

The Effect of Male and Female Age on *Lymantria dispar* (Lepidoptera: Lymantriidae) Fecundity

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ABSTRACT Insects that reproduce sexually must locate a suitable mate, and many species have evolved efficient communication mechanisms to find each other. The number of reproductively viable individuals in a population can be an important constraint in the growth of populations. One factor that can affect insect fecundity is the age of mating adults, as fecundity tends to decline with age. Field observations collected annually on *Lymantria dispar* (L.) from 2001 to 2007 and 2009 consistently revealed a small proportion of egg masses (generally <10% in each year) in which >0 but <5% of eggs were fertilized in an egg mass consisting of ≈200–500 eggs. In these studies, male age was unknown but female age was fixed at <24 h, which, according to previous studies on the effect of female *L. dispar* age on reproductive success, should have been optimal for fertilization. In this article, we analyzed field data (2001–2007 and 2009) to explore patterns in the occurrence of low-fertilized egg masses. We supplemented these data with laboratory experiments that examined the interacting role of male and female age, and multiple male matings. We observed that increases in male and female age reduce the rate of fertilization, which is furthermore reduced, as males mate multiple times as they age. This article highlights the importance of both female and male age at the time of mating in an invading species, with ramifications to low-density populations in this and other sexually reproducing insect species.

KEY WORDS aging, biological invasion, gypsy moth, low-density population, mating success

As nonnative species continue to be introduced into novel environments, it remains important to understand the factors that affect establishment success and subsequent population growth. Some of these factors include host availability, climate suitability, and interactions with competitors and natural enemies (Lockwood et al. 2007). A particularly important constraint in the successful establishment of a nascent population across taxa is the number of individuals introduced into a novel environment (Lockwood et al. 2005, Liebhold and Tobin 2008, Simberloff 2009). In species that reproduce sexually, the number of reproductively active adults available at a given point in space and time, as well as their ability to locate suitable mates, can be particularly important drivers (Calabrese and Fagan 2004, Gascoigne et al. 2009). This can be especially true in species in which the adults are not long-lived, which limits the temporal window in which mates can be located. Among insects, most of which are relatively short lived, most species reproduce sexually and have evolved efficient means of locating mates, such as through aggregation, contact, or sex pheromones.

Despite the relatively short adult life span of most insects, the age of adults at the time of mating could still be an important determinant of fecundity (Clutton-Brock 1988).

The gypsy moth, *Lymantria dispar* (L.) (Lepidoptera: Lymantriidae), was introduced into North America in 1869 and continues to spread beyond its current distributional range (Tobin et al. 2012). In spring, larvae hatch from overwintering egg masses and are able to feed on >300 host tree species including several preferred hosts such as *Betula* (birch), *Populus* (aspen), *Quercus* (oak), and *Salix* (willow) species (Liebhold et al. 1995). Adults emerge in summer, with males generally emerging before females, as females undergo six instars while males only have five. Females are flightless and attract flying males through a sex pheromone, mate once, and oviposit a single egg mass consisting of 200–500 eggs. There is one generation per year. Generally, *L. dispar* spread, as well as the spread in many invading nonnative organisms, occurs according to stratified dispersal in which local growth and diffusive spread is coupled with stochastic long-distance dispersal (Liebhold et al. 1992, Sharov and Liebhold 1998, Tobin and Blackburn 2008). The result of these “jumps” through long-distance dispersal is that new colonies can be initiated far from the established range and often at low-population densities (Shigesada et al. 1995). Consequently, the spread of this and many other invading species essentially in-

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corporate the first two phases of the biological invasion process: arrival through long-distance dispersal to a new area devoid of conspecifics, and establishment (Liebhold and Tobin 2008).

New colonies that arrive beyond the established range either do or do not successfully establish, and a number of factors can affect the rate of establishment (Lockwood et al. 2007, Liebhold and Tobin 2008, Simberloff 2009). In *L. dispar*, female mating success has been consistently shown to be strongly influenced by the background male moth density, such that at low male densities, females are rarely mated while at higher densities, nearly all females are mated (Sharov et al. 1995; Tcheslavskaia et al. 2002; Contarini et al. 2009; Tobin et al. 2009, 2013). Consequently, it is not surprising that *L. dispar* population success has been shown to be linked to population size (Liebhold and Bascombe 2003, Whitmire and Tobin 2006). Another important consideration is the spatial and temporal overlap between males and females, regardless of population size (Robinet et al. 2007). Owing to temporal variability in the timing of sexual maturation within a sex, and protandry, the separation in time can be especially critical in affecting mating success, and consequently, the persistence and reproductive success of new colonies, especially when populations are at low densities (Robinet et al. 2008).

