A female preference for experienced males in the almond moth, *Cadra cautella*

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Abstract

Male mating status can affect female reproductive output if male ejaculate investment declines over consecutive matings. Accordingly, females are predicted to mate preferentially with virgin males. In mildly polyandrous lepidopterans, female fitness is less affected by reduced male investment than in more polyandrous species, and so the predictions for female mating preferences are less clear. We examined female mating preferences in the mildly polyandrous almond moth, *Cadra cautella*, in which ejaculate size does not affect female reproductive output. First, we allowed females to mate with virgin or once-mated males, in which the males were presented individually, or simultaneously. We recorded the latency to mating, and in the case of the simultaneously presented trials, the identity of the successful, copulating male. We found females mated more frequently with mated males (when simultaneously presented with both males), yet females did not differ in the time taken to initiate copulation with any male. We further examined if this mated male advantage was due to differential mate detection or locomotory behaviour of the male treatments. We tested the ability of virgin and mated males to locate a receptive female within a wind tunnel using long-distance pheromone cues, and recorded their activity budget. We found no difference in the ability of mated or virgin males to locate or approach a receptive female, or in their activity levels. These data suggest a female preference for mated males in this species, a preference that may minimise other potential costs of mating.

Keywords: *Ephestia cautella*; mate choice; polyandry; monandry

Introduction

In many insect taxa, males provide a substantial, nutritious ejaculate at mating (for a review, see Vahed 1998) which are costly to produce (Dewsbury 1982; Wedell et al. 2002). Typically, a male's previous mating investment reduces, at least temporarily, the quality of his future mating investments (Vahed 1998). In many insect species, the depletion of sperm and accessory gland material over consecutive copulations can result in a reduction in subsequent female reproductive output (for a review, see Torres-Vila and Jennions 2005; South and Lewis 2011). Thus, male mating experience can have significant consequences for female fitness (Milonas et al. 2011), with costs potentially more severe for monandrous species (Jones 2001; Velde et al. 2011). In such cases, there is expected to be strong selection for females to mate preferentially with males that maximize their reproductive output and/or minimize any costs associated with mating (Andersson 1994; Andersson and Simmons 2006). Despite this, patterns of female mating preferences for males of different mating experiences in insects are equivocal. Research demonstrates increased copulation success for both virgin (Markow et al. 1978; Klepetka and Gould 1996) and experienced (Cook 1995; Krupke et al. 2008; Iyengar 2009) males, and in other cases, the absence of any mating advantage (Schwartz 1991; Kaitala and Wiklund 1995; Velde et al. 2011).

Despite the potential reduced direct benefits (ejaculate size) typically associated with mated males, a mating advantage for experienced male may arise for several reasons. First, it may simply reflect a female preference for traits indicating genetic quality in males, and thus these males are likely to acquire more matings. For example, mated males are a subset of the male population which are on average superior to the general population given their demonstrated ability to secure matings, as not all males are able to copulate. Interestingly, the indirect benefits of mating with attractive males can outweigh the demonstrated direct costs of such matings, as shown in the house cricket, *Acheta domesticus* (Head et al. 2005). Second, however, the higher mating success of mated males may not necessarily be female-driven; the experience of courting or mating itself may increase the male's future copulation success (Molina and Christenson 2008). For example, experienced males 'learn' which females are best to court in *Drosophila melanogaster* (Dukas 2005); while mated, and therefore lighter yellow dung fly males, *Scatophaga stercoraria* may have

an advantage in obtaining copulations through scramble competition (Liggett et al. 1993).

Separating mechanisms of female choice from male-male competition is challenging in the Lepidoptera, in which stationary females broadcast sex pheromones. That is, the extent to which female ‘preference’ is simply a reflection of male activity levels, or success in scramble competition, is frequently unclear. For example, in the polyandrous green-veined white butterfly, virgin males induced a longer refractory period, but females do not appear to discriminate between mated and virgin males, instead mating with the most active males (Kaitala and Wiklund 1995). Thus, differences in mate detection and general activity should be taken into account in mate preference studies. Ultimately, and unlike many other insect taxa, females have control over whether a mating is successful as they can thwart male copulation attempts by simply lowering their abdomen (Phelan and Baker 1986).

