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Research article

Effects of Cyclic Feeding and Starvation, Mating, and Sperm Condition on Egg Production and Fertility in the Common Bed Bug (Hemiptera: Cimicidae)

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Abstract

Bed bugs (*Cimex lectularius* L.) are now endemic in most major cities, but information regarding their basic biology is still largely based on research done over four decades ago. We investigated the effects of starvation, mating, sperm storage, and female and male age on egg production and hatch. Egg production cycles varied with the number of bloodmeals that females received. Once-mated females fed every 5 d had constant egg production for ~75 d followed by a monotonic decline to near zero. Percentage egg hatch was high and constant, but declined after ~30 d to near zero. To determine whether the age of the female, male, or sperm affected these patterns, we mated newly eclosed females to 60-d-old virgin males, 60-d-old mated males, or newly eclosed males. Females produced the most eggs when mated to young males, followed by old mated males, and then old virgin males; percentage hatch followed a similar pattern, suggesting that sperm stored within males for long was deficient. To examine effects of sperm stored within females, we mated newly eclosed females, starved them for 30 or 60 d, then fed them every 5 d. The 60-d starved group produced fewer eggs than the 30-d starved group, and both produced fewer eggs than young females mated to old or young males. Longer periods of sperm storage within females caused lower corresponding percentage hatch. These findings indicate egg production and hatch are governed by complex interactions among female and male age, frequency of feeding and mating, and sperm condition.

Key words: bed bug, *Cimex lectularius*, oviposition, mating, sperm quality

Although practically eradicated from developed countries in the 1960s, bed bugs (*Cimex lectularius* L.) have resurged globally in the past 20 yr (Doggett et al. 2004, Hwang et al. 2005, Reinhardt and Siva-Jothy 2007, Romero et al. 2007, Bencheton et al. 2011, Wang and Wen 2011). Our knowledge of bed bug basic biology and behavior is largely based on research done over four decades ago. Despite the corresponding resurgence in research on bed bugs in the past couple of decades, many elements of the basic biology and behavior of this pest are still poorly understood.

In longer lived insects, it has been shown that adult food quality and quantity affect egg production (Joern and Behmer 1998). Bed bugs are obligatorily hematophagous, and all life stages require a bloodmeal to develop and molt to the next life stage (Usinger 1966). Feeding regimes and host blood type have been investigated in several studies (Usinger 1966, Barbarin et al. 2013), which showed that survival of bed bugs is significantly influenced by the quality of the blood diet and the female's mating status (Barbarin et al. 2014). The impacts of defined cycles of feeding and starvation on fecundity and egg hatch have not been investigated in bed bugs. We hypothesized in this study that newly emerged adult females fed regularly would display constant and maximal egg production. However, if the food supply is interrupted by a starvation period, we hypothesized that egg production would be affected by the number of bloodmeals between starvation periods; females fed only once would have limited resources for egg production and might allocate more nutrients to somatic functions. More bloodmeals after a starvation period would be expected to support greater egg production. The dynamics of the patterns of egg production and the effects of these treatments on egg hatch are also of interest.

Bed bugs mate via traumatic insemination, where the needle-like highly sclerotized male aedeagus pierces the cuticle of the female's abdomen and delivers sperm into the female's hemocoel (Tatarnic et al. 2014). Understandably, this mating strategy can carry significant costs to the female, including the introduction of pathogens present on the aedeagus (Reinhardt et al. 2003) and water loss through the wound caused by piercing (Benoit et al. 2012). Bed bug

females are mated more frequently than necessary to maintain fertility (Siva-Jothy 2006), and higher mating frequencies lead to a higher death rate in females (Stutt and Siva-Jothy 2001). We therefore sought to describe patterns of egg production and egg hatch in bed bugs under controlled conditions where mating frequency and the feeding cycles are clearly defined.

Sperm age and sperm storage also affect egg production and hatch in insects. Females often have specialized cells in their spermathecae that maintain sperm in an optimal environment and provide nutrition, and in some insect species stored sperm remains fertile for several decades (Simmons 2001). Bed bug females can store sperm for up to 4 wk in the seminal conceptacles, after which they begin to lay infertile eggs (Davis 1956). This difference in sperm storage duration may be due to differing embryonic origins of spermathecae (ectodermal) and seminal concepticals (mesodermal; Cragg 1920). When males are older, sperm counts and the ability to deliver sperm to females can be reduced (Jones et al. 2006). Additionally, studies in other species have demonstrated that sperm senescence can occur within a male before ejaculation, especially when stored for long periods of time (Pizzari et al. 2008). These relationships have not yet been investigated in male bed bugs.

Here we investigate the effects of 1) starvation, number of feeding events, and mating, and 2) sperm age and sperm storage in males and females on egg production and egg hatch in bed bugs. Delineating these dynamics in bed bugs is important for understanding the evolution of bloodmeal processing strategies and traumatic insemination. This study will also be useful for comparative reproductive biology studies in other hematophagous insects.

Materials and Methods

Insects

An insecticide-susceptible strain of *C. lectularius* was used (Harold Harlan strain, collected in Ft. Dix, NJ, in 1973 and maintained for 35 yr on human blood, then on defibrinated rabbit blood in our lab since 2008). Bed bugs were maintained in an incubator at 27 °C and a photoperiod of 12:12 (L:D) h. Relative humidity within colony jars was ~50%. Virgin females and males were obtained by separating fifth instars from the colony and keeping them in isolation as they molted into adults.

