red coloured harborages.

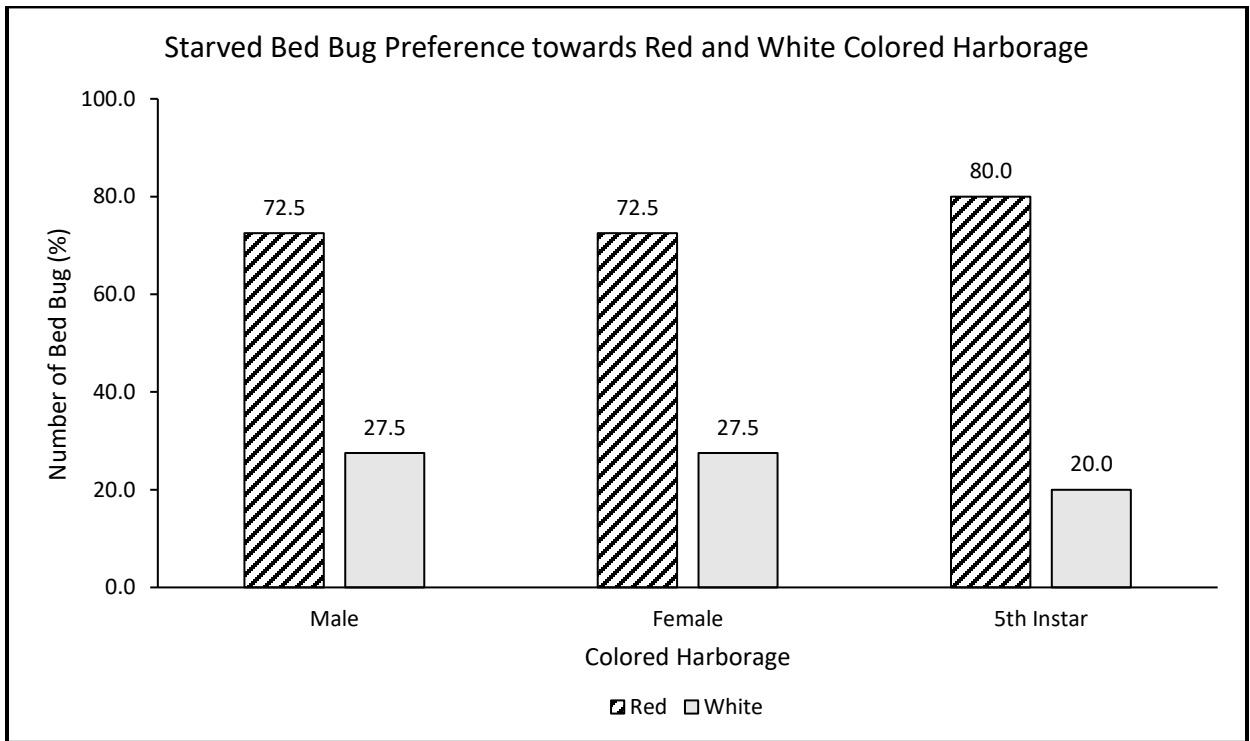


Figure 6. The responses of starved bed bugs to red or white coloured harborages.

