Increasing Coffee Berry Borer (Coleoptera: Curculionidae: Scolytinae) Female Density in Artificial Diet Decreases Fecundity

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ABSTRACT Three experiments were conducted to determine the influence of number of coffee berry borer, *Hypothenemus hampei* (Ferrari) (Coleoptera: Curculionidae: Scolytinae), females (one, two, or five) reared in artificial diet on fecundity and subsequent development of larvae, pupae, and adults. Our results demonstrated that increasing female density from one to two or five individuals did not result in the expected two- or five-fold increase in progeny, despite ample food resources available. Instead, decreased fecundity was observed with increasing density for all experiments. The mechanism reducing fecundity was not identified, but possibly, volatiles are being produced (e.g., host-marking pheromones). The decrease in fecundity may explain why infestations of only one colonizing female per berry are the norm in the field.

KEY WORDS artificial rearing, bark beetles, *Hypothenemus hampei*, host-marking pheromones

The coffee berry borer, *Hypothenemus hampei* (Ferrari) (Coleoptera: Curculionidae: Scolytinae), is the most devastating insect pest of coffee (*Coffea arabica* L. and *Coffea canephora* Pierre ex Froehner) worldwide and has now been reported in most coffee-producing countries (Vega 2008). Damage begins after a female bores a hole in the berry and builds a gallery in the endosperm (i.e., seed), where eggs are deposited. After hatching, the larvae start consuming the seed (each coffee berry has two seeds), thus reducing both the yield and the quality of the marketable product. The larvae pupate within the berry, followed by sibling mating inside the berry, where there is a 10:1 sex ratio favoring females. Males never leave the berry, whereas inseminated females emerge and search for another berry in which to oviposit (Vega 2008). Females can oviposit throughout their entire life span (Corbett 1933) and can live for up to 282 d (Bergamin 1943).

In nature, coffee berries are attacked by only a single female (Jaramillo et al. 2006), and finding a berry bored by more than one female is rare (F.E.V., personal observation). The only report we are aware of where more than one female per berry is reported was written by Tothill (1940) about Uganda. He stated, "During intense infestation more than one female beetle (with corresponding number of entrance holes) will attack a single berry and the damage is much accelerated." One might argue that the number of coffee berries in a coffee plantation is not a limiting resource, and that this abundance explains the lack of multiple infestations per berry; i.e., with many berries and few beetles, if the females' distribution is random (counts per berry follow a Poisson distribution), the probability that berries harbor more than one female is low. However, if the limiting resource for a female and its progeny is the seed inside the berry, it is advantageous for a female (to maximize the survival of her brood) to be the sole colonizing female for that berry. Reports on the maximum number of eggs laid per female within a berry vary. Corbett (1933) reported 60 eggs; Bergamin (1943) found 74 eggs; and more recently, Jaramillo et al. (2009) reported nearly 300 eggs in a single berry. Thus, there is ample demand by the progeny for a limited resource, i.e., the endosperm, to complete development from egg to adult.

Baker (1984) reported that field infestations of the coffee berry borer tend to occur in aggregations, whereas Ochoa Milian and Decazy (1987) found that when infestations exceed 10%, the infestation is no longer aggregated and beetles infest berries at random. However, neither of the preceding two reports indicated that berries were infested by more than one insect, even under aggregated infestations. Working in Mexico, de Kraker (1988) conducted experiments that showed that infested berries seemed to repel other coffee berry borers. Vega et al. (2009) hypothesized that one possible explanation for the lack of multiple colonizing females inside one berry could be the production of a host-marking pheromone produced by the initial colonizing female. Other possible explanations are that female coffee berry borers avoid berries with a physical hole (bored by another female), that

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they are sensitive to volatiles from feces or other life stages present in an occupied berry, or that the berry itself releases volatiles when attacked and occupied.

