

Are virgin male lepidopterans more successful in mate acquisition than previously mated individuals? A study of the European corn borer, *Ostrinia nubilalis* (Lepidoptera: Pyralidae)

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Abstract: Male phenotypic quality may significantly influence female reproductive success. Depletion of sperm and accessory-gland secretions with successive matings represents a reduction in male phenotypic quality and is known to decrease female reproductive output in several lepidopteran species, including the European corn borer (ECB), *Ostrinia nubilalis*. We therefore tested the hypothesis that female ECBs, given the simultaneous choice of an experienced male and a virgin male, preferentially mate with the virgin. However, contrary to prediction, females mated significantly more often with experienced males. Experienced males were significantly lighter than their virgin counterparts, the result of producing three spermatophores that were transferred during previous matings. However, differences in body mass or wing-loading did not appear to play an important role, for within either the experienced or virgin classes, heavier males obtained more matings than lighter ones. Why would females prefer to mate with sexually experienced males? Females may not be exercising any precopulatory choice, and the greater mating success of previously mated males may be related to previous experience. Behavioral observations, however, suggest that female choice occurred. In the process of selecting experienced males, the number of consecutive matings was correlated with low fluctuating asymmetry of the forewing (R-L). This suggests that males who acquired 3 consecutive matings were of above-average quality and were actively selected by females.

Résumé : La qualité du phénotype mâle peut influencer significativement le succès de la reproduction chez une femelle. La réduction du sperme et des sécrétions des glandes accessoires à la suite d'accouplements répétés représente une diminution de la qualité du phénotype mâle et entraîne une réduction du rendement reproducteur des femelles chez plusieurs espèces de lépidoptères, dont la Pyrale du maïs (ECB), *Ostrinia nubilalis*. Nous avons éprouvé l'hypothèse selon laquelle des femelles de la pyrale mises simultanément en présence de mâles vierges et de mâles expérimentés choisiraient des mâles vierges. Cependant, contrairement aux prédictions, les femelles ont choisi significativement plus souvent des mâles expérimentés. Les mâles expérimentés étaient significativement moins lourds que les mâles vierges à la suite du transfert de trois spermatophores au cours d'accouplements précédents. Cependant, les différences de masse ou de charge sur les ailes ne semblaient pas jouer un rôle important, puisque, aussi bien chez les mâles expérimentés que chez les mâles vierges, les plus lourds se sont accouplés significativement plus souvent que les moins lourds. Pourquoi les femelles préfèrent-elles s'accoupler avec des mâles expérimentés? Il se peut que les femelles ne fassent pas vraiment un choix et que le succès plus grand des mâles déjà accouplés soit relié à leur expérience préalable. Les observations du comportement ont toutefois révélé que les femelles font véritablement un choix. Au cours du processus de sélection en vue de l'obtention de mâles expérimentés, nous avons constaté que le nombre d'accouplements consécutifs était relié à une faible asymétrie fluctuante des ailes antérieures (R-L), ce qui indique que les mâles qui se sont accouplés trois fois de suite étaient de qualité supérieure et ont donc obtenu les préférences des femelles.

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Introduction

Male quality, of either genotypic or phenotypic origin, is one factor that can affect female reproductive success in lepidopterans. Male phenotypic quality may vary as the result of mating history (Outram 1971; Brower 1975; Svård

and Wiklund 1986; Rutowski et al. 1987; Lederhouse et al. 1990; Delisle and Bouchard 1995; Delisle and Hardy 1997) or the quantity or quality of larval food (Delisle and Bouchard 1995; Delisle and Hardy 1997). These previous studies showed that females mated with males of phenotypically poor quality may have significantly lower fecundity and fertility than

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those mated with high-quality mates. This reduced reproductive performance is associated with a decline in sperm and other resources that influence egg production or somatic maintenance which are transferred by the male at the time of mating (e.g., Boggs and Gilbert 1979; Engebreston and Mason 1980; Pivnick and McNeil 1987; Rutowski et al. 1987; Boggs 1990; Svärd and McNeil 1994; Delisle and Hardy 1997).