The almond moth *Cadra cautella* (also known as *Ephestia cautella*) (family: Pyralidae) provides an interesting model to explore potential mechanisms of female choice. Although 20% of females mate more than once, the number of spermatophores received by a female does not affect her reproductive output (McNamara et al. 2008). Moreover, while male ejaculate size (including both apyrene and eupyrene sperm number) decreases dramatically with mating experience (McNamara et al. 2007), this reduction has no effect on female fecundity. Male ejaculate size in this species does, however, affect female mating behaviour and longevity. Spermatophore size is an important determinant of female post-mating receptivity as virgin males that transfer larger ejaculates reduce female re-mating rate more effectively compared with small ejaculate producing experienced males (McNamara et al. 2009). Furthermore, attached to each spermatophore is a large chitinous spermatophore process (CSP), the function of which is unclear (Norris 1932). CSP size (which also decreases dramatically in experienced males) entails costs for females: monandrous females that received relatively larger CSPs showed reduced longevity (McNamara et al. 2007). Combined, these data suggest that there may be both costs to mating with virgin males (reduced longevity) and benefits (increased potential to re-mate) to mating with experienced, mated males. The almond moth, therefore, provides an interesting model to explore potential mechanisms of female choice in the absence of a clear selection pressure for females to choose between mated and virgin males based on maximising reproductive output.

In this study we looked for evidence of non-random mating between females and males that differed in mating experience. We then explored whether variation in the mating success of virgin and mated males was attributable to differences in their mate-location ability or general activity levels.

Methods

Adults and larvae of *Cadra cautella* were obtained from the CSIRO, Canberra. This population, initially sourced from a wild Queensland population, had been maintained in the laboratory for four years (approximately 36 generations). Larvae were reared on a diet of bran, yeast, and glycerol (ratio = 10:1:1). Adults and larvae were maintained at 28°C, on a 12h:12h light:dark regime. Virgin individuals were obtained by isolating adults shortly after emergence, before they had the opportunity to mate. Following removal, adults were weighed, sexed, and stored in 5ml polypropylene vials without food or water until required. All mating trials commenced at the start of the scotophase (dark phase).

Newly-emerged males were haphazardly assigned to either a ‘mated’ or ‘virgin’ treatment. ‘Mated’ males were assigned a randomly chosen newly-emerged, virgin female. The couple was placed in a 240ml plastic vial and observed until copulation had ceased. The female was discarded, while the male was removed to a clean 120ml vial until he was required on the following day. Couples that failed to mate were discarded. Males assigned to the ‘virgin’ treatment were placed in one side of a 240ml plastic vial that had been separated into two compartments using a mesh barrier. A newly-emerged virgin female was placed in the other half of the container for 1h. The mesh allowed the transfer of any chemical and auditory cues, but prevented tactile communication. This meant that both ‘virgin’ and ‘mated’ males were of the same age and had developed experience and expended energy on courting females.

Does male mating status or a female’s opportunity for choice affect the likelihood or time to copulation?

To explore whether copulations occur more readily with virgin or mated males or when females are permitted a choice, we set up three mating treatments in which 71 individual virgin females were exposed to either a single virgin male (single-virgin