Artificial Feeding System

Bed bugs were maintained on defibrinated rabbit blood (Quad Five, Ryegate, MT) using an artificial feeding system. Custom built water-jacketed glass feeders connected to a thermal circulator water bath (B. Braun Biotech, Inc., Allentown, PA) heated to 38 °C were used to feed bed bugs. Each feeder can hold up to 4 ml of blood, which was held in place by a membrane (NESCOFILM, Karlan, Cottonwood, AZ) stretched across the bottom of the feeder.

Feeding, Mating, and Starvation Assays

Virgin females 7–10 d after eclosion and males drawn at random from a colony were grouped into treatments in colony jars and allowed to feed on blood for 1 h. Colony jars were constructed by removing the bottom of 5-cm-diameter clear polystyrene wide-mouth threaded round jars (Consolidated Plastics Company, Inc., Stow, OH), and in its place heat-sealing plankton netting fabric (0.3-mm mesh opening, 0.2-mm fabric thickness; BioQuip Products, Inc., Compton, CA), through which bed bugs could feed. A piece of accordion-folded manila folder cardboard (6.6 by 4.4 cm) was placed inside each jar, so bed bugs could crawl up to the mesh and feed. Fully engorged females were placed individually into 20-ml borosilicate glass scintillation vials (Thermo Fisher Scientific, Waltham, MA) with a hole (~1 cm diameter) in the cap to allow for ventilation. Plankton netting fabric underneath the cap prevented insects from escaping. An insert made from black cardstock (Staples, Farmingham, MA) cut to 40 by 14 mm was placed in each vial as an oviposition substrate. In order to ensure oviposition on only one side of the insert, a piece of transparency film (3M, Minneapolis, MN) was affixed to one side of the insert using double-sided adhesive tape (Scotch Brand, 3M). One recently fed male was paired with each female, and left in the vial for 5 d. We did not observe or record the number of matings that occurred during these experiments.

For feeding, individual females from each respective treatment were grouped in a colony jar and allowed 1 h to feed. After feeding, females were re-sorted individually into clean 20-ml vials with a fresh insert for oviposition. Importantly, this design precluded the tracking of each individual female.

Treatment *a* (pink in Fig. 1A): females were fed and mated, then fed every 5 d. Every 20 d, females were re-mated as described above. There were 50 females in this treatment.

Treatment *b* (red): females were fed and mated, and then fed every 5 d, but only re-mated once around d 65. There were 35 females in this treatment.

Treatments *c* (blue), *d* (green), and *e* (black): females were fed and mated, fed 5 d later, and then subjected to a 10-d starvation period. In treatment *c*, females were fed at three consecutive times, 1 h every 5 d, followed by a 10-d starvation period. Treatment *d* females were fed only twice then experienced the 10-d starvation period, and treatment *e* females were fed only once between 10-d starvation periods. There were 35 females in each treatment *c*–*e*.

Bed bugs were maintained in an incubator at 27 °C and a photoperiod of 12:12 (L:D) h. Female mortality was monitored once daily and females that could no longer grasp the insert were considered dead. Eggs were maintained in the incubator for 15 d after females were removed from vials to allow all fertile eggs to hatch. Eggs and nymphs were then frozen for 24 h and subsequently counted. The experiment was terminated at 130 d.

Sperm Quality Assays: Storage in Female

Virgin females 7–10 d after eclosion were grouped into two treatments and mated as described above, but not fed. Females in Treatment *i* (orange; Fig. 4A) were starved for 30 d after the start of the assay to prevent egg production, and subsequently fed every 5 d until d 130 as described above. Females in Treatment *j* (teal) received the same procedure, but were instead starved for 60 d following the start of the assay. There were 50 females in Treatments *i* and *j*, and females were not re-mated during the remainder of the experiment. Mortality was monitored and frozen eggs and nymphs counted as described above. This experiment was also terminated at 130 d.

Sperm Quality Assays: Males

Virgin males 7–10 d after eclosion were separated into two groups (Treatments *g* and *h*; Fig. 4A) and fed every 10 d for 60 d. Males were fed and housed individually in 9-mm screw thread 2-ml borosilicate glass auto injection vials (Thermo Fisher Scientific). Males were allowed to feed until they fully engorged on a bloodmeal. In Treatment *h* (maroon), males were maintained without females and on d 60 of the experiment, each of these "old" virgin males was mated to a 7-10-d-old virgin female as described in previous assays.

