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RESEARCH ARTICLE

Tracking bed bugs (*Cimex lectularius*): a study of the effect of physiological and extrinsic factors on the response to bed bug-derived volatiles

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SUMMARY

The common bed bug, *Cimex lectularius*, feeds on the blood of mammal and bird hosts, and is a pest of global importance. Semiochemicals are chemicals involved in animal communication that may affect behaviour and/or physiology. Attractive semiochemicals that play a role in mediating bed bug behaviour could be exploited for the development of a highly effective novel monitoring device. Tracking software was used to record the response of bed bugs to volatiles from paper previously exposed to conspecific bugs in a still-air olfactometer illuminated by infrared lights, through a variety of activity variables. The effect of time of day as an extrinsic factor, and sex, stage, mating status and nutritional status as physiological factors on the response of bed bugs to the volatiles was examined. Bed bugs of both sexes and all stages responded to the volatiles from bed bug-exposed papers, showing significant attraction and orientation towards the volatile source whether they were starved or engorged. Confirmation that the physiological factors examined do not affect the response of bed bugs to the volatiles from bed bug-exposed papers provides evidence that these bed bug-derived volatiles contain aggregation cues, as semiochemicals that promote aggregation should by definition be detected by both sexes and all life stages. A device baited with such semiochemicals could play a major role in limiting the impact of the current bed bug resurgence by enabling timely detection of infestations, along with quantitative evaluation of control and effective surveillance of the geographical distribution of the pest species.

Key words: Cimicidae, olfactometer, semiochemical, EthoVision.

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INTRODUCTION

The common bed bug, *Cimex lectularius* (Linnaeus 1758; Hemiptera: Cimicidae), which feeds upon the blood of human hosts, is an important public health pest worldwide (Eddy and Jones, 2011). In recent decades, bed bugs have re-emerged globally as an insect of public health importance (Harlan, 2006). Current routine monitoring is limited to visual inspections by trained personnel for live bed bugs and other evidence of infestations such as dead bed bugs, exuviae, eggs and faeces (Harlan, 2006). Such visual inspections are both labour and time consuming. The use of bed bug-detecting dogs is increasing in popularity; trained dogs are able to discriminate between active and inactive bed bug refuges (Pfiester et al., 2008). Whilst a good alternative to a visual inspection, the use of bed bug-detecting dogs can be expensive and is not a suitable method for routine surveillance. There are monitoring devices available of both active and passive design. However, there are no commercial products with proven efficacy that are also suitable for wide scale routine surveillance (Weeks et al., 2011a). A better understanding of the chemical ecology of bed bugs could enable the use of semiochemicals, i.e. chemicals involved in animal communication that may affect behaviour and/or physiology, to manipulate bed bug behaviour for improved trap efficacy.

Attraction of bed bugs to filter papers that have been exposed to bed bugs (bed bug-exposed papers) has been demonstrated in several studies (Levinson and Bar Ilan, 1971; Parashar et al., 2003; Siljander et al., 2007; Siljander et al., 2008; Olson et al., 2009; Weeks et al., 2011b). However, a blend of chemicals that is attractive in the absence of contact and therefore suitable for use as bait in a trap, is yet to be identified. A recent study, using a behavioural bioassay design, that separated responses due to olfaction from those due to contact chemoreception and thigmotaxis, demonstrated that contact is not necessary for attraction to bed bug-exposed papers (Weeks et al., 2011b). Using this assay the identification of volatile chemicals that bed bugs use to locate their refuges may be possible. However, the evaluation of semiochemical-baited trap in the field must be preceded by a better understanding about bed bug chemical ecology and behaviour.

The response of insects to pheromones and other semiochemicals may be affected by many factors, including the physiological state of the insect (e.g. mating and nutritional status) and extrinsic factors such as time of day and temperature (Wertheim et al., 2005). Therefore, it is important to understand the role of such factors on bed bug behaviour for design of further experiments that investigate semiochemicals. In addition, knowledge of the factors that affect the response will give an indication of the behavioural role of the semiochemical and enable the development of a trap with optimal design. Previous studies investigating aggregation behaviour, where the information has been provided, use bed bugs that have been starved beyond their usual feeding regime (Siljander et al., 2008; Weeks et al., 2011b). As the preference to aggregate could decrease during periods of starvation, when bed bugs must begin to locate a host, testing starved bed bugs may result in the evaluation of host-

seeking bed bugs against aggregation cues. Extrinsic factors such as the time of day may also affect the behavioural response of an insect to a semiochemical.

If bed bugs are to be studied during the scotophase then it is important that any light used to permit observation of bed bug behaviour during the bioassay is perceived as darkness, so that the insect exhibits normal nocturnal activity. Direct observation is also undesirable, as a human observer will produce attractive host volatiles that could influence the response of the bed bugs, possibly introducing a directional bias. Previous studies have utilized infrared (IR) photography or video recordings to monitor and then score bed bug behaviour removing the potential influence of light and host volatiles (Olson et al., 2009; Romero et al., 2010; Harraca et al., 2012). Although the potential volume of data produced by this method is large, the format is unmanageable and therefore the data are often under utilized. One previous study used tracking software to record the behavioural responses of bed bugs to putative host location cues (Harraca et al., 2012). Tracking software, such as EthoVision (Noldus Information Technology, Wageningen, The Netherlands), could be used to track bed bug behaviour under IR light. The software detects the insect through changes in the image from a background view and monitors movement by comparing subsequent images. The software records the position of the insect under observation as co-ordinates, which can then be converted into variables once all the data have been accumulated. *Post hoc* conversion of the raw data enables the user to explore the effect of recorded factors on behaviour through more variables than would be possible to record manually through direct observation.

The aim of this study was to investigate the suitability of adapted olfactometry bioassays and EthoVision software for the study of bed bug orientation behaviour in response to volatiles and whilst doing so identify physiological and extrinsic factors, such as sex, time of day, life stage and mating and nutritional status, that affect the behavioural response of bed bugs to volatiles from bed bug-exposed paper.

MATERIALS AND METHODS

Insects

Cimex lectularius were obtained from a culture at the University of Sheffield (UK), then reared at Rothamsted Research in plastic colony pots (60×40 mm, height × diameter) with a plastic screw top lid. A hole (diameter 20 mm), was made centrally in the lid to permit ventilation. Mesh was secured between the pot and the lid with a band (elastic), to prevent escape and allow the bed bugs to be fed easily. The rearing room was maintained at 25±1.5°C, 80±5% relative humidity (RH) and the light regime was set to 14 h:10 h light:dark. During the scotophase (10:00–20:00 h), darkroom safelights (>700 nm, Jessops, UK) were used to illuminate the rearing room. Bed bugs were given access to heparinized sheep blood (TCS Biosciences, Botolph Claydon, UK) once per week via an artificial blood-feeding system (Montes et al., 2002). The glass feeders were cleaned by washing with warm soapy water (1% Teepol 12-20 unperfumed detergent, Hertfordshire Supplies, Welwyn Garden City, UK) followed by acetone (≥99.5%, Sigma-Aldrich, Dorset, UK) and placing in an oven (150°C) for 12 h.

