Female bed bugs (*Cimex lectularius* L) anticipate the immunological consequences of traumatic insemination via feeding cues

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Not all encounters with pathogens are stochastic and insects can adjust their immune management in relation to cues associated with the likelihood of infection within a life cycle as well as across generations. In this study we show that female insects (bed bugs) up-regulate immune function in their copulatory organ in anticipation of mating by using feeding cues. Male bed bugs only mate with recently fed females and do so by traumatic insemination (TI). Consequently, there is a tight temporal correlation between female feeding and the likelihood of her being infected via TI. Females that received predictable access to food (and therefore predictable insemination and infection cycles) up-regulated induced immunity (generic antibacterial activity) in anticipation of feeding and mating. Females that received unpredictable (but the same mean periodicity) access to food did not. Females that anticipated mating-associated immune insult received measurable fitness benefits (survival and lifetime reproductive success) despite laying eggs at the same rate as females that were not able to predict these cycles. Given that mating is a time of increased likelihood of infection in many organisms, and is often associated with temporal cues such as courtship and/or feeding, we propose that anticipation of mating-associated infection in females may be more widespread than is currently evidenced.

reproductive immune anticipation | immune priming | traumatic insemination | bed bug

The bed bugs (Hemiptera: Cimicidae) are unique among gonochorists (species with separate sexes) because they are obligate traumatic inseminators (1, 2). Males use their needle-like intromittent organ to pierce the female's abdomen (Fig. 1*A*) and inseminate into a specialized copulatory organ (the spermalege) despite the fact that females have a separate, and functional, genital tract (1) (Fig. 1*B*) that is required for egg laying. Males of the bed bug, *Cimex lectularius*, only pierce the female's body wall in the section that lies over (Fig. 1*B*) this specialized copulatory organ (3) (Fig. 1*C*). The spermalege has been shown to be critical in localizing and neutralizing the fitness-reducing opportunistic pathogens that are introduced during each traumatic insemination (TI) (4). Mating is directed at recently fed females (5) and occurs at 20× the frequency that is needed to prevent sperm limitation (6). The events leading up to traumatic insemination in *C. lectularius* are temporally and behaviorally stereotypical (5) and of particular importance is the intimate relationship between feeding and mating behavior. To manufacture and lay eggs, females need to take regular (ca. every 7 d) blood meals (e.g., refs. 7 and 8) the size of which determines the number of eggs a female lays after feeding (9). Immediately after feeding, females are engorged with blood and males concentrate their mating effort on these females (5). Under ad libitum feeding female bed bugs receive traumatic insemination weekly (5) and iteratively throughout life (8, 10), but contingent on feeding. Consequently, there is a predictable sequence of events for females, starting with initiating host searching, feeding, and finally traumatic insemination and the associated infection (8). We know that insects can predict, and consequently, manage their immune systems to defend against, the likelihood of immune insult across generations (11) and during ontogeny (12). Recent work on *Drosophila melanogaster* has shown that females can use male courtship song as a cue to up-regulate immunity in anticipation of mating (13) which improves female fitness (14) over relatively short time frames. It therefore seems possible that in the presence of reliable cues, and predictable behavioral cascades, immune anticipation of mating-induced infection may be particularly important in female bed bugs (15) because of TI.

In this study we examine anticipatory immune system management over a 7 d feeding cycle that is associated with TI. We start by examining whether associative learning drives anticipatory immunity under standard feeding cycles. We then explore how the temporal association between feeding, mating, and immune insult in *C. lectularius* is associated with infradian (greater than a day) cycles in immune responsiveness when individuals experience predictable vs. unpredictable feeding cycles with the same mean periodicity. Finally we measure the fitness consequences of such anticipatory immune cycles.

Results

We tested whether female bed bugs that were fed and then mated and immune challenged at regular intervals learned to

Significance

We show that female insects can subtly change the management of their immune system contingent on infradian feeding cycles that act as cues to immune insult during mating. We experimentally reject the possibility that this is learned behavior, and show instead that it is dependent on the predictability of feeding which in turn is a cue for mating-induced infection. Although evidence exists for insect immune anticipation over lifetime scales, this study links the temporal features of feeding to the insect's mating behavior in the context of a system with infection caused by traumatic insemination. We predict similar mating ecology in other animals is likely to select for similar reproductive immune anticipation (RIA).

