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Does Male Sexual Experience Influence Female Mate Choice and Reproduction in the Melon Fly (Diptera: Tephritidae)?

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Abstract. The mating experience of male insects can have significant effects on female fitness, particularly if prior copulations deplete stores of sperm and/or accessory gland products. Females of several tephritid fruit fly species appear to incur a cost - in terms of reduced longevity or reproductive success – by mating with non-virgin over virgin males. Despite these fitness costs, however, females do not always discriminate among males having different sexual experience. The present study had two objectives. First, mating trials were performed to determine whether virgin females of the melon fly *Zeugodacus cucurbitae* (Coquillett) discriminated between virgin and non-virgin (thrice-mated) males in selecting a mate. Second, survivorship, egg production and egg hatch rate were measured for females mated to virgin or non-virgin males to assess the impact, if any, of male mating experience on female lifespan and reproduction. Results showed that females did not discriminate among males based on their sexual experience and that female survival and reproduction were unaffected by male mating experience. These findings are compared with results obtained for other tephritid species.

Like several other species of tephritid fruit flies (Diptera: Tephritidae), the melon fly *Zeugodacus cucurbitae* (Coquillett), displays a lek mating system in which (i) males form aggregations that serve as mating arenas (termed leks), (ii) males do not control any resources critical to females and provide only sperm, and (iii) females are free (i.e., not coerced) to compare and select mates at the arena (Kuba et al. 1984, Iwahashi and Majima 1986, Mir and Mir 2016). In the apparent absence of material benefits, female choice in lek species is presumably based on male phenotypic cues indicative of genetic quality. A common feature of lek species, including tephritids (e.g., Poramarcom and Boake 1991, Robacker et al. 1991, Whittier et al. 1994) is a highly skewed distribution of mating frequency among males, where a few males achieve a large proportion of all matings, and most males mate rarely or not at all (Höglund and Alatalo 2005).

Owing to this mating pattern, *Z. cucurbitae* females arriving to a lek are likely to encounter preferred males that have mated previously, perhaps multiple times. Males of certain insect species may mate repeatedly over relatively short intervals (i.e., within a daily period of sexual activity) and may deplete sperm and/or accessory gland products transferred during copulation, and this reduced ejaculate may, in turn, result in the reduction of female fecundity, fertility (% egg hatch), and longevity (e.g., Helinski and Harrington 1991, Fox et al. 1995, Kant et al. 2012). This possibility, however, seems unlikely for the *Z. cucurbitae*, because (i) daily sexual activity is limited to a brief interval just before sunset, (ii) matings are long-lasting, with pairs coupled until the following sunrise, and, as a result, (iii) males do not mate more than once a day, providing ample time to replenish sperm and seminal fluid components (Kuba et al. 1984, Kuba and Soemori 1988). Consistent with these observations, males of a related species with similar mating behavior, *Bactrocera tryoni* (Froggatt), were found to transfer similar numbers of sperm to females during their first mating and all four successive matings (i.e., on the four consecutive days immediately following the initial copulation; Radhakrishnan et al. 2009). The reproductive output of females was not monitored in this study, and the possibility remains that, although sperm supply did not decrease with repeat matings, other components (e.g., proteins) of the seminal fluid did decrease, having negative effects on female reproduction.

Evidence from two other tephritid species suggests that females may incur fitness costs by mating with a previously mated male, even when the male remating interval was 24 h or longer. In the Mediterranean fruit fly, *Ceratitis capiata* (Wiedemann), females mated to virgin males had higher fecundity and greater survival than females mated to males that had mated 5 d earlier, which presumably allowed sufficient time to replenish sperm supplies (Whittier and Kaneshiro 1991). Likewise, in the olive fly, *Bactrocera oleae* (Rossi), the fecundity and fertility of females mated to thrice-mated males (i.e., one mating on each of the three days immediately prior to mating with the test female) were both significantly lower than recorded for females mated to virgin males (Gerofotis et al. 2015).

The present study addressed two questions regarding mate choice and subsequent fitness consequences in *Z. cucurbitae* females. First, mating trials were performed to determine whether melon fly females discriminated between virgin and non-virgin males in selecting a mate. Second, survivorship, egg production and egg hatch rate were measured for females mated to virgin or non-virgin males to assess the impact, if any, of male mating experience on female lifespan and reproduction.