trials, $n = 24$); a single mated male (single-mated trials, $n = 26$), or to one virgin and one mated male simultaneously (paired trials, $n = 21$). For each trial, virgin females were placed into individual 120ml plastic containers at the onset of the scotophase. Females were allocated to either a single male or paired male treatment. For the single-male treatments, a single virgin or mated male was placed in the female's container. For the paired-male treatment, a virgin and a mated male, matched for initial weight (mean weight difference $\pm$ SE = 0.22 ± 0.05 mg) were simultaneously placed in the container with the female. For all females, the time taken to commence copulation (if it occurred) was recorded. Females that had not commenced copulation within 5hr were discarded. After copulation had ceased, the couple was separated and maintained until death to assess female reproductive output. Females were dissected post-mortem and the spermatophore removed. The CSP was separated from the sperm-containing ampulla and laid flat in a drop of glycerol on a glass slide prior to being photographed at 40x magnification using a *Moticam 480* Camera (Motic China Group Co. Ltd.). The CSP image was then measured using a digital image measurement program *Motic Images 2000 (Version 1.3)*. The region extending from perpendicular to the lateral process to the tip of the primary process was delineated and the area (mm^2) was recorded (see McNamara et al. 2007). For paired trials, the identity of the male that mated with the female was inferred from the size of the CSP as males that have previously mated transfer significantly and unambiguously smaller CSPs (mean $\pm$ SE size of first CSP = 0.19 ± 0.01 mm^2 ; second CSP = 0.07 ± 0.02 mm^2) and fewer sperm (McNamara et al. 2007).

Does male mating status or a female's opportunity for choice affect their reproductive output?

In several cases for each of the mating treatments (single-virgin ($n = 2$), single-mated ($n = 3$) and paired treatments ($n = 3$)), the female was dissected immediately after mating and the CSP size assessed; these females were not included in subsequent analyses of female reproductive output. Remaining females (single-virgin trials ($n = 17$), single-mated trials ($n = 20$), paired trials ($n = 17$) (14 paired-mated and 3 paired-virgin females) were placed in 30ml vials and allowed to oviposit until their deaths. Eggs were collected from the bottom of the vial, separated from the female with a

piece of mesh, and poured over sticky paper. This allowed an accurate assessment of fecundity and fertility, but prevented eggs being eaten by cannibalistic larvae.

Do virgin or mated males locate receptive females more quickly?

We used a wind tunnel (height = 0.5 x width = 0.5 x length = 1.5m) to assess whether a male's mating status affected his ability to locate a sexually mature female. The upwind and downwind ends of the wind tunnel each had a 20cm hole covered by two removable pieces of mesh. A constant-speed air source pushed air via the upwind hole into the wind tunnel. A 'calling' stimulus female (with abdomen extended and pheromone gland extruded) was suspended in a 30ml cage made of mesh from the ceiling of the wind tunnel so that the cage was approximately 10cm in front of the centre of the wind source. One-day old virgin and mated males (obtained as above) were placed at the opposite end of the wind tunnel to the female in upturned 30ml vials (each approximately 5cm equidistant from the beginning of the downwind entrance). Pairs of virgin and mated males were matched closely according to weight (paired t-test: $t_{22} = 0.34$, $p = 0.73$). We ran the air source for one minute prior to each trial and then the two males were released simultaneously and allowed to move freely through the wind tunnel. The time taken for each male to touch or land on the female's cage, the frequency of landing events, and the time taken to fly within 5cm of the female's cage were recorded. Males never physically interacted with each other in the wind tunnel. Males that landed on the female's cage, or approached within 5cm of the female were considered 'successful'. For a sub-set of trials ($n = 15$) an activity budget for both males was also recorded; this included time spent stationary, walking, and flying. Trials were conducted for 20min, however, if both males had not commenced any movement after 10mins the males were returned to their upturned vials and the trial restarted. Trials were restarted once only for each pair of males and, if they failed to move a second time, the trial was discarded. Between trials, the wind tunnel was cleaned with ethanol and the fan was turned on for two minutes to remove any residual odours. The identity of the male within each trial that 'successfully' reached the female the greatest number of times was recorded. In one trial, mated and virgin males approached the female equally; this trial was subsequently discarded from analysis.

Statistics

Data were analysed in JMP (version 9.0.0; SAS Institute Inc. 2000) In two single-virgin trials the couples successfully commenced copulation, but failed to separate. These data were used to assess the likelihood of copulation and latency to copulation, but were excluded from subsequent reproductive output data. Copulation latency data could not be normalized and were examined with a Kruskal-Wallis, non-parametric analysis. Models exploring variation in the proportion of fertilized eggs laid by a female used a binomial error distribution with a logit link function that incorporates the total number of eggs laid by the female as the binomial denominator. Data exploring the frequency with which males successfully approached the female in the wind tunnel and male activity in the wind tunnel were examined with non-parametric Wilcoxon Signed Ranks Tests. Medians [interquartile range] are supplied for non-parametric tests and means $\pm$ standard error for parametric tests.