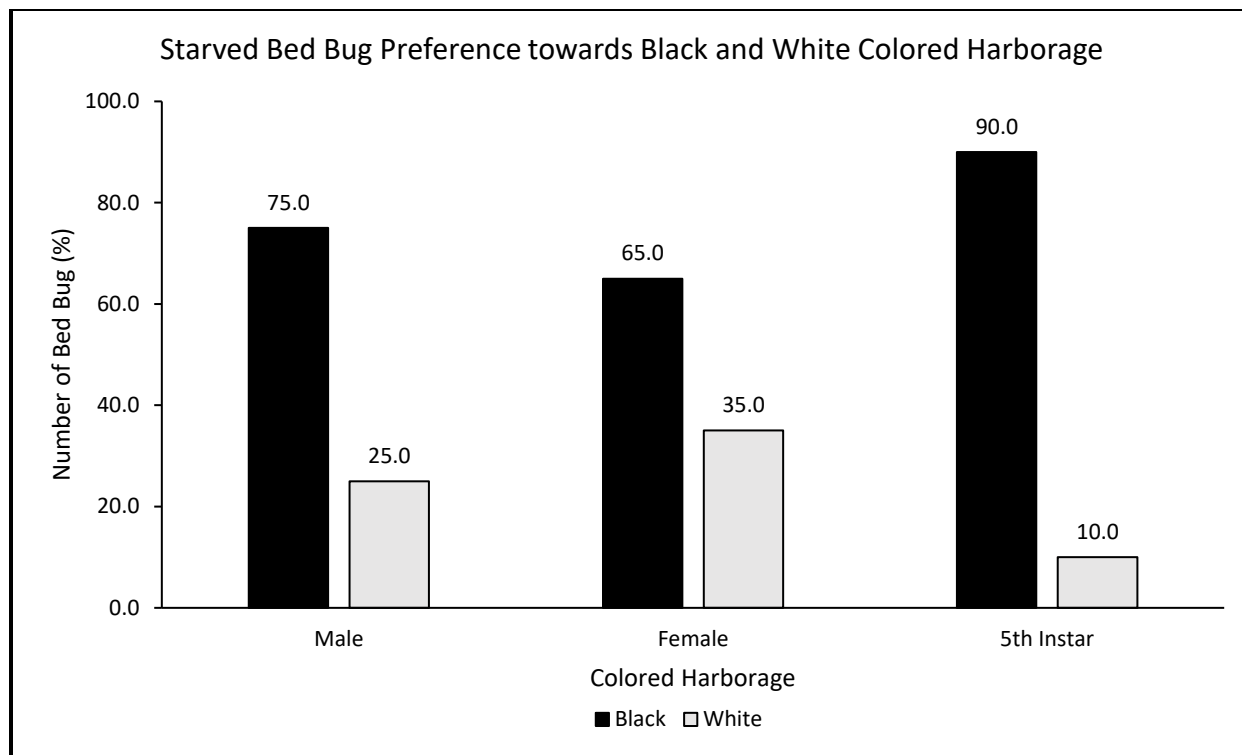


Figure 7. The responses of starved bed bugs to black or white coloured harborage.

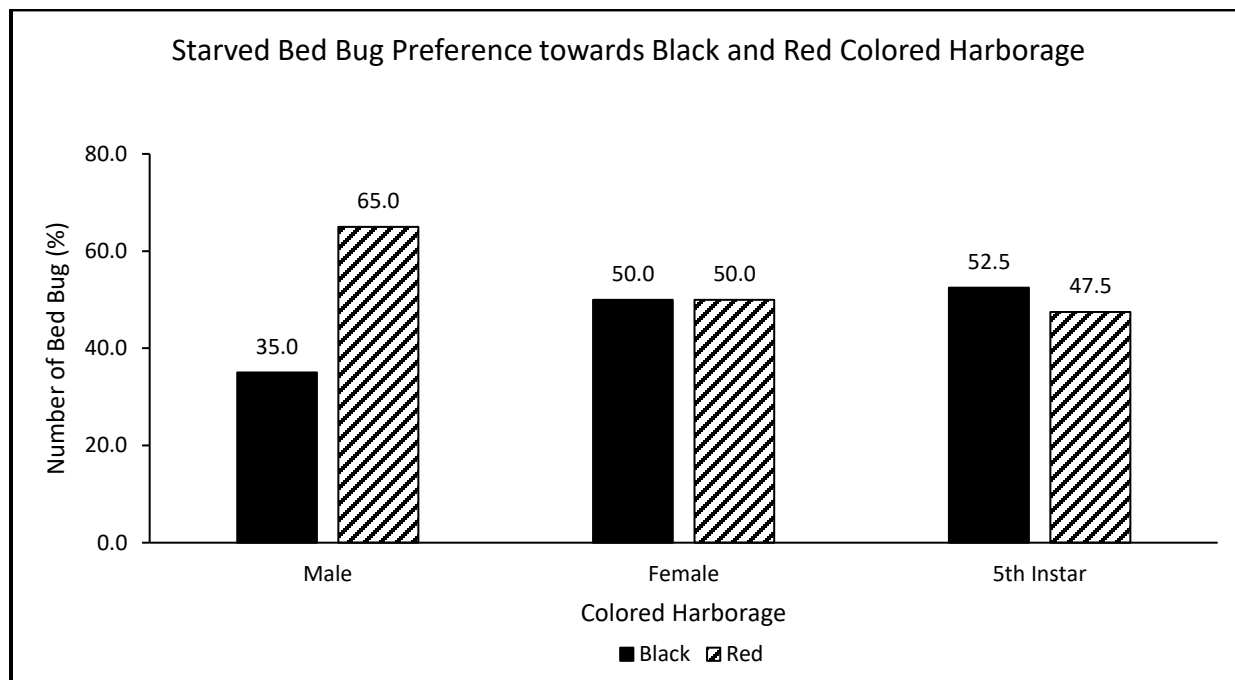


Figure 8. The responses of starved bed bugs to black or red coloured harborage.