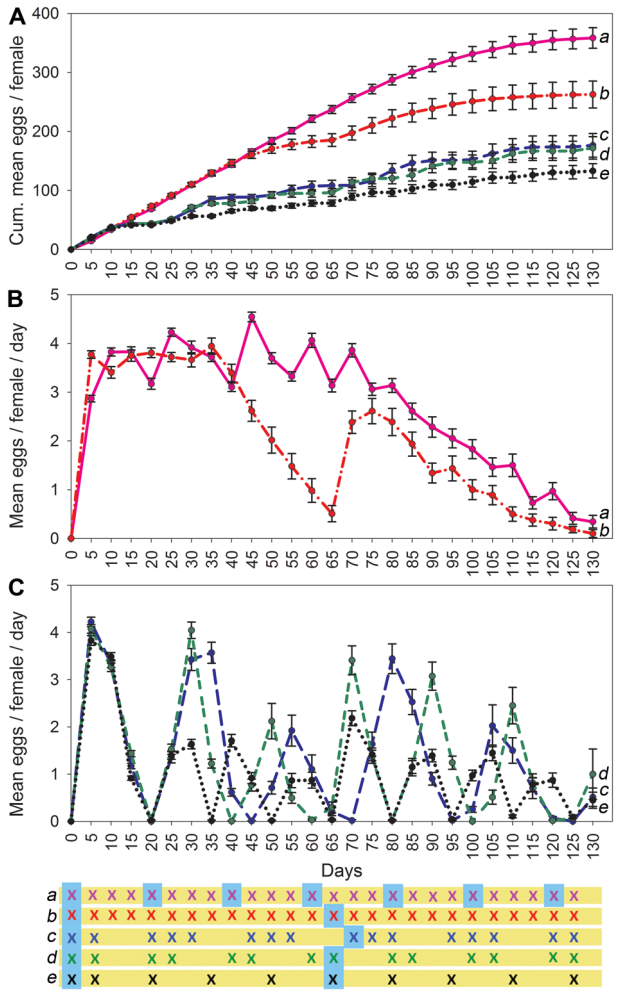


Fig. 1. Egg production in *C. lectularius* females over the 130-d experiment. (**A**) Cumulative mean (± SEM) number of eggs oviposited per female. (**B, C**) Mean (± SEM) daily eggs per female. Each curve represents a different treatment group. Females in the overall positive control group (*a.* pink, solid line: panels A, B) were fed every 5 d and allowed to re-mate every 20 d. Females in the continuous feeding control group (*b.* red, dashed line: panels A, B) were fed every 5 d and allowed to re-mate only once on d 65. All other females were also allowed to re-mate only once between d 65 and 70, but varied in the number of feeding events before and after 10-d starvation periods: females received three feedings events separated by 5 d (*c.* blue, long dashes: panels A, C), two feeding events separated by 5 d (*d.* green, short dashes: panels A, C), and one feeding between recurrent 10-d starvation periods (*e.* black, dotted line: panels A, C). X represents feeding, blue boxes represent presence of males for 5 d (mating), and yellow bars represent starvation periods. Beginning sample size was 50 females for the *a.* (pink, solid line) group and 35 for all other groups.

In Treatment *g* (light green), males were allowed to mate with non-experimental females every 20 d by cohousing one female with each male in 4-ml vials for 24 h. On d 60 of the experiment, each of these "old" males was mated to a 7–10-d-old virgin female as described in previous assays. Treatment *f* (purple) served as a control, where each virgin female was allowed to mate with a "young" 7–10-d-old virgin male for 5 d. Females were housed and treated in the same manner as described in previous assays, and fed every 5 d. Mortality was monitored and frozen eggs and nymphs counted as described above. There were 35 males and females per treatment, and females were not re-mated during this experiment. This experiment was also terminated at 130 d.

Data Analysis

Statistical analysis was completed using SAS version 9.4 (SAS 2013). To assess the effects of treatments, generalized linear models assuming negative binomial counts were used on log-transformed cumulative egg count data. These models were fit using PROC GLIMMIX separately for each 5 d feeding point, and *F*-ratios were reported to test for treatment effects. Models were fit separately because females were pooled by treatment during feeding; thus egg production in each specific individual female could not be tracked throughout the experiment. Pairwise comparisons among cumulative counts were made using *t*-tests, with the variance of a difference of estimated cumulative sums (diff) estimated using the sum of the feeding point and treatment-specific variances. That is, the variance of a cumulative sum is the sum of its component variances. Differences over time were considered significant when $t > 2$ consistently. For treatments where female feeding was delayed 30 and 60 d, we considered d 30 and d 60 to be d 0 for this analysis.

Eggs per female per day comparisons at specific feeding points were done using *t*-tests or an ANOVA with a Tukey HSD test for multiple comparisons. Data were square root transformed before analysis. For percentage hatch, data were arcsine transformed before ANOVA or *t*-tests were performed at specific feeding points.

To assess cyclicity in egg production, mean eggs oviposited per day were calculated for each female (replicate). Data were then Fourier transformed and a time series analysis was obtained by averaging over all replicates. Spectral densities were then estimated from the time series. To test whether a treatment effect existed, a pairwise comparison of the four periodograms was conducted. Normalized cumulative periodograms were generated as suggested by Diggle and Fisher (1991), and Kolmogorov-Smirnov distances were calculated between the normalized cumulative periodograms. A randomization procedure was used to shuffle labels for each treatment pair comparison for 10,000 random shufflings, and randomization *P*-values were generated.