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Conflict of interest statement: R.N. owns "the Cimex store," which conducts commercial research and activities related to bed bugs. He conducted the work in this paper as part of his PhD (prior to owning the store).

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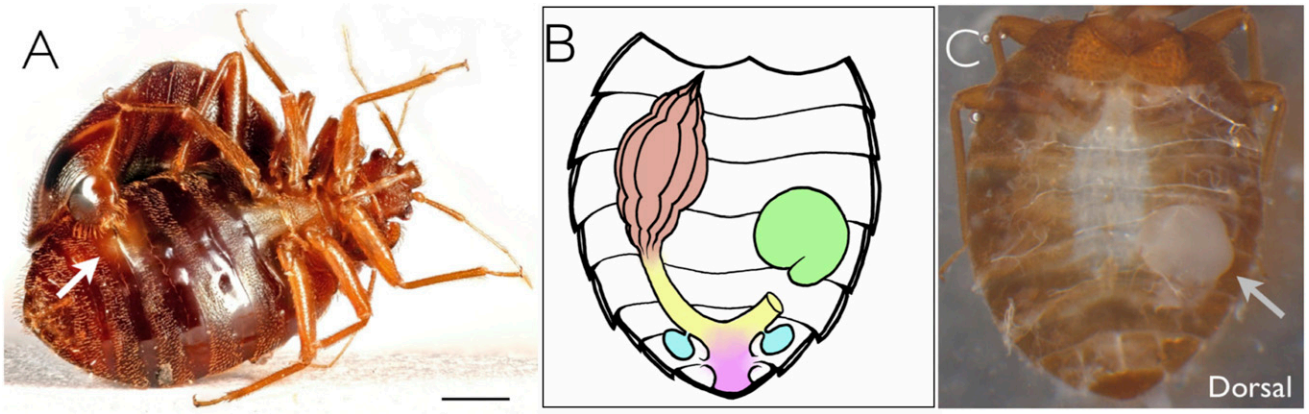

Fig. 1. (*A*) Ventral view of bed bugs flash-frozen during traumatic insemination. The female's underside is visible and the arrow indicates the point at which the male has inserted his intromittent organ into the specialized groove in the female's abdominal wall. The intromittent organ is visible as a dark pin-like structure: the point at which it has penetrated the female's abdomen shows a distinct light-colored mass (the ejaculate) under the female's cuticle. (Scale bar: 1 mm.) (*B*) Schematic of the reproductive system of the female bed bug (dorsal view). The spermalege (green) is unique to bed bugs and is an organ filled with hemocytes that lies in the body cavity at the site of traumatic insemination (see also *C*). The "standard" insect female genitalia are composed of the genital tract (purple), the oviducts (yellow), and the ovaries (brown). Bed bugs do not have a spermatheca—instead they have temporary sperm stores attached to the genital tract called seminal concepticals (blue)—they have a different embryonic origin to true spermathecae (1). (*C*) A dorsal view of the inside of the female's abdomen with the dorsal surface of the specimen removed and all of the organs in the abdomen, except the spermalege (arrow) removed. During traumatic insemination, the ejaculate (and microorganisms on the male intromittent organ) are introduced into this organ; hence its moniker as a "paragenital" organ: it is the functional copulatory organ of the female, despite the fact she has a conventional reproductive tract (purple in *B*, which is used only for egg laying). Sperm swim out of the spermalege, through the blood system, and penetrate the seminal concepticals (blue in *B*) at the base of the female's reproductive system (1) from where they swim up the oviducts (yellow in *B*) and fertilize eggs in the ovaries (brown in *B*).

