



## The social consequences of sexual conflict in bed bugs: social networks and sexual attraction

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Sexual conflict is ubiquitous across the animal kingdom and often involves costly sexual harassment of females by males. An overlooked outcome of sexual conflict is its potential impact on social behaviour. Due to their seemingly harmful mode of copulation (traumatic insemination) and tendency to form aggregations, bed bugs are an ideal model for studying the social implications of sexual conflict. Repeated traumatic inseminations are known to reduce some aspects of female fitness, so we expected the benefits to males and the high costs for females of frequent mating to result in divergent social preferences between the sexes. To examine the impact of sexual harassment on social structure, we devised a novel experimental arena with either two shelters or 12 shelters and continuously tracked sexual and social interactions between individually marked bed bugs over 6 days. By constructing aggregation networks, we examined whether female bed bugs occupied more peripheral network positions compared to males as well as whether females preferentially associated with other females as a strategy to reap the benefits of group living while mitigating the costs of unsolicited sexual attention. We found no evidence that females shape their social environment to evade associating with males. However, when tested individually in a follow-up experiment, mated females showed a strong preference for social cues from females over social cues from males. Our results therefore suggest that males and females may be in conflict over the composition of social associations and highlight the importance of both examining behaviour at the individual level and tracking larger groups of freely interacting populations in more complex environments.

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The past two decades have seen a gradual shift in our perception of animal social behaviour with the growing appreciation that many species traditionally considered solitary possess complex social lives. Individuals of the apparently solitary species clearly do not live in integrated social groups such as social hymenopterans (Kapheim et al., 2015; Michener, 1974; Seeley, 2010; Wilson, 1971), social mammals (Cheney & Seyfarth, 2008; Clutton-Brock, 2016; Sherman et al., 1991) and cooperatively breeding birds (Brown, 1987; Koenig & Dickinson, 2004). Nevertheless, numerous 'solitary' species have parental care that involves an extended period of life within a group, aggregation pheromones that bring together dispersed individuals, food sharing and communal antipredator behaviours modulated via alarm pheromones (Caro, 1994; Costa, 2006; Elbroch et al., 2017; Prokopy & Roitberg, 2001; Wertheim et al., 2005).

While there are clear advantages to life in groups, the close proximity to other individuals also increases the opportunity for a variety of antagonistic interactions. One source of such tension is sexual conflict, which occurs when the reproductive interests of the two sexes are at odds with one another (Chapman, 2006; Parker, 1979). This conflict is pervasive among sexually reproducing animals and often results in sex-specific behaviours and adaptations that provide benefits to one sex at the detriment of the opposing sex (Chapman et al., 2003). A common manifestation of sexual conflict is sexual harassment, where males pursue females through coercive tactics to gain access to reproductive opportunities (Parker & Clutton-Brock, 1995). Well-documented costs of sexual harassment to females include physical injury (Baniel et al., 2017), reduced foraging efficiency (Pilastro et al., 2003; Stone, 1995) and increased predation rates (Arnqvist, 1989). All these costs can decrease female fitness (den Hollander & Gwynne, 2009; Dukas & Jongsma, 2012; Sakurai & Kasuya, 2008). Consequently, females of many species have evolved physiological, morphological and behavioural strategies for evading harmful male pursuit (Brennan

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et al., 2007; Crudgington & Siva-Jothy, 2000; Lessells, 2006; Morrow & Arnqvist, 2003; Siva-Jothy et al., 2019).

Most studies on social behaviour do not consider sexual conflict, and much of the research on sexual conflict does not address its ramifications for the evolutionary biology of social behaviour. There are, however, tight interactions between the two disciplines because living in groups increases the opportunities for antagonistic interactions between males and females, and such sexual conflict can reduce the benefits that females incur from living in groups. Indeed, a few studies indicate that male harassment causes females to engage in social avoidance. For example, in response to sexual harassment, female water striders (*Aquarius remigis*) reduce their activity in the centre of experimental pools where large numbers of males are found and spend most of their time on the edge of pools and out of water (Krupa & Sih, 1993). Likewise, in the Trinidadian guppy, *Poecilia reticulata*, exposure to male harassment drives females to select lower-quality habitats, leading to segregation of the sexes (Darden & Croft, 2008), and results in females forming more disparate social networks (Darden et al., 2009). Other female avoidance strategies include altering social distance from conspecifics (Dadda, 2015) and forming strategic alliances with other females or dominant males to shield themselves from unwanted male attention (Fox, 2002; Martens & Rehfeldt, 1989). Overall, these behavioural responses to harassment have the potential to critically influence social dynamics and the structure and composition of social groups.

The studies just noted suggest that the interdependent dynamics of social behaviour and sexual conflict deserves further investigation. To this end, we used common bed bugs, *Cimex lectularius*, a species often cited as an extreme model of sexual conflict as they have obligate traumatic insemination. During traumatic insemination, males use their needle-like copulatory organ to pierce through females' abdomens and deposit sperm directly into the body cavity (Carayon, 1966). Although traumatic insemination is relatively rare, it has evolved independently several times within invertebrates. Its benefits to males may be related to sperm competition (Lange et al., 2013; Tatarnic et al., 2014). In bed bugs, repeated traumatic inseminations have been shown to reduce female longevity and lifetime reproductive output, likely due to the energetic costs of wound healing and increased frequency of infection (Stutt & Siva-Jothy, 2001).

