Mating Frequency and Fecundity in the Emerald Ash Borer *Agrilus planipennis* (Coleoptera: Buprestidae)

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ABSTRACT The consequences of single versus multiple mating on the longevity, fecundity, and fertility of female emerald ash borers *Agrilus planipennis* (Fairmaire) were examined. In the first treatment, dissections of the common oviduct showed that 43 of 52 singly-mated females had received spermatophores. In the next two treatments, females were observed to mate one time, then housed either alone (observed separate) or with their mate (observed together). In the fourth treatment, females were paired with a randomly chosen male (unobserved together). Weight (0.0428 ± SE 0.0008 g) and longevity (50.5 ± SE 1.6 d) of female beetles did not differ among treatments. Fecundity, but not fertility, had a significant positive correlation with longevity in all treatments. Almost all of the females 'observed together' laid eggs (87%, $N = 31$), while significantly fewer females 'unobserved together' (61%, $N = 31$) and 'observed separate' (54%, $N = 31$) did. The fecundity of females that did lay eggs did not differ among treatments. Based on our results a single mating may be sufficient to ensure maximal fecundity for females, but there is potential for failure of any one mating, and no apparent cost to multiple mating. Thus, multiple mating is likely the best strategy for female emerald ash borers to maximize fecundity. The implications of results for laboratory rearing, and potential population level effects are discussed.

KEY WORDS multiple mating, fecundity, fertility, Allee effect

In theory, female insects obtain enough sperm from a single mating to fertilize all of their eggs, while males maximize their fitness by mating multiple times (Thornhill and Alcock 1983). Nevertheless, multiple mating by female insects is the norm. In a meta-analysis of experiments comparing female fitness in multiply- versus singly-mated female insects, Arnqvist and Nilsson (2000) found that multiple matings in the great majority of species tested resulted in an increased lifetime production of viable offspring for females. There are potential benefits and costs to multiple mating. Benefits may include sufficient, or fresh sperm (Dunn et al. 2005); gonadotropins derived from the male seminal fluid (Yamane and Miyatake 2010); diverse genetic material to increase the fitness of offspring and avoid genetic incompatibility (Jennions and Petrie 2000); and nutritional benefits (Arnqvist and Nilsson 2000, Edvardsson 2007). Costs can include: physical damage from the mating process (Morrow and Arnqvist 2003, Jones et al. 2010); toxicity from substances in the male ejaculate (Eady et al. 2007); exposure to parasites and diseases from the male (Reinhardt et al. 2005, Siva-Jothy 2006); and ecological danger from predators and parasites during the mating itself because of increased conspicuousness (Magnhagen 1991, Lafaille et al. 2010).

Another potential cost of multiple mating is the need to find multiple mates. Traditionally, females are thought to be the limiting sex with female fitness dependent on factors such as body size and condition, and access to resources (Thornhill and Alcock 1983, Kokko and Rankin 2006). Access to mates is rarely considered as a limiting factor for female fitness (Rhainds 2010). However, at low densities, there may be a mate-finding Allee effect where individual fitness decreases as population density decreases because of inability to find mates (Allee et al. 1949). Life history factors that intensify the mate-finding Allee effect include, dispersal from natal site, maturation feeding before mating, and maximal fitness through multiple mating (Gascoigne et al. 2009).

Emerald ash borer, *Agrilus planipennis* (Fairmaire) (Coleoptera: Buprestidae) is an invasive wood-boring beetle from Asia. Emerald ash borer was first discovered in Detroit, MI, in 2002 and has since killed many millions of ash trees in 15 states as well as two Canadian provinces (Mercader et al. 2011). All native ash trees (*Fraxinus* sp.) are vulnerable, and none appear to have resistance to the beetle (Rebek et al. 2008). The beetle is predicted to spread throughout eastern North America and to severely impact ash populations (Muirhead et al. 2006, McCullough and Siegert 2007, Mercader et al. 2009, Kovacs et al. 2010). Knowledge of its mating ecology will help to understand the pop-

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ulation dynamics and spread of this beetle, especially in low density, isolated populations, or both (Elam et al. 2007). In addition, the ability to efficiently rear emerald ash borer in quarantine for the mass production of biological control agents will be crucial to any control efforts (Keena et al. 2009). Understanding reproductive behaviors can aid in improving mating success and maximizing fecundity in the laboratory.