associate the negative immunological consequences of traumatic insemination with their predictable feeding schedule. We fed two groups of virgin adult females to satiation once every 7 d (reflecting observed feeding cycles; ref. 8). The treatment group received artificial septic immune insults (a conditioning stimulus that mimicked traumatic insemination; ref. 3) after feeding, while the control group received no immune insult. After 3 wk of treatment, we assessed an induced antibacterial humoral immune response (lysozyme-like activity; LLA) in the spermalege just 1 d before (day −1) and just before feeding (day 0). Contrary to prediction, both the associative-learning group ($t = -4.4$, $df = 38$, $P < 0.0001$) as well as the group with no opportunity to learn (the control; $t = -10.16$, $df = 38$, $P < 0.0001$) showed significant anticipation in LLA titers in the spermalege before feeding (Fig. 2). Since females in the control group had no opportunity to associate feeding with an immune insult, we conclude that negative associative learning does not underpin the anticipatory increase in immunity observed in the spermalege in both groups.

In infestations with a resident host, cimicids feed ad libitum (e.g., ref. 16) and so females will have predictable feeding cycles (i.e., they control their feeding rate). We tested whether a predictable feeding periodicity (and therefore predictable traumatic insemination and infection) drives anticipatory immunity in female bed bugs (compared with unpredictable feeding cycles). During juvenile development (from egg to imago), females were either fed on a predictable cycle (with 7 d intervals), or on an unpredictable cycle, (with intervals of five, seven, or 9 d per instar, but with a mean of 7 d across all five juvenile instars). A week after imaginal eclosion, both groups of females were fed and received a fixed-duration mating (to ensure controlled insemination; ref. 17). After a further 7 d they received another feed and an immune insult (day +7: see *SI Appendix*, Fig. S2 for a schematic of the experimental logistics). The predictability of feeding had a significant impact on LLA titers ($F_{(1)} = 41.67$, $P < 0.0001$; Fig. 3). Females in the "predictable" feeding group showed an 18.5% increase in LLA titers between day −2 and mating (day 0) ($t = 2.34$, $df = 16$ $P = 0.033$), and a 55% increase between day +5 and feeding/mating on day +7 ($t = 5.9$, $df = 16$, $P < 0.0001$; Fig. 3). By contrast, females in the "unpredictable" feeding group showed no change in LLA titers before feeding and mating on day 0 ($t = 1.52$, $df = 18$, $P = 0.14$) and showed a significant decrease (−51%) before day +7 ($t = 5.02$, $df = 18$, $P < 0.0001$) induction of LLA in this group occurred after feeding and insult.

Our results show that female bed bugs can synchronize the management of their immune system with the predictability of their feeding cycles. We propose that this synchronization functions, at least in part, to up-regulate induced immune effector systems before the immune insult associated with traumatic insemination: by up-regulating induced immunity, females are better able to respond to the microbial insult (4) associated with traumatic insemination. If this proposal is correct we predict that reproductive immune anticipation will have a positive fitness consequence. We measured fecundity and survival in females in a separate experiment where they were subjected to both feeding treatments (predictable vs. unpredictable) as well as sterile and septic challenges (e.g., ref. 3). Following imaginal eclosion and initial feeding and controlled mating, females in both groups received four consecutive feeding/immune insult events (each with a 7 d periodicity). We included a treatment control (nonseptic immune challenge) to identify potential costs associated with anticipatory immunity. The feeding predictability and immune insult treatments had no effect on the rate of egg production (Fig. 4*B*): fertile eggs were produced at the same rate in all groups (ANOVA; $F = 0.18$; $df = 3,114$; $P = 0.91$; combined mean across treatments = 17.4 ± 0.9 eggs per week). Since manipulation of blood-meal size is highly correlated with egg production rate ($r^2 = 0.909$, $F_{1,52} = 521.3$, $P < 0.0001$) (*SI Appendix*, Figs. S3 and S4), we conclude that the lack of an effect of treatment on egg-laying rate indicates that our feeding treatments did not a priori affect reproductive output. There was a significant effect of treatment on survival (Accelerated Failure Time Survival Analysis: Wald $\chi^2 = 68.5$, $df = 3,116$; $P < 0.001$; Fig. 4*A*): the septic treatment reduced survival (Wald $\chi^2 = 49.5$, $df = 2,116$; $P < 0.001$), and predictable feeding increased it (Wald $\chi^2 = 16.6$; $df = 2,116$; $P < 0.001$). There was a significant interaction between feeding predictability and immune insult treatment (Wald $\chi^2 = 5.5$; $df = 1,116$; $P < 0.05$): females with predictable feeding showed a 22% increase in longevity compared with females fed

