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IMPACT OF CONGENERS AND HARBOURAGE MATERIAL ON BED BUG RESPONSE TO A VOLATILE AGGREGATION PHEROMONE LURE

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Abstract Volatile lures are useful tools for the detection and monitoring of bed bug infestations. Developing such lures entails finding out, in the laboratory, compositions that maximize lure attractivity to bed bugs. A standard behavioural test consists in releasing a number of bed bugs in an arena and counting after a given amount of time how many have been captured by a lure versus a control harbourage or trap. In such a test, high variability in control and lure catches may be observed for various reasons (lack of oriented search behaviour under still air conditions, aggregation behaviour, variation of behaviour over time), which limits the statistical power and the capacity to observe differences in attractivity among lure compositions. We used video tracking to explore whether the analysis of bed bug trajectories could offer a more robust testing paradigm as well as whether bed bug's aggregation behaviour may influence the outcome of the test. We found that bed bugs explore with greater intensity areas close to the volatile lure, whether or not harbourages are present. In the presence of harbourages, bed bug behaviour once inside the harbourage was no longer dependant on lure presence or absence . The use of video tracking allowed to reliably evidence the attractivity effect of a volatile lure and can provide a platform for a deeper investigation of the interplay between chemical cues (volatile and non-volatile) and tactile cues (thigmotaxis) in mediating bed bug's search for harbourage.

Key words bed bugs, aggregation, pheromone, volatile lure, harbourages, video tracking

INTRODUCTION

Bed bug pheromone lures have been a part of the pest control's toolkit for more than a decade. With the increasing use of connected traps for the early detection of bed bug infestations, it becomes more and more important to understand what motivates a bed bug to visit a place. Bed bugs use aggregation pheromones to locate suitable harbourage sites, marked by the presence of their congeners, fecal spots and exuviae (Knudsen and Ignell, 2024). They also display a thigmotactic behaviour, where they come in physical contact with and actively explore any irregularity of the substrate or object encountered while walking. Gries et al. (2014) have shown that the aggregation pheromone is comprised of both volatile and non-volatile components. The distance attraction potential of volatile components makes them more interesting as lures for early infestation detection. However, the aggregation behaviour in itself may pose challenges to the study of bed bug responses to volatile signals and the development of efficient volatile lures.
A standard behavioural test consists in the release of bed bugs in a still air arena where one or more harbourages are offered and count, after a given amount of time, of how many are sitting inside lured versus control harbourages. Such tests must be performed on a large number of bed bugs in order to obtain sufficient statistical power to even have a chance to observe an attraction.

It is therefore common practice to test bed bugs in groups. However, the fact that bed bugs are inherently attracted to each other implies that the presence of congeners may bias the way they choose their harbourage. Furthermore, histamine, the non-volatile component of the aggregation pheromone, is extremely persistent in the environment (de Vries et al., 2018) and poses a particularly high risk of contamination of the behavioural test environment.
Here, video tracking was used to record the responses of bed bugs to a synthetic volatile pheromone lure in a still air olfactometer. We investigated how the presence of congeners and harbourage material influences this response.

MATERIAL AND METHODS

Bed bugs. We used adult male bed bugs (*Cimex lectularius*) of a London Field strain obtained from Cimex Store, UK. The bed bugs were reared on filter paper in containers closed with bed bug-proof netting, under ambient conditions of ~22°C, 50-77% relative humidity, on a 12:12 hours light-dark cycle (light on between 8:00 am and 8:00 pm). They were fed once a week on CPDA-preserved human blood. Trials took place during the bed bug's photophase. The males to be used were separated out from the rest of the colony ahead of time, fed 3 to 7 days before the trials. The were left to acclimate in the experimental room for at least 30 minutes, in a dark box, before the onset of each trial.