Emerald ash borer's reproductive biology seems to be typical of its genus (Chapman 1915, Barter 1957, Cote and Allen 1980, Akers et al. 1986, Akers and Nielsen 1990). Adult males emerge before adult females (Rodriguez-Saona et al. 2007, Wei et al. 2007). Both sexes require ≈5–7 d of maturation feeding on the host leaves before mating (Poland and McCullough 2006, Pureswaran and Poland 2009). Mating typically takes place on the host trees. Males locate females by sight, often dropping out of the air directly on top of females (Lelito et al. 2007), and further identify females via a contact sex pheromone (Lelito et al. 2009, Silk et al. 2009). Mating is prolonged, lasting 50 min on average (Pureswaran and Poland 2009) as compared with 5–12 min for other *Agrilus* species (Chapman 1915, Barter 1957, C.E.R., unpublished data). After mating in other *Agrilus* species, pairs part ways with no mate guarding (Chapman 1915, Barter 1957), and this appears to also be the case for emerald ash borer (C. E. Rutledge, unpublished data). Although Barter (1957) contends that female *Agrilus anxius*, bronze birch borer will avoid second matings, laboratory studies of bronze birch borer showed that females will mate multiple times (Akers 1985), as will emerald ash borer females (Lyons and Jones 2005). Given the lack of pair bonding, it is likely that additional matings are with new partners. Females begin to lay eggs a week to 10 d after mating, and can continue to lay eggs over the next 4–6 wk. Females can lay up to 200 eggs, although average is ≈70 (Wei et al. 2007).

Nothing is known about the impact of multiple matings on female emerald ash borer longevity, fecundity (number of eggs), and fertility (number of viable eggs). In the only *Agrilus* species studied on this question, bronze birch borer, no difference in fecundity, fertility, or longevity was found among females mated once, twice, or housed with males and allowed to mate multiple times (Akers 1985).

In this experiment, we allowed males and females to choose partners and mate. After mating, a subset of females was frozen and dissected to ascertain the rate of successful spermatophore transfer. Another subset of mating pairs either was separated or housed together. In an additional treatment, arbitrarily chosen random pairs were housed together and the same parameters were measured as for the observed pairs.

Materials and Methods

Insects. Beetles were provided by USDA APHIS PPQ personnel at the experimental station in Brighton, MI 48116. Beetles were reared from infested ash bolts (up to 30 cm in diameter and 70–72 cm long) collected in the Brighton, MI area, and held in a refrigerated box (4.0 ± 0.1°C) until they were needed and then put at room temperature (20–23°C) in rolled cardboard tubes with a clear plastic cup attached to one end (see Myers et al. 2009 for photograph, and Barak et al. 2010 for more detailed description of emergence tubes). Adults were attracted to the light and collected from the cups daily and housed 10 per petri dish (90 diameter by 15 d mm) with 2–3 clipped ash leaves until shipped under a valid permit to the United States Forest Service quarantine facility in Ansonia, CT. The petri dishes were placed inside a Tyvek (DuPont, Wilmington, DE) envelope in a small cooler (25 liters by 20 h by 20 d cm) for shipment. Upon arrival, beetles were sexed by examining them for the 'beard' of short golden setae found only on the male (Fairmaire 1888). They were then placed in single-sex, 7.6-liter containers for at least 6 d of maturation feeding before use in experiments. The 7.6-liter Clear Rubbermaid (Winchester, VA) Square Containers (no. 6308, 22.2 liters by 21.1 w by 22.2 h cm) have a single 7.5-cm hole cut in the tight fitting lid and the same sized hole cut in two opposite sides of the container. The side holes have white no-see-um polyester netting hot glued over the holes and a square (30 cm per side) of the same fabric is placed over the container opening and then secured by snapping the lid on over it. Beetles were supplied hardened-off foliage of *Fraxinus uhdei* (Wenzig) Lingelsh and water. The foliage was cut in the green house then gently washed (hand swishing and brushing with a 5-cm-wide paint brush along the leaflet veins) in a dilute soap solution (1–2 drops of dishwashing soap to 4 liters of water) to remove any arthropods that might be on it, rinsed twice with water, and allowed to air dry before use. The ends of 3–5 leaflets were placed through a 5-mm hole in the lid of a 236.5-ml plastic container (Berry Plastics T31408CP, Evansville, IN) with water in it before being put in the 7.6-liter containers. Water was supplied by sticking a 5-cm piece of dental wicks through a hole in the lid of a 29.6-ml squat plastic container (Solo Cup Company P100 cup with a PL1 lid, Lake Forest, IL). A paper towel was placed in the bottom of the container to absorb excess moisture. Beetles were held at 25 ± 2°C, 65 ± 5% RH, and a photoperiod of 16:8 (L:D) h. These conditions have been shown to be ideal for emerald ash borer (Keena et al. 2009). Foliage was changed twice a week. To accumulate a sufficient sample size, beetles from nine different shipments were used over the course of a 9-mo period. Shipments were 100–200 beetles each and beetles that died during shipment or within 3 wk of initial mating were not used in the studies. Voucher specimens were deposited at the Entomology Division, Yale Peabody Museum of Natural History, New Haven, CT.