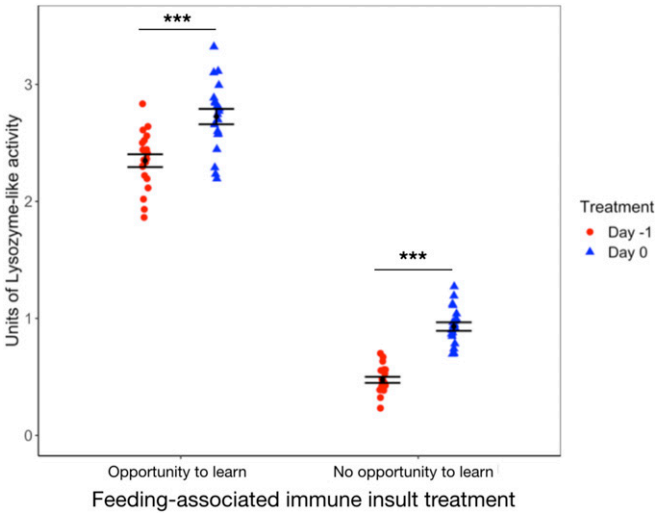

Fig. 2. Lysozyme-like activity on day −1 and day 0 in female *C. lectularius* that were experimentally exposed to systemic immune insult when fed (Opportunity to learn), and females that were not (No opportunity to learn) ($n = 20$ in each group). If females learn to associate feeding with wounding we predicted an increase between day −1 and day 0 in the "opportunity to learn group" but no difference between day −1 and day 0 in the "no opportunity to learn" group. There are significant (anticipatory) increases in the spermalege in the "opportunity to learn" group (t = −4.4, df = 38, $P < 0.0001$) as well as the group with no opportunity to learn (the control) (t = −10.16, df = 38, $P < 0.0001$). The increase in the control group between day −1 and day 0 (despite their lower overall response) indicates an ability to anticipate feeding and/or mating occurs via a route other than associative learning. The larger responses overall in the group that had the opportunity to learn (i.e., was exposed to systemic immune insult) is most likely the effect of immune priming (e.g., see ref. 11) via the treatment.

in the unpredictable feeding regime (median lifespans, unpredictable feed = 25.5 ± 1.6 d, predictable feed = 31 ± 1.6 d). Because egg-laying rates were the same in all four treatments (Fig. 4*B*) the consequence of variation in survival was that fitness (total viable eggs laid in their lifetime) varied significantly between treatments (Fig. 4*C*). Consequently, females in the unpredictable feeding group (i.e., that did not anticipate insult) had lower fitness (because their cumulative fecundity was reduced by their shorter lifespan). There was no survival difference between feeding groups in the sterile immune treatment (z = 1.31; $P = 0.19$), but a significant difference in survival between feeding predictability groups in the septic treatments (z = 3.81; $P < 0.001$), suggesting that the costs associated with mounting anticipatory immune responses may not be that high compared with the benefits. Our examination of the fitness effects of anticipatory immunity showed no effect on reproductive rate but a significant impact on survival. This strongly suggests that the immune anticipation (and potentially other unmeasured traits) we have observed under these conditions is adaptive.