Results

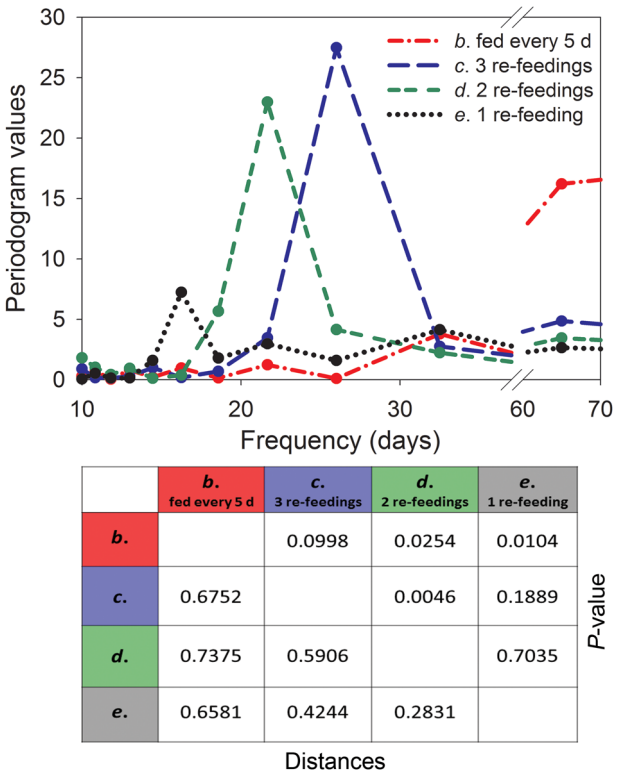
The number of feeding events following a 10-d starvation period affected egg production in bed bugs. Cumulative egg production at the end of the experiment (d 130; Fig. 1A) was generally greater with more feeding events. Control females fed every 5 d and allowed to re-mate every 20 d (*a.* pink in Fig. 1A) oviposited 358.3 ± 17.47 (SEM) eggs per female over 130 d. This group produced significantly more eggs than similarly fed females that were allowed to re-mate only once on d 65 (*b.* red in Fig. 1A; pink–red diff = 95.80, $t = 8.77$, $P < 0.0001$), which produced 262.5 ± 22.87 eggs per female. Egg production in other treatment groups, which were also allowed to re-mate only once between d 65 and 75, varied by the number of feeding events before and after the recurrent 10-d starvation periods. Generally, more feeding events resulted in greater cumulative egg production. Thus, females fed three and two times (*c.* blue and *d.* green, respectively, in Fig. 1A) produced only 176.3 ± 19.96 and 172.01 ± 18.98 eggs per female by d 130, respectively, significantly fewer than females fed every 5 d and allowed to re-mate every 20 d (*a.* pink) ([pink–blue diff = 181.99, $t = 15.90$, $P < 0.0001$] and [pink–green diff = 186.29, $t = 15.34$, $P < 0.0001$]), but these two treatments did not differ from each other (blue–green diff = 4.30, $t = 0.41$, $P = 0.681$). However, these treatment groups differed significantly at d 130 from females that were fed only once between the 10-d starvation periods (*e.* black in Fig. 1A; [blue–black diff = 43.23, $t = 5.16$, $P < 0.0001$] and [green–black diff = 38.93,

$t = 4.18$, $P < 0.0001$]), which oviposited 133.1 ± 12.14 eggs per female over 130 d. Differences among these treatments became evident early in this experiment. The effect of more frequent re-mating was evident on d 60, as females that mated every 20 d (*a*. pink) produced significantly more eggs than females re-mated once on d 65 (*b*. red) (pink–red diff = 38.65, $t = 4.70$, $P = 0.0008$). Females fed less frequently (one [*e*. black], two [*d*. green], and three [*c*. blue] re-feedings) diverged from the control group (*a*. pink) even earlier on d 15 ([pink–blue diff = 8.94, $t = 5.27$, $P < 0.0001$], [pink–green diff = 8.74, $t = 5.14$, $P < 0.0001$], and [pink–black diff = 11.46, $t = 6.83$, $P < 0.0001$]). The treatment effects of negative binomial models were highly significant at each time point, with all *F* ratios exceeding 13.03 (df = 5,103; $P < 0.0001$).

The 10-d starvation periods imposed a cyclic pattern on egg production, and the feeding regime appeared to modulate the frequency and amplitude of these cycles (Fig. 1B and C). When fed optimally every 5 d and allowed to re-mate every 20 d (*a*. pink in Fig. 1B) females rapidly attained a steady state, producing ~3.5 eggs per day per female until ~d 80, and egg production then monotonically declined to near zero by d 130. Fourier transform analysis did not detect any cyclicity in this treatment group. Preventing females from re-mating every 20 d caused a monotonic decline in egg production beginning about 40 d after the first mating, but this decline was rescued by allowing females to re-mate on d 65 (*b*. red in Fig. 1B). Thus, re-mating once imposed a clear second cycle in optimally fed females with a period of 65 d, coincident with the period of re-mating (Fig. 2).

The 65-d period in once-re-mated females was further disrupted by recurrent 10-d starvation periods in all three treatments, but the frequency of egg production cycles varied with the frequency of re-feeding females after each starvation period. Females fed only once exhibited rapid attenuated cycles of egg production (*e*. black in Fig. 1C) with a fundamental frequency at 16.2 d (Fig. 2). Females fed twice before the next 10-d starvation period had cycles of longer periods, with a fundamental frequency at 21.6 d, each with higher amplitudes, i.e., greater egg production per d (*d*. green in Fig. 1C, Fig. 2). Finally, females fed three times over 15 d between 10-d starvation periods exhibited even longer cycles with a fundamental frequency of 26 d (*c*. blue in Fig. 1C, Fig. 2). An overall comparison among periodograms was significant ($D = 19.2$; df = 2; $P = 0.0002$). Kolmogorov–Smirnov distance (K–S) post hoc comparisons of periodograms revealed that the periods for females fed every 5 d (*b*. red) and females re-fed three times after each 10-d starvation period did not differ (K–S = 0.6753, $P = 0.998$), but the former did differ significantly from the periods for females re-fed only once (*e*. black; K–S = 0.6581, $P = 0.0104$) or twice (*d*. green; K–S = 0.7375, $P = 0.0254$) after each 10-d starvation period. Females re-fed only twice also exhibited a significantly shorter period than females re-fed three times (*c*. blue; K–S = 0.5906, $P = 0.0046$), but not relative to females re-fed once (*e*. black; K–S = 0.2831, $P = 0.7035$).