Mating Procedure. Up to 20 males and 20 females were placed together in a 7.6-liter plastic container with ash foliage and water, as described previously. Only males and females that had had at least 6 d of maturation feeding were used (mean = 9.32 ± SE 0.33 d, range 6–19 d). We were concerned that females who had had less time for maturation feeding may not

have gained sufficient weight to be sexually mature, however, there was no relationship between age at mating and weight at mating ($R^2 = 0.002, F = 1.10, P = 0.920$). The mating cage was placed in direct sunlight coming through a window to encourage mating. As natural pairs formed, they were removed from the cage and held separately in petri dishes (90 diameter by 15 d mm) to monitor time in copula. After mating, individuals were assigned numbers, and weighed.

Background Level of Mating Success. To assess the success rate of spermatophore transfer in behaviorally-complete matings, 52 pairs of beetles were allowed to mate as described above. Individuals in each pair were assigned numbers and weighed. Females were then frozen, dissected, and inspected under a dissecting scope for the presence of a spermatophore in the common oviduct.

Mating Frequency Treatments and Pair Maintenance. Beetles were placed in one of three remaining treatments. Two treatments comprised beetles that had been observed mating once. For the 'observed together' treatment, pairs that had been observed mating were housed together until the female died. In the 'observed separate' treatment, mated females were housed alone and followed until the female died. Finally, in the 'unobserved together' treatment an arbitrarily chosen male and female were housed together and followed until the female died. After mating or pairing (unobserved treatment), the beetles were assigned numbers, and weighed.

Experimental beetles were housed in wide mouth 0.9-liter glass jars with a mesh lid (same mesh as used for the larger containers), foliage, water, and an egg-laying substrate. The stem of the foliage, 1–2 leaflets of *F. uhdei* prepared as described previously, and a piece of dental wick were placed through a hole in the lid of a 29.6-ml squat plastic container (Solo Cup Company P100 cup with a PL1 lid, Lake Forest, IL). The egg laying substrate was a 20-cm-long piece of 1.9-cm-diameter solid PVC bolt wrapped first with white butcher paper and then with a strip of 1.9-cm-wide purple curling ribbon (spaced 1 cm apart so it did not overlap). One-third of a white trifold paper towel or a 9-cm-diameter filter paper was placed in the bottom of the jar to soak up excess water. The females often used the paper in the bottom for egg laying in the folds or adjacent to the water reservoir. Beetles were checked 2–3 times a week for mortality. The foliage was changed twice a week and the bolts were changed and checked for eggs once a week. Any eggs were removed and checked daily (except weekends) for hatching. The eggs were held in petri dishes placed on a platform over water in the bottom of a transparent plastic box (30 liters by 70 w by 20 h cm) to maintain high humidity and held at the same conditions as the adults. At the end of 3 wk, any unhatched eggs were assessed to see if they were fertilized (eggs turn brown as the larva develops) or not (unembryonated eggs remain yellow in color and often desiccate). For both the 'unobserved together' and the 'observed together' treatments, any dead males were replaced with an arbitrarily chosen male of the same cohort so that a male was present throughout the life of the female. Females that did not survive at least 3 wk after the mating date were not included in the analyses because they may not have had time to start laying eggs, a critical parameter in this study.