Still air olfactometer test. The trials were performed in round arenas 44 cm in diameter. Three holes, 3 cm diameter were cut into the arena floor. A container (volume 110 ml) was attached under each hole. One of the three containers was loaded with a synthetic volatile aggregation pheromone lure (Nattaro Scout®) while the other two were left empty and served as controls. The position of the lured and control containers was randomized between trials. The lure was kept in place 30 minutes prior to the start of each trial. This allowed a build up of volatiles in and above the container. The arena floor was covered with filter paper 40 cm in diameter, carefully attached with painter's tape to avoid bed bugs crawling under. The filter paper was punctured with about a hundred 0.5 mm diameter holes above each hole, which allowed diffusion of the volatile lure from the containers to the portion of arena right above. These unctured areas will hereafter be referred to as targets. A depiction of the resulting arena can be seen in Figure 1.a. In some trials, harbourage material was added to each target in the form of plastic crosses 6 mm high, centered on the target and topped with a transparent watch glass (5 cm diameter, flat, resulting arena depicted on Figure 1.b.). This height was sufficient to prevent bed bugs from climbing onto the watch glasses. The arena was kept in a dark room and only illuminated with IR light. At the beginning of each trial, either one or ten bed bugs were released at the center of the arena. Their activity was monitored for 30 minutes. After each trial, we exchanged the filter paper floor, lure containers, watch glasses and plastic crosses to prevent build up of volatile and non-volatile residues from the lures and the bed bugs which may influence bed bug behaviour.

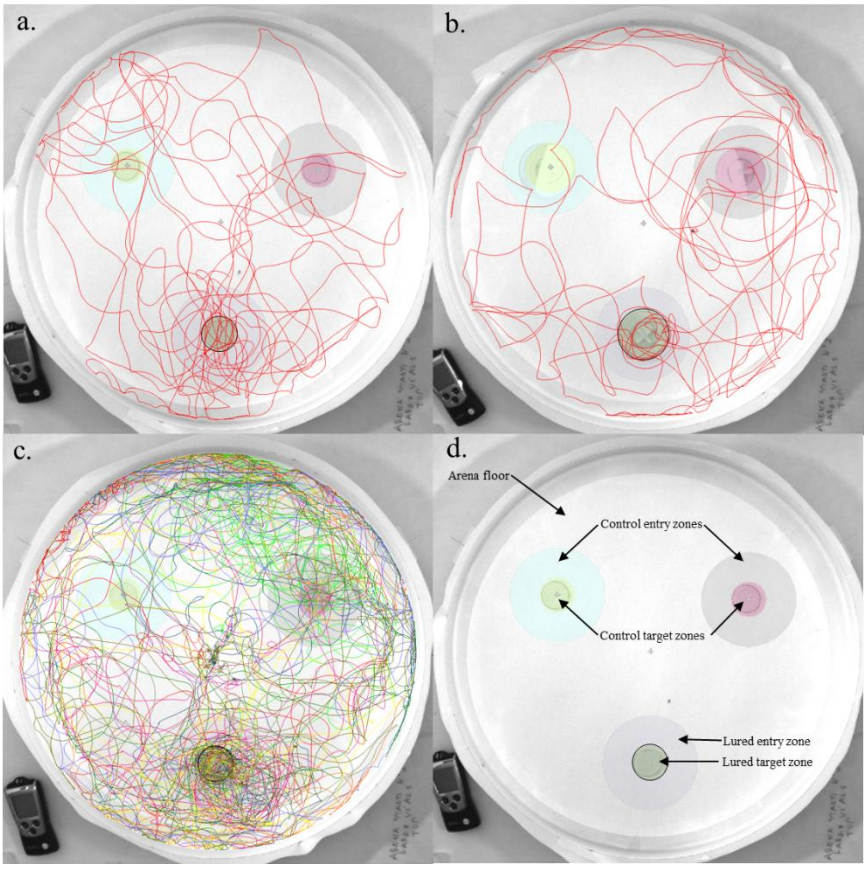

Figure 1. Images from the video feed showing arena settings, with overlayed examples bed bug tracks. a. Arena without harbourage, bed bug tested alone b. Arena with harbourage (6 mm high cross topped with a watch glass) and bed bug tested alone. c. Arena without harbourage and one group of 10 bed bugs tested together. d. Location of arena floor, entry zones and target zones.