Although cyclical patterns of egg production are created by feeding, starvation, and mating frequency, the hatch rates of nymphs were largely unaffected by the different feeding regimes (Fig. 3). At d 5 the percentage hatch for all treatment groups was nearly 100% and the groups did not differ from each other ($F = 0.51$; df = 4; $P = 0.73$). As percentage hatch declined around d 30 in all groups that were not re-mated (i.e. *a*. pink group re-mated every 20 d excluded), there were likewise no differences among groups at d 55 ($F = 1.04$; df = 3; $P = 0.38$), when hatch rate was ~30%. Although significant divergence became apparent at d 95 ($F = 6.97$; df = 2; $P = 0.0017$) and a Tukey HSD test revealed that hatch rate was higher in females re-fed twice (*d*. green) than in the control females



	b. fed every 5 d	c. 3 re-feedings	d. 2 re-feedings	e. 1 re-feeding
b.		0.0998	0.0254	0.0104
c.	0.6752		0.0046	0.1889
d.	0.7375	0.5906		0.7035
e.	0.6581	0.4244	0.2831	

P-value

Distances

Fig. 2. Periodograms of eggs oviposited by *C. lectularius* females over the 130-d experiment. Each curve represents a different treatment combination, as in Fig. 1: females in the continuous feeding control group (*b*. red) were fed every 5 d and allowed to re-mate only once on d 65. All other females were also allowed to re-mate only once between d 65 and 70, but varied in the number of feeding events before and after 10-d starvation periods: females received three feeding events separated by 5 d (*c*. blue), two feeding events separated by 5 d (*d*. green), and one feeding between recurrent 10-d starvation periods (*e*. black). Data were Fourier transformed and analyzed using a time series analysis. Differences among groups were assessed with Kolmogorov–Smirnov distances. The *a*. group (pink in Fig. 1) was excluded from this analysis because no cyclic pattern of oviposition was apparent. Beginning sample size was 35 for all groups.

(that fed every 5 d [*b*. red] or fed every 5 d and allowed to re-mate every 20 d [*a*. pink]), sample sizes were small at that point due to female mortality.

To separate the effects of sperm age when stored in the male, we 1) mated young females but prevented egg production to age the sperm within the female, or 2) mated females to older virgin males. Both female age and sperm age affected egg production (Fig. 4). Frequently re-mated and re-fed females (*a*. pink in Fig. 4A) oviposited 256.5 ± 7.43 eggs per female over 70 d. However, when sperm storage was imposed on young mated females for 30 d (*i*. orange) or 60 d (*j*. teal) before they were fed, cumulative mean egg production decreased dramatically to 89.6 ± 10.40 and 22.8 ± 7.99 eggs per female by d 120 and d 130, respectively (Fig. 4A). Female age per se was not as important as the aging sperm within the female—virgin females first mated at age 60 d produced fewer eggs per day per female over the next 70 d (see below). Likewise, the 30 d (*i*. orange) and 60 d (*j*. teal) sperm stored in female groups began to diverge very early from the male stored sperm groups and fed every 5 d, allowed to re-mate every 20 d group (*a*. pink), both at d 5 ([pink–orange diff = 12.38, $t = 21.59$, $P < 0.0001$] and [pink–teal diff = 14.14, $t = 25.86$, $P < 0.0001$]). The treatment effects of negative binomial models were highly significant at each time point, with all *F* ratios exceeding 21.83 (df = 9,271; $P < 0.0001$).

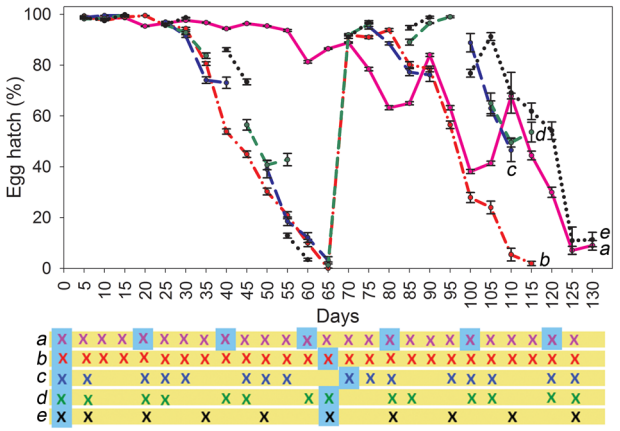


Fig. 3. Mean percentage (± SEM) egg hatch in *C. lectularius* females over the 130-d experiment. Treatments are as in Fig. 1: Females in the overall positive control group (*a*. pink) were fed every 5 d and allowed to re-mate every 20 d. Females in the continuous feeding control group (*b*. red) were fed every 5 d and allowed to re-mate only once on d 65. All other females were also allowed to re-mate only once between d 65 and 70, but varied in the number of feeding events before and after 10-d starvation periods: females received three feedings events separated by 5 d (*c*. blue), two feeding events separated by 5 d (*d*. green), and one feeding between recurrent 10-d starvation periods (*e*. black). X represents feeding, blue boxes represent presence of males for 5 d (mating), and yellow bars represent starvation periods. Gaps on the graph resulted when fewer than 10 females in a given treatment produced eggs (usually corresponding to 10-d starvation periods). Beginning sample size was 50 females for the *a*. (pink) group and 35 for all other groups.