Bed bugs show moderate social behaviour. In natural infestations, they are typically found in mixed-sex aggregations within protective crevasses (Johnson, 1941; Reinhardt & Siva-Jothy, 2007). Their social attraction is driven by volatile and nonvolatile chemicals as well as tactile cues (Gries et al., 2015; Reinhardt & Siva-Jothy, 2007; Siljander et al., 2007, 2008). Finally, bed bugs emit an alarm pheromone in response to cues of danger, and this leads nearby bed bugs to disperse (Levinson et al., 1974). The social and sexual features of bed bugs provide us with a unique opportunity to study how the presence of intense sexual conflict and harassment differentially affect the social tendencies of the two sexes, and how these differences are reflected at the population level.

To track the social and sexual dynamics of bed bugs, we developed a novel naturalistic arena, which allowed us to continuously observe populations of freely interacting bed bugs over several days. We experimentally manipulated the intensity of sexual conflict by providing the bed bugs with either two shelters or 12 shelters. We expected females to experience higher rates of sexual harassment and traumatic insemination when given two shelters rather than 12 shelters due to the limited opportunities for avoiding males. Furthermore, we predicted that in the 12-shelter treatment, females would take advantage of the large number of shelters to adopt male avoidance strategies. To detect patterns of female social

avoidance, we used social network analysis, a powerful toolkit of statistical and graphical techniques used to analyse and visualize social relationships (Croft et al., 2008; Webber & Vander, 2019; Whitehead, 2008), to create networks based on how often we observed individuals in the same aggregation. First, we predicted that females would evade unwanted sexual advances from males by occupying less central network positions in these aggregation networks and by exhibiting lower levels of sociality overall, as quantified by their network strength. Second, we predicted that the bed bugs would show phenotypic assortment by sex, because preferentially associating with females would allow females to gain the benefits of aggregation without enduring the increased costs of harassment and traumatic insemination by males.

Bed bugs showed weaker tendencies than expected to form aggregations when provided with many shelters, and no evidence for social avoidance by females. Hence, we conducted a follow-up experiment to critically test bed bugs' specific social attraction to and avoidance of conspecifics of distinct sex and mating status. We allowed each focal bed bug to choose between two shelters that varied in their occupation history. First, as a baseline, we verified that both males and females would strongly prefer shelters previously occupied by females over shelters that had never harboured bed bugs. Second, we expected that previously mated males and females would prefer shelters formerly occupied by females over shelters previously occupied by males. This is because males should be highly attuned to cues that indicate potential mating opportunities, while females should avoid males owing to costly harassment and traumatic insemination. Finally, we predicted that males would prefer shelters previously occupied by virgin females over shelters formerly harbouring mated females. This could be owing to either mated females suppressing the emission of aggregation cues as a social avoidance strategy, or males' acute sensitivity and preference for virgin over mated females.

## METHODS

### *Ethical Note*

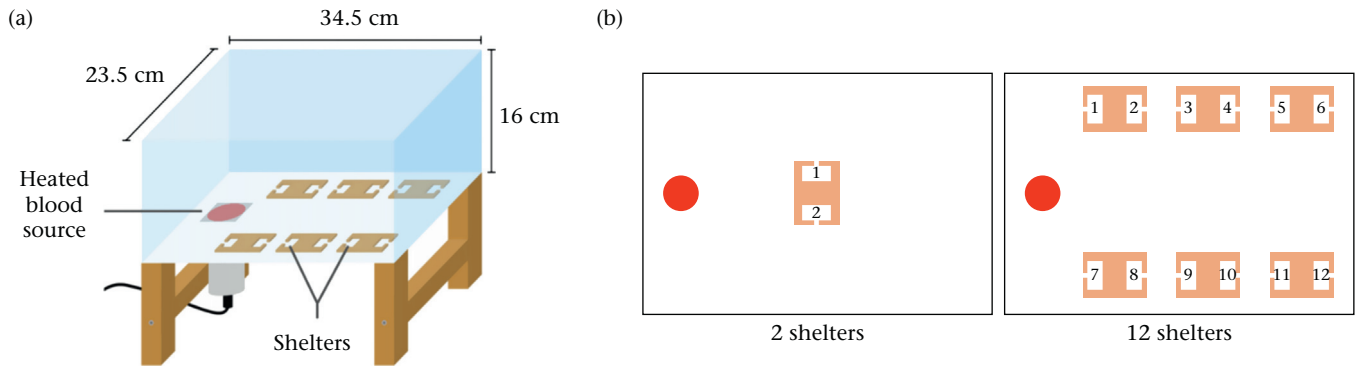
Our research complied with all applicable laws and did not require approval from an ethics committee. While we do not require formal ethics approval, we treat our subjects in accordance with strict animal ethics standards under the assumption that they experience emotion in general and pain in particular.

### *Study Population and Maintenance*

We used descendants of *C. lectularius* collected from four sites in southern Ontario between October 2019 and January 2020. We maintained the colony in a small room kept at  $27 \pm 0.5$  °C at 40% relative humidity with lights off at 0900 hours and on at 1700 hours. We housed bed bugs in 85 ml spice jars containing strips of folded filter paper to provide a rough surface for walking and oviposition. Each jar contained roughly 50–150 bed bugs of the same life stage. We fed the colony weekly under red light with defibrinated rabbit blood (Hemostat Laboratories, Dixon, CA, U.S.A.) using a Hemotek membrane-feeding system (Discovery Workshops, Accrington, U.K.).