Unless otherwise stated, experimental insects were adults of both sexes, which had been blood-fed 7–14 days previously. As the bed bugs were normally fed weekly, this would have allowed enough time for the bug to have digested the blood meal but not to become malnourished (Mellanby, 1939a). Recently engorged and malnourished bed bugs exhibit a low level of activity in olfactometers (Olson et al., 2009). At least 12 h before use, experimental insects were transferred into a pot for transporting to the bioassay room the following day. Bed bugs were chosen randomly from colony pots, identified as adults and sexed under a dissecting microscope (Usinger, 1966). The bioassay room was maintained at the same temperature and light cycle as the rearing room. However, in the bioassay room red light was used only for manipulation of equipment between replicates, otherwise the room was without illumination. The RH in the room was maintained at 50±5%. Bed bugs were introduced into the room at least 60 min prior to use, to give them time to acclimatize to the change in RH and any other differences in environmental conditions.

Olfactometer development

A Petri dish olfactometer developed by Weeks et al. (Weeks et al., 2011b) was adapted for use with video recording equipment and EthoVision software and is, hereafter, described as the Still-air olfactometer I (Fig. 1A). Modifications to the olfactometer were as follows: the base of the Petri dish (140 mm diameter) was used as the base of the olfactometer, which increased the volume of the arena by a factor of 1.7, decreasing the chance that the arena will be saturated with volatiles during the bioassay duration. The substitution of the polystyrene Petri dish lid for a Perspex lid reduced reflections, increased focus for tracking and created a better seal. Furthermore, when using EthoVision, it was not necessary to have markings on the arena to separate the zones, as these can be added digitally to the captured image. A central hole enabled the introduction of bed bugs to the arena without removal of the lid. The hole (diameter 10 mm) was covered during experiments with a glass coverslip (22×22 mm) to prevent bed bugs from escaping.

Problems detected by tracking the insects in the Still-air olfactometer I led to the development of an alternative olfactometer design, that was symmetric: hereafter described as the Still-air olfactometer II (Fig. 1B). The original distance between the pots (64 mm) was maintained, but the distance between the pots and the centre, and the pots and the edge, was equal (32 mm). Additionally, it was possible to dismantle these olfactometers, making them easier to clean. Further modifications were as follows: the olfactometer design consisted of a circular arena (diameter 190 mm) comprising a Perspex base (220×220 mm), wall (height 24 mm) and lid. Therefore, the volume of the arena was increased by a factor of 2.7 from Still-air olfactometer I. The lid and the base were graduated such that they created a tight seal with the wall and the pots, respectively. The arena was lined with fine mesh (pore size 500 µm) that was held in place on the base with pins; the wall was positioned inside these pins and the lid slotted on top of the wall.

In both designs, two pots (plastic, 60×40 mm, height × diameter) were placed under the holes in the olfactometer arena. When the test volatiles were present, a pot that contained a bed bug-exposed paper and a pot that contained clean filter paper were used. During controls (test volatiles absent), two pots with clean filter paper were used.

Bed bug-exposed papers were filter papers (Whatman, 70×40 mm) that had each been exposed to 100 bed bugs for 1 month. All bed bugs, exuviae and eggs were taken off and the papers were placed individually into a clean pot, hereafter described as the ‘odour’ pot (O). Therefore, bed bug-exposed papers, consisted of faeces and any additional material deposited through contact with bed bugs. The ‘no odour’ pot (NO) contained clean unexposed filter paper (Whatman, 70×40 mm). The unexposed filter paper was replaced after each replicate. In both treatments (volatiles present and control) the position of the O pot was randomly assigned.

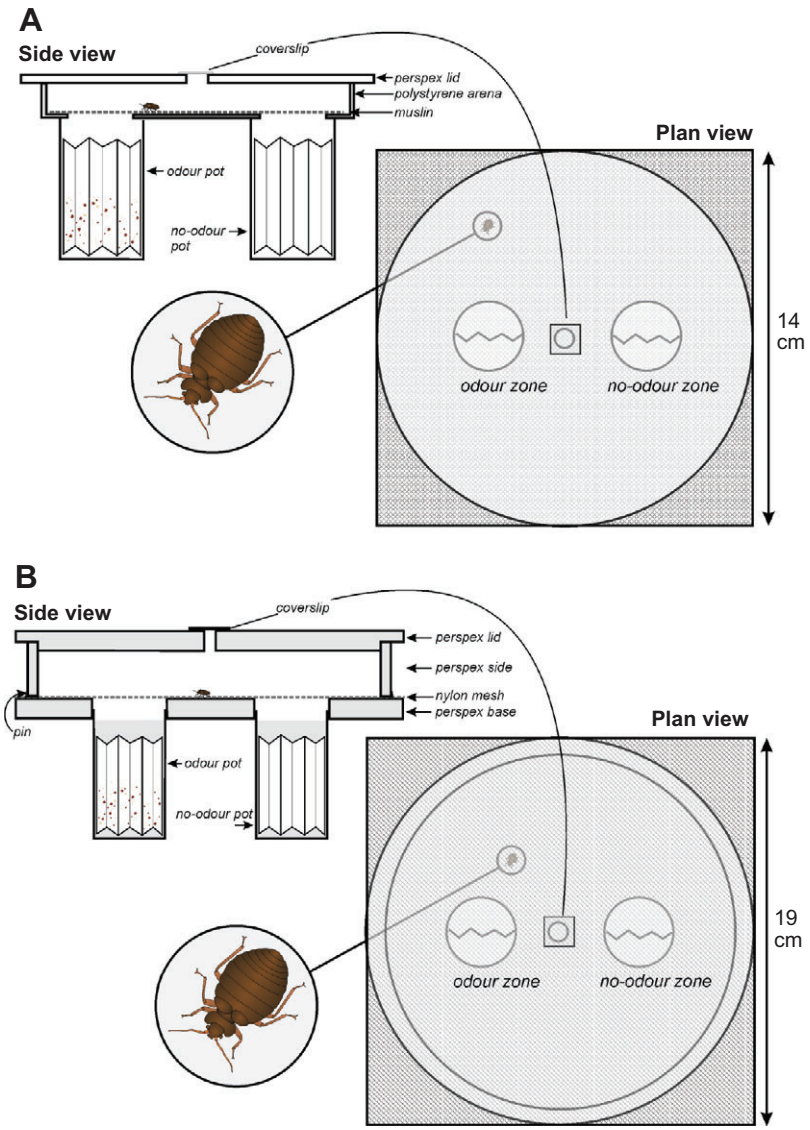

Fig. 1. Illustration of the Still-air olfactometers used to investigate bed bug behaviour. (A) Still-air olfactometer I, internal diameter 140 mm, hole diameter 26 mm, distance between holes 64 mm, distance to edge 12 mm. (B) Still-air olfactometer II, internal diameter 190 mm, hole diameter 26 mm, distance between holes 64 mm, distance to edge 32 mm. Pots were plastic and measured 60×40 mm (height × diameter). Odour pot contained bed bug-exposed paper. No-odour pot contained clean unexposed filter paper.

Fifteen minutes after setting up the equipment, an individual bed bug was introduced into the centre of the arena using a fine paint brush. The bed bug was then tracked for 15 min.