Results

Do females copulate more readily or frequently with virgin or mated males?

A successful copulation was observed in the majority (64/76) of all trials. Four single-virgin, three single-mated, and one paired couple did not mate. These 8 trials were excluded from further analysis.

Females were more likely to mate with a mated than virgin male, when presented with a choice of mate in the paired trials (16/20 trials; $\chi^2_1 = 7.20$, $p = 0.007$). However, in a no-choice setting, the likelihood of a copulation successfully occurring was comparable regardless of whether the male was a virgin (21/27 trials) or had previously mated (23/26 trials: Fisher's exact $p = 0.467$).

The median time (mins) taken for females to commence copulation was comparable for all females regardless of the mating status of her mate or whether he was presented individually or simultaneously with a rival (single-virgin = 11.0 [2.00-15.00], $n = 21$; single-mated = 8.00 [4.00-13.50], $n = 23$; paired-virgin = 3.50 [2.75-5.50], $n = 4$; paired-mated = 5.50 [3.00-14.25], $n = 16$; $\chi^2_3 = 1.53$, $p = 0.67$).

Does male mating status or a female's opportunity for choice affect their reproductive output?

Two single-virgin trials, in which the couples could not separate after copulation (and therefore died) are excluded from this analysis. The mean number of eggs laid by a female was not affected by the mating status of the male when females were given no choice in mating partner (single-virgin = 81.47 ± 11.41 ; single-mated = 71.05 ± 7.64), or when they were able to choose between the two simultaneously presented males (paired-virgin = 90.66 ± 11.93 ; paired-mated = 63.71 ± 9.75 ; $F_{3,48} = 1.13$, $p = 0.34$). However, females laid more eggs when they were heavier ($F_{1,48} = 20.04$, $\beta = 9.84$ (2.16), $p < 0.001$) and when the male with which they mated was heavier ($F_{1,48} = 4.56$, $\beta = 7.37$ (3.43), $p = 0.04$).

The mean proportion of fertilised eggs produced by a female was not affected by the mating status of the male when females were given no choice in mating partner (single-virgin = 0.79 ± 0.07 ; single-mated = 0.73 ± 0.073), or when they were able to choose between the two simultaneously presented males (paired-virgin = 0.89 ± 0.04 ; paired-mated = 0.68 ± 0.08 ; $F_{3,48} = 0.20$, $p = 0.89$). The number of fertilised eggs produced was also not affected by female weight ($F_{1,48} = 0.02$, $\beta = 0.003$ (0.02), $p = 0.89$), or male weight ($F_{1,48} = 0.01$, $\beta = -0.005$ (0.04), $p = 0.90$).

CSP size differed significantly across the four groups of males ($\chi^2_3 = 43.20$, $p < 0.001$; Fig. 1), with virgin males providing larger CSPs.

Do virgin or mated males locate receptive females more quickly?

We found no evidence that virgin and mated males differed in their ability to locate a female in the 22 trials in which a male successfully approached the female. The first male to enter the 5cm zone or land on the female's cage was equally likely to be mated or virgin (13 mated vs 9 virgin males; $\chi^2_1 = 0.73$, $p = 0.40$). Moreover, there was no difference in the median time for mated (61 [29.5-347.5] secs) or virgin [50 (34.5-211.5] secs), males to make contact with the female ($U = 0.09$, $df = 1$, $p = 0.76$). Mated and virgin males did not differ in the number of times they successfully approached the female (Table I). We also scored which male approached the female the most within a trial: of the 21 trials in which there was a winner, we found no difference in the likelihood that either male approached the female the greatest

number of times (13 mated vs 8 virgin males; $\chi^2_1 = 1.19$, $p = 0.27$). Furthermore, mated and virgin males did not differ in their activity budgets over the ten min trial period (Table I).