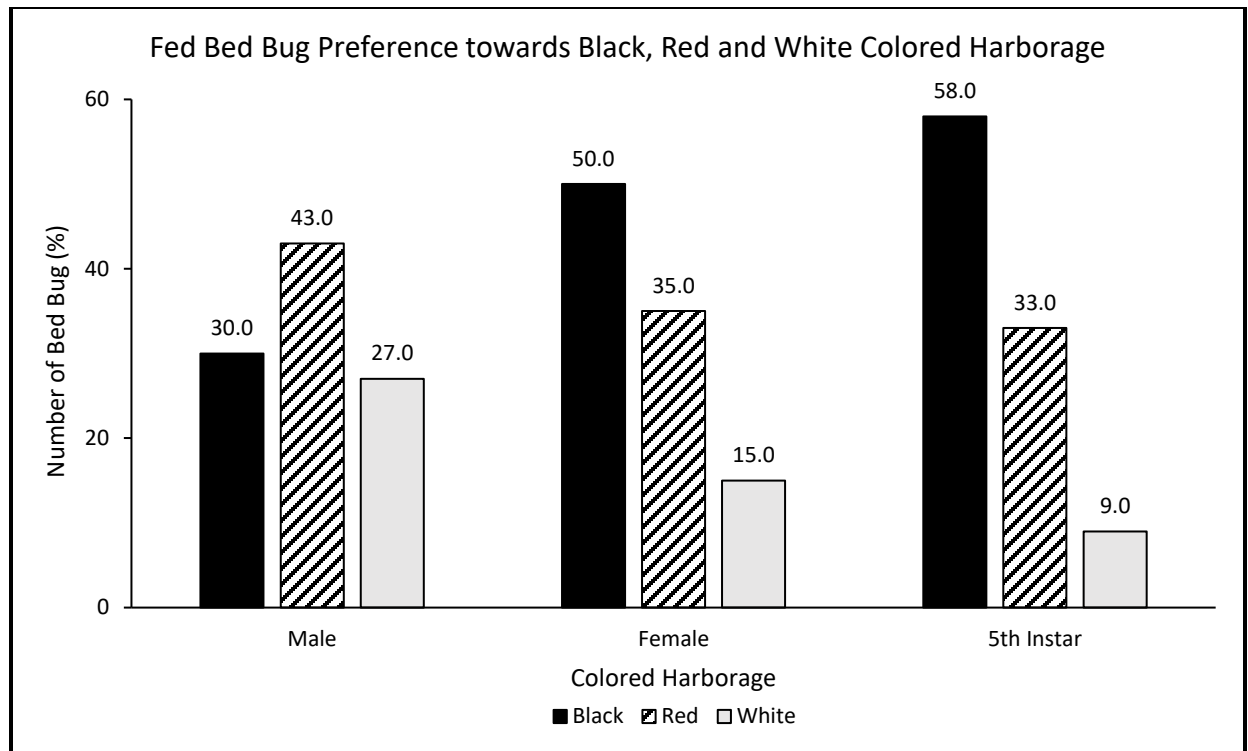


Figure 9. The response of fed bed bugs to black, red and white coloured harborage.