In *Drosophila melanogaster*, females will avoid mating with recently mated, sperm-depleted males (Markow et al. 1978). Given the potential reproductive costs of mating with a phenotypically inferior male, one might expect that female lepidopterans would also discriminate against previously mated males, although this was not the case in the green-veined white butterfly, *Pieris napi* (Kaitala and Wiklund 1995). However, we reexamined this question in a moth by measuring the relative reproductive success of virgin and previously mated male European corn borers (ECBs), *Ostrinia nubilalis*, placed simultaneously with one receptive virgin female. The ECB is an ideal experimental model with which to test this hypothesis because (i) spermiogenesis and spermatogenesis are completed before adult males emerge (Chaudhury and Raun 1966), (ii) previous male mating has a significant negative effect on female fecundity and fertility (Royer and McNeil 1993), (iii) females generally only mate once or twice under field conditions (Elliott 1977), and (iv) there is a male pheromone that plays a role in mating (Royer and McNeil 1992). We predicted that females would be able to discriminate between an experienced and a virgin male and, given the choice, would preferentially mate with the virgin. Even if female choice did not play a significant role in mate choice, we anticipated that virgin males would fare better in male-male interactions, owing to larger energy reserves and greater vigor.

Materials and methods

The (Z)-strain ECBs were reared at $25 \pm 0.2^\circ\text{C}$, $70 \pm 10\%$ R.H., under a 16 h light : 8 h dark photoperiod. Pupae were sexed and males and females held in separate cages. Moths were weighed upon emergence on a Mettler AE100 balance and kept in individual cages (150 cm^3) with an unlimited source of water. At the time of emergence males were alternately assigned to the virgin or mated/experienced category. Males assigned to the virgin category were never given the opportunity to mate until they were used in female-choice experiments. In the experienced-male selection process, we paired a 1-day-old male with a 1-day-old virgin female and then provided another 1-day-old virgin on alternate days until they had acquired 3 consecutive matings, following the protocol of Royer and McNeil (1993). This level of prior mating experience was chosen because female reproductive output was significantly less by the male's fourth mating (a mean reduction of 13 and 14% in fecundity and fertility, respectively) than that of females mated with virgins (Royer and McNeil 1993). Of the 200 males assigned to the experienced-male selection process, 52 successfully mated 3 consecutive times and were used in a mating-choice trial 48 h later. The wings of all males assigned to the mating-selection process were frozen and stored for future use (see below).

Twenty-four hours prior to a mating trial, an experienced male and a naive virgin of the same age were randomly selected as competitors for a single virgin female. Both males were anaesthetized with CO_2 (10–15 s at 10 L/min) and one, chosen at random, was marked with a small white dot of water-soluble Mistake Out® on

the thorax following removal of scales with a fine paintbrush. The marking of the males did not affect the outcome of mating trials ($2 \times 2 \chi^2$ (marking \times outcome of mating trial), $df = 1$ ($\chi^2 = 0.21$, $P = 0.64$). All males were weighed before and after a mating trial to obtain average adult body masses. A mating trial consisted of two males and a 1-day-old virgin female placed in a large inverted plastic cup (350 cm^3) with a water source for 24 h. Observations were made during the last 5 h of the scotophase, the normal mating period, using a flashlight covered with two layers of tissue paper and a Kodak® Wratten No. 29 red gelatin filter. Behavioral observations and the identity of the mating male (marked or unmarked) were noted. Later, the experience class (virgin or experienced) of the mating male was confirmed by the size of the spermatophore dissected from the female's abdomen as a male ECB's first spermatophore is ca. 8 times larger than his fourth one (Royer and McNeil 1993). If no mating had been observed, but a spermatophore was present in the female's reproductive tract, its size was used to determine which male had mated during the mating trial. If neither male mated with the female (confirmed by dissection) the same males were retested the following night with a new 1-day-old virgin female. In the event of a second night without a successful mating, the trial was terminated.