Statistics. To evaluate if the males and females that were allowed to choose their partners among a large group of potential partners were using size as a factor in choosing their mates, a linear regression was used. Data from all treatments where the beetles had a choice of partner ('background success', 'observed together', 'observed separate') were pooled for this analysis.

A *t*-test was used to determine if the weight of males, females, or the length of time in copula differed between pairs in which dissection showed that a spermatophore had been successfully transferred, and pairs in which no spermatophore had been transferred.

A chi-squared test was used to assess if the number of females who laid at least one egg differed among the 'observed together', 'observed separate' and 'unobserved together' treatments. The three egg-laying treatments were compared further using analysis of variance (ANOVA) with treatment and egg laying status (whether or not the female had laid at least one egg or no eggs) as independent factors. The dependent factors tested were female weight, longevity, fecundity, and fertility. Fecundity was measured as the total number of eggs laid. To normalize the fecundity data, which was strongly skewed, it was ranked in all analyses. Fertility was considered to be the proportion of viable eggs (embryonated, whether they hatched or not), and the values were transformed by taking the arcsine of the square root of the proportions to normalize the data. Male weight also was tested as a dependent factor against treatment and egg laying status for the three treatments in which the females had a single male partner.

Finally, linear regression was used to test for correlations between longevity and fecundity of egg-laying females, longevity and fertility of egg-laying females, weight and fecundity of egg-laying females, weight and fertility of egg-laying females and between fecundity and fertility of egg-laying females. All analyses were done using SYSTAT nine (SPSS 1999).

Results

Partner Choice. Weight was not a factor in mate choice. In the mating treatments where male and female beetles were allowed to choose their partners from a large group of individuals, there was no correlation between male and female weights ($R^2 = 0.013$, $F_{1, 60} = 0.762$, $P = 0.386$).

Background Level of Mating Success. Forty-three of the females dissected after mating had spermatophores in their reproductive tract, whereas nine females had no spermatophore, a success rate of 83%.

The weight of females in successful matings did not differ from the weight of females in unsuccessful matings (*t*-test $t_{24.6} = 1.064$ $P = 0.298$). Males who suc-

Table 1. Weight (± SE), lifespans (± SE), fecundity (± SE), and fertility of *Agrilus planipennis* subjected to 4 mating-frequency treatments

Treatment	Laying status[a]	N	Lifespan (d)[b]	Female weight (g)	Fecundity[c]	Fertility (%)[d]	Male weight[e]
Background success rate	Sperm	43	NA	0.0379 (±0.0017)	NA	NA	0.0313 (±0.0010)
	No sperm	9	NA	0.0351 (±0.0019)	NA	NA	0.0258 (±0.0025)
Observed together	Layers	27	50.59 (±2.86)	0.0432 (±0.0012)	46.70 (±8.32)	65.0	0.0312 (±0.0012)
	Non-layers	4	35.25 (±2.93)	0.0458 (±0.0044)			0.0348 (±0.0015)
Observed separate	Layers	17	49.12 (±2.17)	0.0448 (±0.0020)	40.00 (±10.83)	58.2	0.0305 (±0.0011)
	Non-layers	14	48.93 (±3.96)	0.0366 (±0.0016)			0.0289 (±0.0013)
Unobserved together	Layers	19	57.47 (±4.03)	0.0440 (±0.0021)	53.16 (±11.56)	52.2	0.0300 (±0.0013)
	Non-layers	12	47.92 (±6.13)	0.0439 (±0.0019)			0.0303 (±0.0017)

[a] Laying status indicates whether the female laid any eggs during her lifetime. For background success rate, females were dissected to determine if sperm was successfully transferred to the female.
[b] Lifespan is days since beetle shipment arrival.
[c] Fecundity is number of eggs laid over the course of a lifetime by laying females only.
[d] Fertility is percent of viable eggs. Eggs damaged during transfer or damaged by mites or mold were excluded from the calculation.
[e] Weight of first male partner, subsequent male weights are not incorporated into averages.

cessfully transferred spermatophores were not significantly heavier than those who did not (*t*-test $t_{10.7}$ = 2.049, P = 0.066) (Table 1). The duration of mating was not different between pairs that successfully transferred a spermatophore (mean = 62.19 ± SE 3.06 min) and those that did not (mean = 42.25 ± SE 3.06 min) (*t*-test $t_{8.8}$ = 2.084, P = 0.068).