Our objective was to determine whether forced cohabitation by coffee berry borers had an effect on the number of eggs laid, and the number of larvae, pupae, and adults (all globally referred to as "fecundity" hereafter). If so, this might explain why, in the field, females prefer noncolonized berries. The availability of artificial diets to rear the insect in the laboratory (Villacorta and Barrera 1993, Pérez-López et al. 1995, Portilla-Reina 1999, Portilla and Streett 2006) is ideal for testing this hypothesis. We conducted three separate experiments aimed at assessing fecundity differences of a single female versus those forced to cohabit in groups of two or five.

Materials and Methods

Insect Rearing. Coffee berry borers were reared using Villacorta and Barrera (1993) artificial diet, with the following modifications: 21 g of agar (A9915, Sigma-Aldrich, St. Louis, MO), 0.6 g of cholesterol (101380, MP Biomedicals, Inc., Solon, OH), 0.23 g of sorbic acid (S7420, Sigma-Aldrich), 0.5 g of Vanderzant vitamin mixture for insects (V1007, Sigma-Aldrich), and no formaldehyde. Another difference is that we used sterile 20-ml clear glass liquid scintillation vials for rearing (28 mm o.d. by 61 mm height, with cap attached; 986561, Wheaton Science Products, Millville, NJ). Insects were reared in the dark in a growth chamber set at 28°C.

Adult females used in the experiments were obtained by individually placing colony females in diet-containing vials and then keeping track of their progeny. This allowed aging the progeny by keeping track of the first adult emergence until sufficient adults were available for each experiment. This setup allowed for mating with siblings before initiating the experiment. For each experiment, female adults were mixed (randomized) before placing into vials. For all experiments, ≈6 ml of diet was placed in each vial; this amount of diet was sufficient to run the experiments without having to replenish the diet. Here and in the experiments described below, vials were kept in the dark in a Series 101 growth chamber (Percival Scientific, Inc., Perry, IA) set at 25°C. Under these conditions, adults start emerging 30–40 d after the eggs are laid.

Experiments. Due to the difficulty in obtaining a large number of females of a known age, we conducted experiments on three different dates, and at each time, we had different numbers of females available. The three experiments were focused in testing the same null hypothesis (see Statistical Analysis).

Experiment 1. Adult females at the initiation of the experiments were ≤72 d old. One, two, or five females (the three treatments) were placed in each vial, with 10 replicates per treatment. Each treatment was repeated two times, i.e., 60 vials total were used in the experiment. At 40 d postinitiation, eggs, larvae, pupae and adults in 20 vials per treatment were counted by carefully dissecting the diet.

Experiment 2. Adult females at the initiation of the experiments were ≤142 d old. Either one or two females (the two treatments) were placed in each vial, with 20 replicates per treatment. The one-female vial treatment was repeated two times, and the two-female vial treatment was repeated three times, i.e., 100 vials total were used in the experiment. At 40 d postinitiation, eggs, larvae, pupae and adults were counted by carefully dissecting the diet.

Experiment 3. Adult females at the initiation of the experiments were ≤73 d old. One, two, or five females (the three treatments) were placed in each vial. For days 10–40 postinitiation, nine vials per treatment were destructively dissected every 10 d, and the total numbers of eggs, larvae, pupae, and adults were counted. Thus, there were 108 vials in total, with 27 vials (nine vials for each one of the three treatments) at each one of the four sampling dates. For days 50–120, eight vials per treatment were destructively dissected every 10 d. Thus, there were 240 vials in total, with 27 vials (nine vials for each one of the three treatments) at each one of the eight sampling dates. The entire experiment consisted of 348 vials. Mean fecundity for this experiment (Fig. 1) is presented on a per original female basis to better depict how fecundity was affected by forced cohabitation and to be consistent with the statistical analysis.

Statistical Analysis. The counts of the various life stages were modeled using a quasi-Poisson distribution in the generalized linear models framework. The quasi-Poisson distribution assumes that the counts are over-dispersed relative to a Poisson distribution (as occurs for most real biological count data). In a Poisson distribution, the mean and variance are equal while in the quasi-Poisson distribution, a scale parameter (a multiplier for the variance) is separately estimated. Because the counts of the life stages were destructive, i.e., an individual was not followed over time, the tests for each life stage and at each time point were independent, and do not require a multiple testing adjustment. However, the total counts are sums of the other counts, so a test using total counts is not independent from tests of their components and was not made.