A post-hoc test was conducted to determine whether there was a relationship between the number of consecutive matings acquired in the selection process for experienced males and male quality, using fluctuating asymmetry (FA) of the forewings as an indicator of quality (e.g., Palmer and Strobeck 1986; Møller 1992; Thornhill 1992b). The wings of all males used in the selection process for experienced males were grouped according to the number of matings obtained (after failing to mate once, or after a mating trial) and then frozen for subsequent measurements. Wings broken during handling or infected by fungus during storage (129 of 200) were not included in the FA analysis. The forewings were removed from the thorax using fine forceps and placed under a transparent glass slide and their area was measured twice, with a 24-h interval, using a DIAS II video digitizer (Decagon Devices Inc.; 512×488 pixels resolution). The wings of virgin males used in the mating trials were not kept and thus were not available for FA analysis.

Statistics were carried out on the MINITAB software program (Minitab Inc. 1996). Measurements are reported as the mean \pm 1 SD. All χ^2 analyses with $df = 1$ were corrected for continuity using Yates' method (Zar 1984); t tests were performed using separate variances when these differed between groups (Zar 1984). A mixed two-way ANOVA ((R-L) \times individuals) was used to ensure that between-individual variation in wing asymmetry was significantly larger than could be accounted for by errors in measurements (Palmer and Strobeck 1986; Swaddle et al. 1994). Levene's test for homogeneity of variances was used to test for differences in wing-asymmetry variation between males in the different mating categories (Palmer 1994), as it is less sensitive to non-normality than Bartlett's test (Levene 1960).

Results

Experienced males obtained significantly more matings in the trials than virgins (Table 1). We detected no significant differences in body mass of males that successfully mated in their trials (pooled virgin and experienced) and those that did not (adult body masses at the time of the trial: 21.7 ± 3.4 vs. 20.7 ± 2.4 mg, $df = 78$, $P = 0.13$; body masses at emergence: 34.6 ± 5.1 vs. 34.3 ± 4.1 mg, $df = 78$, $P = 0.83$). Experienced and virgin males did not differ in emergence mass (Table 1), but in 31 of the 40 trials the experienced male was lighter than his virgin counterpart. Overall, therefore, experienced males were significantly lighter than vir-

Table 1. Outcome of 40 mating trials, together with the corresponding male body masses (mean \pm SD) at emergence and during the trials, when an experienced male (3 previous matings) and a virgin male European corn borer, *Ostrinia nubilalis*, were placed with one virgin female.

	Virgin male	Experienced male	Test	df	P
No. of successful matings	13	27	$\chi^2 = 4.9$	1	<0.05
Emergence body mass (mg)	34.8 \pm 4.4	34.2 \pm 4.9	$t = 0.52$	78	0.59
Trial body mass (mg)	22.6 \pm 3.1	19.8 \pm 2.3	$t = 4.47$	78	<0.001

gins at the time of testing (Table 1). Given that experienced males were more successful in mating than virgins, one might expect that being lighter conferred an advantage. However, within each mating category, males that succeeded in mating were heavier than those that did not. This difference was significant in the case of virgins (25.1 \pm 3.1 vs. 21.3 \pm 2.2 mg, $n = 13$ and 27, respectively, $t = 3.9$, $df = 38$, $P < 0.0001$) but not for experienced males (20.0 \pm 2.1 vs. 19.3 \pm 2.5 mg, $n = 27$ and 13, respectively, $t = 0.81$, $df = 20.6$, $P = 0.42$). Furthermore, of the 13 virgin males that succeeded in mating, only 1 was lighter than its experienced male counterpart. Collectively, therefore, previous mating history was a better predictor of the outcome of a trial than male mass.