Olfactometers were cleaned after each replicate and re-used; new unexposed pots were used for each replicate and cleaned before use. Still-air olfactometer II had removable mesh, so this was replaced with new unexposed and cleaned mesh between replicates. Olfactometers were washed with warm water and detergent (1% Teepol 12-20), rinsed with a 1:1 solution of ethanol:distilled water (≥99.8%, Sigma-Aldrich) followed by distilled water, and dried between replicates. Unexposed pots and mesh were washed with warm water and detergent (1% Teepol 12-20), rinsed with a 1:1 solution of ethanol:distilled water (≥99.8%) followed by distilled water, and dried. Clean cotton gloves were worn when handling olfactometers and other equipment.

Effect of sex and time

Using Still-air olfactometer I, temporal variation in activity and response to volatiles from bed bug-exposed papers was investigated by completion of bioassays at six time points. The times included a single time point at the end of the photophase, 09:00 h, and five time points during the scotophase: 11:00, 13:00, 15:00, 17:00 and 19:00 h. Treatment (i.e. presence or absence of the volatiles), sex of the bed bug and odour pot position were randomized to each time point. Each treatment/sex combination was completed at each time point over a block of 4 days (n=3).

Effect of sex, mating status and stage

Still-air olfactometer II was used to investigate the variation in response to volatiles from bed bug-exposed papers between males, females and nymphs. The females were further separated into mated and virgin individuals to investigate the effect of mating status. Mated females were chosen randomly from colony pots that had been fed 7–14 days previously; as bed bugs mate when the female is engorged, the majority of females in these pots should have mated. Virgin females were obtained by isolating engorged fifth instar nymphs into vials for moulting into adults. After moulting, females were kept separately from males. The nymphal instar tested was of the fifth stage. In each block (comprising 1 day) each bed bug sex/stage type was tested in the presence and absence of the volatiles from bed bug-exposed paper. The order of testing and the odour pot position was randomized. Each block was tested on a different

bed bug-exposed paper (n=13). The bioassays were completed in the early scotophase, between 10:00 and 14:00 h.

Effect of nutritional status

Still-air olfactometer II was used to investigate the effect of nutritional status on the response to volatiles from bed bug-exposed papers by testing bed bugs that were starved and engorged. Starved bed bugs had not been fed for 7–9 days prior to being used in tests. Engorged bed bugs had a blood meal 3–5 days previously. In each block (comprising 1 day), each bed bug sex/nutritional status combination was tested in the presence and absence of the volatiles (n=10). The order of testing and the odour pot position was randomized. The bioassays were completed in the early scotophase, between 10:00 and 14:00 h.

Video equipment

A high-resolution monochrome camera (Sanyo B/W CCD) with a varifocal lens (5–50 mm, manual iris) and an IR pass filter was used to record data. The camera was suspended 60 cm above the olfactometer using a stand. Light for the recordings was provided by two IR LED arrays, positioned 68 cm from the camera and 10 cm from the olfactometer. The lights were facing downwards, which provided indirect lighting to reduce reflections on the camera lens. The whole set-up was contained in a white screen (45×45×100 cm, length × width × height) and the base of the stand was covered with white paper, which reflected light to improve contrast.

EthoVision software

EthoVision version 3.1 software (Noldus Information Technology) was used to capture video images to track bed bugs during bioassays. The detection method used for acquisition was subtraction, as described below. The detection thresholds were set so that all objects that were different from the background image by less than nine or greater than 255 pixels were ignored and, therefore, recognized as part of the background. To reduce problems caused by background noise, a scan window was used (100 pixels), which was set to search the complete arena after five missed samples. The sampling rate was 5 samples s^{-1}. For each sample the software detects the bed bug as a difference from the background image and records its position. The spatial resolution was 25.56 pixels cm^{-1}. The arena was divided into zones by two different zone definitions, i.e. zone and pot (see Fig. 2). A standard calibration of the arena was completed to enable EthoVision to convert distances between two points from pixels to x,y co-ordinates.

Data analysis and variables

The x,y co-ordinates were analysed in EthoVision to calculate orientation and activity variables. In experiments with Still-air olfactometer I, data analysis was completed at two levels, zone (i.e. a semi-circle comprising half of the arena) and pot (i.e. area above the pot), to identify the best level to use for future data collection and analysis. Data for all relevant variables were analysed in order to identify those appropriate for use in future studies. For experiments with Still-air olfactometer II, in response to the results with Still-air olfactometer I, only the time spent in each zone and the number of visits to each pot were recorded.

The difference was calculated between the odour and no-odour data, i.e. in most cases O–NO. Therefore, a positive difference indicated a greater mean value for the odour than the no-odour zone or pot. Variables calculated at both the zone and pot level were speed, proportion of activity and angular velocity. Speed, i.e. the distance moved by the bed bug per unit time, was calculated in

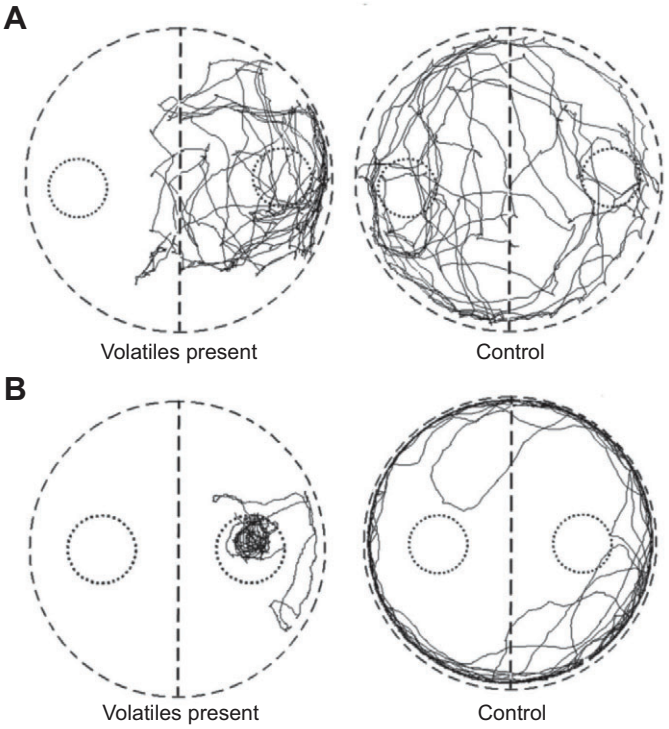

Fig. 2. Example tracks recorded by EthoVision of *Cimex lectularius* in Still-air olfactometer I (A) and II (B), in the presence and absence (control) of volatiles from bed bug-exposed paper. The odour pot is positioned on the right-hand side of the arena. Arena and zones (two sides of the arena) are marked with a dashed line, pots (i.e. areas directly above pots) with a dotted line and the path of the bed bug during a behaviour bioassay with a continuous black line (duration, 15 min).