Discussion

Female bed bugs up-regulate induced immunity in the copulatory organ (the spermalege) when they have predictable access to food: they do so in anticipation of traumatic insemination and the associated infection. Our study identifies a fitness trait, reproductive immune anticipation (RIA; ref. 15), and provides an ecological context for it. A study of a wild cimicid (16) showed that these insects fed at a rate that ensured females were not food limited. This is likely to occur in most cimicids because they are faithful to a relatively large host (e.g., ref. 1). Consequently, they have no spatial or temporal constraints on access to their host and so likely experience ad libitum feeding conditions in infestations because of the relative size, and predictable availability, of the host. Bed bug females will feed at a rate that maintains maximum egg production and so will be subjected to the associated high levels of traumatic insemination (6) in a predictable manner (although it is possible that the optimum feeding interval may not always be 7 d and could be dependent on local conditions). By contrast, a female bed bug in an infestation where the host is periodically absent will have unpredictable access to food and therefore unpredictable mating-associated infection. This study shows that (*i*) predictable, periodic feeding induces LLA in the female's copulatory organ before the next feeding event and (*ii*) in unpredictable feeding conditions females do not anticipate immune insult: in these females the response is likely to be a "classic" induced (i.e., delayed) response. The relatively long delay in LLA in the nonpredictable group could be due to two nonexclusive, hypothetical immune management tactics. First, in the nonpredictable scenario females may rely on (*i*) constitutive cellular defense (the spermalege is membrane bound and replete with hemocytes) for immediate defense and (*ii*) induced specific pathways upon infection. The delay in the generic induced system (LLA) may represent a "mopping up" phase. Moreover, a rapid induced generic effector may be more effective in the predictable scenario because although the timing is predictable, the qualitative nature of the insult is not.

Although predictability of feeding did not result in a difference in survival under sterile conditions, the data (Fig. 4) suggest that in the predictable feeding group survival was lower: there may therefore be a measurable cost of maintaining immunity in the absence of an immune insult over a longer time frame.

Since the observed immune effector up-regulation was not dependent on experiencing an immune challenge, our results allow us to dismiss the possibility that the anticipation is a learned phenomenon (see also ref. 18). Instead it appears to be driven by cycles of feeding (and/or hunger). We suggest that as digestion of her last blood meal begins to limit her ability to produce eggs, a female is stimulated to begin feeding. DeVries et al. (19) have shown several *C. lectularius* metabolic traits with predictable dynamics over a 7 d cycle that could act as "hunger" cues, and therefore also potentially stimulate RIA. Metabolic cues that result in rhythmic physiological or behavioral responses have

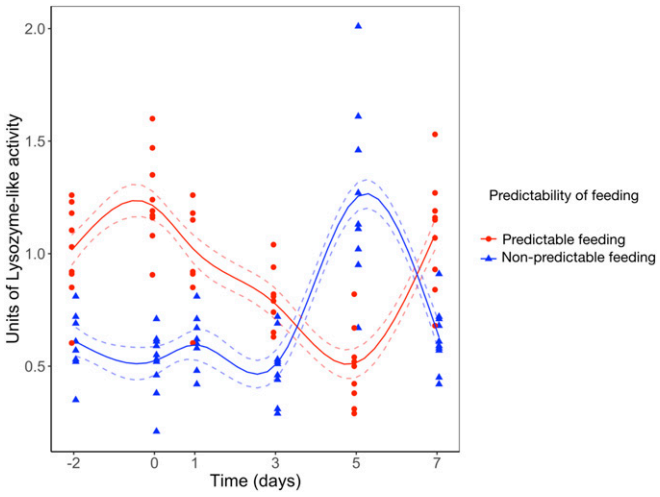

Fig. 3. Female *C. lectularius* in the predictable feeding regime showed increased LLA before feeding on day 0 and day 7, while females in the nonpredictable regime did not increase LLA before day 0 and showed a significant decrease in LLA on day 7. Fitted line (±SE) is based on the predicted fit from a General Additive Model ($R^2 = 65.5\%$), significance of fit for predictable and nonpredictable feeding is F = 12.27, $P < 0.0001$ and F = 20.74, $P < 0.0001$, respectively.