We were motivated initially by field observations in which egg masses, when collected and examined, would consistently reveal very low rates of fertilization, in which <20 eggs, and sometimes as few as 1–2, out of up to 500 eggs would be fertilized. This was unusual in our experience because in most cases, the overwhelming majority of eggs were either all fertilized, or all unfertilized. These field studies were conducted to evaluate mating disruption tactics against *L. dispar* (Tcheslavskaia et al. 2005; Thorpe et al. 2007; Onufrieva et al. 2008, 2010). In these studies, plots were treated with aerial applications of a mating disruption product, and deployed tethered females, all of which were <24 h old, were used to measure mating success when compared with untreated control plots.

A prior laboratory study demonstrated that *L. dispar* female age at the time of mating affects their reproductive potential such that older females (>48 h old) produced fewer viable eggs than younger (<48 h old) females; however, in this case, all males were <24 h old (Proshold 1996). Another study reported that males, which, unlike females, are not monogamous, transfer less sperm at each successive mating when using males and females <48 h old (Proshold and Bernon 1994). In field observations, on which this study was motivated, only young females (<24 h old) were used, while male age and the number of prior male matings were not known. In this study, we were collectively motivated by field observations of very low rates of egg mass fertilization, prior research on the role of female *L. dispar* age (Proshold and Bernon 1994, Proshold 1996), and a lack of understanding regarding the role of male age and its interaction with female age. Specifically, we first examined field data collected annually from 2001 to 2007 and 2009 to determine if there

were patterns in the occurrence of low-fertilized egg masses, such as a tendency to occur early or late in the flight season, or in treated or untreated control plots. We then examined, under laboratory conditions, the role of both female and male age, and the effect of male and female age when coupled with multiple male matings, on the rate of egg mass fertilization.

Materials and Methods

Field Data. Field studies were conducted each year from 2001 to 2007 and 2009 at sites in the Appomattox-Buckingham State Forest, Cumberland State Forest, and Goshen Wildlife Management Area (all in Virginia). These studies were designed to measure the effect of mating disruption tactics, such as product formulation and dose, against *L. dispar* populations (Tcheslavskaia et al. 2005; Thorpe et al. 2006, 2007; Onufrieva et al. 2008, 2010). Mating disruption products were Hercon Disrupt II (Gelva 2333, Solutia Inc., Springfield, MA), SPLAT GM (ISCA Technologies, Riverside, CA), and 3M MEC-GM Sprayable Pheromone (3M Canada Co., London, ON, Canada) applied at various doses, from 15 to 37 g/ha. In these studies, *L. dispar* male and female pupae were obtained from the U.S. Department of Agriculture–Animal and Plant Health Inspection Service (USDA–APHIS), Center for Plant Health Science and Technology, Buzzards Bay, MA. Briefly, adult females (<24 h) were deployed in untreated control plots and plots treated with an aerial application of a mating disruption product and dose, and returned to the laboratory after 24 h. Females were placed individually on trees and were bound by a band of duct tape covered with a Tanglefoot bird repellent (The Tanglefoot Company, Grand Rapids, MI) to minimize their loss to predation and maximize their recovery.

Across all studies, females were deployed two to four times per wk over the 6–10 wk generally corresponding to the male moth flight period. In addition to background densities of male moths, which were unknown at the time of the study, laboratory-reared males (50–150) were released twice a week at the time of female deployment. Males were released to ensure moth densities within the plots, and to extend the data collection period beyond the natural male moth flight period when background male moth populations were 0. Females were allowed to oviposit under laboratory conditions (females usually, but not always, oviposit whether they have been mated or not). Egg masses were then maintained under laboratory conditions for at least 60 d to allow for embryonation and then examined to quantify the proportion of fertilized eggs per egg mass (Tcheslavskaia et al. 2002).