Of the 40 matings, 29 were observed. A mating sequence in *O. nubilalis* usually consisted of the following consecutive steps: (i) the male approached a calling female; (ii) his wings were held vertically above the abdomen, fluttering slightly; (iii) his claspers were extruded; and (iv) he aligned his thorax 90° to that of the female and attempted to copulate by bending his abdomen in her direction. Male sex pheromones probably also play a crucial role in courtship and mating, particularly in closer interactions (Royer and McNeil 1992). Female rejection of males was observed at least once in 12 of the 29 observed copulations. The female either moved away or bent the tip of her abdomen towards the substrate, making it inaccessible to the male. Females rejected seven experienced and five virgin males. Rejected males never successfully mated, which supports the idea that female choice plays a role in ECB mating. In the remaining 17 observed matings, the first male to attempt a copulation was ultimately successful (5 virgins, 12 experienced). Once a male began copulating, he was never dislodged by the other male, although such attempts were observed in several trials.

Of the 200 males from which experienced males were selected, 74 (37%) never mated, 43 (21.5%) mated only once, 31 (15.5%) mated twice, and 52 (26.0%) mated 3 times. There was no correlation between the emergence mass of a male and the number of consecutive matings he subsequently obtained ($n = 200$, $r^2 = 0.001$, $F = 0.2316$, $P = 0.630$). Forewing asymmetry was normally distributed ($n = 71$ pairs of wings, Lilliefors's probability = 0.16) and nondirectional (0.025 \pm 1.29 mm², $t = 0.16$, $P = 0.87$; Table 2). The average wing size of an individual did not correlate with absolute FA ($n = 71$, $df = 1$, $r^2 = 0.004$, $P = 0.26$; Table 2). ECB forewings are small (51.04 \pm 3.76 mm²) and mean asymmetry represented only 1.7% of mean wing area (Table 2). Nevertheless, our measurements were accurate enough to reveal differences in each wing's area within an individual because

Table 2. Description of forewing area asymmetry ($n = 71$ pairs of wings) in the European corn borer.

Mean wing size (mm ²), (R+L)/2	51.04 \pm 3.76
Net asymmetry (mm ²), (R-L)	0.025 \pm 1.29
Skew	-0.0085
Kurtosis	2.77
Absolute asymmetry (mm ²), R-L	0.9 \pm 0.9
Slope (mean wing size vs. R-L)	0.553 \pm 0.491*

Note: Values are given as means \pm 1 SD.

* $P = 0.26$.

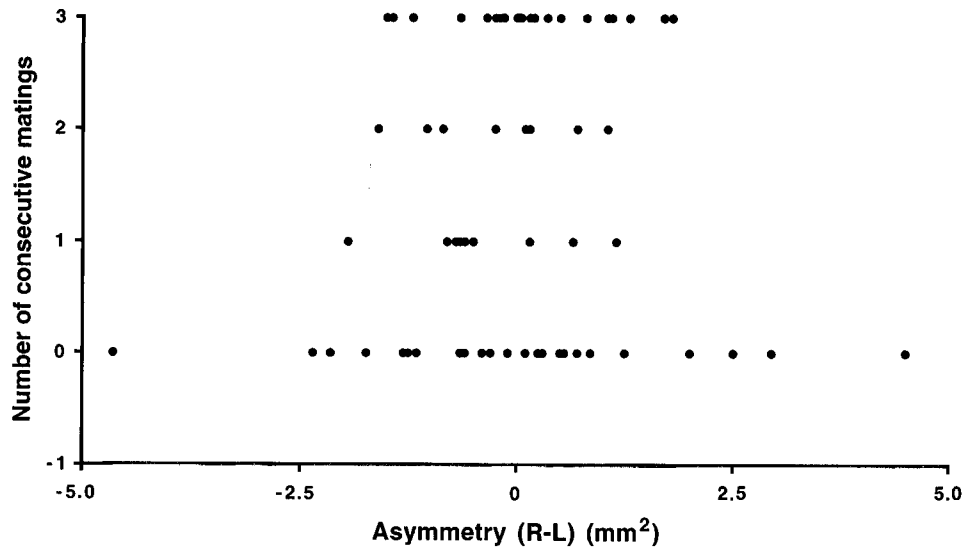
the error term of the mixed two-way ANOVA (which includes the sides \times replicate and sides \times replicates \times individual interactions) was significantly smaller than the sides \times individuals interaction term ($F_{[70,140]} = 7.15$, $P < 0.001$). Thus, we could test for differences in FA variances between mating groups and this test was significant ($n = 71$ pairs of wings, $F_{[3]} = 2.84$, $P = 0.044$). A subsequent Tukey's pairwise comparison revealed significant differences between the forewing FA variances between mating categories 0 and 3 ($P = 0.043$; Fig. 1).