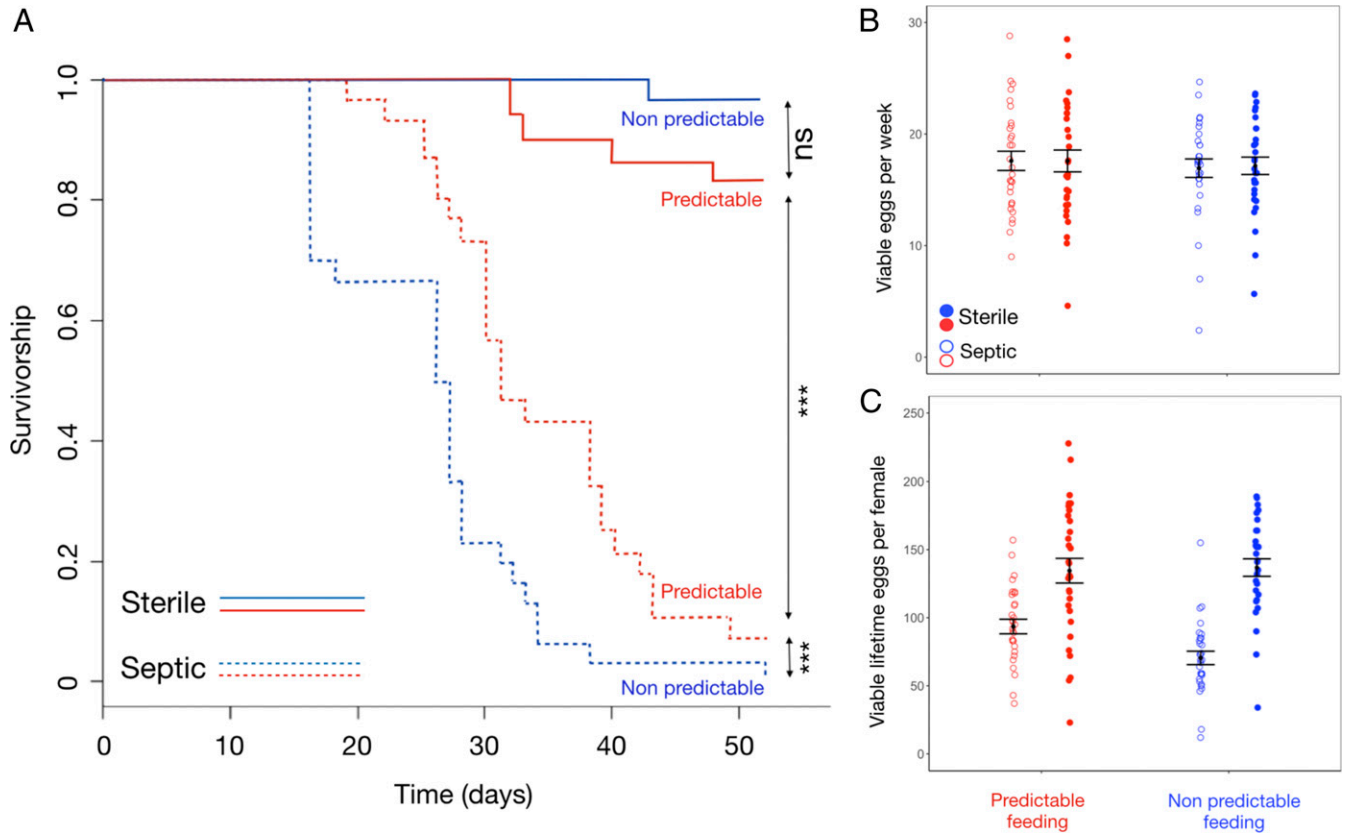

Fig. 4. (*A*) Survival curves for females in each of four treatment groups. Day 0 represents the first day on which a female imago was wounded during traumatic insemination. There was a significant effect of treatment (Accelerated Failure Time Survival Analysis: Wald $\chi^2 = 68.5$, df = 3,116; $P < 0.001$). Septic treatments reduced survival (Wald $\chi^2 = 49.5$, df = 2,116; $P < 0.001$) while predictable feeding increased it (Wald $\chi^2 = 16.6$; df = 2,116; $P < 0.001$). There was also a significant interaction between the predictability of feeding and the immune insult treatments (Wald $\chi^2 = 5.5$; df = 1,116; $P < 0.05$). There was no difference between sterile treatments (z = 1.31; $P = 0.19$). (*B*) The egg-laying rate of each female in *A*: There is no difference in egg- laying rate between treatments (ANOVA; F = 1.54; df = 3,116; $P = 0.21$). (*C*) There were significant differences in the total number of viable egg produced per female (i.e., fitness) between treatments (ANOVA; F = 23.83; df = 3,116; $P < 0.0001$). Fitness in the septic predictable treatment was significantly higher than in the septic nonpredictable treatment (Tukey, Q = 3.9, $P = 0.029$), there was no difference in fitness between the sterile groups (Tukey, Q = 0.31, $P = 0.89$).