More than 900 females were deployed in each year and site, and across all years and plots, 29,592 females were deployed, of which 20,487 were recovered and oviposited an egg mass. In most egg masses, we observed that eggs were generally either completely unfertilized or completely fertilized; however, we also observed low rates of fertilization in which >0 and ≤5% of the eggs were fertilized in an egg mass. We

thus explored patterns in the field-collected data associated with low rates of fertilization, which we defined as an egg mass in which >0 and $\leq 5\%$ of the eggs in the egg mass were fertilized ($N = 626$ egg masses). Across all years and sites, there were 14 and 47 untreated control and treated plots, respectively. When pooling across all years, we used logistic regression to test the main effects of treatment (i.e. plots treated with mating disruption vs. untreated control plots) and ordinal date, and their interaction, on the occurrence of low-fertilized egg masses relative to the total number of egg masses recovered by treatment and on each ordinal date. The total number of egg masses collected per ordinal date ranged from 8 to 98 in control plots (median = 54), and from 8 to 543 in treated plots (median = 281). Overdispersion was corrected using the scaled Pearson chi-square, and significance of effects was based on the likelihood ratio chi-squared, G^2 . Statistical analyses were conducted in R (R Development Core Team 2013).

Laboratory Experiments. We examined the effect of male and female age and multiple male matings in a controlled laboratory experiment. We obtained *L. dispar* male and female pupae from the USDA-APHIS, Center for Plant Health Science and Technology, Buzzards Bay, MA. Male and female pupae were reared separately under laboratory conditions ($21 \pm 1.5^\circ\text{C}$ and a photoperiod of 14:10 [L:D] h) in $\approx 875\text{ cm}^3$ cardboard containers (≈ 25 per container) until adult emergence.

Upon emergence, we transferred individual adults into plastic cups (volume $\approx 136\text{ cm}^3$) containing a small piece of a paper towel to provide a resting spot. We performed a full factorial experiment using males and females that were 1, 2, 3, 4, or 5 d old at the time of mating, for a total of 25 treatment groups (i.e. 1 d male + 1 d female, 1 d male + 2 d female, ..., 5 d male + 5 d female) and 20–32 replications per treatment group. We extended the age to 5 d based on past work suggesting that the majority of adult males are thought to survive up to 2 d in nature, with some capable of living up to 5 d (Robinet et al. 2008). Males and females were randomly assigned to a treatment group. Cups were marked daily to keep track of age. Males and females were allowed 24 h to mate, after which the male was removed. Females remained in the cup until an egg mass was produced, or the replication was repeated if the female died before ovipositing. In a separate experiment, we also randomly selected males to mate multiple times, once per day, from 2 to 5 d. Moreover, males were 2, 3, or 4 d old at the time of the second mating, while females were 1, 3, or 5 d old at the time of mating. There were 20–35 replications per treatment group.

After oviposition, egg masses were collected and placed individually into 70-mm petri dishes and maintained under laboratory conditions for at least 60 d to allow for embryonation (Tcheslavskaya et al. 2002). Following this period, egg masses were vacuumed through a fine mesh screen ($280\text{ }\mu\text{g}$) to remove setae and to help separate individual eggs. Eggs were ex-

amined and counted to determine the number of fertilized and unfertilized eggs; unfertilized eggs remain white while fertilized eggs become dark due to the presence of an embryo. In total, 940 egg masses were analyzed in the laboratory experiment.

In the single mating experiment, a fertilized egg was considered as a binary variable (i.e. fertilized or not) while controlling for the number of eggs in the egg mass ($N = 525$ total egg masses). We used logistic regression in R (R Development Core Team 2013) to test the main effects of male and female age (1–5 d), and their interaction, on the rate of egg fertilization. Overdispersion was corrected using the scaled Pearson chi-square, and significance of effects was based on G^2 .

We also used quantile regression (Koenker and Hallock 2001, Cade and Noon 2003) to quantify the conditional response at different percentiles of the distribution of egg fertilization rates associated with male and female age using the quantreg package in R (R Development Core Team 2013). We used the quantile regression approach because of our interest in quantifying the conditional response at different quantiles, such as egg fertilization rates of the bottom or top 10th percentile of the distribution, instead of only the conditional mean response that is estimated using conventional regression techniques (see Cade and Noon 2003 for an excellent treatment of this technique). In this case, the proportion of fertilized eggs in an egg mass was regressed over the combined male and female age, from 2 to 10 d, to estimate predicted probabilities of fertilization at the 10th, 25th, 50th, 75th, and 90th quantiles (the estimates at the 50th quantile are analogous to the conditional mean response extracted from conventional least squares regression). In this analysis, we combined male and female age for two primary reasons. First, from our analyses on the main effects of male and female age, we observed that fecundity declined considerably when males and females were both >3 d old. Second, because we already considered the independent effects of male and female age, we combined male and female age in our quantile regression analysis to provide a generalized description of the effect of their combined age on fecundity.