Discussion

Contrary to our original hypothesis, the mating success of naïve male ECBs was not greater than that of experienced individuals despite the possible cost to females of mating with previously mated individuals (Royer and McNeil 1993). However, while no significant difference was observed in the mating success of naïve and experienced male green-veined white butterflies (Kaitala and Wiklund 1995), experienced male ECBs were significantly more successful at mate acquisition than virgins. A similar trend was noted in the winter moth in that on any given night, previously mated males had a greater probability of mating than naïve individuals (Van Dongen et al. 1999). If repeated matings generally decrease male phenotypic quality, which has the potential to reduce female reproductive success, then why do females mate with experienced males? There are at least two possible explanations: superior competitive ability of experienced males in the absence of precopulatory choice by females, or active female choice favoring experienced males.

In the absence of precopulatory female choice, the competitive ability of previously mated males could have been superior, as a result of greater mobility or of experience gained during previous matings. As body mass declines with

Fig. 1. Signed forewing fluctuating asymmetry (R-L) (mm^2) in male European corn borers, *Ostrinia nubilalis*, that had acquired different numbers of consecutive matings ($n = 27, 10, 9,$ and 25 pairs of wings in the categories of 0, 1, 2, and 3 consecutive matings). Significant differences in variation were detected between categories 0 and 3.



repeated matings but wing size remains constant, mated males will have lower wing-loading and possibly greater aeronautical abilities. Liggett et al. (1993) observed a positive correlation between low wing-loading and higher mating success in the yellow dung fly, *Scatophaga stercoraria*. However, we do not think that low wing-loading explains the high success of experienced males, as trials took place in small cages, where the flight component was largely removed. This claim is supported by data for a related pyralid species, *Parapediasia teterrella*. In this species smaller males, with lower wing-loading, had a high rate of mating success in the field but lost this advantage in the laboratory, where the flight component was removed (Marshall 1988). Furthermore, our results show that within a given class (experienced or virgin), heavier males were generally more successful in acquiring mates than lighter ones. Alternatively, experienced males may be more active. Although we did not quantify activity, we saw no evident differences in the behavior of mated and naïve individuals. Even in large cage experiments, Kaitala and Wiklund (1995) found no significant differences between the activity of naïve and mated male green-veined white butterflies, so one would not expect this to have been a determinant factor in our small experimental arenas.

We did observe female rejection behavior during our mating trials, which suggests that active mate choice by female ECBs could explain the results obtained. It is possible that experienced males were, on average, of higher quality than virgin males and therefore females chose to mate with them. Indeed, the rigorous selection process for obtaining experienced males in the mating trials (only 52 from an initial 200 met the requirements) may have inadvertently selected higher quality males. One way that females may assess the quality of potential mates is through male pheromones, as these may be of considerable importance for male mating success (e.g., Fitzpatrick and McNeil 1988; Birch et al. 1990), including that of the ECB (Royer and McNeil 1992). In the true armyworm, *Pseudaletia unipuncta*, there is considerable inter-

individual variability in both the total quantity of male pheromone and the relative proportion found in the bilateral left and right abdominal hair pencils (Fitzpatrick et al. 1985). Furthermore, after a mating sequence the male only loses about 6% of his pheromone (Fitzpatrick and McNeil 1988), which cannot be replaced. Thus, if at emergence a high-quality male had twice the pheromone of low-quality one, then even after three matings, his pheromone titer would still exceed that of a poor-quality virgin.