We used contrasts, contrasting the results from vials with one female to those with more than one female. If the females do not interfere with each other (i.e., they behave independently), then the progeny count of two females should be double that of one female, and five times for the vials with five female. Thus, the contrasts testing the null hypothesis of no interference among females compares the progeny count from one female vials to half the progeny count of two female vials, and to one fifth the progeny count from five female vials. To reject the null hypothesis there must be significantly fewer than twice the number of progeny in two female vials, or five times the number of progeny in five female vials, compared with one female vials. This is in the spirit of traditional null hypothesis formulation, with a null hy-

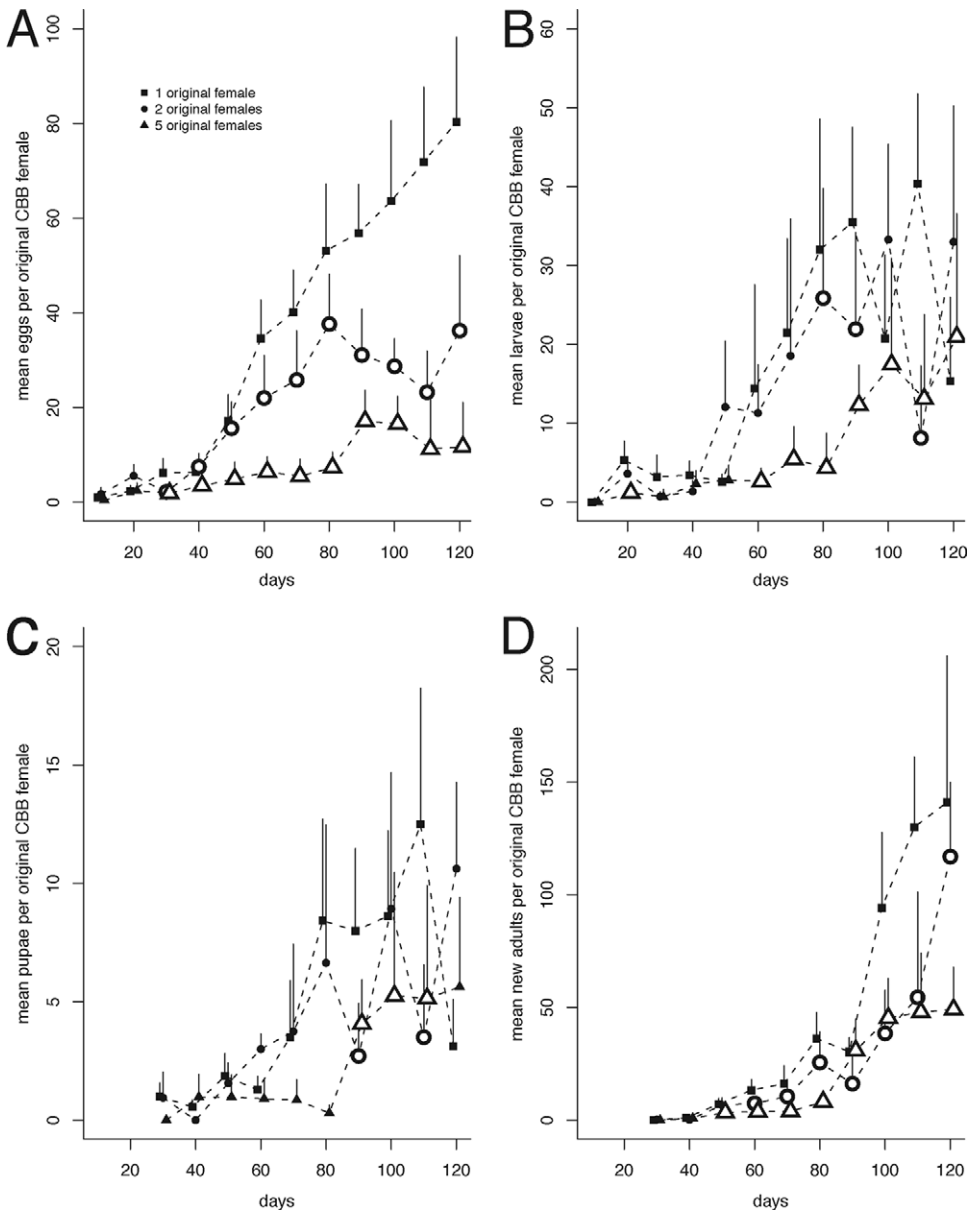

Fig. 1. Mean + SEM per original coffee berry borer (CBB) female for eggs (A), larvae (B), pupae (C), and adults (D) per original female, 10–120 d postinfestation. Larger hollow symbols indicate significant contrasts with the one-female vials at $\alpha = 0.05$. To avoid clutter, only the upper SEM bar is depicted.

pothesis of no treatment (cohabitation) effect. The power of these tests is reduced when the counts are small, and made additionally conservative because we have allowed for overdispersion (so average differences must be greater to reject the null hypothesis). We used the glm function of the R statistical program (R Development Core Team 2009), with the gmodels package to create and test the contrasts.

For ease of interpretation, all figures and tables present data on the original scale (counts), with the means and standard errors calculated in the usual way, except for totals, where the SEs were calculated by summing over variances for each life stage, weighted by contributed proportions. However, the modeling for this kind of count data, in the generalized linear models framework, uses the log link, i.e., the linear function predicts the expected log counts. This can, and does, lead to apparent discrepancies between functions of data (e.g., means) on the original scale and model testing results (on the log scale), because the log of a mean of counts is not equal to the mean of logs of counts, and the difference between the quantities increases with increasing overdispersion of