In the multiple male mating experiment, the binary condition of an egg (fertilized or not) was considered relative to the eggs in the egg mass ($N = 410$ total egg masses). We tested the main effects of male age (2–5 d), female age (1, 3, or 5 d), the number of prior male matings (2–5), and male age at time of the second mating (2–4 d), as well as all possible interaction effects. Because of the number of main and interaction effects, we used step-wise logistic regression to first define a subset of variables to test. Following this procedure, the significance of main and interacting effects was based on G^2 , and overdispersion was corrected using the scaled Pearson chi-square. Statistical analyses were conducted in R (R Development Core Team 2013).

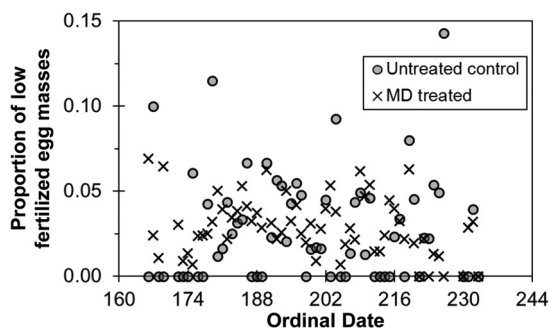


Fig. 1. Proportion of low-fertilized egg masses (>0 and $\leq 5\%$ of eggs in an egg mass) collected from untreated control plots and plots treated with mating disruption, 2001–2007 and 2009, regardless of formulation or dose. Across all years, there was a median of 54 and 281 egg masses per sampling day in untreated control and treated plots, respectively.

Results

Field Data. Of the 20,487 females that were recovered from field studies and those that oviposited an egg mass, eggs were generally either completely unfertilized or completely fertilized in 96.9% of these egg masses. However, in 626 egg masses, we observed a low rate of fertilization in which >0 and $\leq 5\%$ of the eggs were fertilized, including 345 egg masses that were >0 and $\leq 0.5\%$ fertilized; in other words, several egg masses contained only one to two fertilized eggs in an egg mass of 200–500 eggs. This phenomenon was observed in all years from 2001 to 2007 and 2009. When examining the occurrence of low-fertilized egg masses relative to all egg masses, there was no significant effect of treatment (i.e. untreated control plots vs. plot treated with mating disruption, regardless of formulation or dose; $G^2 = 0.07$; $df = 1$; $P = 0.80$), ordinal date ($G^2 = 0.13$; $df = 1$; $P = 0.71$), or a day by treatment interaction ($G^2 = 0.05$; $df = 1$; $P = 0.83$). Low-fertilized egg masses occurred throughout the adult period, and in both treated and untreated plots (Fig. 1). Overall, we observed a low rate of fertilization in 3.1% of egg masses from all years; however, we also observed dates in which $>5\%$ and up to 14.3% of egg masses contained a low rate of fertilized eggs (Fig. 1).

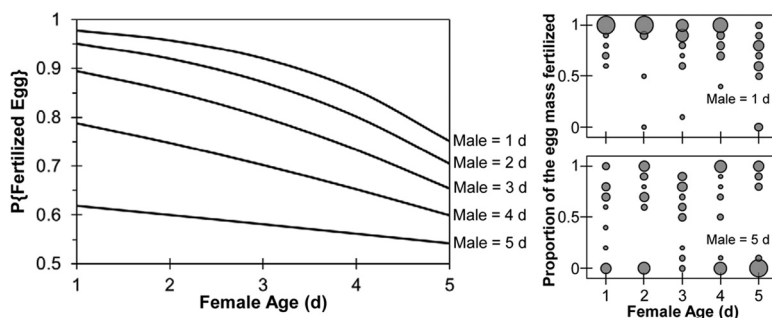


Fig. 2. Predicted probabilities of an egg being fertilized by male and female age, and when males are mated only once. As an example of the distribution of raw data, the insert proportional scatter plots show the proportion of eggs fertilized in an egg mass by female age when male age is either 1 or 5 d (the size of the circles represents the number of observations; the smallest-sized circle is one egg mass).