In the Japanese scorpionfly, *Panorpa japonica*, females discriminated between males on the basis of their pheromone emissions, and successful males had, on average, a lower wing-length FA (Thornhill 1992b). FA represents departures from ideal symmetrical development of a bilateral trait, and low FA has been correlated with different measures of fitness such as high heterozygosity, greater longevity, high fecundity, dominance, and faster growth rates (see reviews by Palmer and Strobeck 1986; Leary and Allendorf 1989; Parsons 1990; Palmer 1994; Watson and Thornhill 1994; but also Johnstone 1994; and Fowler and Whitlock 1994). Numerous studies have correlated low FA with high mating success (birds, Møller 1988, 1990, 1992; mammals, Malyon and Healy 1994; coleopterans, Ueno 1994; dipterans, Liggett et al. 1993; McLachlan and Cant 1995; Møller 1996, but also see Markow and Ricker 1992; mecopterans, Thornhill 1992a; odonates, Harvey and Walsh 1993; orthopterans, Simmons 1995) and in at least one study, low FA has also been shown to be heritable (Thornhill and Sauer 1992), suggesting that FA of relevant traits may serve as a measure of overall fitness.

A similar situation could exist for the ECB, as the males who acquired 3 successive matings had significantly lower FA than those who never mated. As noted above, under our small-enclosure experimental conditions, any flight-performance advantages that might have been conferred by low FA would have been largely removed. Furthermore, while visual assessment of wing symmetry may serve in a diurnal

species (i.e., birds; Møller 1992), it is unlikely that visual cues play an important role for nocturnal lepidopterans such as the ECB, especially when the differences in wing FA are very small (<2%). However, low FA of male forewings may correlate with other traits reflecting male quality, such as pheromone titers in the bilateral pheromone glands.

Females may also exercise postcopulatory mate choice, where male quality influences females' postmating behaviors. This may be reflected in the duration of the refractory period and the probability of subsequent remating (Delisle and Bouchard 1995; Kaitala and Wiklund 1995; McNeil et al. 1995; Delisle and Hardy 1997; Van Dongen et al. 1999), as well as in sperm precedence once remating occurs. Following multiple mating, the success of sperm from the different males can vary (e.g., Svärd and McNeil 1994; Wendell and Cook 1998) depending on factors such as spermatophore size (LaMunyon and Eisner 1993, 1994, although a larger spermatophore does not always reflect high quality, see Delisle and Hardy 1997) or the quantity of sperm and (or) the male-derived peptides that modify the female endocrine system (Kingan et al. 1995; Fan et al. 1999), which are transferred by each male at the time of mating. If there is a positive relationship between wing FA and male pheromone titers, could one also exist between FA and the male resources (sperm and accessory-gland secretions) transferred to females at the time of mating? There is some evidence to support this, for in a study of fitness components and FA in the winter moth (*Operophtera brumata*), the probability of a female remating increased with increasing asymmetry of the first male and not with his body size (Van Dongen et al. 1999).

In conclusion, we have shown that males who successfully completed 3 previous matings had a higher probability of acquiring an additional mate than did a virgin of similar age, when they were simultaneously in the presence of one virgin female. We have postulated that this is due to precopulatory mate choice by females and that there may be a correlation between wing FA and male pheromone titer. Furthermore, we raised the possibility of a correlation between wing and (or) male pheromone titer and characteristics of the male ejaculate. It is clear that the results of studies examining the relationships between FA and individual fitness or quality are varied, to the point where the generality of FA as a valid indicator of individual fitness is questioned (see Palmer 1999; Van Dougan et al. 1999; and references therein). However, in light of the results obtained in this study, we believe that examining FA in male moth pheromones (see Fitzpatrick et al. 1985), from the perspective of both pre- and postcopulatory mate choice, could prove fruitful. Olfactory cues such as male pheromones will change with each successive mating and the level of FA could provide females with a reliable indicator of male quality. However, this idea cannot be tested with the ECB, as the male pheromone has not yet been identified. In future work using the true armyworm, we will examine to what extent FA actually reflects male quality, as measured by pheromone titer and the production of resources (sperm and accessory-gland secretions). We will also determine how male quality affects the reproductive success of both sexes, by examining parameters such as the quality of progeny produced, the probability of remating, and the degree of sperm precedence when females remate repeatedly.

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