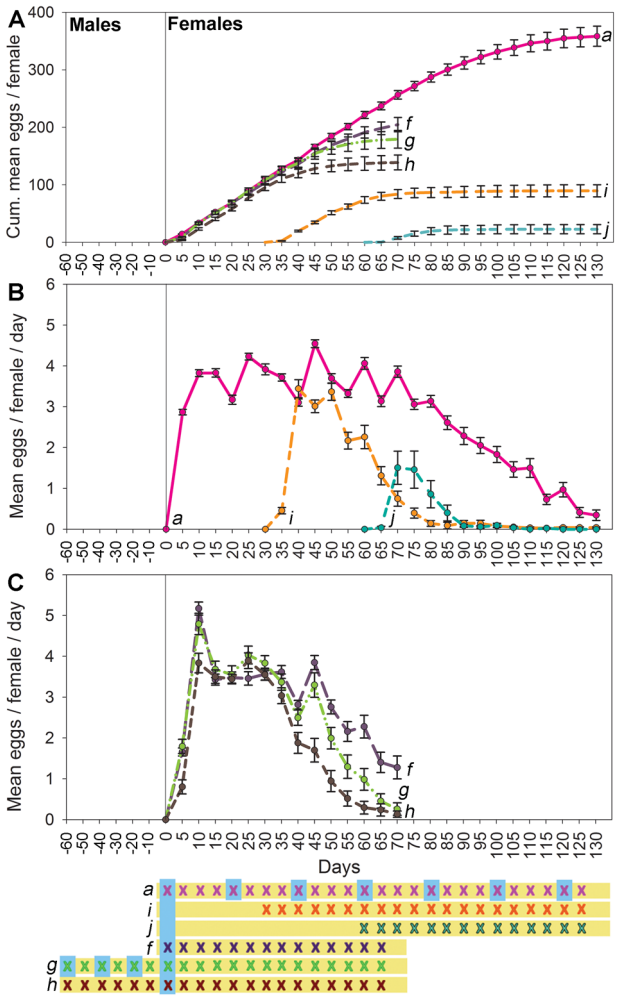


Fig. 4. Egg production in *C. lectularius* females in relation to sperm storage and quality. (**A**) Cumulative mean (± SEM) number of eggs oviposited per female. (**B, C**) Mean (± SEM) daily eggs per female. Each curve represents a different treatment group: Females in the overall positive control group (*a*. pink: panels A, B) were mated to young males, fed every 5 d and allowed to re-mate with young males every 20 d. Young females mated to young virgin males and fed every 5 d (*f*. purple: panels A, C). Young females mated to 60-d-old males that were allowed to mate every 20 d (*g*. light green: panels A, C). Young females mated to 60-d-old virgin males (*h*. maroon: panels A, C). Females mated to young males at d 0, starved to d 30, and fed every 5 d beginning on d 30 (*i*. orange: panels A, B), and females treated as above, but fed beginning on d 60 (*j*. teal: panels A, B). X represents feeding, blue boxes represent presence of males for 5 d (mating), and yellow bars represent starvation periods. Where male treatments are not indicated, young virgin males were used. Beginning sample size was 50 females for the *a*. (pink) and *i*. (orange) groups, and 35 for all other groups.

A significant effect of frequent re-mating was indicated at d 70 (pink–purple diff = 51.79, $t = 4.99$, $P < 0.0001$; Fig. 4A) by lower egg production in females mated to young males and then fed regularly every 5 d (*f*. purple in Fig. 4A), compared with similarly fed females that were allowed to re-mate every 20 d (*a*. pink).

The effect of sperm age in males was also examined. Two male groups were compared: Males that were mated every 20 d, and then mated to young virgin females (*g*. light green in Fig. 4) were compared to same age (60 d) virgin males (*h*. maroon in Fig. 4). Females mated with the old virgin males produced fewer eggs by d 45 (132.9 ± 10.10) than females mated with multiply mated males (164.2 ± 11.03; light green–maroon diff = 26.11, $t = 4.71$, $P < 0.0001$). Cumulative egg production in females mated to young virgin males (*f*. purple) was higher than in females mated to old virgin males (*h*. maroon) starting on d 45 (155.2 ± 6.35; purple–maroon diff = 27.10, $t = 5.20$, $P < 0.0001$). These results suggest that males that were able to recycle their sperm through recurrent mating events produced higher quality sperm that resulted in greater egg production.