Discussion

Our data revealed that in the absence of choice, females were equally likely to mate with a virgin male as an experienced male; however, when two such males were presented simultaneously the experienced male was three times more likely to mate with the female compared to his virgin rival. This difference in mating success apparently arises as a result of female preference for mated males, as there are no forced copulations (Phelan and Baker 1986) and no observable differences between mated and virgin males in their general activity levels, or mate location abilities. Our study also confirms previous findings that suggest that females derive no immediate fitness benefit through increased fecundity or fertilisation success from their choice (McNamara et al. 2007).

In general, evidence for female mating preference with respect to male copulation experience is inconsistent. In species where males are likely to be sperm limited, arguably the vast majority of species (Wedell et al. 2002), it is predicted that females should prefer to mate with virgin or infrequently mated males. Accordingly, female preference for, or increased mating success of, virgin males has been demonstrated in numerous species (Sanders 1975; Markow et al. 1978; Nakatsuru and Kramer 1982; Sato and Goshima 2007). However, there is also evidence that females will readily or preferentially mate with previously mated males, despite the potential fertility costs of their reduced ejaculate size (van Dongen et al. 1998; Kendall and Wolcott 1999; Schlaepfer and McNeil 2000; Edvardsson et al. 2008; Krupke et al. 2008). This disparity may be due to species' differences in the probability of future mate detection and the costs of rejecting certain males. In many lepidopteran species, for example, the depletion of sperm and accessory gland material over consecutive copulations typically results in a reduction in female reproductive output (for a review see Torres-Vila and Jennions 2005). Yet the strength of selection to avoid these males may depend on the species' mating system; female reproductive output is more

strongly tied to the amount of ejaculate received by highly polyandrous species compared with monandrous or mildly polyandrous species (Torres-Vila et al. 2004).

There are two mutually non-exclusive proximate explanations for the increased mating success of previously mated males. First, mated males may be superior competitors, either through inherent differences in their quality (indicated by their proven copulation success), or by a change in phenotype caused by the act of mating, which increases their ability to locate and copulate with receptive females faster than virgin males (Schlaepfer and McNeil 2000). Alternatively, the greater reproductive success of previously mated males may reflect an active female preference for this phenotype. Three lines of evidence lead us to reject the first explanation in favour of a female preference for mated males. First, it is unlikely that variation in courtship experience accounts for the observed difference in mating success as we controlled for any benefits of previous experience on future copulation success by allowing males in the virgin category to actively court females prior to testing in their trials. Second, it is conceivable that mated males have a potentially greater flying speed or mobility because they have transferred a large amount of body mass with their ejaculate during mating and thus have a lower wing-loading (Kaitala and Wiklund 1995). However, we matched virgin and mated males for weight, removing any potential differences in wing-loading between males. Moreover, mated males were not more active, and were not able to locate or reach receptive females faster within the wind tunnel. Finally, although mated males comprise a group with a demonstrated ability to acquire matings (100% mating success compared with only 68 percent [$n = 75$] mating success for virgin males; K.B.M. unpublished data), females actively reject males by raising their abdomen and thus any given mating is a product of both male and female willingness to mate. Interestingly, this preference for mated males also exists for mated females; non-virgin females mated more often with experienced males when they had previously mated with a virgin male, but expressed no preference when previously mated to an experienced male (McNamara et al. 2009).