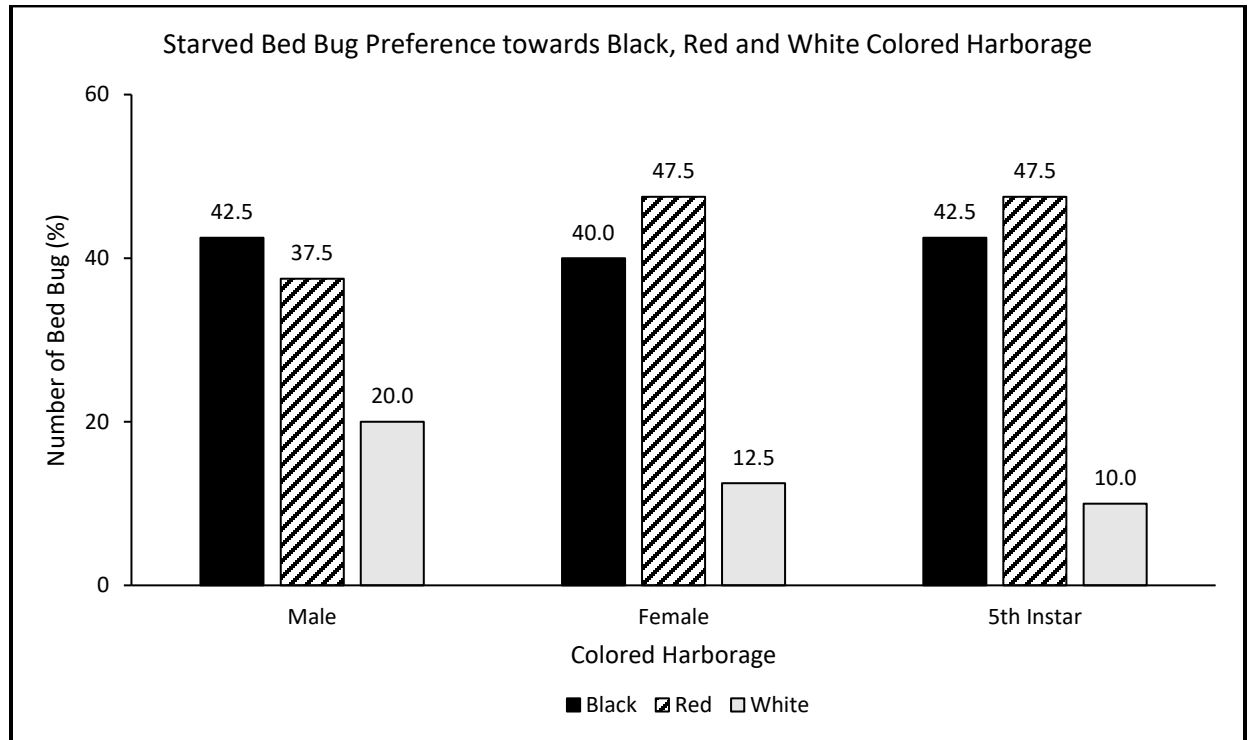


Figure 10. The response of starved bed bugs to black, red and white coloured harborage.

Oviposition Assays

A one-way ANOVA was performed to evaluate the relationship between coloured harborage and the proportion of eggs that was deposited on each coloured harborage. Mean of eggs laid on black coloured harborage was 4.8 ± 1.645 , followed by eggs laid on red coloured harborage with 6.4 ± 1.746 , and white coloured harborage recorded the highest eggs laid with 7.7 ± 1.693 (Fig. 11). The ANOVA was not significant at the 0.05 level, $F(2, 27) = 0.734$, $p = 0.489$. A post hoc Tukey HSD test also indicated there were no significant differences between the mean eggs laid on black and red coloured harborage ($p = 0.784$), between black and white coloured harborage ($p = 0.458$) or between red and white coloured harborage ($p = 0.851$). Mean of eggs laid on black coloured harborage was 4.8 ± 1.645 , followed by eggs laid on red coloured harborage with 6.4 ± 1.746 , and white coloured harborage recorded the highest eggs laid with 7.7 ± 1.693 (Fig. 11). The ANOVA was not significant at the 0.05 level, $F(2, 27) = 0.734$, $p = 0.489$. A post hoc Tukey HSD test also indicated there were no significant differences between the mean eggs laid on black and red coloured harborage ($p = 0.784$), between black and white coloured harborage ($p = 0.458$) or between red and white coloured harborage ($p = 0.851$).