The patterns of daily oviposition in these treatments are shown in Fig. 4B, C. Females fed every 5 d and allowed to re-mate every 20 d achieved a steady rate of ~3.5 eggs per day per female (*a*. pink in Fig. 4C). When unfed females were forced to store sperm for 30 d and then re-fed (*i*. orange), they achieved similar mean daily egg production on d 40 ($F = 0.05$; df = 1; $P = 0.82$) but declined soon after at d 45 ($F = 40.87$; df = 1; $P < 0.0001$) and beyond. Females that stored sperm for 60 d (*j*. teal) oviposited up to 1.5 eggs per day per female, significantly fewer eggs than same age females that were fed every 5 d and allowed to re-mate every 20 d (at d 70, the peak of egg production for the former group: $F = 56.73$; df = 1; $P < 0.0001$), even though the latter had oviposited many more eggs before d 60. Percentage egg hatch followed a similar pattern, with similar maximum hatch for the 30 d stored sperm group and the control ($F = 1.61$; df = 1; $P = 0.208$) of females the same age (Fig. 5A). As for egg production, the maximum egg hatch for females that stored sperm for 60 d was <60%, compared to ~80% for same aged control females ($F = 10.38$; df = 1; $P = 0.0021$).

Male age had a less pronounced effect on egg production and egg hatch. The peaks of egg production and egg hatch rate were similar in all three treatment groups (young males, 60-d-old virgin males, 60-d-old males allowed to re-mate every 20 d; Fig. 4B). Differences among these treatments became evident around d 40 when egg production was significantly lower in females mated to old virgin males ($F = 8.00$; df = 2; $P = 0.0007$). By d 50, females mated to the 60-d-old frequently mated males (*g*. light green in

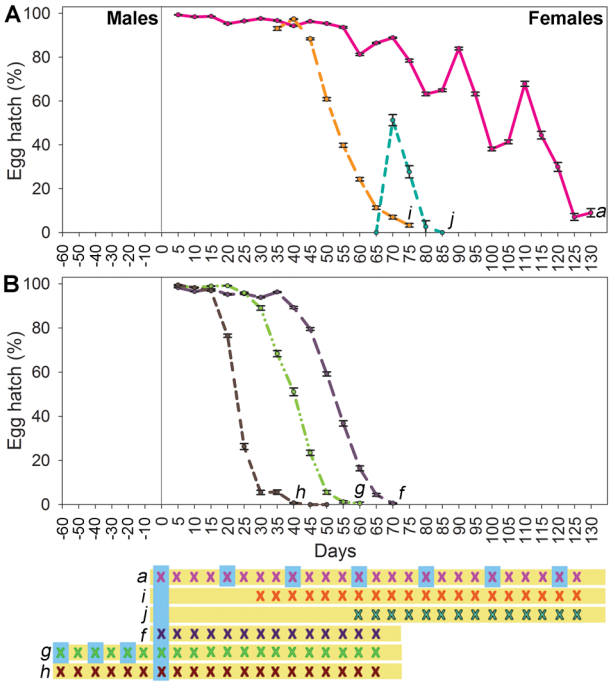


Fig. 5. Mean percentage (± SEM) egg hatch in *C. lectularius* females in relation to sperm storage and quality. Each curve represents a different treatment group: Females in the overall positive control group (*a.* pink: panel **A**) were mated to young males, fed every 5 d and allowed to re-mate with young males every 20 d. Young females mated to young virgin males and fed every 5 d (*f.* purple: panel **B**). Young females mated to 60-d-old males that were allowed to mate every 20 d (*g.* light green: panel B). Young females mated to 60-d-old virgin males (*h.* maroon: panel B). Females mated to young males at d 0, starved to d 30, and fed every 5 d beginning on d 30 (*i.* orange: panel A), and females treated as above, but fed beginning on d 60 (*j.* teal: panel A). X represents feeding, blue boxes represent presence of males for 5 d (mating), and yellow bars represent starvation periods. Where male treatments are not indicated, young virgin males were used. Beginning sample size was 50 females for the *a.* (pink) and *i.* (orange) groups, and 35 for all other groups.

Fig. 4B) produced fewer eggs per day per female than females mated to young virgin males ($F = 6.46$; df = 1; $P = 0.0138$). These results suggest that young males produced the most fertile sperm, sperm quality declined during long-term storage in males, and that mating allowed males to replenish more fertile sperm. Egg hatch rate showed the same pattern, but with clearer separation among the treatments beyond d 20 (Fig. 5A). The older virgin male group diverged from the other groups around d 20 ($F = 11.23$; df = 2; $P < 0.0001$) and beyond, and the older mated male group diverged from the young virgin male group at d 35 ($F = 12.03$; df = 2; $P = 0.001$).

Discussion

Egg production in bed bugs is governed by feeding–starvation patterns, mating status, and mating frequency of the female, and sperm quality, which in turn is affected by its time in storage within the male and within the mated female. Our experiments were designed to uncouple these effects. Females that were fed every 5 d and allowed to re-mate every 20 d produced eggs at a steady rate of ~3.5 eggs per female per d for ~70 d, followed by a gradual senescence to d 130 (when we terminated the experiment), resulting in 130 d (approximating "lifetime") fecundity of ~360 eggs per female. Preventing females from re-mating for 65 d caused a rapid decline in egg production after d 40, but egg production was substantially rescued by allowing females to re-mate for 5 d between d 60 and d 65. The latter females had substantially lower cumulative egg production (~260 eggs per female) than frequently re-mated females. Egg hatch followed a similar pattern as egg production. These results are consistent with previous findings that the mating rate of female bed bugs does not affect the overall rate of egg production as long as females are mated a minimum of every 4 wk to maintain fertility (Davis 1964, Stutt and Siva-Jothy 2001).