been well studied in relation to their interactions with the classic circadian clock (20, 21) as well as nutrient sensitive circadian clocks in tissues such as the liver of mammals (22) or the fat body of insects (23); these cues seem to be essential for homeostasis (24). However, many organisms from diverse taxa have feeding cycles of infradian (greater than a day) periodicity (25). In these cases, the circadian clock is unlikely to provide the endogenous signal that governs periodic physiological responses. Instead, biological rhythms may be achieved via other mechanisms that are linked with feeding status, such as oxidation-reduction cycles of metabolites, which generate physiological responses without transcription of circadian clock genes (26).

The association of these feeding cycles with an immune response may have its origins in how hematophagous insects control the proliferation of gut flora following large blood meals (27). Previous studies of hematophagous hemipterans (28, 29) identified lysozyme activity in the midgut as central to the defense of the gut epithelium against vectored parasites (although bed bugs do not vector any known parasites). The potential co-option of this immune response by the spermalege, and its alteration to peak on the day of feeding, (not 1–3 d after, as is the case in the midgut of *Triatoma brasiliensis*; ref. 28) is compatible with the idea that the blood meal can activate immune responses in other organs (30, 31), and play a controlling role in the feedback between feeding, immune and reproductive cycles (32). A recent study found that blood feeding in *Anopheles gambiae* served as trigger for an anti-*Plasmodium* anticipatory immune response in the hemolymph, even in naïve individuals (33), suggesting that selection pressures distinct from feeding (such as vector-borne disease infection, or in the case of the bed bug traumatic insemination) could lead to the evolution of anticipatory immune responses.

Although the bed bug RIA system described here is unlikely to have direct parallels in other mating systems, the notion that feeding and mating cycles may be linked to immune insult probably has wider applicability (5). Mating is associated with the opportunity for infection by specialist as well as opportunist pathogens and is often associated with physical damage to the female's integument (e.g., ref. 34). Moreover, fed females have greater reproductive potential and so males should ensure they mate with females who have, or are about to, feed (35). Given that females usually determine the time and place of copulation, often in relation to their fecundity, we predict that RIA is unlikely to be confined to bed bugs.

Methods

Study Organism. *C. lectularius* were maintained in an incubator at 26 ± 1 °C, at 70% relative humidity with a cycle of 12 L: 12 D. Bed bugs were fed on sterile blood using the protocol of Davis (36). After hatching from eggs, bed bugs have five larval instars before eclosing into adults. Each eclosion requires a blood meal (see *SI Appendix*, Fig. S1 for details of the insect's life cycle). Bed bugs are sensitive to variation in ambient gas partial pressures, temperature, and vibration. We minimize this variation in all protocols and make sure it is

replicated across all treatments. All procedures adhered to the UK's Animals (Scientific Procedures) Act 1986, and were covered by UK Home Office licenses.

Artificial Immune Insult. The immune insult associated with mating was replicated by piercing the cuticle and spermalege with a pulled glass capillary tube (made using a Narishige PC-10) with the same terminal dimensions as the male's paramere (3). Before each challenge, glass needles were sterilized with 100% ethanol and allowed to air dry before being dipped in a solution of microbes derived from the bed bugs' refugia (see ref. 4). Sterile treatment controls used an ethanol-sterilized glass needle.

Dissections. Spermalege samples were dissected from bed bugs at set time points during experiments. The spermalege was washed twice in ice-cold PBS and samples were homogenized in 2 μL of PBS for use in lytic zone assays.