$cm\,s^{-1}$. Time spent moving (s) was calculated by comparing the bed bug speed with a pre-set threshold of greater than 0.1 $cm\,s^{-1}$. The threshold was set based on the speed of bed bug movement recorded in preliminary trials. The time spent moving was divided by the time spent in the zone to give activity as a proportion. The absolute angular velocity (turning rate, the change in direction of the bed bug per unit time) was calculated in $deg\,s^{-1}$. The time spent in each zone (s) was also calculated. The number of visits to each pot was calculated as a frequency and for analysis at the pot level the time spent per visit (s) was also considered. A visit was defined as a period of time spent in the area directly above a pot, and one visit lasted from the time of entry to this area until exit. The distance to pot (cm) was calculated by taking the average of the distance of the bed bug from the centre of each pot over the duration of the bioassay. The distance from the odour pot was then subtracted from the no-odour pot (NO–O) to give a positive value when bed bugs spent more time closer to the odour pot.

All results were analysed using GenStat version 11.0 (VSN International, Hemel Hempstead, UK) (Payne et al., 2008). Residual plots for each variable indicated that the data were normally distributed, but as the data contained missing values that made the final design unbalanced, a restricted maximum likelihood analysis method was used, followed by approximated F-test. The least significant differences at the 5% level were used to determine significance within the treatment factors using an approximated t-test.

The latency to first visit (s) was also recorded and used to calculate the pot that was visited first for each replicate (i.e. the pot with the lowest latency). These data were analysed by paired t-test.

The null hypotheses for all statistical tests were that there were no significant differences in bed bug behaviour in the presence or absence of the volatiles from bed bug-exposed papers, and that this response was not affected by time or the sex, mating status or nutritional status of the experimental insect.

RESULTS

Olfactometer development

Bed bugs were tracked successfully in Still-air olfactometer I using the camera with IR lighting and EthoVision software. Visualization of the tracks showed differences between the orientation of bed bugs in the presence and absence of volatiles from bed bug-exposed paper (Fig. 2A). When the volatiles were present, bed bugs spent the majority of time between the edge of the olfactometer and the area directly above the pots, suggesting that volatiles were accumulating in this area. A possible explanation for the build-up is that the distance between the pots and the edge was relatively short compared with the distance from the pots to the centre of the olfactometer. Still-air olfactometer II was designed to be symmetrical to reduce volatile build-up in the area between the edge of the olfactometer and the area directly above the pots. Bed bugs were tracked successfully in the modified olfactometer (Fig. 2B). In Still-air olfactometer II, in the presence of the volatiles from bed bug-exposed paper, bed bugs left the edge of the arena and spent the majority of the bioassay duration directly above the odour pot. In comparison, in control experiments, bed bugs spent more time in contact with the edge of the arena.

Behavioural responses to volatiles from bed bug-exposed paper in Still-air olfactometer I

In the presence of test volatiles from bed bug-exposed paper, a significantly higher proportion of bed bugs (76%; t_{66}=–2.43, P=0.018) visited the odour pot first compared with controls. In the absence of test volatiles (controls), there was no significant difference in choice of first pot visit (54%).

There were significant differences in speed ($F_{1,40}$=4.18, P=0.048) and time spent ($F_{1,33.5}$=4.64, P=0.038) in the two zones dependent on treatment (Table 1). Bed bugs moved faster and spent more time in the odour zone compared with the no-odour zone when the volatiles from bed bug-exposed paper were present than during controls. There were no significant differences in the proportion of activity or angular velocity between the two zones caused by the treatment or any of the other factors or the corresponding interactions.

Bed bugs were significantly closer to the odour pot than the no-odour pot in the presence of the volatiles from bed bug-exposed paper than during controls (Table 2; $F_{1,33.3}$=5.41, P=0.026). In the presence of the volatiles, there was also significantly more time spent per visit ($F_{1,40.2}$=4.91, P=0.032) and a greater proportion of activity ($F_{1,22.4}$=8.92, P=0.005) over the odour pot than over the no-odour pot. None of the other factors or corresponding interactions was significant for these variables.

Table 1. Behaviour of *Cimex lectularius* in the zones of the Still-air olfactometer I

Variable	Volatiles present	Control	P
Speed (cm s^{-1})	0.058±0.057	–0.031±0.056	0.048
Activity (proportion)	0.048±0.071	–0.028±0.070	0.189
Time spent (s)	218.700±81.900	4.100±80.400	0.038
Angular velocity (deg s^{-1})	–4.530±17.460	–16.630±17.100	0.528

Mean differences (±s.e.m.) of each variable calculated between odour and no-odour zones (O–NO).

Table 2. Behaviour of *Cimex lectularius* in the pot zones of the Still-air olfactometer I

Variable	Volatiles present	Control	P
Speed (cm s^{-1})	0.180±0.100	–0.030±0.100	0.061
Frequency of visits	8.410±1.940	0.660±1.900	0.010
Distance to pot (cm)	1.820±0.620	0.130±0.610	0.026
Activity (proportion)	0.260±0.100	–0.110±0.090	0.005
Time spent per visit (s)	1.450±0.850	–1.190±0.870	0.032
Angular velocity (deg s^{-1})	55.810±19.430	–27.860±19.010	0.005

Mean differences (±.s.e.m.) of each variable calculated between odour and no-odour pot zones (O–NO for all variables except distance to pot, NO–O).

In the presence of volatiles from bed bug-exposed papers, bed bugs visited the odour pot more frequently ($F_{5,34.2}$=3.58, P=0.01), and in the pot area, displayed a greater angular velocity ($F_{1,42}$=9, P=0.005). However, the response was dependent on a high-order interaction between treatment, sex and time. There was no significant difference in speed between the two pots dependent on treatment.

Effect of sex

As an independent factor, sex had no significant effect on any variable. However, when the data were analysed as subsets by sex, only males walked significantly faster over the odour pot than the no-odour pot in the presence of the volatiles from bed bug-exposed papers (Fig. 3A; $F_{1,20}$=4.37, P=0.05). Males were also significantly closer to the odour pot than the no-odour pot in the presence of the volatiles from bed bug-exposed papers (Fig. 3B; $F_{1,12.9}$=5.68, P=0.033), which they visited more frequently (Fig. 3C; $F_{1,20}$=5.31, P=0.032) and for longer (Fig. 3D; $F_{1,12.8}$=7.81, P=0.015). Whilst in the odour zone, males showed a greater proportion of activity (Fig. 3E; $F_{1,20}$=6.21, P=0.022) and a greater turning rate (Fig. 3F; $F_{1,14.3}$=6.34, P=0.024). When the data were analysed in this way, female bed bugs showed no significant behavioural responses, either positive or negative, to the volatiles. For example, females did not differ significantly in their speed between pots dependent on treatment ($F_{1,11.9}$=0.5, P=0.494).

Temporal variation in response to volatiles from bed bug-exposed paper

Data were analysed to identify temporal trends in bed bug behaviour in the presence and absence of the volatiles from bed bug-exposed papers. The only variables that were dependent on the time of day were dependent on high-order interactions between multiple factors. The average difference in speed of bed bugs between the odour and no-odour pots was dependent on an interaction between sex and time ($F_{5,36.7}$=2.77, P=0.032). Males walked significantly faster than females over the odour pot at 09:00, 11:00 and 17:00 h. The frequency of visits to pots ($F_{5,34.2}$=3.58, P=0.01) and the angular velocity or turning rate ($F_{5,42}$=13.97, P=0.029) were dependent on treatment, sex and time in a high-order interaction. In general, in the presence of the volatiles, males made significantly more visits to the odour pot than the no-odour pot in the early scotophase between 11:00 and 13:00 h (Fig. 4A). At these times, males also turned at a significantly greater rate over the odour pot in the presence of the volatiles than during controls (Fig. 4B). At 13:00 h, females also made significantly more visits to the odour pot (Fig. 4A) and, whilst there, turned at a significantly faster rate (Fig. 4B) in the presence of the volatiles than during controls. In addition, females made a significantly greater number of visits to the odour pot than the no-odour pot at 19:00 h.