Treatments that imposed a starvation period and limited the number of feeding events dramatically affected egg production. Starvation imposed a cyclic pattern of egg production. Frequent feeding events (three feedings at 5-d intervals) resulted in high amplitude long cycles of egg production, whereas infrequent feeding events (one or two feedings) attenuated the amplitude of more frequent cycles. As expected, cumulative egg production reflected the pattern of feeding frequency. Likewise, egg hatch followed the pattern of egg production so that as egg production declined, so did viability of the eggs.

A constraint of this research was that feeding our large number of experimental females individually on such a rigorous schedule would have been too time consuming. Therefore, female identity was lost when females were grouped by treatment for group feeding and then separated again. In future analyses, it would be instructive to follow patterns of egg production in individual females throughout her lifetime. Moreover, we binned egg production into 5-d intervals from which we estimated eggs per female per day. In future studies, it would be useful to understand individual variation in daily egg production and egg hatch.

In our starvation and mating assays, mean eggs per female per day was approximately double that found in another recent study (Polanco et al. 2011b). There are several reasons for this difference. For example, we used a laboratory strain that had been maintained in colony for over 40 yr, whereas Polanco et al. (2011b) used a field-collected strain. Different strains can differ in life table parameters (Barbarin et al. 2014). Most importantly, however, Polanco et al. (2011b) fed bed bugs every 11 d to allow females to complete an entire oviposition cycle, whereas we fed females every 5 d; as our findings show, bugs fed less often oviposit fewer eggs daily than more frequently fed bugs. In addition, to allow females to re-mate, each female was housed with a male for recurrent 5-d intervals in our study, instead of continuously. The continuous presence of males in the Polanco et al. (2011b) study could have lessened egg production by harassing females and imposing on them a potentially significant cost of traumatic insemination (Stutt and Siva-Jothy 2001). When males are present, females are mated more often than necessary and live shorter than when mated optimally (Siva-Jothy 2006). Overall, results of lifetable and reproductive potential experiments in bed bugs are highly variable among studies, partly because different bed bug strains have been used, also with differing levels of insecticide resistance, but mainly because different environmental conditions (e.g., temperature), feeding regimes (e.g., blood type, feeding frequency), and mating routines (e.g., frequency, number of males, male age) were used (Johnson 1941; Davis 1964; Polanco et al. 2011a,b; Barbarin et al. 2014; Gordon et al. 2015). Our results underscore the importance of optimizing these conditions for lifetable studies.

Sperm age and sperm storage substantially affected egg production in bed bugs. Because egg production is intimately linked to bloodmeals, when females were starved for 30 or 60 d after mating, sperm was not utilized and instead was stored within the female until she was fed so egg production could commence. Under these conditions, the quality of sperm stored within the female

deteriorated rapidly, as evidenced by low egg production in these females. This finding is consistent with the observations that bed bug females become infertile as oxygen radicals increase in stored sperm (Reinhardt and Ribou 2013). In natural situations, however, it may be unnecessary for bed bugs to store sperm for long periods, as they live in aggregations where females are likely mated frequently by males (Stutt and Siva-Jothy 2001).

Sperm quality deteriorates at a slower rate when sperm is stored in males rather than in females. Young males have the highest quality sperm and lead to the greatest egg production in both young and older females. Females mated to virgin males that had been prevented from mating for 60 d produced fewer eggs. This effect could be attributed either to male age (60 d old) or to the accumulation of some senescent sperm in the absence of mating. The latter was supported by the observation that sperm quality could be partially rescued by allowing males to mate every 20 d before mating with the experimental young virgin female. Although females in both groups produced eggs nearly at the same level as females that had been mated to young males, clear differences in egg production were evident ~40 d after mating, and significant differences became evident even sooner in egg hatch rate, with better performance by females that mated with males that had mated every 20 d. These differences were likely due to older senescent sperm being cycled out in males during frequent matings. Indeed, the need to recycle sperm may explain the frequent observation of male–male matings (Ryne 2009), especially in male bed bugs older than 50 d.

The results of this study could shed light on real life situations where bed bugs experience longer periods without access to a host, as for example in a hotel or hospital room, or an apartment or home that are vacated periodically. Even with intermittent feeding, bed bugs can achieve significant egg production, albeit less than when they have regular access to a host, which accentuates the speciousness of the strategy of vacating a room or home with the hopes that all bed bugs will die. A central theme in life history theory is the trade-off between reproduction and survival, where reproduction reduces life span (Stearns 1992, Travers et al. 2015). It is possible that under conditions of periodic starvation and intermittent egg production in the field, females live longer and distribute their eggs over a longer time period than optimally fed females.

Females can store sperm and produce fertile eggs for ~60 d after access to a male for 5 d. Thus, a single mated foundress of a new infestation can produce ~180 eggs during this time, while her early progeny become adults. Bed bugs are particularly resilient to deleterious effects of inbreeding (Fountain et al. 2015), so the foundress can mate with one or more of her sons and regain full reproductive potential. Additionally, sperm quality declines in males that have not had the opportunity to mate for up to 60 d, independent of the male's normal ageing. Yet these males are still able to fertilize some, though fewer, eggs. Thus, lone founder males would be able to retain some sperm quality over several months until females arrive.

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