### *Arena Design and Treatments*

To observe sexual and social dynamics, we constructed a  $34.5 \times 23.5 \times 15$  cm Plexiglas arena with a 3 cm diameter circular hole cut into one of the shorter ends to perfectly fit a Hemotek feeding reservoir (Fig. 1a). To prevent escape, we secured a layer of mesh fabric that bed bugs could feed through over the feeding hole.



**Figure 1.** (a) Schematic overview of the Plexiglas experimental arena with dimensions. (b) Diagrams depicting overhead views of how shelters were arranged for the two-shelter and 12-shelter treatments.

We lined the arena floor with filter paper and further prevented escape by applying a layer of Fluon to the walls.

We manipulated shelter availability with two treatments, a two-shelter treatment, which limited opportunity for social avoidance, and a 12-shelter treatment, which provided ample opportunity for female behavioural avoidance strategies (Fig. 1b). The choice of two shelters or 12 shelters was based on our preliminary observations that two shelters could readily accommodate 24 bed bugs while 12 shelters provided sufficient opportunities for social avoidance. On average, shelters in the two treatments were at a similar distance from the blood source. Shelters were constructed from  $5 \times 7 \times 0.3$  cm balsa wood slat segments covered with glass microscope slides. Each segment of balsa wood contained two shelters created by cutting  $1.5 \times 3$  cm cavities, each with a narrow 0.5 cm entrance. Each of these shelters were sufficiently spacious to accommodate all 24 adult bed bugs included in each replicate. In the two-shelter treatment, shelters were placed in the centre of the arena while for the 12-shelter treatment, the six segments of balsa wood were evenly spread out in the arena (Fig. 1b). We ran three replicates of each treatment.

### Behavioural Observations

For each replicate, we continuously observed 24 virgin, adult bed bugs (12 males, 12 females) for 24 h/day over six consecutive days. We collected virgin focal individuals by isolating recently fed fifth-instar juveniles until they emerged as adults. We then placed the newly emerged adults in same-sex groups and individually marked each bed bug with Sharpie oil-based paint markers. We released the focal individuals at the centre of the arena 30 min before the start of the dark phase (0830 hours). The focal bed bugs typically remained highly active during the first couple of hours and explored much of the arena before settling into their shelters. We provided heated blood between 1300 hours and 1500 hours on the first, third and fifth days of each replicate and stimulated foraging behaviour by exhaling into the arena at the beginning of each feeding period. All focal individuals fed at least once during the experiment. Throughout the dark period (0900–1700 hours), when much of the bed bug activity occurs, we live-observed the bed bugs under red light and recorded all instances of mounting and traumatic insemination. Then, using a Canon VIXIA HF R800 camera, we videorecorded the bed bugs during the light period, and later scored from the videos all mountings and inseminations that occurred during the light period. Overall, we determined the identities of both bed bugs for 2271 out of 2286 mountings and all 319 inseminations that occurred during the dark period. We also determined the identities of both bed bugs for 344 out of 355 mountings and 45 out of 46 inseminations that occurred during the

light period. Mounting and traumatic insemination are highly stereotyped and distinctive behaviours. A mount consists of a male ‘jumping’ onto a female and then dismounting within 5 s (Stutt & Siva-Jothy, 2001). Insemination is characterized by the male mounting the female, then remaining securely attached with his abdomen curled underneath the female’s right abdomen for up to 5 min (1–5 min, Carayon, 1966, p. 103; 30–300 s, Figure 2 in Siva-Jothy & Stutt, 2003). In a data set including 193 insemination durations recorded in our laboratory for another experiment, the average  $\pm$  1 SD insemination duration was  $102.4 \pm 53.9$  s and the range was 18–406 s. Based on the literature, and because only two inseminations in our data set lasted less than 30 s, we chose 30 s as the minimum duration for a mounting to be considered insemination.

To validate our insemination criterion, we compared offspring production in two groups of 25 recently fed, 7-day-old virgin females. Each female of the inseminated group received a single traumatic insemination, while each female of the no-insemination group did not interact with males. We then held all the females individually inside 35 mm petri dishes lined with filter paper. While 92% of the once-inseminated females produced eggs and hatchlings, no female of the no-insemination group laid eggs.

As for social associations, we carried out scans at the start of each hour during the dark phase for a total of nine scans per day, where we documented the location and social partners of each bed bug. We considered bed bugs to be aggregated based on whether two individuals were touching or in a group of continuously touching bed bugs. We excluded one female and one male from two different 12-shelter replicates from our analyses as both bed bugs died within the first day of the experiment. One additional male from a two-shelter replicate was removed from the analyses due to both behavioural and physical abnormalities – the male was unable to properly mount females and we later observed under a microscope that it had deformed genitalia.

### Social Network Analyses

We created all network visualizations and ran our analyses with R v.4.1.1 (R Core Team, 2021). Using the ‘igraph’ package (Csárdi & Nepusz, 2006), we constructed social networks where weighted edges represented association indices between dyads based on how often they were observed in the same aggregation. Specifically, we used the simple ratio index (SRI) to calculate association indices, which is recommended for when nearly every individual can be reliably recorded in every sampling period (Hoppitt & Farine, 2018). Then, to quantify individual sociability, we extracted strength values from the aggregation networks. Strength is equivalent to the sum of all edge weights connected to a node and represents how

often and with how many others an individual bed bug was seen aggregating with.

