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Discrimination between lineage-specific shelters by bat- and human-associated bed bugs does not constitute a stable reproductive barrier

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Abstract The common bed bug *Cimex lectularius*, has been recently shown to constitute two host races, which are likely in the course of incipient speciation. The human-associated lineage splits from the ancestral bat-associated species deep in the history of modern humans, likely even prior to the Neolithic Period and establishment of the first permanent human settlements. Hybridization experiments between these two lineages show that post-mating reproductive barriers are incomplete due to local variation. As mating takes place in off-host refugia marked by aggregation semiochemicals, the present investigation tested the hypothesis that bed bugs use these semiochemicals to differentiate between refugia marked by bat- and human-associated bed bugs; this would constitute a pre-copulation isolation mechanism. The preference for lineage-specific odors was tested using artificial shelters conditioned by a group of either male or female bed bugs. Adult males were assayed individually in four-choice assays that included two clean unconditioned control shelters. In most assays, bed bugs preferred to rest in conditioned shelters, with no apparent fidelity to shelters conditioned by their specific lineage. However, 51 % of the bat-associated males preferred unconditioned shelters over female-conditioned shelters of either lineage. Thus, bed bugs show no preferences

for lineage-specific shelters, strongly suggesting that semiochemicals associated with shelters alone do not function in reproductive isolation.

Keywords Pre-copulation reproduction isolation · Pheromones · Parasites · Aggregation behavior

Introduction

The bed bug (*Cimex lectularius*) was recently shown to constitute two distinct host lineages (Balvín et al. 2012) which are genetically isolated (Booth et al. 2015), a bat-associated (BA) lineage and a human-associated (HA) lineage. Evidence suggests that ancestral BA *C. lectularius* populations gave rise to HA populations (Horváth 1913) which have subsequently differentiated in association with humans. This host-associated differentiation appears to represent a unique example of incipient, and possibly even sympatric, speciation. Balvin et al. (2012) showed considerable morphological differentiation between these two lineages, mostly in sensory and locomotory structures. Divergent morphology likely represents adaptations to the native host and may thus result in lower fitness on non-native hosts (Wawrocka and Bartonička 2013). Extensive use of insecticides within homes since the introduction of DDT has imposed extremely strong selective pressure, leading to the evolution of various mechanisms of insecticide resistance (e.g., Zhu et al. 2010; Mamidala et al. 2011; Adelman et al. 2011). The uneven distribution of different resistance-associated haplotypes across the planet (Zhu et al. 2010; Booth et al. 2015) suggests that bed bug populations are differentially selected and that populations are not well connected. Moreover, the prominence of *kdr* mutations (a target site resistance mechanism to DDT and pyrethroid insecticides) in HA bed bugs in Europe and N. America (Zhu et al. 2010; Booth et al. 2015), and their complete

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absence in BA *C. lectularius* in Europe supports the notion of differential selection and low gene flow between these two lineages (Booth et al. 2015).

Molecular dating of the split of the BA and HA lineages suggests that restrictions in gene flow between the lineages developed deep in bed bug history, tens or hundreds thousand years ago most likely predating the Neolithic revolution (Balvín et al. 2012). Before humans settled in permanent dwellings, they were unlikely to supply the bed bug a favorable and stable environment. Prior to the Neolithic Period, bed bugs likely parasitized humans only in stable cave dwellings, where the switch from bats to humans most likely took place (Povolný and Usinger 1966) and where humans and bats likely coexisted. It is therefore plausible that reproductive isolation barriers between BA and HA *C. lectularius* might have arisen in the common cave environment, and a HA lineage accompanied humans out of caves.