Table 1. Mean ± SEM per female for each insect stage 40 d after placing one, two, or five females in vials containing artificial diet

Developmental stage	No. females in vial		
	1	2	5
Eggs	7.1 ± 1.72 (143)	6.1 ± 2.66* (243)	2.6 ± 3.81* (262)
Larvae	13.1 ± 3.01 (263)	4.1 ± 2.15* (164)	1.7 ± 2.76* (168)
Pupae	1.3 ± 0.38 (27)	0.9 ± 0.55 (36)	0.1 ± 0.28 (14)
Adults	3.6 ± 0.84 (73)	1.8 ± 0.65* (74)	1.3 ± 0.54* (127)
Total	25.1 ± 2.38 (506)	12.9 ± 2.21 (517)	5.7 ± 2.99 (571)

Each treatment had 10 replicates and was repeated two times, for a total of 60 vials. Numbers in parenthesis represent total numbers for each insect stage summed over vials. Contrasts tested whether there were significantly fewer than twice or five times the number of progeny in two females or five females per vial, respectively, when compared with one female per vial. An asterisk indicates that the null hypothesis was rejected ($\alpha = 0.05$).

the data. We point out in the Results an example of such a discrepancy.

Results

Experiment 1. Null hypotheses were rejected in six of the eight tests (Table 1). The mean numbers of eggs, larvae, and adults in vials containing two or five females were significantly fewer than expected, compared with one female vials. Compared with all other stages the number of pupae was always very low; consequently, significant differences were not detected. Overall, the total number of insects counted at the end of the 40-d period was very similar for one (506 insects), two (517 insects), or five (571 insects); that is, totals were similar irrespective of the number of original females per vial.

Experiment 2. The null hypothesis was rejected for eggs and larvae (two of four tests; Table 2), indicating that the mean numbers of eggs and larvae in vials containing two females were significantly fewer than expected, when compared with vials containing one female. The mean number of insects produced for one female per vial was 13 and eight when two females were present. In total, 530 insects were counted at 40 d for the 20 vials containing one female, and 982 were counted in vials containing two females (Table 2). In this experiment, two-female vials produced about twice as many pupae and adults as one-female vials (i.e., pupae and adult progeny were not reduced by cohabitation, although eggs and larvae were).