Treatment, Fecundity, and Fertility. Treatments strongly influenced how many females laid at least one egg, with significantly more females in the 'observed together' treatment (87%, N = 31) laying eggs than females in either the 'observed separate' (54%, N = 31) or the 'unobserved together' (61%, N = 31) treatments (X^2 = 8.27, df = 2, P = 0.016) (Table 1).

When females that laid no eggs were removed from the analyses, there was not a significant difference in fecundity among the three treatments ($F_{2,60}$ = 0.425, P = 0.656). Nor did the proportion of viable eggs differ among treatments ($F_{2,60}$ = 2.012, P = 0.143). There was a slight, but significant correlation between the number of eggs and the percentage of eggs that were viable (R^2 = 0.103, $F_{1,61}$ = 7.022, P = 0.010) with a slightly greater percentage of eggs being viable as the number of eggs increased.

Beetle Size and Fecundity. *Female Weight.* There was no significant difference among treatments in the weights of females ($F_{2,87}$ = 2.169, P = 0.135), nor were females that laid eggs significantly heavier than those that did not ($F_{1,87}$ = 1.079, P = 0.302). There was a significant interaction between treatment and laying status (whether or not a female had laid at least one egg) ($F_{2,87}$ = 3.604, P = 0.031). In the 'observed separate' treatment, there was a larger weight differential between females that laid eggs and females that laid no eggs than in the other two treatments (Table 1). Weight was not correlated with fecundity among laying females (R^2 = 0.014, $F_{1,61}$ = 0.876, P = 0.353). However, there was a slight, but significant correlation between weight and fertility (% egg hatch) (R^2 = 0.070, $F_{1,61}$ = 4.568, P = 0.037).

Male Weight. There was no significant difference among treatments in the weights of males ($F_{2,87}$ = 1.182, P = 0.311), nor was there a significant difference between the weights of males who were partnered with females who laid eggs and those that did not ($F_{1,87}$ = 0.049, P = 0.825). Unlike female weights, there was no significant interaction between treatments and partners' laying status in male weights ($F_{2,87}$ = 0.635, P = 0.532). Paternal weight was not correlated with fecundity (R^2 = 0.010, $F_{1,61}$ = 0.636, P = 0.428), or fertility (R^2 = 0.003, $F_{1,61}$ = 0.214, P = 0.645).

Longevity. Treatment did not impact longevity ($F_{2,87}$ = 1.924, P = 0.152). There was a significant difference between the lifespan of females that laid eggs and those that did not ($F_{1,87}$ = 4.830, P = 0.031), with laying females living an average of 5.5 d longer than females who did not lay eggs. There was no interaction between treatment and laying status, indicating that the relationship between laying status and lifespan was similar among all three treatments ($F_{2,87}$ = 1.380, P = 0.257). Among laying females, there was a significant positive correlation between lifespan and fecundity (R^2 = 0.258, $F_{1,61}$ = 21.168, P = 0.000), although there was none between longevity and fertility (R^2 = 0.007, $F_{1,61}$ = 0.443 P = 0.508). Weight and longevity were not correlated (R^2 = 0.005, $F_{1,61}$ = 0.284, P = 0.596).

Discussion

Multiple mating can have a variety of costs and benefits for female insects (Arnqvist and Nilsson 2000). However, under the conditions of this experiment, there were no costs associated with multiple mating. Females that cohabited with males lived as long as females that did not (Arnqvist and Nilsson 2000, Morrow and Arnqvist 2003, Jones et al. 2010). Nor was there a cost of fecundity to female longevity (Legaspi and O'Neil 1993, Arnqvist and Nilsson 2000). The longer the females lived, the more eggs they laid. Females in this study had free access to food and water during their lives. The relationship between fecundity and longevity might change under more stressful conditions (Fox 1993). In addition, potential environmental costs of multiple mating, such as exposure to pred-

ators (Jersabek et al. 2007, Lafaille et al. 2010), were not assessed.

Many more females in the 'observed together' treatment laid eggs than females housed with a random male, or singly-mated females. However, there was no concomitant increase in egg number. If a female did lay eggs, the number of viable eggs did not differ among treatments.