The filter paper floor and lure containers were treated as consumable. Watch glasses and crosses were cleaned with soap and water at the end of each day and reused the next day. A fresh lure was used every day.

Video tracking and track analysis. A video feed of the arena was captured using an infrared camera (Basler). Bed bug activity was tracked live at a rate of 3.13 frames/s and processed using Ethovision XT17 (Noldus Information Technology). Individuals that climbed on the arena's edge or crawled under the filter paper were excluded from analysis. Tracks were manually corrected to ensure that every individual included in the analysis was detected for at least 1700s out of a total trial duration (1800s). Body swaps (exchange of identity between two individuals) were manually corrected when they generated artefactual movement at a speed exceeding the maximum observed for naturally moving bed bugs. The tracks were smoothed (Lowess method, 10-point averaging) to eliminate artefactual movement due to video noise. Movement parameters were calculated in different zones of the arena (Figure 1.d). Target zones were defined as the area above each container (diameter 3 cm when no harbourage was added, 5

cm when a harbourage was present on the target). Entry zones were defined as the area immediately surrounding each target zone (diameter 10.5 cm). The rest of the arena was designated as "arena floor". For each bed bug, we computed the time spent, the proportion of time spent moving, cumulated track length, mean velocity and mean angular velocity in each zone. The later three parameters were calculated only from the portions of tracks when the bed bug was moving (velocity threshold 0.2 cm/s).

Statistical analysis. all analyses were performed using R version 4.2.0.

Effect of being alone or in a group on response to the lure in the absence of harbourage material. We used analysis of variance to test the effect of trial type (bed bugs alone or in groups), zone (entry zones, targets, and arena floor) and their interaction on each parameter of interest. The data were log-transformed whenever doing so reduced deviation from the normal distribution. For parameters time spent in zone and cumulated track length, the analysis was done separately on arena floor, entry and target zones because their value depends on the surface area of the considered zone. Velocity and angular velocity were compared simultaneously across all zones. Posthoc Tuckey pairwise comparisons were used to compare individual zones with one another.

Effect of presence or absence of harbourage material on response to the lure by bed bugs tested alone. The analysis followed the same principle as above, except for the following differences. For parameters that depend on zone surface area, the factor "zone" was nested into "trial type" to account for the difference in entry and target zone surface area between the two trial types. The parameter proportion of time spent moving was analysed using a binomial generalized linear model, and the significance of factors trial type, zone and their interaction assessed using model simplification.

RESULTS AND DISCUSSION

The total distance covered by the bed bugs included in this study over the course of 30 minutes averaged about 13 m when no harbourages were present and 9.5 m when harbourages were provided. Bed bugs' average velocity was about 1 cm/s, and some of them showed maximum velocities beyond 2.5 cm/s. These numbers are consistent with empirical observations that bed bugs are highly capable of dispersing actively (e.g. Cooper et al., 2015). Figures 1.a-c show example tracks followed by bed bugs. The parameters of their trajectories and the statistical analysis results are summarized respectively in Tables 1 and 2.

Effect of being alone or in a group on response to lure in the absence of harbourage material. The amount of time spent in lured entry and target zones was significantly higher than in the corresponding control zones (Figure 2.a). The same was true of cumulated track length. Average velocity was significantly lower (Figure 3.a, Table 2.b) and average angular velocity higher in lured entry and target zones than in corresponding control zones. In other words, bed bugs spent significantly more time, moved longer distances at lower average velocity and along more convoluted tracks in lured target and entry zones than in the respective control zones. This indicates a more intense exploration of the area where the volatile lure is detected. Such an intensified exploration was reported before by Weeks et al. (2012). They, however, found an increase rather than decrease in average velocity near the volatile source. This discrepancy may relate to a different way of defining the zones of interest or the parameter average velocity (inclusion or exclusion of point below movement threshold), or to the use of a natural rather than synthetic aggregation pheromone.