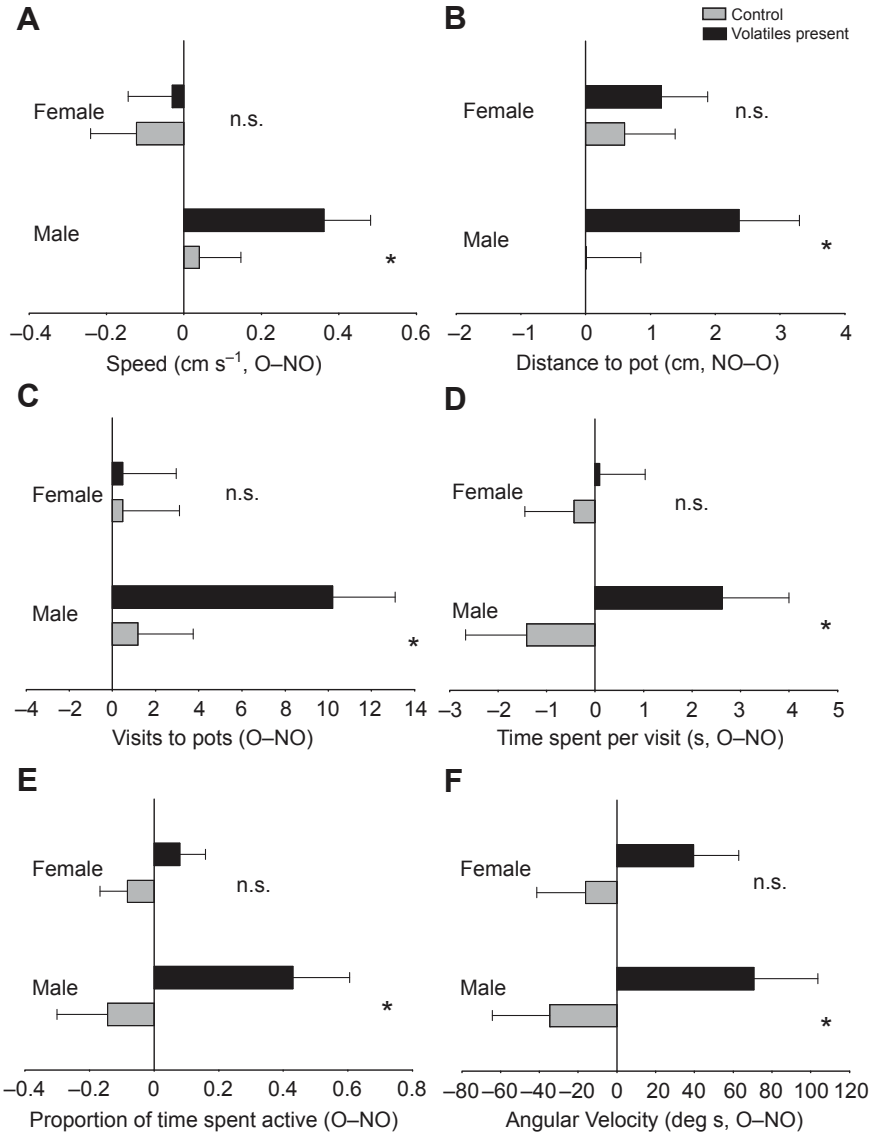

Fig. 3. Behaviour shown by *Cimex lectularius* in Still-air olfactometer I, in the presence and absence (control) of volatiles from bed bug-exposed paper, mean differences in (A) speed (cm s^{-1}), (B) distance to pot (cm), (C) visits to pots, (D) time spent per visit (s), (E) proportion of activity and (F) angular velocity (deg s^{-1}). Bars represent the mean difference (±s.e.m.) calculated between the pots [O–NO for all variables except distance to pot (B), NO–O]. Average s.e.d. for males and females, respectively: (A) 0.1590 and 0.1068 cm s^{-1}, (B) 1.074 and 1.058 cm, (C) 3.83 and 3.13 visits, (D) 1.524 and 1.380 s, (E) 0.2325 and 0.1047, (F) 41.61 and 34.42 deg s^{-1}. Asterisks indicate significant differences between the average response in the presence and absence of the volatiles, analysed by *F*-test: **P*≤0.05; n.s., not significant, *P*>0.05.

Effect of sex, mating status and stage

Bed bugs responded to the volatiles from bed bug-exposed papers. There were significantly more visits to the odour pot ($F_{1,83}$=33.66, P<0.001) and significantly more time spent in the odour zone ($F_{1,95}$=12.65, P<0.001) in the presence of the volatiles from bed bug-exposed paper when compared with controls. The effect of sex and stage of the insect was not significant when considered alone or as an interaction with treatment for either variable. However, for virgin females there was a trend towards fewer visits to the odour pot and less time spent in the odour zone in the presence of the volatiles from bed bug-exposed paper compared with the other sex/stage categories (Fig. 5). Mated females made the most visits to the odour pot in the presence of the volatiles from bed bug-exposed paper, followed by males and nymphs. Nymphs spent the most time in the odour zone, on average, followed by mated females and males.

Effect of nutritional status

Bed bugs responded to the volatiles from bed bug-exposed papers. There were significantly more visits to the odour pot ($F_{1,67}$=20.13, P<0.001) and significantly more time spent in the odour zone ($F_{1,67}$=17.93, P<0.001) in the presence of the volatiles from bed bug-exposed paper when compared with controls. The effect of nutritional status was not significant for either visits to pots or time spent in zones. However, females spent significantly more time in the odour zone than the no-odour zone regardless of treatment ($F_{1,67}$=8.76, P=0.004). The interaction between nutritional status and sex was also significant for the number of visits to pots (Fig. 6; $F_{1,67}$=4.86, P=0.031). Starved males and engorged females made significantly more visits to the odour pot than engorged males, independent of treatment type. Although the behaviour of bed bugs of different sexes and nutritional status did not vary significantly dependent on treatment, starved males and engorged females showed a trend towards increased visits to the odour pot and increased time spent in the odour zone (Fig. 7).