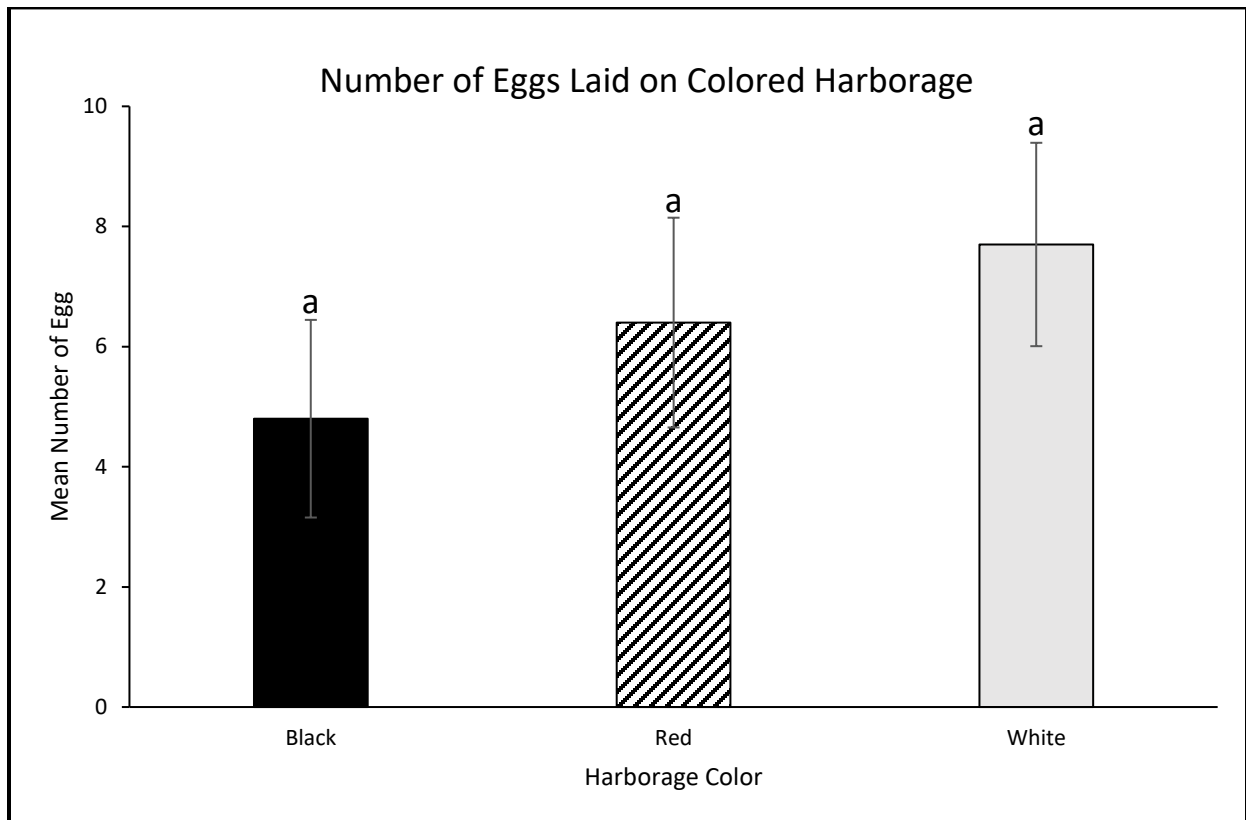


Figure 11. Mean of eggs laid on different coloured harborage.

Rearing using Coloured Harborages

Tropical bed bugs were fed once every week on arm of a human volunteer for 12 weeks. All stages of the tropical bed bug remained attached to the parafilm membrane until the completion of their blood meals. Adult tropical bed bug emerged as early as week 6 for all types of harborage colour. However, the number of males and females were different for all replicates with one replicate of the black harborage recorded the highest number of females (32 individuals) and the lowest number (18 individuals) of female recorded by one replicate of black and white harborage. After 12 weeks, all containers have various life stages, from egg to all nymphal stages and adults.

A two-way ANOVA was performed to determine the effect of harborage colour and time (in weeks) on the live individuals recorded in each week (Fig. 5). There was a statistically significant interaction between the effects of harborage colour and time on the live individuals, $F(22, 72) = 1.017, p = 0.457$. However, there was no statistically significant difference in the mean of live individuals between week ($p = 0.000$) and between harborage colour ($p = 0.001$). The mean of live individuals from the first week until the seventh week showed no significant difference. From the eighth to the tenth week, there was a significant difference of live individuals. Lastly, there was no significant difference of live individuals on the last two weeks.