Table 1. Summary of trajectory parameters of bed bugs tested alone or in groups of 10, in the presence or absence of harbourage. Different letters and bold type indicate, within each trial type and zone group, when the value for the lured zone differs significantly from at least one control zone (posthoc analysis using Tuckey pairwise comparisons, whenever then effect of zone was significant overall). Ns: non-significant differences.

Trial type	Zone group	Zone	Time in zone (s)		% time moving		Cumulated track length (cm)		Mean velocity (cm/s)		Mean angular velocity (deg/s)	
			mean	sd	mean	sd	mean	sd	mean	sd	mean	sd
1 bug no harbourage (n=17 trials)	Arena floor		1469	100 -	76	11 -	1036	272 -	0.91	0.14 -	16	2 -
	Entry	control 1	**80**	**46 ab**	89	21	**71**	**27 ab**	1.13	0.19	12	5
		control 2	**60**	**44 a**	90	17 ns	**59**	**31 a**	1.2	0.2 ns	9	2 ns
		lured	**129**	**63 b**	89	12	**111**	**59 b**	0.99	0.16	16	4
	Target	control 1	**13**	**10 a**	95	12	**13**	**7 a**	**1.2**	**0.28 a**	11	7 a
		control 2	**12**	**10 a**	95	17 ns	**11**	**7 a**	**1.14**	**0.17 a**	8	2 b
		lured	**46**	**28 b**	97	6	**38**	**21 b**	**0.88**	**0.14 b**	15	5 ab
10 bugs no harbourage (n=21 trials, 201 bugs)	Arena floor		1418	155	77	11	982	237	0.9	0.13	16	2
	Entry	control 1	**73**	**69 a**	89	18	**60**	**43 a**	**1.08**	**0.21 a**	13	**5 a**
		control 2	**79**	**81 a**	89	19 ns	**66**	**49 a**	**1.1**	**0.21 a**	12	**5 a**
		lured	**140**	**100 b**	88	16	**102**	**56 b**	**0.93**	**0.19 b**	15	**5 b**
	Target	control 1	**16**	**18 a**	95	15	**14**	**12 a**	**1.1**	**0.3 a**	**10**	**6 a**
		control 2	**21**	**28 a**	94	15 ns	**16**	**16 a**	**1.05**	**0.25 a**	**12**	**7 a**
		lured	**63**	**66 b**	90	17	**38**	**31 b**	**0.84**	**0.22 b**	**16**	**6 b**
1 bug harbourages (n=14 trials)	Arena floor		1223	394	75	14	761	326	0.82	0.19	14	4
	Entry	control 1	103	98	65	29	39	23	0.91	0.17	12	3
		control 2	79	73 ns	69	28 ns	36	30 ns	0.88	0.25 ns	13	4 ns
		lured	111	101	78	27	61	54	0.77	0.15	15	2
	Target	control 1	**57**	**72 ab**	59	36	**16**	**20 ab**	0.67	0.17	13	7 a
		control 2	**21**	**24 a**	78	26 ns	**8**	**5 a**	0.63	0.2 ns	14	6 b
		lured	**292**	**492 b**	61	40	**30**	**26 b**	0.65	0.26	16	5 ab

None of the trajectory parameters differed significantly between bed bugs tested alone and in groups, with the exception of average velocity (Table 2, Figures 1a and 2a). Indeed, bed bugs moved on average slower in the presence of congeners, regardless of their location in the arena (Figure 2.a.), which is most likely the result of them slowing down when they come in contact with one another (they can be seen doing so repeatedly on grouped trial videos). The difference in behaviour between lured and control zones was not influenced by the presence of congeners (interaction terms non significant, see Table 2). This is with the exception of a marginally significant interaction term for mean angular velocity, but the fact that angular velocity was not