DISCUSSION

Olfactometer development

The modified olfactometer (Still-air olfactometer I) used in this study allowed for the measurement of a wide range of bed bug behaviour, including orientation towards a volatile source (Weeks et al., 2011b). It was also possible to observe and record the response of bed bugs to volatiles without interference from thigmotactic responses. EthoVision was a valuable addition to the bioassay, enabling the collection of large amounts of data with consistent

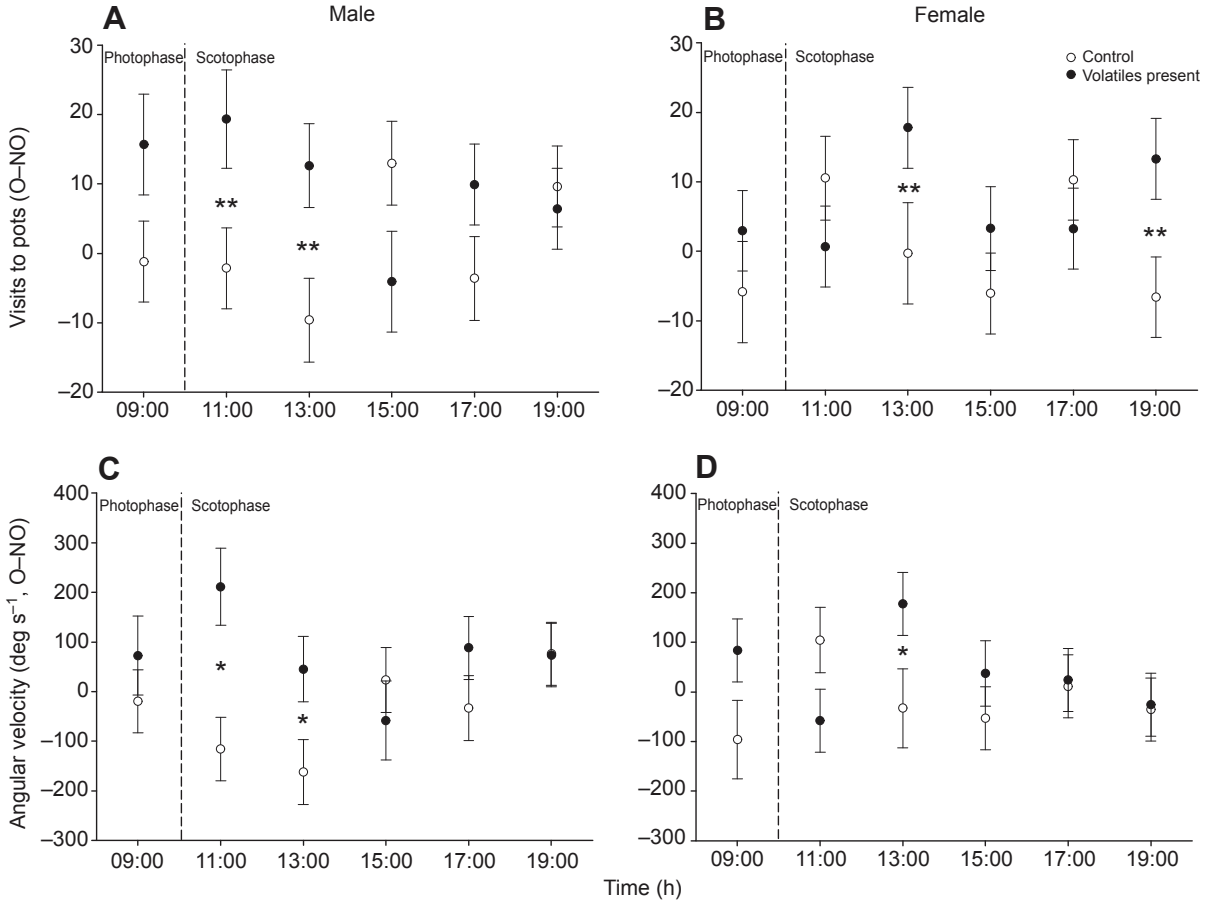

Fig. 4. Temporal effect of volatiles from bed bug-exposed paper on *Cimex lectularius* in Still-air olfactometer I on the number of visits to pots (A, male; B, female) and the turning rate (C, male; D, female). Difference in mean number of visits (s.e.d.=8.669 visits) and turning rate in deg s^{-1} (s.e.d.=95.53 deg s^{-1}) calculated between odour and no-odour pot for males and females of *C. lectularius* (O–NO). Values are means ± s.e.m.; asterisks indicate significant differences between the average response in the presence and absence of the volatiles, analysed by *F*-test: **P*≤0.05, ***P*≤0.01.

interpretation. When analysing recorded tracks using EthoVision, it became clear that the volatiles were accumulating between the edge of the olfactometer and the area directly above the pots. As this could have been due to asymmetry in the design of Still-air olfactometer I, further modifications were completed to produce the symmetric Still-air olfactometer II. The subsequent bioassays produced tracks that demonstrated that the volatiles were no longer accumulating in the area and that bed bugs were able to successfully orientate towards the volatile source throughout the duration of the bioassay. In addition, the Still-air olfactometer II was easier to clean and, therefore, preferable, as contamination from previous replicates could cause bias in later experiments.

Behavioural responses of *C. lectularius* to volatiles from bed bug-exposed papers

The behavioural experiments completed during the present study, using data collected and analysed by EthoVision, have supported conclusions from previous studies that bed bugs are activated by, and attracted to, the volatiles from bed bug-exposed paper (Levinson and Bar Ilan, 1971; Parashar et al., 2003; Siljander et al., 2007; Siljander et al., 2008; Olson et al., 2009; Weeks et al., 2011b). Unlike many other studies evaluating the response of bed bugs to volatiles from bed bug-exposed papers, the present study has recorded detailed behavioural measurements over a short time frame, e.g. the first choice of the bed bug and a range of activity variables. The final choice, the variable that is usually recorded (Levinson and Bar Ilan, 1971; Siljander et al., 2007; Siljander et al., 2008; Olson et al., 2009), has limited relevance when designing semiochemical baits, as the bed bug will be caught in the trap on first contact. Recording the final choice of bed bugs in experiments run over a long time frame gives little information about how the insect located the volatile source. For example, location could occur by chance followed by arrestment due to short range cues. Therefore, as attraction to the trap through chemotaxis is vital, corresponding variables should be recorded in behavioural studies. The present study used EthoVision to record bed bug behaviour, measured by a selection of variables at two levels, zone and pot, for 15 min after initial exposure to volatiles from bed bug-exposed paper. The variables that yielded the most informative and consistent results were the time spent in zones, frequency of visits to pots and the distance from the pots. Whilst the proportion of activity, speed and turning rate were also significantly different in the presence of volatiles, they were too variable to be considered to be reliable indicators of a behavioural response to volatiles. Distance from the pot and visits to the pot were not independent variables. If a bed bug makes many visits to the odour pot, its average distance from the odour pot will be less. It is, therefore, preferable to use the frequency of visits as a variable to measure in future studies, as it is easier to calculate. Time spent in zones and visits to pots should be the variables used in future olfactometer-based behavioural bioassays.