Materials and Methods

Insects. Flies used in this study were derived from a laboratory colony started with 500–600 adults reared from zucchini (*Cucurbita pepo* L.) collected in commercial fields near Kapolei, HI. The colony was maintained in a large screen cage (60 x 40 x 30 cm) and provided a food mixture of sugar and yeast hydrolysate (5:1 v:v) ad libitum and water. Store-bought, rinsed zucchinis were provided for egg-laying, and infested vegetables were placed in opaque plastic boxes on wire-mesh screening over a layer of vermiculite for pupation. Once sifted from the vermiculite, pupae were placed in screen cages for emergence. Adults were separated by sex within 2 d of emergence, well before reaching sexual maturity at 13–17 d of age (Shelly, unpublished data) and placed in cubical (30 cm per side) screen cages (200–250 flies per cage) with food and water as described above. Flies were held at 23–27°C, 50–80% relative humidity, and a natural photoperiod (≈ 12:12 L:D) and were 2–3 generations removed from the wild when used in this study.

To distinguish virgin from non-virgin males in the mating trials, collected pupae were divided into 3 groups, two of which were coated with fluorescent dye of a different color (blaze orange or signal green, DayGlo Corporation, Cleveland, OH, USA), while the remaining group was undyed. Males used in the mating trials were from the dyed batches, while all females used were undyed. For males, the color used for a given treatment was alternated between successive days of mating trials. Upon emergence, the flies generally retain dye particles on the body that can be viewed with a dissecting microscope under UV (black light). However, where external dye was not conspicuous, the head was crushed with forceps to examine the collapsed ptilinum, which picks up dye particles upon emergence from the puparium.

Male mating experience. Non-virgin males were obtained as follows. Twenty-five virgin males (18–20 d old) and 40 virgin females of similar age were placed in individual plexiglass cages (40 x 40 x 30 cm) approximately 2 h before sunset (the period of sexual activity in this species, Kuba et al. 1984). No food or water was provided. Mating pairs were collected by gently coaxing them into perforated plastic vials and left undisturbed overnight (pairs remain coupled until sunrise, Kuba and Soemori 1988). To guarantee that handling did not disrupt copulation, pairs were observed for 1 h after sunset, and any decoupled pairs were discarded. The following morning the mated males were returned to the plexiglass cages (the unmated individuals having been removed) and provided food and water. Then, approximately 2 h before sunset, the food and water were removed, and new virgin females were introduced into the cages with the once-mated males. The aforementioned procedure was repeated to obtain thrice-mated males, which were then used the following sunset in mating competitiveness trials against virgin males. Thus, the non-virgin males mated on three successive days immediately preceding testing. Virgin males (20–24 d old when tested) were obtained from all-male, holding cages.

Mating trials. Mating trials were performed in 2 nylon-screened field cages (2.5 m height, 3 m diameter) placed outside the laboratory in Halawa, Oahu. Three potted guava plants, *Psidium guajava* L. (2–3 m high), were placed in the center of each field cage to provide resting and calling sites for the flies. In all trials, 60 virgin males, 60 non-virgin (i.e., thrice-mated) males and 60 virgin females (19–23 d old) were released per cage approximately 2 h before sunset. Cages were checked continuously from 1 h before sunset to 0.5 h after sunset; a flashlight was used to locate mating pairs at low light levels. Mating pairs were collected by gently coaxing them into perforated plastic vials. At the end of the trials, vials were taken into the darkened laboratory, and mating pairs were left undisturbed overnight. Males were removed from the vials (using an aspirator) early the following morning, killed by freezing, and identified by dye color. Mated females were retained for measurements of survivorship, fecundity, and fertility (see below). Fourteen replicates (two field cages per day over 7 d) were completed during September-November, 2017.

As male size may influence male-male interactions and male mating success (Shelly 2018), it is possible that non-virgin males were larger, on average, than virgin males. Consequently, after mating trials on 3 test days, body size estimates were made for 10 mated and 10 unmated virgin males and 10 mated and 10 unmated non-virgin males. Unmated males were collected the morning after the mating trial. Thus, 60 males in total (10 males/tent x 2 tents/day x 3 days) were collected for size measurements from each category. As an index of body size, the anterior edge of the discal cell of the right wing was measured (to the nearest 0.03 mm) using an ocular micrometer mounted on a dissecting microscope.