One potential reason for the increased success of females who mated multiple times is the observed background rate of failure by males to pass a spermatophore (nine of 52). Based on that statistic alone, about a fifth of females who mated only once would be expected to be uninseminated. This success rate is not significantly different from the rate reported for *Agrilus anxius*, which had 13 successful transfers for 15 matings (df = 1, $\chi^2 = 0.134$, $P = 0.714$) (Akers 1985). Success rates should climb to 95% by the second mating, making mating more than once a sound strategy (Ridely 1998), and it is likely that this is a common strategy in the genus (Chapman 1915, Barter 1957, Cote and Allen 1980, Akers 1985).

More than twice as many females in the 'observed separate' treatment had no eggs than would be expected from just the rate of failure of spermatophore passage (45% versus 17%). Even though females in the 'observed separate' treatment were not smaller than females in the study overall, singly-mated females who did not lay eggs were on average 18% lighter than those that did lay eggs. However, these females were not lighter than females who had successful matings in the background-success-rate study (*t*-test: $t_{13} = -s1.588$, $P = 0.136$), suggesting that low-weight does not interfere with successful spermatophore transfer. Perhaps females cannot use sperm from a mating that occurred before they had completed maturation feeding (and presumably sexual maturity). There may also be some variability in the length of time males need to maturation feed before becoming sexually mature.

Among the 12 females in the 'unobserved together' treatment that did not lay eggs, there is no way to differentiate between pairs that failed to mate and those that mated unsuccessfully. Given the much higher rate of successful egg production in the 'observed together' treatment, than in the 'observed separate' treatment, it is likely that many of the pairs in the 'observed together' group mated more than once. It seems that if a pair mated, they were likely to mate more than once, as was observed in Akers' (1985) study of bronze birch borer. In addition, in the 'unobserved together' group there were no significant differences in weight between females that did lay eggs and those that did not lay eggs, as we saw in the 'observed separate' group, and as we might expect to see if nonlaying females in the 'observed together' group had mated unsuccessfully. Therefore, it is likely that many of the 12 pairs that did not lay eggs in the 'unobserved together' treatment never mated at all. Emerald ash borer females display a degree of choosiness when mating in the laboratory at both low and high densities. Randomly-paired individuals, the lowest possible density, often fail to mate. When couples were paired for 90 min, only 12% of the pairs copulated (12/97 C. E. Rutledge, unpublished data). Mating rates do rise as the time allowed to couple increases, Pureswaran and Poland (2009) found that 70% of pairings resulted in a successful mating when up to 300 min were allowed before copulation occurred. At the high density of 11 females and 22 males, four successful matings, and 25 rejected male-mating attempts occurred over the course of 145 min (suggesting female choice plays a role, C. E. Rutledge, unpublished data). Although pairs in our experiment were housed together for over 3 wk, it is likely that failure to mate was a contributory factor in the higher proportion of non-egg laying females seen in the 'unobserved together' treatment as compared with the 'observed together' treatment. More explicit work on female-mate choice, and it's interaction with population density at realistic field densities, is needed, especially as courtship is apparently limited (Lelito et al. 2007). Size does not seem to be a factor when choosing mates in the laboratory.

Our results have implications for emerald ash borer rearing programs. Females that remain with males over the course of their lifetime are more likely to lay eggs than females mated once and isolated. The male housed with the female should be one with whom she has already mated, not a randomly selected male. Finally, allowing female beetles lifetime access to their mates obviates the need to determine exactly when the females have reached reproductive maturity.

More work needs to be done to understand the role of multiple mating in emerald ash borer reproductive strategies. Although females readily mate multiple times in the laboratory, there is no information on how often females mate in the wild. However, given the background rate of failure for spermatophore transfer, multiple mating seems likely to occur, and is perhaps necessary. Thus, our findings could have implications for the dynamics of emerald ash borer spread and establishment. Emerald ash borer reproductive biology exhibits several of the traits that make mate finding difficult; dispersal from a natal site, a premating maturation period, high-degree of mate choice, and lack of long-distance mate-finding strategies (although this is compensated for in part by host specialization). Thus, the need for multiple matings could intensify the Allee effect (Gascoigne et al. 2009). Although so-called component Allee effects such as mate-finding (Stephens et al. 1999) may not translate into demographic Allee effects, a need for multiple mating may help maintain new, isolated emerald ash borer populations at low densities (Elam et al. 2007). Management efforts to suppress new emerald ash borer populations may be aided by understanding this dynamic.

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