### Statistics

We analysed linear mixed-effects models (LMMs) in R using the package ‘lme4’ v.1.1–27.1 (Bates et al., 2015) and report Wald  $\chi^2$  values generated with the ‘Anova’ function from the ‘car’ package v.3.0–11 (Fox & Weisberg, 2019). We verified model fits by visually inspecting plots of model residuals using the ‘DHARMA’ package (Hartig, 2019). To examine whether the two-shelter treatment resulted in higher levels of sexual harassment compared to the 12-shelter treatment, we constructed two LMMs, one with mounting rate and the other with insemination rate as the dependent factor. Both models included treatment as a fixed factor and replicate as a random factor.

We tested whether males were more social than females within each treatment using an LMM combined with a permutation test. In this model, we used the log of strength values taken from aggregation networks as the dependent factor and included treatment, sex and the treatment\*sex interaction term as fixed factors and replicate as a random factor. Because measures obtained from social networks are inherently nonindependent, thus violating the assumptions underlying most parametric tests (Croft et al., 2011), we performed node-label permutation tests by shuffling and redistributing the nodes among all possible node positions in each of our six observed networks. This is a commonly used approach for assessing whether nodes with different attributes reliably occupy different network positions (central/more social versus peripheral/less social) (Farine & Whitehead, 2015). After obtaining new strength values from the randomized networks, we reran our LMM and extracted *t* ratios from the relevant contrast using the package ‘emmeans’ function in R. By performing 1000 iterations of this network randomization process, we were able to compare observed contrast *t* ratios to a null distribution of *t* ratios representing the null hypothesis that males and females do not differ in their propensity to aggregate. In total, we ran two permutation tests, one for male versus female strength in the two-shelter treatment and one for male versus female strength in the 12-shelter treatment.

To examine whether the six bed bug populations showed positive assortment by sex, we calculated assortativity index (AI), a value between  $-1$  and  $1$  where  $1$  represents perfect assortativity,  $-1$  represents disassortativity, and  $0$  indicates no assortment, for each of the six aggregation-based networks. This was done using the ‘assortnet’ package, which accounts for weighted edges (Farine, 2014). We then performed 1000 iterations of a node-label permutation test for each of the six observed networks. This resulted in a distribution of 1000 new AIs for each of our six bed bug populations representing the null hypothesis that associations between individuals were random or not biased by sex. We obtained two-tailed *P* values by comparing the observed AIs for each network to its respective null distribution of AIs.

### Social Attraction Experiment

To directly assess bed bugs’ specific social attraction to conspecifics of distinct sex and mating status, we conducted a follow-up experiment with five treatments, where focal bed bugs could choose between two shelters that varied in their occupation history. First, as baseline control treatments, we presented either male or female focal individuals with the choice of a shelter previously occupied by mated females versus an unused control shelter. Next, to test whether the social cues of males and females are differentially attractive to the two sexes, we presented either male or

female focal individuals with the choice of a shelter previously occupied by mated females versus a shelter previously occupied by mated males. Lastly, to examine whether mating status alters attractiveness of females, we presented focal males with the choice of a shelter previously occupied by mated females versus a shelter previously occupied by virgin females. We randomized and counterbalanced the position of the shelters and ran five replicates, each including six trials per each of the five treatments. Due to occasional shortages of bed bugs for generating social cues, our final sample size was 29 trials per treatment except for the treatment of focal females choosing between cues of mated females and mated males, where we only had 28 trials.

We created choice arenas by placing two shelters at opposite ends of an 85 mm diameter petri dish, which was lined with filter paper and coated with Fluon around the side (see Results, left side of Fig. 5). We constructed shelters by folding  $15 \times 15$  mm segments of filter paper into triangular tents with floors, each held together by a small piece of masking tape. To manipulate their occupation history, we placed the shelters individually inside plastic vials 2.5 cm wide and 9.5 cm high, with four recently fed (<2 h) adult bed bugs. The bed bugs were either mated males, mated females or virgin females. We obtained the mated females and mated males by collecting adult bed bugs of roughly the same age from our general population, and we obtained virgin females by isolating recently fed fifth-instar juveniles until they emerged as adults. We allowed these stimulus bed bugs 4 days to walk, rest, defecate and lay eggs in and on the shelters. In a few cases, we used three ( $N = 7$ ) or two ( $N = 3$ ) stimulus bed bugs to generate social cues for each of the two shelter options instead of the usual four due to a shortage of age-matched bed bugs from the general population. Immediately before the choice assay, we immobilized the stimulus bed bugs using ice to remove them from the shelters. We ensured focal bed bugs were never housed in the same containers as bed bugs used to produce social cues to control for possible effects of familiarity.

For focal individuals, we generated virgin adult bed bugs as described above, then continued to keep the adults individually isolated for one additional week postemergence. After this week of social isolation, we briefly consolidated the focal individuals in same-sex jars for feeding. The next day, we placed one male and one female in a 50 mm petri dish lined with filter paper for up to 10 min and verified that traumatic insemination had occurred using the same criterion as detailed above. Immediately after insemination, the pair of bed bugs were again isolated to ensure every bed bug had only mated once prior to the choice trial. At 1300 hours on the same day (the middle of the dark period), we placed the focals at the centre of each petri dish. Twenty hours later, at the end of the light phase, an observer blind to treatment recorded the bed bugs’ shelter choice.

We used the ‘lme4’ package to perform generalized linear mixed-effects models (GLMMs). For the two control treatments, we ran a single binomial logistic regression with sex as a fixed effect and replicate as a random effect to assess whether attraction towards the used shelters varied by sex. We then ran a GLMM for each of the three other treatments again using the binomial distribution with replicate as a random factor to assess whether the bed bugs showed significant attraction to one type of social cue over the other.