Female survival, fecundity, and fertility. To assess whether male mating status affected direct fitness benefits, females mated to virgin or non-virgin (i.e., thrice-mated) males were monitored for survival and reproductive output. One day after mating, females were placed in screen cages (30 cm per side, six females per cage); eight cages were established for females mated to virgin or non-virgin males, respectively. The cages were maintained for 7 weeks, with deaths recorded daily for each cage (dead females were removed upon detection). In an earlier study (Shelly and Nishimoto 2017), oviposition was found to be quite low during the week immediately after mating. Consequently, starting 1 week after mating, I introduced longitudinal sections (≈ 10 cm) of zucchini in a Petri dish for oviposition. Zucchini was introduced 2 d per week over 6 consecutive weeks for 6 h per day, with the flesh (below) resting on moist filter paper, the ends covered with transparent plastic wrap, and the skin (above) scored with four parallel slits (using a knife) running the length of the zucchini section. The slits were made, because preliminary observations revealed that females preferentially oviposited in slits over intact areas. Eggs were counted 1 d after oviposition using a dissecting microscope, and although the slits concentrated egg deposition, thus facilitating egg counts and collection, the entire zucchini section was examined for eggs. All eggs were removed from the zucchini using an X-Acto knife (Westerville, OH) and placed on black cloth in Petri dishes. Eggs were then counted, and the Petri dishes were covered to prevent desiccation. Fertility (% egg hatch) was scored 72 h later using a dissecting microscope. Egg deposition and development occurred at 25–26°C under a natural photoperiod. Although the monitoring duration used (i.e., 7 weeks) did not span the complete lifespan of the females, it was considered sufficiently long to assess potential differences in fecundity and fertility, as egg production tapers off rather quickly 7–8 weeks after mating (Shelly and Nishimoto 2017).

Data analysis. The numbers of matings obtained by virgin vs. non-virgin males were compared using raw data in the t-test as the parametric assumptions of normality and equal variance were met. The χ^2 test was used to compare the proportions of females mated to virgin vs. thrice-mated mates that survived until 7 weeks after mating (i.e., when egg collection was stopped). Wing measurements were compared among the four male categories (mated and unmated virgins and non-virgins) using the Kruskal-Wallis test as raw and $\log_{10}$ transformed data were non-normal. Both fecundity and fertility (arc sine transformed) data were normally distributed and were analyzed using a 2-way ANOVA, with week and male mating status as main effects and the Holm-Šídák method used for post hoc multiple comparisons. Means ± 1 SE are presented. Statistical analyses were performed using SigmaPlot 11.0 (Systat Software, San Jose, CA).

Results

Mating trials. Females did not discriminate between virgin and non-virgin males. On average, virgin males obtained 15.7 ± 1.4 matings per replicate compared to 15.0 ± 0.9 for non-virgin males ($t = 0.42$, $df = 12$, $P = 0.68$). Body size did not vary significantly among male categories ($H = 3.10$, $df = 3$, $P = 0.38$). Median wing measurements were 2.35 and 2.36 mm for mated and unmated virgin males, respectively, and 2.38 and 2.33 mm for mated and unmated non-virgin males, respectively.

Female survival, fecundity, and fertility. The majority of females survived the entire 7-week test period regardless of whether they mated with a virgin or a non-virgin male. The proportion of survivors was slightly greater for females mated to virgin males than females mated to non-virgin males, but the difference was not statistically significant (39/48 = 81% vs. 35/48 = 73%, respectively; $\chi^2 = 0.53$, $P = 0.47$).

Fecundity varied significantly over time ($F_{5, 84} = 6.35$, $P < 0.001$) but did not vary with male mating experience ($F_{1, 84} = 0.31$,

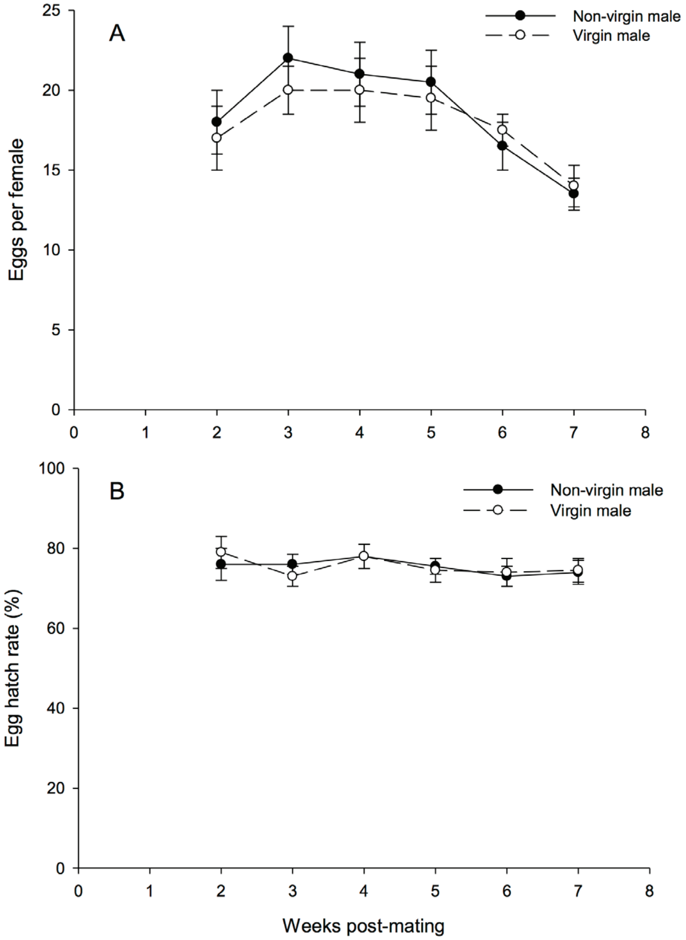

Figure 1. (A) Number of eggs laid per female per week and (B) proportion of hatched eggs per week for *Zeugodacus cucurbitae* females mated to virgin or non-virgin (thrice-mated) males. Symbols represent averages (± 1 SE) over 8 cages per male type.