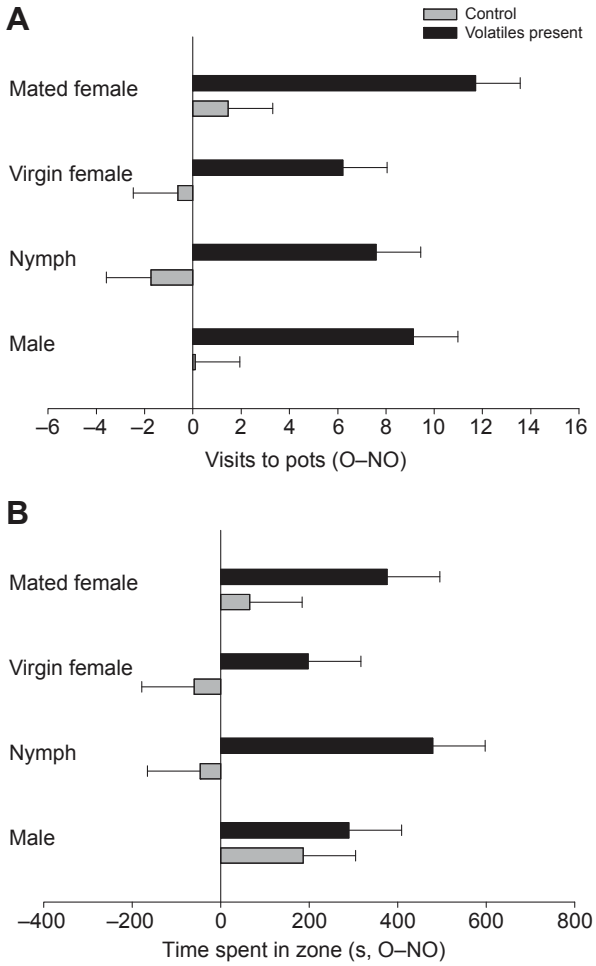

Fig. 5. Effect of volatiles from bed bug-exposed paper on male, female and immature *Cimex lectularius* in Still-air olfactometer II. Bars represent the mean differences in (A) visits to pots (s.e.d.=2.551 visits) and (B) time spent in zones (in seconds, s.e.d.=168 s) between odour and no-odour pots and zones (O–NO), respectively. Values are means ± s.e.m. *F*-test: (A) $F_{3,83}$=0.53, P=0.665 and (B) $F_{3,95}$=1.08, P=0.363.

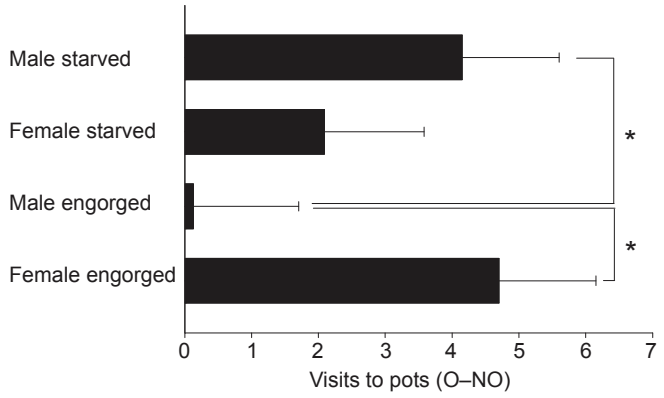

Fig. 6. Visits to pots of male and female starved and engorged *Cimex lectularius* in Still-air olfactometer II. Bars represent the mean differences in visits to odour and no-odour pots (s.e.d.=2.144 visits, O–NO). Values are means ± s.e.m.; asterisks indicate significant differences between the average response in the presence and absence of the volatiles, analysed by *F*-test, *P≤0.05.

Temporal variation in attraction

Although there have been no previous studies into the precise timing of bed bug aggregation behaviour, they are known to aggregate in refuges during the day (Usinger, 1966). The present study investigated the relationship between time and the response of bed bugs to potential aggregation semiochemicals and identified temporal trends in behaviour. The attraction of bed bugs to the area directly above the volatile source and the turning rate was dependent on a high-order interaction between the presence of the volatiles, the sex of the bed bug and the time of day at which the bioassay was performed. For both males and females, there was a significant attraction to the pot containing the potential aggregation volatiles in the early scotophase. During the time period when the volatiles were more attractive there was also a significantly higher turning rate. Alterations in turning rate and direction are used in chemotaxis to orientate towards the source of attractive volatiles (Kennedy, 1978). Bed bugs that are orientating towards a source of volatiles may be using their antennae to detect small changes in chemical input to follow a concentration gradient to the source. This could result in an increased turning rate when volatiles are present compared with during controls.

In a field study of bed bug behaviour using a trap design that caught mainly unfed host-seeking bed bugs, host location activity was found to be concentrated in the late scotophase between 03:00 and 06:00 h (Mellanby, 1939a). Conversely, in laboratory-based studies, the activity peak was recorded to be in the mid-scotophase, just 4 h after the onset of the phase, followed by a slow decline in activity towards the end of the scotophase (Romero et al., 2010). Both studies indicate that during the early scotophase bed bugs remain in their refuges and are aggregated, which supports the results of the present study as during the early scotophase bed bugs were most attracted to putative aggregation cues. As for bed bugs, triatomine bugs (Triatominae: Reduviidae) also show temporal variation of certain activities and a daily rhythm of aggregation has been reported for *Triatoma infestans* (Minoli et al., 2007). The study identified the presence of a 24 h cycle with maximum aggregation at the end of the scotophase and increasing dispersal during the photophase (Minoli et al., 2007). Therefore there are differences in the timing of both aggregation and feeding behaviours between these two biologically similar families, Cimicidae and Triatominae.

The results of this study suggest that the most appropriate time period for future behavioural experiments with bed bugs and potential aggregation semiochemicals is during the early scotophase, 10:00–15:00 h, when attraction is high and less variable.

The effect of physiological factors

Bed bugs aggregate in refuges during the day in their non-feeding inactive state. Just as attraction to kairomones decreases following blood feeding (Aboul-Nasr and Erakey, 1968; Reinhardt and Siva-Jothy, 2007), it would be expected that responses to aggregation cues diminish when bed bugs should leave the aggregation in order to seek a host and obtain a blood meal. It has previously been noted that female bed bugs were less attracted to refuge volatiles, i.e. potential aggregation semiochemicals, during periods of starvation (Marx, 1955). In the present study, all sex/nutritional status combinations of bed bug (male and female, engorged and starved) were significantly attracted to the volatiles from bed bug-exposed papers. However, this result is not unexpected as bed bugs need to aggregate daily to protect themselves from the environment and detection. Therefore, provided testing is completed at the correct time of day, nutritional status should not have a significant impact on behavioural response. In a study where bed bugs preferentially aggregated under bed bug-exposed