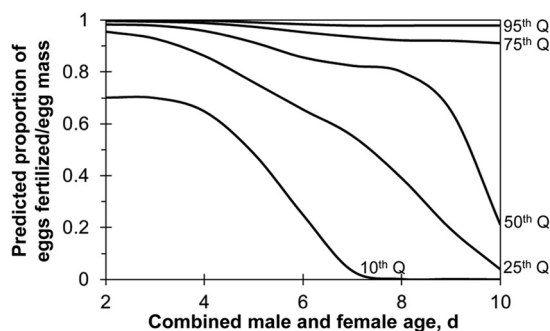


Fig. 3. Quantile regression predictions of the proportion of eggs fertilized in an egg mass by the combined male and female age, and when males mate only once. The 50th quantile (Q) is analogous to the mean conditional response in conventional least squares regression. The 90th quantile suggests that 10% of egg masses are nearly fully fertilized regardless of male and female age, while the 10th quantile suggests that 10% of egg masses are completely unfertilized when the combined male and female age is ≥ 7 d.

Laboratory Experiments. In the single mating experiments ($N = 525$ total egg masses), the main effects of male age ($G^2 = 37.7$; $df = 1$; $P < 0.01$), female age ($G^2 = 26.4$; $df = 1$; $P < 0.01$), and their interaction were significant ($G^2 = 12.9$; $df = 1$; $P < 0.01$). The probability of an egg being fertilized declined with a decrease in both male and female age (Fig. 2). When collapsing over female age, odds ratios suggested that eggs were $1.6\times$ (95% CI = 1.4, 1.8) less likely to be fertilized as males aged by 1 d. When collapsing over male age, eggs were $1.4\times$ (95% CI = 1.2, 1.5) less likely to be fertilized as females aged by 1 d.

The quantile regression analysis revealed considerable variability in the rate of fertilized eggs across all combinations of male and female age (Fig. 3). The predictions at the 50th quantile revealed a steep decline in the proportion of fertilized eggs when the combined male and female age exceeded 8 d. Interestingly, we observed that at least 25% of egg masses were almost completely fertilized even as males and females each approach 5 d (indicated by the 75th

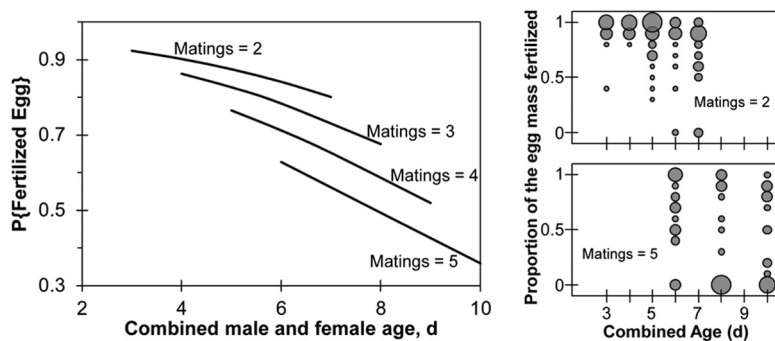


Fig. 4. Predicted probabilities of an egg being fertilized based on the combined male and female age, and the number of prior male matings. As an example of the distribution of raw data, the insert proportional scatter plots show the proportion of eggs fertilized in an egg mass by the combined male and female age when males have mated two or five times (the size of the circles represents the number of observations; the smallest-sized circle is one egg mass).

quantile prediction). Conversely, we observed that at least 10% of egg masses are only $\approx 70\%$ fertilized even when males and females are each only 1 d old, and that 10% of the egg masses contained no fertilized eggs when the combined male and female age exceeded 7 d (indicated by the 10th quantile prediction).

In the multiple mating experiments ($N = 410$ total egg masses), the main effects of male age ($G^2 = 17.5$; $df = 1$; $P < 0.01$), female age ($G^2 = 13.1$; $df = 1$; $P < 0.01$), and the number of prior male matings ($G^2 = 9.8$; $df = 1$; $P < 0.01$) were significant, as was the male-by-female age interaction ($G^2 = 5.4$; $df = 1$; $P = 0.02$). The main effect of male age at time of the second mating and all other possible interaction effects were not significant (all $P > 0.05$). The predicted probability of an egg being fertilized declined with an increase in male and female age, and with an increase in the number of male matings (Fig. 4).

Discussion

Increases in male *L. dispar* age can result in significant decreases in rates of fertilization success, especially when breeding with older females (Fig. 2), or when males have mated multiple times (Fig. 4). This work enhances prior studies on the role of female age on gypsy moth fertilization success (Proshold and Bernon 1994, Proshold 1996) by incorporating the effect of male age, which had not been addressed previously. Results from prior studies (Proshold and Bernon 1994, Proshold 1996) are also consistent with our laboratory observations regarding the role of female age and multiple male matings when male age is < 48 h. In addition, our use of quantile regression reveals considerable variation in fertilization success across male and female age. Some egg masses can be partially fertilized even when adult age is optimal for reproduction, or nearly fully fertilized even when adults are considerably aged (Fig. 3). Our field observations also demonstrate that the phenomenon of low-fertilized egg masses (> 0 and $\leq 5\%$ of eggs per egg mass), albeit rare, occurs consistently throughout the adult period (Fig. 1).