The initial number of individuals for all types of harborage colour were 50 newly hatched first instars per replicate. One-way ANOVA was conducted to determine the effects of different harborage colour on the live individuals after 12 weeks of feeding (Fig. 4). There was no statistically significant difference in the number of live individuals when the tropical bed bugs were reared in containers with different harborage colour, $F(2, 6) = 0.555, p = 0.601$. Containers with red coloured harborage recorded the highest population size (600 ± 89.238), followed by black coloured harborage (473 ± 133.841) and white coloured harborage (458 ± 84.234).

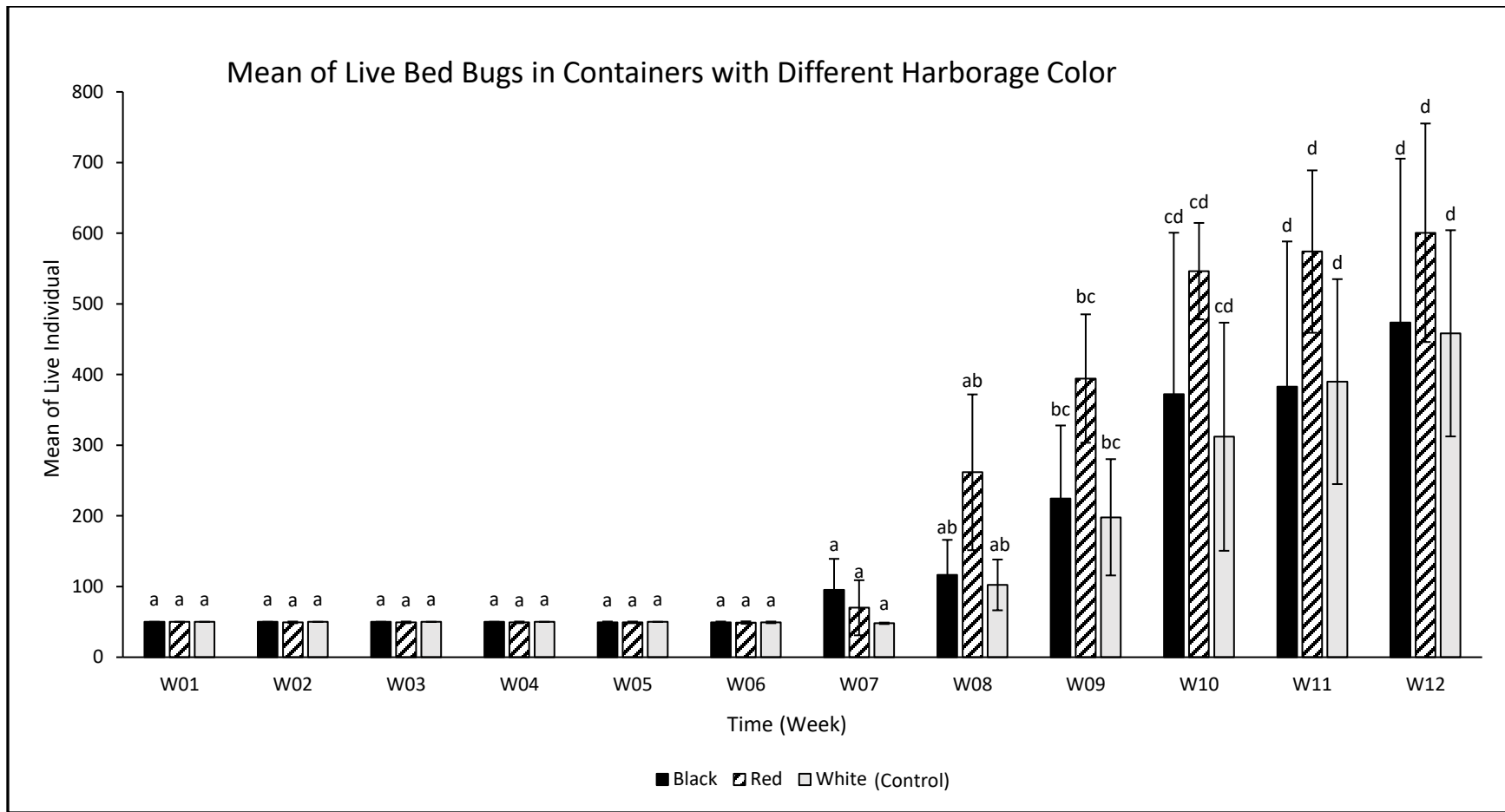


Figure 5. Mean of live bed bugs in the containers containing different harborage colour.

