Little effect of delayed mating on fecundity or fertility of female fungus gnats *Lycoriella ingenua*

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Abstract. The effect of delayed female mating for the mushroom fungus gnat *Lycoriella ingenua* is investigated. We examine the effect of delaying female mating on the fertility and egg viability of female flies that have a mating delay of 0–5 days after emergence. Male fly age is held constant. Female age does not impact male acceptance and most flies copulate within seconds of pairing. We find that female flies experiencing mating delays of 0–4 days after emergence lay a similar number of eggs onto artificial substrates. Females that experience a mating delay of 5 days lay 54% fewer eggs than those that mate on day 0 (day of emergence). There is no effect of mating delay on the percentage of larvae that emerge. The results of the present study indicate that mating delays have little effect on the fertility or fecundity of the mushroom fungus pest *L. ingenua*.

Key words. Delayed mating, fecundity, fertility, fungus gnat, *Lycoriella ingenua*, mating disruption, mushroom pest, Sciaridae.

Introduction

One of the most significant pests of commercially grown white button mushrooms, *Agaricus bisporus* is the sciarid fungus gnat *Lycoriella ingenua* (Dufour) (Diptera: Sciaridae) (Erler *et al.*, 2011). Female *L. ingenua* lay eggs directly into mushroom compost (Cloonan *et al.*, 2016b). This composting media is highly selective for *A. bisporus* and contains a microbial environment suitable for its growth and development (Pecchia *et al.*, 2002; Parati *et al.*, 2011). Several of the fungal species abundant in this mushroom compost are also attractive to gravid female *L. ingenua* (Cloonan *et al.*, 2016b), making mushroom compost an ideal habitat for *L. ingenua* development. Adult *L. ingenua* are also shown to be capable of carrying spores of mycoparasitic green mold *Trichoderma aggressivum* and thus probably vector spores across mushroom-growing beds inside the rooms within mushroom growing houses (Mazin *et al.*, 2018).

No effective monitoring tools exist for mushroom growers to record the arrival of *L. ingenua* into a mushroom growing house and thus growers are unable to accurately predict the onset of fly infestation. Growers rely solely on calendar-based insecticide sprays to control *L. ingenua* populations in a mushroom growing room (Shamshad, 2010). These applications include compost drenches of insecticides (Shirvani-Farsani *et al.*, 2013), although early resistance to some of these insecticides is reported (Brewer & Keil, 1989; White & Gribben, 1989; Bartlett & Keil, 1997). These early reports of insecticide resistance, as well as a desire from consumers for chemical-free mushrooms (Tibbles *et al.*, 2005), contribute to the objective within the mushroom industry for more behaviourally based and less toxic techniques for controlling *L. ingenua*.

Adult female *L. ingenua* flies produce a form of germacradienol, a sesquiterpene alcohol, which serves as a sex pheromone component to attract male conspecifics in laboratory Y-tube bioassays (Andreadis *et al.*, 2015). In gas chromatography–behaviour coupled bioassays, the germacradienol elicits male wing-fanning and abdomen curling (Andreadis *et al.*, 2015). The stereochemistry of this *L. ingenua* sex pheromone component remains to be fully characterized, although work is continuing to definitively identify its structure.

Once fully identified, a synthetic version of this pheromone component could potentially be used for mating disruption. In several moth species, successful mating disruption can occur not because females are prevented from mating over their lifetimes but rather because the pheromone disruptant delays mating

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by females (Rice & Kirsch, 1990; Knight, 1997; Fadamiro *et al.*, 1999) and reduces the fecundity and fertility of females (Knight, 1997; Fadamiro & Baker, 1999). Male moths may still eventually find females over time (Torres-Vila *et al.*, 2002), although pest populations can still be reduced as long as a delay in mating negatively affects the fitness of females (Walker & Allen, 2011).

In anticipation of the *L. ingenua* female-produced sex pheromone component being completely characterized, we consider it important to gather information concerning the degree to which delayed mating might impact female fitness and contribute to mating disruption success in this species. Thus, in the present study, we investigate this aspect of *L. ingenua* biology and report the effects of delayed female mating on their acceptance of mates, as well as fecundity and egg fertility.