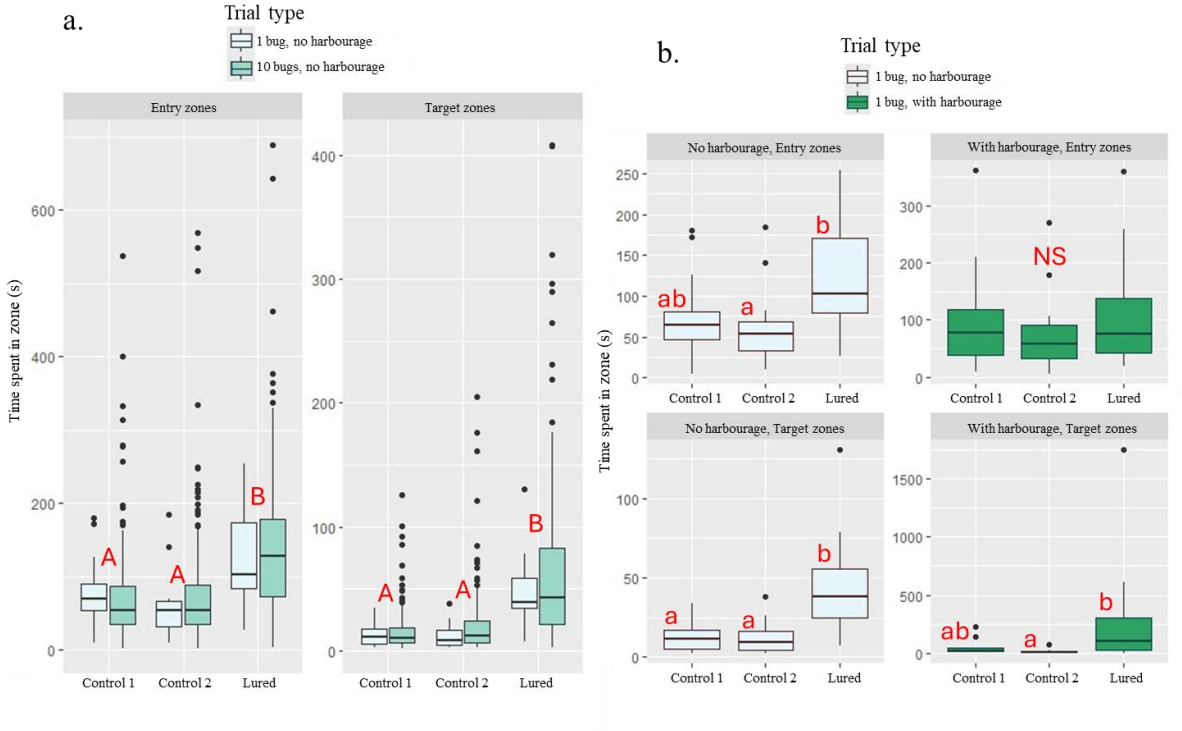

Figure 2. Boxplots of time spent in lured and control entry and target zones by bed bugs tested a. alone or in groups of 10 in the absence of harbourages b. alone in the presence or absence of harbourages. Different red letters indicate conditions that significantly differ from one another.

affected significantly by the presence of the lure in bugs tested alone may be the result of a lack of statistical power rather than a real pattern (see Table 1). We therefore find no evidence that the intensity of the lure-induced foraging of bed bugs was influenced by the presence of congeners in the absence of harbourage material.