Although results from our laboratory studies are consistent with prior laboratory studies (Proshold and Bernon 1994, Proshold 1996), laboratory observations remain different from field observations. For example, laboratory studies demonstrated that although fertilization rates declined with male and female age, these lower rates are still higher than what we observed in the field. This is probably not surprising given that laboratory conditions were designed to exclude mate finding, as males and females were placed together in mating chambers. The difference between laboratory studies, in which low fertilization rates are those egg masses where the majority still have $> 30\%$ of the eggs fertilized, and field observations, in which some egg masses contain $\leq 5\%$ fertilized eggs, could reflect the costs of mate finding under natural conditions, including female calling and male flight behaviors (Cardé et al. 1974; Cardé and Hagaman 1984; Gieblultowicz et al. 1990, 1992).

The effects of male and female age on reproductive success could also be important in populations managed by mating disruption tactics, which is a strategy used against a number of insect pests (Cardé and Minks 1995, El-Sayed et al. 2006, Yamanaka and Liebhold 2009, Witzgall et al. 2010, Suckling et al. 2012). The concept of mating disruption is based on the introduction of synthetic pheromone into the environment at levels that interfere with mate-finding ability through competitive attraction, false trail following, camouflage of the plume produced by calling females, desensitization, habituation, and sensory imbalance (Ridgway et al. 1990, Miller et al. 2006, Yamanaka 2007). Mating disruption does not always prevent mating, and one of the indirect mechanisms of mating disruption tactics is delayed mating (Mori and Evenden 2013). Thus, applications of synthetic pheromones could increase the amount of time that males spend locating calling females, which could result in lower fertilization success due to an increase in the age of one or both mates.

We also note that we did not observe a difference in the proportion of low-fertilized egg masses collected from untreated control plots and plots treated

with mating disruption (Fig. 1), even though past research has consistently shown that mating success declines significantly in plots treated with mating disruption related to untreated control plots (Tchelsavskaia et al. 2005; Thorpe et al. 2006, 2007; Onufrieva et al. 2008, 2010). One reason for the lack of a difference could be the overall rarity at which we observed low-fertilized egg masses, making it challenging to detect a significant difference. Furthermore, males and females likely vary in their respective fitness, regardless of whether they were placed in a treated or control plot. In natural settings, nutritional deficiencies during larval development, such as when larvae consume plants that are less preferred as hosts (Barbosa and Greenblatt 1979, Raupp et al. 1988, Awmack and Leather 2002), could result in background variation in fitness. It is also possible that males are more likely to mate multiple times in untreated control plots, while males in treated plots could mate when they are older due to the increased challenges of finding a mate in the presence of synthetic pheromone.

The effect of age in reproductively active adults is likely most crucial in low-density populations where suitable mates are fewer in number and harder to locate. Low-density populations can also be subject to Allee effects, which refer to a positive relationship between individual fitness and population size (Allee 1938, Stephens et al. 1999, Courchamp et al. 2008). Past work has demonstrated the role of Allee effects in invading *L. dispar* populations (Johnson et al. 2006, Tobin et al. 2007, 2009; Vercken et al. 2011), including the presence of strong Allee effects as the spatial and temporal dispersion between males and females increases (Robinet et al. 2008). However, a prior study (Robinet et al. 2008), the theoretical model, parameterized from field data, assumed that delays in adult male and female emergence did not affect fecundity. Because this work shows an effect of both male and female age on reproductive success, our findings add an additional dimension to the importance of spatial and temporal overlap in reproductive adults.

Although many insect species have evolved efficient mate-finding communication strategies, they have generally done so in native environments where local populations are not necessarily devoid of neighboring populations that are connected by dispersal. Past work has suggested that in *L. dispar*, mate-location strategies are not expected to be as successful in low population densities as they would be in dense populations (Cardé and Hagaman 1984). Moreover, the challenge of mate location could not only be critical in newly invading populations of a nonnative species but also in sparse populations of threatened and endangered species (Wells et al. 1988). This work underscores the importance of adult age, in both males and females, which could also interact with other population-level processes, such as reproductive synchrony and protandry, and environmental conditions, such as habitat fragmen-

tation and disturbance, to affect low-density population persistence and growth.

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