Changes in construction design over the last several centuries have favored synanthropic associations of several bat species in Europe (Horáček 1983) and ecological re-association of BA and HA bed bugs. Thus, in Central Europe, the BA and HA lineages are known to occur in sympatry in human dwellings (Balvín et al. 2012). However, population genetic analysis with both nuclear and mitochondrial genes indicates no significant contemporary gene flow between HA and BA populations, suggesting the existence of ecological or reproduction barriers (Booth et al. 2015). Indeed, Wawrocka et al. (2015) showed post-copulation incompatibility between bed bugs from the two lineages, resulting in their inability to produce hybrids. Recently, however, we showed that hybrids do occur when different combinations of strains are used (O. Balvín, T. Bartonicka, unpublished), and crosses of BA and HA bed bugs, maintained in the lab on a common artificial feeding system, produce viable offspring (DeVries, unpublished). Nevertheless, given the present day differentiation of sympatric HA and BA bed bugs, selection against hybrids is expected to occur and may result in reinforcement and development of pre-mating isolation mechanisms (Servedio and Noor 2003).

Pre-mating isolation barriers are often based on mate recognition, frequently mediated by sex or aggregation pheromones (e.g., Smadja and Butlin 2009). Such barriers are commonly identified between sibling species (e.g., (Bournez et al. 2015). However, several cases of intraspecific differentiation of response to semiochemicals have been reported, mainly for sympatric host races of plant-feeding insects (e.g., (Thomas et al. 2005; Dumas et al. 2015). Intraspecific differentiation of pheromones can also evolve in allopatry, i.e., in association with genetic differentiation (Olivero et al. 2015; Lecocq et al. 2015).

When not feeding on their hosts, bed bugs aggregate in specific dark shelters, where blood meals are processed and males locate females for mating (Usinger 1966). The shelters are marked by aggregation pheromones, which serve to attract bed bug from some distance and arrest their movements within the harborage (Levinson and Bar 1971). Differentiation of their aggregation pheromones would thus serve as an effective pre-mating isolation barrier between the two host lineages. Therefore, the present paper aims to determine the role of aggregation pheromones in preventing gene flow between the BA and HA lineages of *C. lectularius*.

Material and methods

Populations and rearing

We used four bed bug strains collected in the Czech Republic in 2014. Two HA strains were collected from human residences: Beroun town, central Bohemia (HA-B), and Čestmírova street, Prague (HA-C). BA strains were collected in a roost of *Myotis myotis* in Úštěk town, northern Bohemia (BA-U), and a roost of *Myotis emarginatus* in Prudká village, south Moravia (BA-P).

In order to assess independence of collections, we examined the sequence of cytochrome oxidase subunit I, following the protocol of Booth et al. (2015) and using four individuals per collection. Individuals within the collections always exhibited the same haplotype, but haplotypes were unique for each collection. Only the HA-C strain was heteroplasmic, but it consistently showed a combination of the same two haplotypes. Since heteroplasmy is usually stable within local populations (Robison et al. 2015), the HA-C collection could be regarded independent as well.

The insects were reared in 50-ml falcon tubes with the bottom replaced by a screen with 0.5-mm mesh size, at 27 °C, 40–50 % RH on a 12:12 light:dark cycle. They were fed weekly during the scotophase on human blood collected in citrate phosphate dextrose adenine (CPDA, purchased from the Faculty Hospital Královské Vinohrady, Prague). The construction of the artificial feeding system followed Aak and Rukke (2014). Insects from at least the fourth generation since collection in the field were used in the assays.

Assay design

The study used a method of conditioning harborages which has been previously used in many studies (Levinson and Bar 1971; Siljander et al. 2007; Olson et al. 2008; Olson et al. 2014). Briefly, dark matte paper (2×4.8 cm; 180 g/cm^2) was formed into a tent, as shown in Fig. 1, and placed in a 4-cm glass Petri dish. Immediately after feeding, either 10 adult females or 10 adult males were introduced into the Petri dish, with the shelter being the only surface suitable for walking and resting in the photophase. After 7 days, the bugs were removed, and the shelter was used in an assay within 2 h.
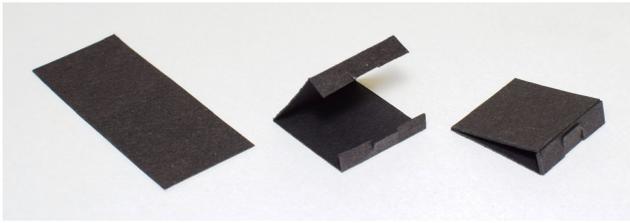
Fig. 1 Construction of the shelter. Photo by O. Balvín

Eggs were carefully removed from the female-conditioned shelters.