Effect of presence or absence of harbourage material on response to lure by bed bugs tested alone. Bed bugs spent more time in the target zones when harbourages were provided. For instance, they spent an average of 5 min, resp. 45 s, in the lured target zone with and without harbourage (Table 1), a difference that is too large to be accounted for solely by the change in target zone's surface area. Significantly more time was still spent in lured than target zones. On the contrary, the amount of time spent in entry zones was no longer significantly different between lured and control entry zones when harbourages were provided (Figure 2.b). A

lack of statistical power may contribute to the difference between lured and control entry zones being harder to evidence with bed bugs tested alone (n=17 and 14 bed bugs respectively), but observation of Figure 2.b. suggests that in this case there is actually no pattern. The same was found for cumulated track length (Table 1). The addition of harbourages also impacted bed bug mean velocity in entry and target zones as well as the difference in velocity between lured and control zones (Table 2b, trial type and interaction term significant). Bed bugs generally moved slower in the presence than in the absence of harbourages.

Table 2. Significance of the effect of trial type, zone and their interaction on bed bug trajectory parameters. Bold type is significant differences. F values and p-values obtained by analysis of variance, except for % of time moving (generalized linear model with binomial distribution).

a. Parameters depending on zone surface area

Comparison	zone group	effect	Time in zone (s)		Cumulated track length (cm)	
			F	p-value	F	p-value
1 vs 10 bed bugs (no harbourage)	arena	trial type	1.8	0.18	0.8	0.37
	entry zones	trial type	0.36	0.55	0.42	0.52
		zone	**44**	**$<2.10^{-16}$**	**48**	**$<2.10^{-16}$**
		interaction	0.41	0.67	0.6	0.55
	target zones	trial type	0.9	0.34	0.02	0.89
		zone	**112**	**$<2.10^{-16}$**	**95**	**$<2.10^{-16}$**
		interaction	0.46	0.64	0.55	0.39
no harbourage vs harbourage (1 bed bug)	arena	trial type	2.5	0.13	0.37	0.55
	entry zones	trial type/zone	**3**	**0.022**	**1.7**	**0.014**
	target zones	trial type/zone	**9.3**	**$4.26.10^{-6}$**	**8.6**	**$1.1.10^{-5}$**

b. Parameters independant of zone surface area

Comparison	effect	% time moving	Mean velocity (cm/s)		Mean angular velocity (deg/s)	
		p-value	F	p-value	F	p-value
1 vs 10 bed bugs (no harbourage)	trial type	not tested	**7.9**	**$4.9.10^{-3}$**	1.4	0.25
	zone		**55**	**$<2.10^{-16}$**	**20**	**$<2.10^{-16}$**
	interaction		0.3	0.94	**2.2**	**0.04**
no harbourage vs harbourage (1 bed bug)	trial type	**$4.5.10^{-4}$**	**96**	**$<2.10^{-16}$**	2.8	0.094
	zone	0.9	**7.6**	**$2.5.10^{-7}$**	**3.9**	**0.001**
	interaction	0.34	**4.4**	**$3.1.10^{-4}$**	0.88	0.51

The mean velocity they adopted no longer differed between lured and control zones (Figure 3.b.). Bed bugs spent a significantly lesser proportion of their time moving in the entry and target zones with than without harbourages (respectively 59-78% and 89-97%). This proportion was not influenced significantly by presence or absence of a lure. Average angular velocity was not impacted by the presence of harbourages (Table 1).

In summary, the addition of harbourages did not change the tendency of bed bugs to explore lured areas with a greater intensity. They spent more time and moved longer distances near the lure, but this enhanced activity was more focused on the target zone, which may be due to either the target zone being wider, or to a thigmotactic response to the harbourage material.

The fact that bed bugs moved slower and spent a greater proportion of their time immobile near or under harbourages than when the same areas were harbourage-free further shows that they did respond to the harbourages themselves. It seems although they did spend more time in the lured harbourages, once any harbourage found, their behaviour was primarily influenced by contact with the harbourage itself, regardless of the presence or absence of volatile signals. Indeed, the difference in total time spent in lured versus control target zones does not seem greater in the presence than in the absence of a harbourage. Besides, the fact that bed bugs moved on average slower on the arena floor than in control entry and target zones in the absence of harbourages (Figure 3) further indicates that thigmotactism (in this case towards the outer wall of the arena) does have a strong influence on their behaviour.