Experiment 3. The null hypothesis was rejected in most instances for eggs (Fig. 1A), larvae (Fig. 1B), pupae (Fig. 1C), and adults (Fig. 1D); and for many pupae (Fig. 1C), where counts were much lower. The mean numbers of eggs per original female from day 30 to 120 in vials originally containing two or five females were significantly fewer than expected, compared with vials containing one female (Fig. 1A). For mean number of larvae per original female (Fig. 1B), vials containing five females had significantly fewer larvae than expected at day 20 and from day 60 onward. For vials containing two females, there were significantly fewer larvae at 80, 90, and 110 d. There were fewer instances where the mean number of pupae per original female was fewer than expected (Fig. 1C). The mean number of new adults per original female was significantly fewer than expected for vials containing two or five females from day 60 onward (Fig. 1D).

Note that for larvae on day 120, the mean of the five-female vials (calculated from data on the original scale) is closer to the mean of the one-female vials than the mean of the two-female vials; however, it is the one-female–five-female contrast that was significant. This is the result of testing on the log scale, where extreme values (in two-female vials) are greatly pulled in, but depicting the data in the figure on the original scale.

Table 2. Mean ± SEM per female for each insect stage 40 d after placing one female or two females in vials containing artificial diet

Developmental stage	No. females per vial	
	1	2
Eggs	6.3 ± 1.49 (253)	3.8 ± 1.99* (454)
Larvae	4.9 ± 1.06 (196)	2.8 ± 1.37* (299)
Pupae	0.8 ± 0.42 (34)	1.0 ± 0.6 (91)
Adults	1.1 ± 0.62 (47)	1.1 ± 1.0 (138)
Total	13.1 ± 1.24 (530)	8.1 ± 1.59 (982)

Trials with one female were repeated twice (40 vials total) and trials with two females were repeated three times (60 vials total). Numbers in parenthesis represent total numbers for each insect stage, summed over vials. Contrasts tested whether there was significantly fewer than twice the number of progeny in two females per vial compared with one female per vial. An asterisk indicates that the null hypothesis was rejected ($\alpha = 0.05$).

Discussion

The results from the third experiment (Fig. 1) demonstrate that the effects of initial numbers of females on fecundity are not limited to the first 40 d (as shown in experiments 1 and 2) and that these effects can be detected in subsequent generations. Data for the third experiment were collected every 10 d for 120 d; therefore, two additional generations could have been produced inside the original vials. Because the coffee berry borer does not exhibit cannibalistic behavior, this factor is not responsible for the observed decrease on fecundity for cohabitating females. Similarly, due to the relatively copious starting quantities of the artificial diet, competition for food was not a factor. The artificial diet was never depleted in any of the three experiments.

Intraspecific competition due to increased population density (crowding) in insects in culture could have effects on size, developmental rate, survival, behavior, longevity, and fecundity, and these effects also might become evident in subsequent generations (Barbosa et al. 1972, Peters and Barbosa 1977, Sørensen and Loeschcke 2001). Our experiments were designed to assess effects on fecundity, and overall, the results show that forced cohabitation by two or five female coffee berry borers typically resulted in decreased fecundity compared with just one colonizing female (Tables 1 and 2; Fig. 1). Reduced female fecundity in other bark beetles species (e.g., *Dendroctonus*, *Ips*, and *Tomicus* spp.) also has been reported when their density in trees increases (McMullen and Atkins 1961, Anderbrant et al. 1985, Sauvard 1989, Anderbrant 1990, Reeve et al. 1998, Amezaga and Garbisu 2000, Sallé and Raffa 2007).