## RESULTS

### *Effect of Shelter Availability on Harassment Received by Females*

On average, females were mounted approximately 4.14 times per day and inseminated approximately 0.89 times per day. Females in the two-shelter treatment were mounted more frequently



than females in the 12-shelter treatment (LMM: Wald  $\chi^2_1 = 26.58$ ,  $P < 0.0001$ ; Fig. 2a). However, we did not detect any differences in traumatic insemination rates between the two treatments (LMM: Wald  $\chi^2_1 = 0.73$ ,  $P = 0.39$ ; Fig. 2b).

#### Male versus Female Strength of Aggregation

Overall, bed bugs of both sexes spent more time aggregating with conspecifics in the two-shelter treatment than in the 12-shelter treatment (LMM: Wald  $\chi^2_1 = 336.74$ ,  $P < 0.0001$ ; Fig. 3a, b). Within the two-shelter treatment, females displayed higher levels of sociality than males ( $P_{\text{rand}} < 0.01$ ; Fig. 3a, b, Appendix, Fig. A1a) while sex differences in network strength were not detected in the 12-shelter treatment ( $P_{\text{rand}} = 0.13$ ; Fig. 3a, b, Appendix, Fig. A1b).

#### Assortativity by Sex

In each of our six bed bug groups, observed assortativity indexes were close to zero, indicating no preference for aggregating with same-sex versus opposite-sex individuals (Fig. 4). Additionally, our network randomization tests revealed that only one out of the six bed bug populations showed significant, although low, positive assortment ( $P_{\text{rand}} < 0.05$ ; Fig. 4). The remaining five networks were not more assorted than expected by chance, indicating no significant tendency for individuals to aggregate with same- or opposite-sex conspecifics (Fig. 4).

#### Individual Choice Assays

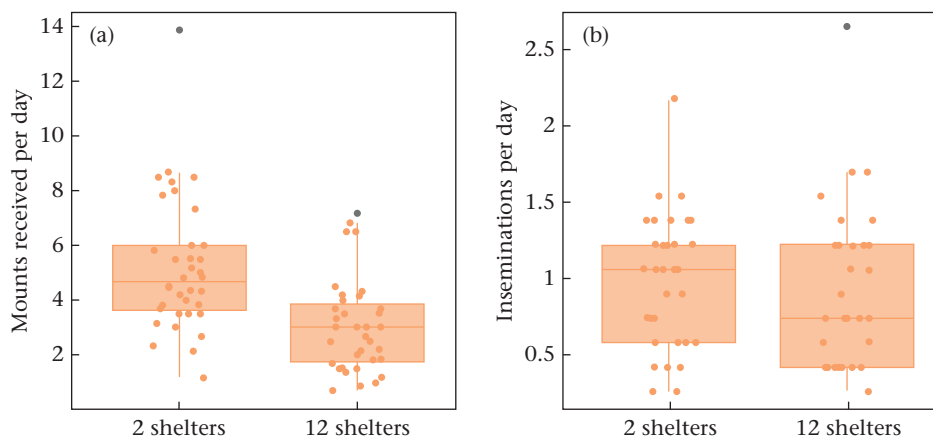
Focal mated males and focal mated females both preferred shelters previously occupied by mated females over unused control shelters (GLMM intercept: Wald  $\chi^2_1 = 11.20$ ,  $P < 0.001$ ; sex: Wald  $\chi^2_1 = 0.97$ ,  $P = 0.32$ ; Fig. 5). When presented with the choice between shelters previously occupied by mated males and shelters previously occupied by mated females, focal males showed a nonsignificant tendency towards mated females (GLMM intercept: Wald  $\chi^2_1 = 1.98$ ,  $P = 0.16$ ; Fig. 5) while focal females significantly preferred mated females (GLMM intercept: Wald  $\chi^2_1 = 9.56$ ,  $P < 0.01$ ; Fig. 5). Lastly, when presented with the choice between shelters previously occupied by virgin females and shelters previously occupied by mated females, focal males preferred shelters with cues from virgin females (GLMM intercept: Wald  $\chi^2_1 = 5.40$ ,  $P < 0.05$ ; Fig. 5).