The experimental individual tested in the arena was from a different strain than the group that conditioned the shelters. This way, we made sure we tested the preference for a host lineage, not just preference for the same bed bug colony. At each occasion, a randomized complete block design was used including experimental males from all four strains. The strain for the non-specific shelter was randomly chosen. Although the light intensity across the arenas was equal, the position of the lineage-specific tent was random with respect to orientation of the test room.

The assays were conducted in 10-cm glass Petri dishes with paper lining the bottom in order to allow bed bugs to walk between shelters. The tests were performed in identical climatic and light conditions as the rearing. Each Petri dish contained two conditioned and two unconditioned shelters (control). Adult males were used as experimental animals, as they, unlike females, were shown to respond consistently to aggregation chemicals (Siljander et al. 2008) and, regarding the research question of this paper, represent those searching for mate. The experimental males were fed 1 week prior to the test. A male was introduced in the center of the arena 4 h before the onset of photophase, and its final position was recorded 2 h after the onset of photophase. Each test male was used only once in a single assay.

Statistical analyses

The preference for the conditioned shelter, or the host lineage-specific shelter, or differences in response between strains were tested by Chi-square analysis using Statistica 8.0 (StatSoft 2007). In statistical tests, we compared (a) conditioned (both populations combined = two tents) vs. unconditioned (=two tents) or (b) one population (=one tent) vs. another population (=one tent).

Results

The results of individual choice assays are summarized in Table 1 for each strain and depicted in Fig. 2 for each host lineage. In seven assays, the male was found outside any shelter at the end of the assay; these trials were excluded.

There was a clear preference by most bed bugs for bug-conditioned shelters ($p \leq 0.002$). However, BA-P males tested with both male- and female-conditioned shelters and BA-U males tested with male-conditioned shelters chose the conditioned and unconditioned shelters equally.

In six of the eight treatments, there was no evidence of host lineage-specific preferences (Table 1). In one treatment, however, BA-P males chose the female BA-conditioned shelter significantly more than the female HA-conditioned shelter ($p = 0.013$), and in another, the choice of HA-C males of the female-conditioned HA shelter over the female BA-conditioned shelter was nearly significant ($p = 0.061$).