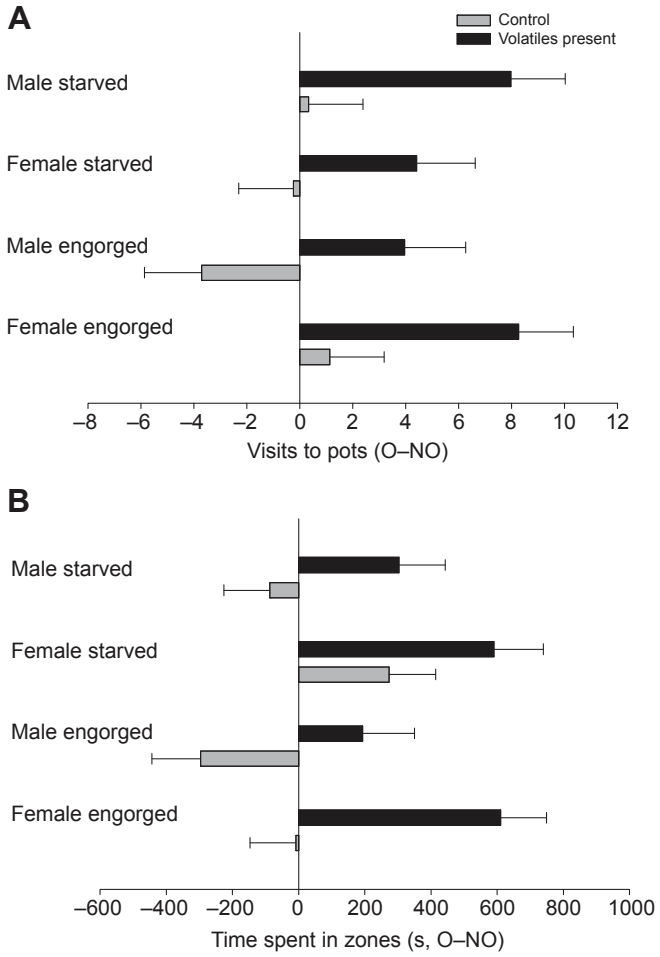

Fig. 7. Effect of volatiles from bed bug-exposed paper on male and female starved and engorged *Cimex lectularius* in the presence and absence of bed bug-exposed paper in Still-air olfactometer II. Bars represent the mean differences in (A) visits to pots (s.e.d. 3.009 visits) and (B) time spent in zones (in seconds, s.e.d. 203.2 s) between odour and no-odour pots and zones (O–NO). Values are means ± s.e.m. *F*-test: (A) $F_{1,67}$=0.17, P=0.683 and (B) $F_{1,67}$=0.35, P=0.554.

discs, the response was not affected by nutritional status (Olson et al., 2009). However, in general, aggregation decreased with increased time since the last blood meal (Olson et al., 2009). A recent study clarified that fed bed bugs aggregated for more of the scotophase than unfed bed bugs (Reis and Miller, 2011). However, all bed bugs, regardless of nutritional status, returned to the aggregation 2 h before the start of the photophase (Reis and Miller, 2011).

Differences in aggregation behaviour between the sexes have been recorded in other studies of bed bugs (Levinson and Bar Ilan, 1971; Siljander et al., 2007; Siljander et al., 2008; Pfiester et al., 2009; Weeks et al., 2011b). For example, one study found that females of *C. lectularius* were less likely to aggregate than males and nymphs, and that in populations of increasing male bias, females were less likely to be associated with the main aggregation (Pfiester et al., 2009). Differences between the response of males and females were recorded during the present study. For example, the response varied with sex of the bed bug and time, a result indicating that the timing of aggregation behaviour may vary between the sexes. The peak response for both sexes was in the early scotophase, but in males the peak was at 11:00 h whilst in females the peak response was recorded at 13:00 h. However, there was an overlap in time that implies that males and females of *C. lectularius* could be aggregating in synchrony, which would be beneficial for mating. Mating is essential throughout the reproductive life of a female bed bug to maintain fecundity, as egg production ceases when mating is prevented (Mellanby, 1939b; Davis, 1964; Usinger, 1966; Stutt and Siva-Jothy, 2001). Interestingly, at 19:00 h, i.e. the late scotophase, females showed significant attraction to the volatiles but the difference in turning rate was not significant, implying that a different type of behaviour was occurring compared with that recorded in the early scotophase, e.g. oviposition.

There was a trend for females to be consistently less responsive to the volatiles than males during the study. However, this was only significant when the data were analysed by subsets. The reason for a reduction in attraction of females to aggregation cues could be due to females being the dispersal stage of the population (Pfiester et al., 2008), but also the behaviour could be an avoidance tactic due to the costs, e.g. wounding and pathogen infection, associated with the high frequency of mating that would occur in a male-biased population. Mating in bed bugs has a survival cost to the female and males typically mate females at a higher rate than necessary for viable egg production (Stutt and Siva-Jothy, 2001). In this situation, females that had already mated should be less attracted to aggregation semiochemicals than newly moulted unmated females (Siljander et al., 2008). However, when the difference between the response of mated and unmated females to the volatiles from bed bug-exposed paper was investigated, all life stages of bed bug responded strongly to the volatiles. Variation in results when testing bed bugs of different physiological states for aggregation behaviour could depend on the stimuli presented. Several studies that have shown differences in attraction between the sexes and stages have included live bed bugs as or within the stimuli (Siljander et al., 2008; Pfiester et al., 2009). However, in those studies, including the current study, where live bed bugs were removed from the refuge-based volatiles, the physiological state of the insect appears to have less of an effect on the behavioural response (Olson et al., 2009). Several other studies have found bed bugs themselves to not attract conspecific bed bugs (Marx, 1955; Weeks et al., 2011b). The presence of volatiles from bed bugs themselves may affect the behaviour of the experimental insects due to the production of an alarm pheromone. Furthermore, the absence of volatiles produced directly by bed bugs may make the refuge equally attractive to bed bugs of both sexes, as females may be unable to determine the male composition and assess the mating cost they are likely to incur.

All bed bug life stages aggregate in refuges. Therefore, bed bugs of both sexes and all life stages should be able to locate a refuge through attraction to semiochemical cues that promote aggregation. Furthermore, bed bugs of different nutritional status should show little variation in attraction as regardless of when they last fed, bed bugs return to their refuges during the daylight hours. In the current study, bed bugs of both sexes and all stages responded to the volatiles from bed bug-exposed papers, showing significant attraction and orientation towards the volatile source whether they were starved or engorged. This is in concordance with a previous study that showed no effect of life stage or feeding on aggregation behaviour (Olson et al., 2009). The results of this study imply that the volatiles produced from bed bug-exposed papers are highly likely to act as aggregation cues.

Conclusions

The development of a still-air olfactometer for investigating bed bug behaviour has provided a robust assay for testing potential bed bug attractants and other semiochemicals. Furthermore,

modifications enabled the use of tracking software, i.e. EthoVision, which permitted recording under IR light and collection of a dataset with great potential for subsequent analysis. Using this behavioural bioassay, bed bugs responded significantly to the volatiles from bed bug-exposed papers, showing attraction and orientation towards the volatile source. There was no significant, independent effect of time, sex, mating status or nutritional status on this response. Bed bugs that were males, virgin females, mated females and nymphs, engorged and starved, all showed significant attraction to the volatiles from bed bug-exposed papers by visiting the odour pot more frequently and spending more time in the odour zone in the presence of the volatiles. The confirmation that these physiological factors are not affecting the response of bed bugs to the volatiles from bed bug-exposed papers provides evidence that the volatile profile contains aggregation cues, as semiochemicals that promote aggregation should by definition be detected by both sexes and all life stages. The identification of these volatiles now needs to be undertaken using advanced chromatographic and spectroscopic approaches, i.e. coupled gas chromatography-electroantennography and coupled gas chromotography-mass spectrometry so that compounds identified as physiologically active can be tested for attractiveness in olfactometer assays and then bed bug monitoring devices. An optimized bed bug monitor is urgently needed to improve the management of this pest, and a device baited with attractive semiochemicals could be used for both pre- and post-treatment surveillance to improve control efficacy as well as a means of control in itself through population suppression.

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