$P = 0.58$; Fig. 1A). The interaction term was not significant ($F_{5,84} = 0.40$, $P = 0.84$). The multiple comparisons test revealed significant differences in fecundity between each of weeks 3, 4, and 5 and week 7 ($P < 0.001$ in all cases).

Fertility did not vary significantly with time ($F_{5,84} = 1.16$, $P = 0.33$) or male mating status ($F_{1,84} = 0.01$, $P = 0.94$; Fig. 1 B). The interaction term was likewise not significant ($F_{5,84} = 0.17$, $P = 0.97$).

Discussion

In trials conducted under field-cage conditions, *Z. cucurbitae* females did not discriminate between virgin and non-virgin (thrice-mated) males when selecting a sexual partner. Moreover, male mating experience had no detectable effect on female survival, fecundity, or fertility. Thus, random mating with respect to male sexual experience had no major positive or negative consequences on the longevity or reproductive output of females. The robustness of this claim is, however, constrained by the design of the experiment. For instance, the study continued only 7 weeks after the females mated, and it is possible that differences in survival and reproduction might have emerged over a longer sampling interval. While this possibility cannot be dismissed, a decline in weekly egg deposition was observed 5–6 weeks after mating in both this study and an earlier one study (Shelly and Nishimoto 2017), suggesting that the interval of data collection spanned the period of peak egg laying (similarly, egg laying decreased markedly 3–4 weeks after mating in *B. oleae* females, Gerofotis et al. 2015). In addition, a negative effect on female fitness might have been observed if the non-virgin males had mated on a larger number (> 3) of consecutive days prior to mating with the test females. While possible, data from related species (Radhakrishnan et al. 2009; Gerofotis et al. 2015) reveal no decline in sperm transferred during matings on increasing numbers of consecutive days (i.e., separated by 24 h).

The finding that the survival and reproduction of *Z. cucurbitae* females did not vary with male mating status is consistent with data for *A. ludens* females (Pérez-Staples et al. 2010) but differs from several other tephritid species. In *B. oleae*, for example, females mated to non-virgin males had significantly lower longevity, fecundity, and fertility (Gerofotis et al. 2015). Although no differences were found for fecundity or fertility, females of *A. striata* that mated with virgin males had greater longevity than females mated to non-virgins (Pérez-Staples and Aluja 2004). Mating with virgin males also conferred fitness advantages to females of *C. capitata* (Whittier and Kaneshiro 1991).

Fewer studies have performed both choice tests, in which females are offered equal numbers of virgin and non-virgin males as potential mates, and measurements of direct fitness benefits conferred by mating with virgin or non-virgin males. Since these benefits vary independently of male mating experience in *Z. cucurbitae* (this study) and *Anastrepha ludens* (Loew) (Pérez-Staples et al. 2010), it is not unexpected that male mating status does not influence female mate choice in *Z. cucurbitae*, but it is surprising that *A. ludens* females mate preferentially with non-virgin males (Pérez-Staples et al. 2010). These authors suggest that this pattern may reflect, not so much female preference, but the increased ability of sexually experienced (and likely older) males to overcome female resistance to mating. Data from *C. capitata* suggest that females are unable to discriminate between virgin and non-virgin males. Even though females enjoyed increased fecundity and longevity via mating with virgin males (Whittier and Kaneshiro 1991), they were found to mate equally with virgin and non-virgin males (Shelly and Whittier 1993). Similar results have been obtained for the endemic Hawaiian species, *Drosophila silvestris* (Perkins) (Schwartz 1991).

In sum, under the experimental protocol and conditions used, the survival, fecundity, and fertility of *Z. cucurbitae* females did not differ significantly following matings with virgin vs. non-virgin males, a result that differs from findings for several other tephritid species. Females of *Z. cucurbitae*, and perhaps tephritid species in general, appear incapable of distinguishing between virgin and non-virgin males in selecting a mate (unlike females of *Drosophila melanogaster* Meigen, Saleem et al. 2014). Such indiscriminate mating appears to have no major fitness consequences for *Z. cucurbitae* females but may have negative effects for females of other fruit fly species.

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