Test protocol optimization. The results presented above show that video tracking of bed bugs in a flat arena in the absence of harbourages provides a suitable platform for the documentation of bed bug responses to volatile lures. Because their response to pheromone components is not affected by the presence of congeners (similar results with one and 10 bed bugs in this study), bed bugs may be tested in groups in such a setting, in order to maximize data collection. The addition of harbourage material may not be more informative when the focus is the response to the volatile cues and may pose practical challenges to video tracking (bed bug detection may be hindered by reflections on watch glasses or by sitting against a cross or each other). In a still air setting, arena dimensions and air movements may influence odour spatial distribution or the likelihood that bed bugs interact with the arena's edge (Weeks et al, 2012). Such factors therefore deserve close attention. Finally, bed bugs will deposit volatile and non-volatile residues in the arena, that may build up and bias the behaviour of their congeners if the same material is recycled from one test to the next. Attention should be paid to decontamination protocols. We solved this by having as many disposable elements as possible.

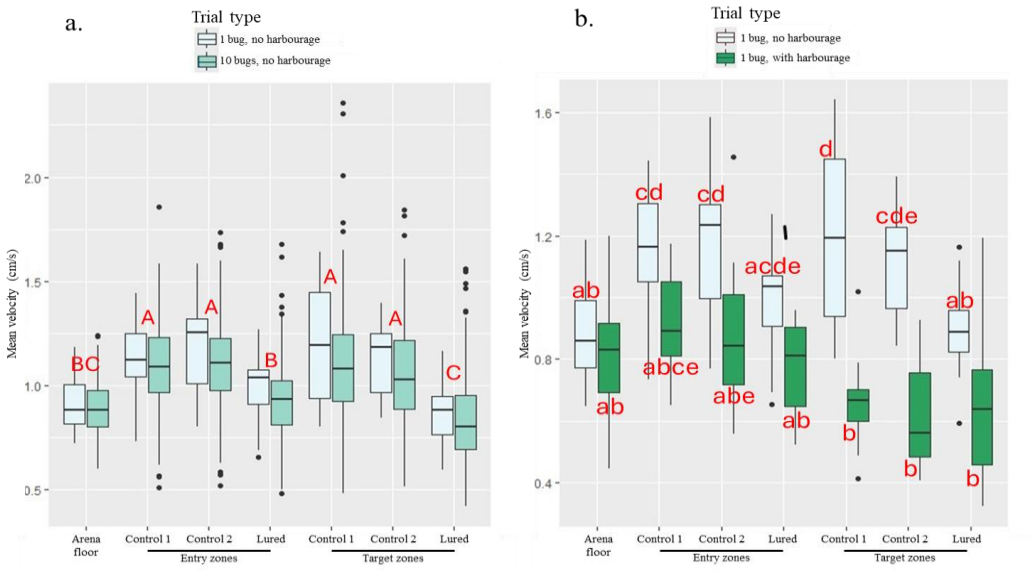

Figure 3. Boxplots of mean velocity of bed bugs in different zones, when tested a. alone or in groups of 10 in the absence of harbourages b. alone in the presence or absence of harbourages. Different red letters indicate

CONCLUSION

In the absence of harbourage material, the presence of congeners did not affect male bed bug response to a volatile aggregation pheromone lure, an observation consistent with a gregarious rather than social behaviour. When harbourages were added, although bed bugs did interact more with the lured than control harbourages, it seemed like the thigmotactic response took precedence over the volatile lure, and the presence of the harbourage did not enhance the contrast between activity in the lured versus control areas. How the presence of congeners may interfere with the volatile lure's impact on harbourage selection should be evaluated as well. Sex and feeding status have been shown to influence bed bug responses to volatile aggregation cues (Weeks et al., 2012) and the conclusions above may not fully apply to females. More generally, video tracking offers an interesting platform to gain a deeper insight into how the volatile, non-volatile and thigmotactic aspects of interaction between bed bugs and their harbourages interplay.

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