Measuring LLA—the Lytic Zone Assay. A lytic zone assay measured the lytic activity of a homogenized sample against the peptidoglycan cell walls of *Micrococcus luteus* (37). Agar (10 mg/mL), *M. luteus* (5 mg/mL), Tween 20 (0.001%), and streptomycin sulfate (1 mg/mL) were heated until dissolution in distilled water and a thin layer (ca. 1 mm) poured into plastic plates. Wells (2 mm diameter) were cut in the agar to hold homogenized samples of spermalege and incubated at 30 °C for 48 h. The lytic zone surrounding samples were photographed using a digital camera and measured using Image-Pro Plus 5.1 image analysis software. Measures of LLA were standardized to units of lysozyme activity using a time-specific standard curve (38) and all measures were controlled for body size (LLA titers were adjusted acording to pronotum width measured with the same digital camera set up described above).

Negative Associative Learning. We generated two groups of virgin adult females that were fed weekly from hatching through to adulthood. After each adult blood meal (1 wk apart for 3 wk), females in the treatment group received septic needle challenges (see "immune insult" above), and while females in the control group received no treatment after feeding. Dissections of the spermalege we carried out on days 6 and 7 after the third treatment: lytic zone assays were used to quantify LLA titers.

The Effect of Feeding Predictability. Two groups of females were generated. The control group was reared from hatching through to adulthood (6 wk) on a 7 d feeding cycle (*SI Appendix*, Fig. S1). The treatment group was reared on feeding intervals of 5, 7, and 9 d in such a way that the mean cycle was always 7 d, but there was no predictable feeding date. Following imaginal eclosion, all females were fed to satiation and then received a 60 s mating (ensuring consistent ejaculate size; ref. 6). Seven days later, they were fed again and exposed to a septic needle challenge. LLA titers in the spermalege were measured at day −2, 0 (just before feeding), and days 1, 3, 5, and 7 (again just before feeding) after the challenge (see *SI Appendix*, Fig. S2 for schematic).

Longevity and Fitness Associated with Anticipatory Immunity. The study to measure the fitness consequences of anticipating traumatic insemination used the same protocol as above to generate feeding "predictability." After feeding, females in each group were subjected to either the septic treatment or a sterile treatment control. All females were mated for 60 s every 4 wk to maintain fecundity (6). We measured survival and fecundity in each of the four groups of females on a daily basis.

Blood Meal Size and Egg-Laying Rate. The quantity of blood ingested by a female was manipulated by interrupting feeding at different time intervals. Feeding duration was timed from the moment the female stylet was inserted until it was removed. We measured the difference in the female's wet body weight before and after feeding. All females were subsequently mated with a virgin male for 60 s and housed individually in 5 mL plastic tubes with a strip of filter paper on which they laid eggs. The total number of eggs laid by each female over 14 d was counted (egg laying had ceased in all tubes by this time).

Statistical Analyses. All statistical analyses were carried out using R (39). To deal with variance increasing with the mean, all statistical models were run as generalized linear models (GLMs) with gamma correction. Where interaction effects are described, the test statistics are preceded by the term being expressed, e.g., "day: challenge." There were two exceptions: first, when comparing spermalege lysozyme in septic treatment individuals between days 0 and 1. In this instance, variance did not increase with the mean and a paired *t* test was sufficient in analysis of the results. Second, we estimated lysozyme-like activity as a function of time and feeding predictability by fitting a flexible generalized additive model (GAM) using the *gamm4* package (40) treating LLA as the response variable. The use of GAMs allowed for nonlinearities in the relationship between time and feeding predictability on LLA and, in this instance, significantly outperforms the equivalent GLM ($\chi^2_{(111)} = 715.23$, $P < 0{\cdot}0001$). The model was specified as a tensor product smooth (41) constructed from the smooths of individual covariates time and feeding predictability. Minimum adequate models were determined from the stepwise exclusion of parameters from a full model using log likelihood tests (42). Model fit was assessed using the diagnostics specified within the *gamm4* package. This model allows the effects of each variable to be assessed and formally allows visualization of peaks, ridges, and valleys in LLA activity for each entrainment treatment.

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