## DISCUSSION

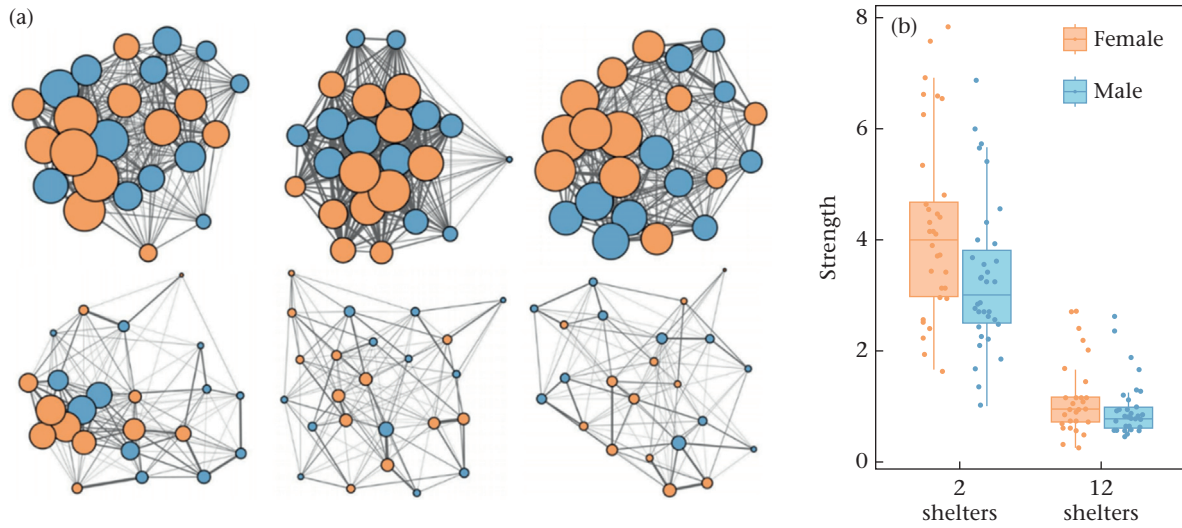
Using a novel seminaturalistic arena, we tracked aggregation and traumatic insemination patterns of replicate bed bug populations over six consecutive days. As traumatic insemination in bed bugs is often cited as an extreme example of sexual conflict (Reinhardt & Siva-Jothy, 2007; Siva-Jothy, 2006; Stutt & Siva-Jothy, 2001), we constructed aggregation networks to assess whether we would see signs of social avoidance strategies used by females at the population level to avoid sexual harassment as seen in other species (Dadda, 2015; Darden & Croft, 2008; Krupa et al., 1990; Stanley et al., 2018). Contrary to our predictions, we found that females were not less social than males overall and that social networks were not assorted by sex. The lack of observed female social avoidance patterns may suggest that female bed bugs are well counteradapted for mitigating potential costs of repeated inseminations, as suggested by Morrow and Arnqvist (2003). Given the mixed empirical evidence on how harmful traumatic insemination is to females (Morrow & Arnqvist, 2003; Stutt & Siva-Jothy, 2001), additional research into the actual fitness consequences of different traumatic insemination rates is needed. Nevertheless, our fine-scale continuous observation of bed bugs revealed several novel insights about both their sexual and social dynamics.

First, we predicted that reducing shelter availability would dramatically increase sexual conflict intensity through sexual harassment, which we quantified using mounting and insemination rates. However, we found that only mounting (Fig. 2a), but not insemination rate (Fig. 2b), was higher in the two-shelter treatment versus 12-shelter treatment. Furthermore, our data revealed that the majority of mounts did not result in successful insemination (Fig. 2a and b). The high proportion of unsuccessful mounts suggests that insemination rate is not as male-controlled as previously thought (Reinhardt et al., 2009; Stutt & Siva-Jothy, 2001). Accordingly, we often observed females running away from sexually harassing males or assuming a refusal posture as described by Siva-Jothy (2006). Thus, although our aggregation networks did not reveal patterns of female avoidance at the population level, our documentation of general avoidance behaviour highlights the importance of fine-scale continuous observations as well as the importance of studying sexual conflict in more complex, realistic environments, which allow females to perform their full range of evolved avoidance strategies.

Another key consideration and likely explanation for the lack of difference in insemination rate between our two treatments is



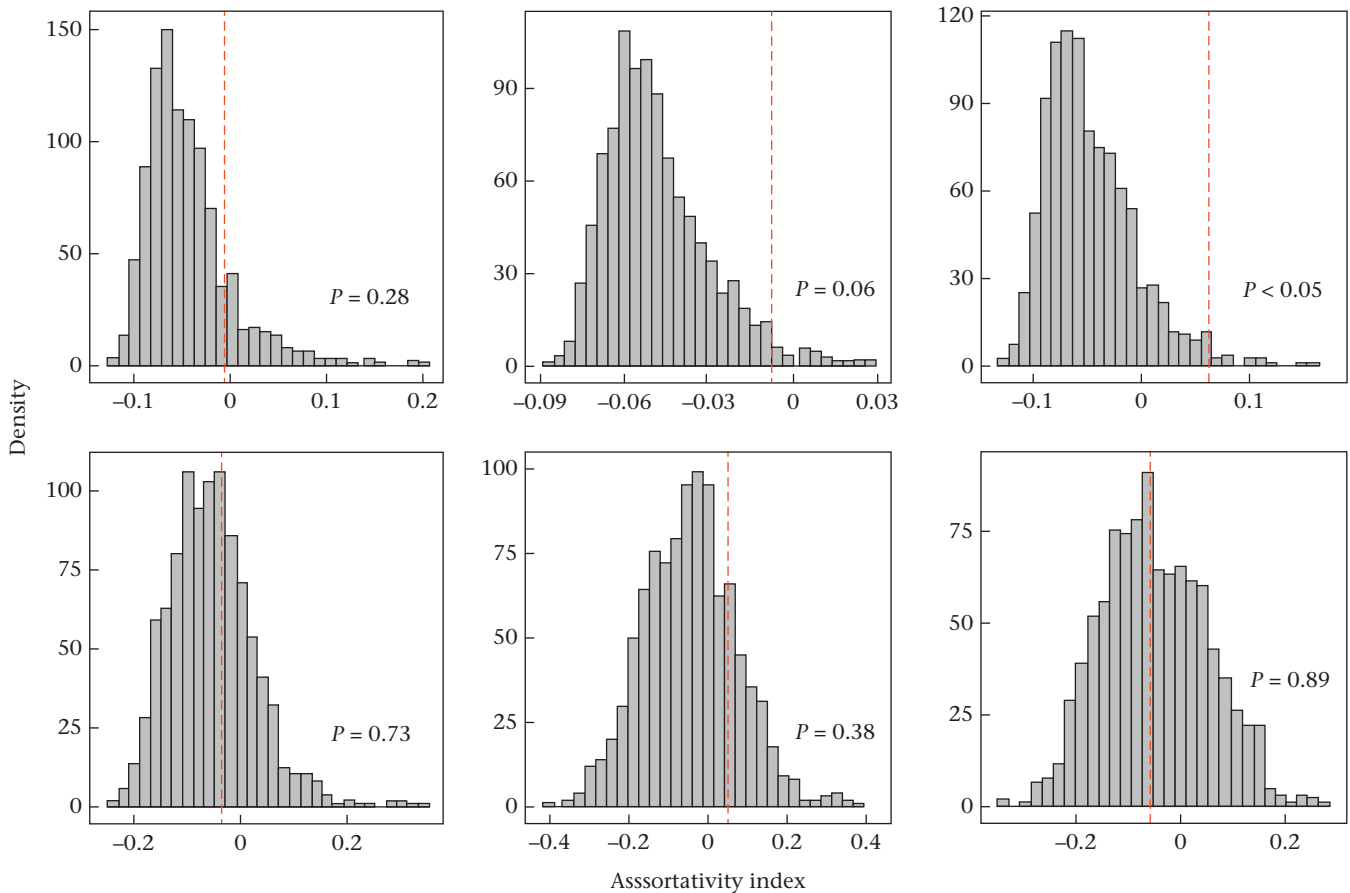
**Figure 2.** Effect of treatment on the amount of sexual harassment received by females. The daily rate of (a) mounts and (b) traumatic inseminations received by females in the two-shelter ( $N = 36$ ) versus 12-shelter ( $N = 35$ ) treatments. Bold horizontal lines indicate the medians, the boxes represent the interquartile range (IQR) between the first and third quartiles, and the vertical lines extend to the minimum and maximum values. Outliers are shown in black.