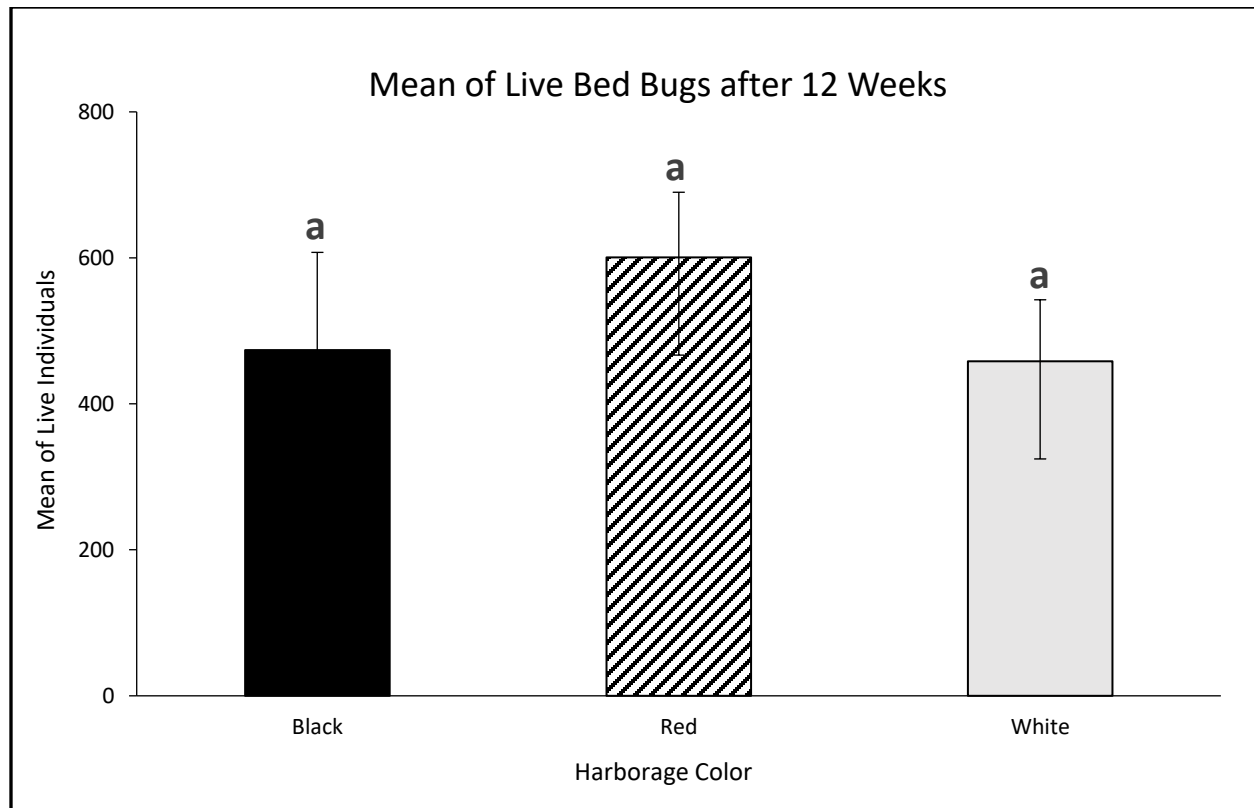


Figure 13. Mean of live individuals after 12 weeks according to the harborage colour.

DISCUSSION

Bed bugs can be found worldwide because they are easily transported on or in luggages, furnitures, boxes and clothes. These thin and tiny insects can be found in residential houses, hotels and public transports, and they have sparked major concerns to the hospitality and tourism industry. Bed bugs are mostly active during the night, however more significant activities has been observed under low light condition compared to under complete dark condition (Singh *et al.* 2015). Romero *et al.* (2010) explained that this peak of activity was because of the bed bug's harborage-seeking behaviour. Therefore, understanding the physiological colour 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.