Discussion

In the present paper, we investigated the fidelity of bed bugs to semiochemicals produced by lineage-specific aggregations. We tested the hypothesis that bat- and human-associated populations of *C. lectularius*, which are significantly genetically differentiated, may use aggregation pheromones as pre-mating reproductive isolation mechanisms. As a necessary first step, bed bug preference was tested in two-choice assays for bed bug-conditioned vs. clean unconditioned shelters. In choice assays, HA adult males significantly preferred either male- or female-conditioned shelters over unconditioned shelters. BA males also preferred male-conditioned shelters over unconditioned shelters, but they showed no preference for female-conditioned over unconditioned shelters. The response of BA males is consistent with the result of Siljander et al. (2007) who showed male aggregation responses to male-exposed paper discs, but not to female-conditioned discs. In contrast to Siljander et al. (2007), however, our results with HA males show that they respond to female-conditioned shelters of both lineages. These disparate results may be attributed to different designs and methods; Siljander et al. (2007) used a still-air olfactometer, whereas we used a sheltering assay. Moreover, Siljander et al. (2007) documented a loss of the differential responses by juveniles and females when the paper discs were inaccessible to them, suggesting that contact with the discs was required for sustaining the behavioral response. Our assays integrated both volatile and contact signaling systems in a small arena-based sheltering assay and showed that shelters marked by mated females attracted and arrested males within the shelters. Our results with adult males of the HA lineage thus do not support the idea of a sex-specific deposition of aggregation pheromone, in agreement with earlier studies (Levinson and Bar Ilan 1971). Nevertheless, it is important to note that both early studies (e.g., Levinson and Bar Ilan 1971) and some recent studies (e.g., Olson et al. 2014) either did not report the stages
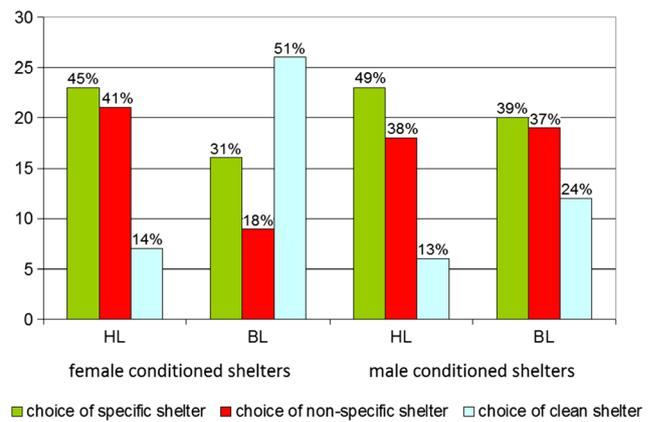
Fig. 2 Result of tests of preference of lineage-specific shelters. *Bars* represent numbers of males with a particular choice; respective percentage is given above each

(juvenile, male, female) or the status (mated vs. virgin, fed vs. starved) of bed bugs that conditioned shelters or combined stages and sexes, so these disparities may be related to biological effects as well.

Our main motivation for this investigation was the question whether *C. lectularius* exhibits lineage-specific shelter preferences. Contrary to our expectation, bed bugs did not prefer shelters marked by their lineage vs. the other lineage; that is, overall, bat-associated and human-associated bed bugs made no significant differentiation between shelters conditioned by their own or the other lineage. Nevertheless, it is relevant to point out that in four of the eight treatments bed bugs choosing lineage-specific shelters outnumbered those choosing non-specific shelters by ~2:1 (average 2.8:1). In the remaining four treatments, however, this ratio was 0.68:1, suggesting a preference for the non-specific shelters. While it is likely that more trials will reveal significant differences in aggregation responses of the two lineages, or by some strains and not others, we emphasize that overall we found no clear behavioral differentiation. Therefore, it is very likely that with so many bed bugs choosing non-lineage-specific shelters, shelter fidelity does not function as a reproductive barrier between these two host-associated lineages.

While pre-mating barriers appear to be minimal or none between these two host-associated lineages, the existence of mating and post-mating barriers remains unsettled. Usinger (1966) observed no reduction in fertility of cross-lineage matings between HA and BA bed bugs. Wawrocka et al. (2015), on the other hand, obtained no offspring from reciprocal crosses between two HA strains and two BA strains. However, DeVries et al. (in prep.) showed compatibility among several HA and BA bed bug populations. It is possible that some strains have differentiated more than others and/or that maintenance in the lab for multiple generations under identical conditions homogenized the differences that separated freshly collected bed bugs of the two lineages.