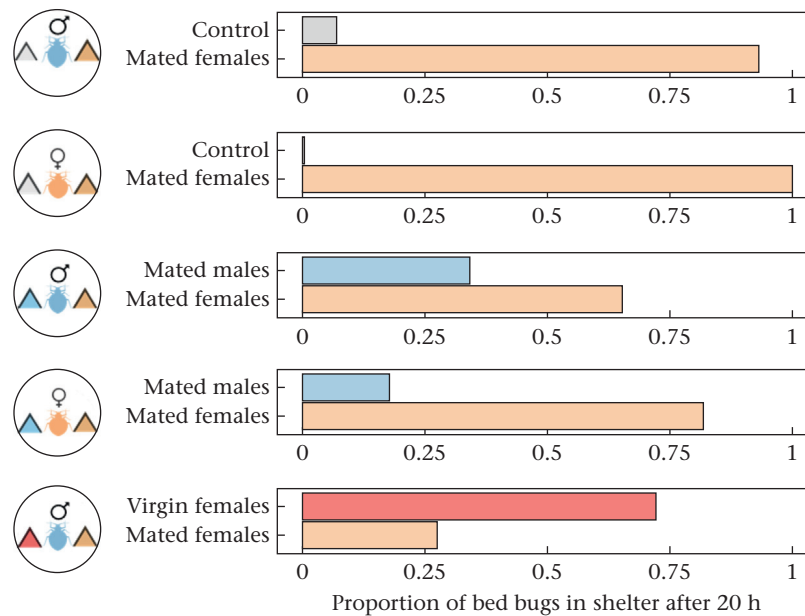
**Figure 3.** (a) Aggregation networks of bed bug groups. The top row represents networks from two-shelter treatment groups while the bottom row represents networks from 12-shelter network groups. Orange nodes denote females while blue nodes denote males. Edge width represents the strength of association between dyads and node size corresponds to strength (total sum of edge weights). For clearer visualization, node size for the second two-shelter network is scaled to half the size of nodes relative to all the other networks. (b) Strength comparison between males and females within each of the two treatments.

sperm and/or seminal fluid constraint in males, which has been previously documented in a range of taxa including bed bugs (Birkhead, 1991; Linklater et al., 2007; Preston et al., 2001; Radhakrishnan et al., 2009; Reinhardt et al., 2011). Because male bed bugs are known to experience seminal fluid depletion and can

gauge the recent mating history of a female using their copulatory organ (Siva-Jothy & Stutt, 2003), mounts that do not result in insemination could be the result of males adaptively aborting insemination attempts based on indicators of female attractiveness or potential sperm competition. Closer investigation of how males



**Figure 4.** Distribution of assortativity indexes from permuted networks. The top row represents randomized networks from the two-shelter treatment while the bottom row represents randomized networks from the 12-shelter treatment. Red dashed lines represent assortativity indexes of the observed networks.



**Figure 5.** We gave individual focal adult bed bugs a binary choice between two filter paper shelters that varied in their occupancy history. Occupation history of shelters varied between the five treatments denoted by the colour of triangular shelters in each diagram, where blue represents mated males, orange represents mated females and red represents virgin females. The first two treatments included an unused control shelter as one of the two shelter options and is shown as a grey triangle. Bars correspond to the occupancy history of shelters; bed bug colours correspond to the sex of focal individuals, with blue representing mated adult males, orange representing mated adult females and red representing virgin females.

differentially pursue females that vary in traits such as recent mating history can reveal new insights into male mate choice, sexual selection and mating system evolution.