A study conducted by McNeill *et al.* (2016) found that common bed bugs (*Cimex lectularius*) have different colour preferences for their harborage and oviposition sites. Similarly, our results showed that tropical bed bugs (*Cimex hemipterus*) both fed and starve strongly preferred black and red coloured harborage compared to white coloured harborage. Fed males and females preferred black over red coloured harborage. On the other hand, when starved, male bed bugs preferred red over black coloured harborage. Nymph, however, did not showed preferences towards red or black harborage, either fed or starved. Colour is also an important clue that hematophagous insects use to seek shelter (Steverding & Troscianko 2004). They

documented that traps with a blue exterior and black and red interior were very effective at attracting and optimising tsetse fly trap captures. Similarly, a study by Hoel *et al.* (2007) and Mann *et al.* (2009) recorded that *Phlebotomus papatasi* and *Lutzomyia shannoni* have been shown to be attracted to red colour. 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 colours.

Both adult and immature bed bugs are able to differentiate between different colours, and preferentially select harborages based on colour-specific visual cues. Typically, bed bugs tend to feed and find harborages during dark periods, so colour preferences may not be important in those cases (Singh *et al.* 2015). However, these harborage colour 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 colour experiments show that bed bugs do not hide in just any harborage; rather they will select a harborage based on its colour when moving in the light.

McNeil *et al.* (2016) theorised that specific colours may represent an opportune oviposition site and mating arena or for safety purpose due to the presence of other animals and limits the visibility of predators. Oviposition assays showed that female bed bugs laid more eggs on red coloured harborages compared to black coloured harborages. Black (and blue) colours 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 bed bugs will oviposit their eggs on a wide range of coloured harborage, they will avoid ovipositing under green coloured harborages (McNeill *et al.* 2016). This avoidance behaviour may be because of green or yellow colours are usually associated with an outdoor environment having plants and bright light, which is not where bed bugs are normally found.

Bed bugs that have been reared in the containers with red paper as their harborage recorded the highest number of individuals compared to black and white paper after 12 weeks. The life span of bed bugs are significantly influenced by their diet and mating frequencies. We fed our bed bug colonies once every week (7 days interval) because this is the optimal interval for bed bug feeding session. Bed bugs will hide in their harborage after feeding on their host for molting and reproduction. Usually it takes about 6 to 7 days for molting process to complete and female adults typically able to lay eggs up to 10 days. Although bed bug can live up to months without blood meal, they would not be able to molt or produce offspring when they did not have a blood meal as blood is required for those process to happened. These conditions are not favourable to the researchers who need lots of bed bugs colonies for their tests and experiments. Our preliminary study which to compare two feeding interval (7 days vs. 14 days) on the reproduction of bed bugs showed that 14 days feeding interval would took longer time to produce large scale bed bug colonies in the laboratory.

In addition, we started our colonies with 50 first instars for each replicate. After 6 weeks, the fifth instars started to molt and emerged as adults. However, the number of males and females

were different for each replicate. This seem to have an effect towards the reproduction rate of the tropical bed bugs. For example, replicate with higher number of females (i.e., 32 females and 18 males) would produced more offsprings (>500) compared to replicate with lower number of females (i.e., 18 females and 28 males) which produced less offsprings (<200) after 12 weeks of observation. Bed bug reproduction process involved very unique process called traumatic insemination where the male pierces the female's abdomen (Harlan 2006; Reinhardt & Siva-Jothy 2007; Usinger 1966). This process caused damages to the female body. This could explain why colonies with more males than females produced less offspring. Female bed bugs would have more traumatic inseminations from more than one male which would later damages the female body and affect their ability to produce and lay eggs. On the other hand, colonies where a lot of females rather than male, produced more offsprings as they only received one traumatic insemination after each feeding session.

CONCLUSION

Tropical bed bugs have different colour preferences for their harborage and oviposition sites which influence by gender and nutritional status. Black and red coloured harborages seem to be the most attractive harborages. These findings indicate that bed bugs may have a mechanism to discriminate colours and should be useful in bed bug trap design as an attempt to enhance trap captures. Futher studies may include other colours and prolong the rearing experiment.

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AUTHORS' CONTRIBUTIONS

Abd Hafis Abd Rahim: Methodology, investigation, data curation, formal analysis, writing– original draft, writing – review and editing.

Abdul Hafiz Ab Majid: Conceptualisation, supervision, project administration, resources, funding acquisition, writing – review and editing.

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