Table 1 Results of assays of preference of adult male bed bugs of lineage-specific conditioned shelters and control unconditioned shelters. Italicized values are significantly different preferences

Strain of test males		Human-associated (HA) test males						Bat-associated (BA) test males					
		Female-conditioned shelters			Male-conditioned shelters			Female-conditioned shelters			Male-conditioned shelters		
		HA-B	HA-C	Both	HA-B	HA-C	Both	BA-P	BA-U	Both	BA-P	BA-U	Both
No. of assay lineage-specific shelter chosen		7	16	23 (45 %)	12	11	23 (49 %)	11	5	16 (31 %)	5	15	20 (39 %)
No. of assay lineage non-specific shelter chosen		13	8	21 (41 %)	12	6	18 (38 %)	2	7	9 (18 %)	11	8	19 (37 %)
No. of assays unconditioned shelters chosen		4	3	7 (14 %)	2	4	6 (13 %)	14	12	26 (51 %)	8	4	12 (24 %)
Total no. assays, N		24	27	51	26	21	47	27	24	51	24	27	51
Preference for conditioned shelters	χ^2	*10.667*	*16.333*	*26.843*	*18.615*	*8.048*	*26.064*	0.037	0.000	0.020	2.667	*13.370*	*14.294*
	p	*0.001*	*<0.001*	*<0.001*	*<0.001*	*0.005*	*<0.001*	0.847	1.000	0.889	0.102	*<0.001*	*<0.001*
Preference for lineage-specific shelter	χ^2	1.800	2.667	0.091	0.000	1.471	0.610	*6.231*	0.333	1.960	2.250	2.130	0.026
	p	0.180	0.061	0.763	1.000	0.225	0.435	*0.013*	0.564	0.162	0.134	0.144	0.873

The most common cause of local incompatibility that could be eliminated through homogenization is the *Wolbachia* bacterium (Werren 1997). Preliminary results suggest that HA and BA bed bugs are in a stable mutualistic relationship with the same *Wolbachia* strain and so far no differences have been found even in the variable *Wolbachia* surface protein sequence (O. Balvin, unpublished). Nevertheless, the genetic divergence of HA and BA lineages is deep and genetic analysis indicates no contemporary gene flow between HA and BA populations (Booth et al. 2015). It remains possible that (a) there is an undocumented divergence between the *Wolbachia* populations infecting HA or BA or (b) that culturing bed bugs in the lab homogenizes their *Wolbachia* symbionts, obscuring in situ differences.

Aggregation pheromones of bed bugs are associated with exuviae and feces (Usinger 1966; Gries et al. 2015; Choe and Campbell 2014). It is possible that the lack of lineage differentiation in our assays was because populations of both host lineages were reared in the lab on a uniform diet. It is possible that if the bat-associated strains were fed on bat blood and the human-associated strains on human blood, the marked shelters might have been more differentiated based on fecal metabolites. Differences in fecal metabolites may be related to differences in the processing of bat and human blood, connected with their biochemical differences (e.g., Condo et al. 1989) or differences in gut microbiomes that decompose organic material. Since some of the aggregation pheromone components of bed bugs appear to be of bacterial origin (Gries et al. 2015), gut microbiomes could differentiate shelters marked by BA and HA bed bugs. Indeed, recent research demonstrated that the aggregation pheromone of the German cockroach is influenced by gut bacteria (Wada-Katsumata et al. 2015), suggesting that local differentiation in shelter preferences could be due to local differences in environmental microbes that colonize the gut. We also could not dismiss the idea that other bed bug semiochemicals might be affected by the host blood. For example, diet has been shown to influence the composition of cuticular hydrocarbons (Jennings et al. 2014; Etges and de Oliveira 2014) that often serve as aggregation cues. It is important to note, however, that contact between BA and HA bed bugs would most likely occur after bats vacate a home-associated roost, causing BA bed bugs to leave their shelter and feed on humans. In this case, the diet-related differences between the two lineages would rapidly disappear.

In conclusion, the present study has shown a large variation in aggregation responses between human- and bat-associated *C. lectularius*. Overall, the results show strong preferences of adult males for bed bug-conditioned shelters over clean unconditioned shelters. Although some strains showed strong lineage fidelity, other strains did not. These results strongly suggest that aggregation pheromones do not serve as reproduction barriers between these two host-associated lineages.

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