Females did not use the increased number of shelters in the 12-shelter treatment to occupy more peripheral network positions to avoid males. Moreover, to our surprise, bed bugs in the 12-shelter treatment formed relatively sparse social networks with low strength values. That is, when given a choice among a dozen high-quality shelters, the bed bugs did not form the anticipated large aggregations. Rather, the average group size was about two (Fig. 3a and b). This was unexpected because natural infestations of bed bugs typically comprise large, mixed-sex aggregations (Johnson, 1941; Mellanby, 1939; Reinhardt & Siva-Jothy, 2007). Furthermore, our social preference test revealed that both male and female adult bed bugs show a strong preference for occupying shelters with social cues from conspecifics over identical shelters with no social cues (Fig. 5), echoing results from previous studies on bed bug social attraction (Gershman et al., 2019; Levinson & Bar Ilan, 1971; Weeks et al., 2011, 2013). This apparent contradiction between our social network study and follow-up experiment could be explained by the absence in the arena of pre-existing physical and chemical stimuli including faeces, exuviae, eggs and pheromones, which may be crucial for facilitating aggregation formation in bed bugs.

As for the two-shelter treatment, we found that females were more social than males (Fig. 3b; Appendix, Fig. A1a). However, our networks alone cannot tell us whether higher female strength values are the result of females themselves showing a higher propensity to seek others or whether other individuals preferentially associate with females over males. With our social attraction experiment, we directly addressed this question and found that mated females strongly preferred shelters with cues from other females over other males and that males too, tended towards a preference for females (Fig. 5). Therefore, females occupying more central network positions in the two-shelter treatment likely reflect a strong tendency for both females and males to associate

with females over males. However, despite females' preference for shelters previously occupied by females as opposed to males, we still found that bed bug networks from both treatments generally showed no assortment by sex (Fig. 4). This suggests that females are incapable of engineering their social environment to reduce levels of sexual harassment, most likely because males are adept at locating and exploiting females even in relatively large, complex environments.

Lastly, we found that males can discriminate between social cues left by virgin females versus mated females, with a preference for virgin females presumably because of their higher reproductive value (Fig. 5). This suggests that females adjust their deposition of contact pheromone based on their reproductive status as indeed suggested by Siljander et al. (2007). It also tells us that in addition to using their intromittent organ to directly assess a female's mating history (Siva-Jothy & Stutt, 2003), males also possess indirect mechanisms of assessing their reproductive landscape to strategically seek mating opportunities that lessen sperm competition intensity and thus increase reproductive success.

Overall, our seminaturalistic social network experiment revealed that female bed bugs struggled to socially evade males even when provided with several high-quality shelters. Yet, at the individual level, females showed a clear tendency to avoid shelters with social cues from males. We thus conclude that female bed bugs are generally incapable of shaping their social environment in a way that reduces levels of sexual harassment. Further research taking a network-based approach on sexual and social dynamics can better elucidate how competing reproductive interests can shape social behaviour at both the individual and population level.

#### Author Contributions

J.Y. and R.D. designed the experiments, J.Y. conducted the experiments and analysed the data. J.Y. and R.D. wrote the manuscript.

## Data Availability

Our data and code can be found at the attached Github link. [Bed bug social network analysis 2021 \(Original data\)](#) (Github)

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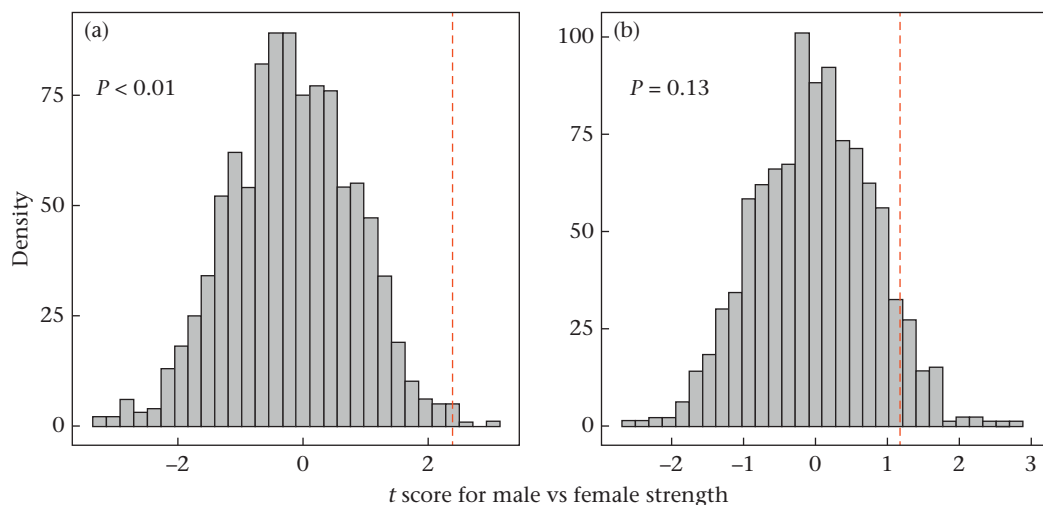
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## Appendix



**Figure A1.** Null distributions of  $t$  scores representing the effect of sex on strength for (a) the two-shelter treatment and (b) the 12-shelter treatment. Null distributions for each treatment are the result of 1000 node-label network randomizations. Red dashed lines are  $t$  scores representing the observed effect of sex on strength for each treatment.