The mechanisms behind this experienced male advantage are not entirely clear. In the almond moth, the most likely mode of attraction by males and discrimination by females is pheromonal. The role of pheromones in mate attraction in insects has gained increasing attention (Svensson 1996; Wyatt 2003; Johansson and Jones 2007) and the importance of pheromone titre in mate discrimination has been

documented in several species. In the tobacco moth, *Ephestia elutella*, females preferentially mate with larger males, which produce twice the quantity of courtship pheromone than smaller males (Phelan and Baker 1986) and in the Caribbean fruit fly, *Anastrepha suspensa*, mated males initiate subsequent copulations faster than virgin males which is thought to be related to the two-fold quantity of sex pheromones released by these mated males compared with virgin males of the same age (Teal et al. 2000). Courtship in the almond moth is complex. Males fan their wings in close proximity to the females, emitting sex pheromones. In the final movements of the courtship routine the male brings his abdominal hairpencils (assumed to be pheromone diffusion structures) into contact with the female's antennae (Grant and Brady 1975). At this point, variation in pheromone titre may provide a potential mechanism for differential mating success. Furthermore, it is not possible to ascertain the courtship intensity of the mated and virgin males, given our experimental design. In the European corn borer *Ostrinia nubilalis*, in which females have reduced fecundity and longevity when mated to experienced males, females reject mated males more often, yet the authors suggest that mated males have a mating advantage over virgin males as they attempt matings more frequently (Milonas et al. 2011). Yet, similarly to our study, this mated male advantage was only evident when the virgin and mated males were presented simultaneously to a female, and not when presented individually. Thus, it also remains possible that mated and virgin almond moth males court differently, with females preferring males of one courtship intensity.

Given that there are no clear costs, but potential benefits, to mating with a mated male, it is also possible that this preference for experienced males may not be driven by differences in male courtship or pheromone quality, but rather an active female preference for mating experience *per se*. Males reduce female re-mating frequency by transferring enlarged ejaculates (Kaitala and Wiklund 1995; Oberhauser 1997; Wedell and Cook 1999; Marcotte et al. 2006; McNamara et al. 2009), explaining why lepidopteran ejaculates are composed mostly (typically 90 percent) of anucleate, 'filler' sperm (Cook and Wedell 1999). Thus, mating with a mated male may mean that females have increased control over their remating behaviour, and therefore the paternity of their offspring. This might be particularly relevant in the Lepidoptera because while females emit pheromones to attract males, these signals are not targeted towards specific mates (Svensson 1996). Thus, multiple mating may be the best (if not only) way for females to exert control over her offspring's

paternity. The impact of female multiple mating on offspring fitness remains to be tested in the almond moth. However, it raises the intriguing possibility that the increased longevity (associated with the reduced CSP) and the opportunity to mate multiply that is afforded to females who mate with experienced males (McNamara et al. 2007; McNamara et al. 2009) may act as a selection pressure for females to discriminate against virgin males.

In conclusion, there is unlikely to be a selective pressure for female almond moths to avoid mating with experienced males to avoid reduced fertility (as may be the case in highly polyandrous species) as there are no costs to females of receiving smaller ejaculates (McNamara et al. 2007). Although there is no clear difference in male mate locating ability and general activity levels, as demonstrated in other species, a mated male advantage may arise through a female preference for increased courtship activity or olfactory attractiveness. The almond moth provides evidence that, even in monandrous species, the fitness costs of mating with sperm depleted males need not necessarily be significant, and raises the intriguing possibility that female mate choice may evolve also to potentially mitigate the costs of mating, rather than to maximise benefits.

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Table I. The median number of times that males approached a caged female, and the median activity values for virgin and mated males in the wind tunnel. Total activity is the sum of flying and walking duration. Medians are presented with their interquartile range.

Activity	Virgin male	Mated male	Statistics	P
Approaches	0.50 [0.00 - 2.75]	1.00 [0.00 - 2.00]	$W = 115.50$	1.00
Flying duration (s)	75.00 [32.00 – 212.00]	53.50 [1.50 – 134.00]	$W = 52.00$	0.43
Walking duration (s)	14.00 [4.50 - 32.25]	37.81 [3.00 - 50.25]	$W = 57.00$	0.44
Total activity (s)	176.00 [47.75 - 268.50]	97.00 [49.50 - 185.80]	$W = 47.50$	0.50

Figure legend

Figure 1. Boxplots of the size of the CSP transferred by virgin and mated males in the singly-presented and paired trials. Boxplot whiskers represent maximum and minimum values. Letters above the boxplots denote statistically different comparisons.

582 Figure 1.

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