Although there are several mechanisms that could have evolved to reduce cohabitation rates, there is scant evidence for any of them in the literature for the coffee berry borer. In contrast, species of *Dendroctonus* and *Ips* produce aggregation pheromones when they attack the phloem of *Pinus* spp. This is followed by the production of defensive resins that, if the tree is healthy, end up killing the insects; but if the tree is under stress, the resins are not sufficient to suppress infestations (Blomquist et al. 2010). When the density of phloem attack (galleries per square meter) starts becoming too high, male *Dendroctonus ponderosae* Hopkins produce the antiaggregation pheromone frontalin, which serves to signal other conspecifics that the phloem is unavailable and therefore attack ceases (Pureswaran et al. 2000). As stated by Blomquist et al. (2010), frontalin "may function as a spacing factor signaling 'the tree is full' to other beetles."

Similar to the situation where *D. ponderosae* males produce frontalin, we hypothesize that due to the limited food resources available inside a coffee berry, once a female enters the berry, a host-marking pheromone is produced that alerts other females that the berry is occupied. Tephritids are known to produce host-marking pheromones after egg laying in fruit (Prokopy 1972, 1975; Prokopy et al. 1978; Prokopy and Papaj 2000), and oviposition deterrence chemicals have been reported for bean weevils in the genus *Callosobruchus* (Bruchidae) (Oshima et al. 1973; Messina and Renwick 1985a,b). The pepper weevil, *Anthonomus eugenii* Cano (Curculionidae), is known to produce an oviposition deterrent contained within the frass of females as well as in the oviposition plug the female places over eggs (Addesso et al. 2007). Other Curculionidae, such as the plum curculio, *Conotrachelus nenuphar* (Herbst) (Butkewich et al. 1987); the boll weevil, *Anthonomus grandis grandis* (Boheman) (Stansly and Cate 1984); and the cabbage seedpod weevil, *Ceutorhynchus assimilis* (Paykull) (Kozlowski et al. 1983) have been shown to discriminate against hosts that have already been oviposited on. More than 60 herbivorous insect species in the orders Coleoptera, Diptera, Hymenoptera, and Lepidoptera have been reported to produce oviposition-deterring pheromones (Anderson 2002). The topic has been reviewed by Roitberg and Prokopy (1987), Nufio and Papaj (2001), Anderson (2002), and Hoffmeister and Roitberg (2002).

Figure 1A shows that eggs laid by two or five females decreased significantly over time, compared with vials containing only one female. It is noteworthy that these changes begin at ≈day 30. One reason for this delayed effect could be that the presence of larvae (or something produced by them) is the signal females are responding to. If the reduction in numbers of eggs were due to a marking pheromone produced by the colonizing female, then the differences would probably occur sooner. Another possibility is that if a marking pheromone is being produced by the colonizing females, the artificial diet might in some way impede its movement throughout the galleries until it reaches the other colonizing females, i.e., it may take awhile for it to build up high enough to affect female behavior. Finally, there is the possibility that in the field, a combination of insect and berry-derived volatiles together will be necessary for a repellent effect at an early stage of colonization. An insect signal may be responsible for reducing fecundity should two females end up in the same berry, but a combination of insect and damaged berry signals may be required for female borers to avoid an infested berry altogether.

For the coffee berry borer, a consequence of not marking the coffee berry could be reduced fecundity (as observed in our study), due to having to share a limited resource with other females and their progeny. The next phase of our research will focus on determining whether volatiles with an effect on fecundity are being produced, because this could result in practical improvements for coffee growers. Such volatiles might be associated with oviposition or might be related to the production of feces or brood-mediated signals (Nufio and Papaj 2001). Elucidation of such a volatile in insects reared in artificial diet eliminates the difficulties in conducting the experiment using coffee berries. Excising berries and taking them to the laboratory for subsequent infestation results in the production and identification of volatiles that have no relevance to situations faced by insects in the field, due to the berries having been excised from the plant. Similarly, infesting berries with different coffee berry borer densities in the field and assessing volatile production in planta is difficult. Any volatile that we suspect might have a potential influence on fecundity will be subsequently tested in electroantennogram assays, as well as in laboratory experiments using insects reared in artificial diet. Volatiles found to affect fecundity in the laboratory will be tested in the field.

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