Materials and methods

Insect rearing protocol

The culturing methods used to rear the *L. ingenua* flies for the present study were similar to those described by Cloonan *et al.* (2016a). Briefly, flies were reared on a mixture of phase II mushroom compost, a slow-release nitrogen supplement, brewer's yeast and agar at a ratio of 100 : 1 : 1 : 1 filled in plastic Solo cups (355-mL) (Solo, Mason, Michigan). The cups were placed in mesh cages (30 × 30 × 30 cm) (BioQuip, Rancho Dominguez, California) and kept in an environmental growth chamber under an LD 12 : 12 h photocycle at 21 °C and 70% relative humidity. Under these conditions, the *L. ingenua* life cycle is approximately 21 days from egg to adult (Lewandowski *et al.*, 2004).

Manipulations to delay mating of females.

For these experiments, we used female flies that were allowed to mate on the day they emerged ('day 0') or were not allowed to mate until 1 day after emergence ('day 1') or else on subsequent days ('day 2', 'day 3', etc.). Thus a mating delay of 1 day will have occurred on day 1 and a mating delay of 5 days will have occurred on day 5. The females were able to be kept alive over this time span in cages provided with sugar water but, after a mating delay of more than 5 days (i.e. day 6 and onward), the females in these experiments began to experience significant mortality (> 50%), as was also noted by Andreadis *et al.* (2016). Because of this mortality, we could only use data from females experiencing a mating delay of up to 5 days (i.e. day 5 or earlier).

Newly-emerged female *L. ingenua* flies immediately mate with males (Binns, 1980) making it difficult to obtain virgin females (Cloonan *et al.*, 2016a). Accordingly, *L. ingenua* pupae were first collected individually from the rearing media and gently placed individually in 10-mL disposable culture tubes (15 × 85 mm) (VWR International, Radnor, Pennsylvania) covered with Parafilm M (Bemis Healthcare Packaging, Oshkosh, Wisconsin). Newly-emerged adults were then easily separated by sex according to visibly sexually dimorphic abdomens (Lewandowski *et al.*, 2004). Male flies have an obvious and pronounced set of claspers at the apical tip of their abdomens, whereas the apical tips of female abdomens are pointed (Lewandowski *et al.*, 2004). Each day for six successive days, 20 or more females < 12 h old were released into six different mesh cages (30 × 30 × 30 cm) (BioQuip) that contained a 354-mL portion cup (Dart Container Corporation, Mason, Michigan) filled with a 10% table sugar solution on a cotton wick for the flies to consume *ad libitum*.

In this way, each of six cages contained > 20 female flies all of the same age, ranging from newly-emerged (day 0) to the fifth day post-emergence (day 5). These cages were held under colony conditions each day for these 6 days, at which time female flies were drawn from each of the six cages and individually placed into separate vials awaiting the addition of two 1–3-day-old virgin males (for mating procedure, see below). This protocol was followed during the course of the experiments such that cages containing groups of 20 females of all six different age classes were available at the same. This ensured that female matings and subsequent egg depositions could occur at the same time across classes to account for possible daily variability.

With respect to the mating protocol, single experimental day 0 to day 5 virgin females were first aspirated individually into 354-mL portion cups. At the bottom of each portion cup, a small disk of water agar approximately 5 mm in diameter (15 g L^{-1} agar) (Difco Agar Technical; Becton, Dickinson and Company, Sparks, Maryland) was placed, which would later serve as a substrate for oviposition. Two 1–3-day-old virgin males were added to each cup containing a female. Typically, when male flies were aspirated into the portion cups containing an individual female fly, courtship and copulation commenced immediately for all treatment groups, regardless of age. Portion cups were placed under colony conditions for 12 h overnight with the assumption that females had mated overnight. In the morning, the two male flies were removed. Each of the cups containing a mated female was placed back under colony conditions and monitored daily for egg deposition onto the water agar disk. Typically, females died after oviposition. The dead females were gently removed with forceps and examined so that any eggs that were stuck to the abdomen of the female were not touched with the forceps and stayed on the water agar disk. The number of eggs deposited was recorded under a stereomicroscope at 10× magnification (Olympus SZ61; Hunt Optics & Imaging Inc. Pittsburgh, Pennsylvania) and the cups were placed back under colony conditions to monitor egg hatching. At 12-h intervals, each portion cup was examined under a stereomicroscope for hatched larvae. Newly-emerged larvae were immediately removed with a size 1 paint brush (Artist's Loft; MSPCI, Irving, Texas) to prevent egg cannibalism by the larvae.

Statistical analysis

All statistics were analyzed using PRISM, version 5.0 (Graph-Pad Software Inc., San Diego, California). The mean number of eggs laid by females, mean number of larvae emerging and

fertility (percentage larvae emerging) were first analyzed for normality via the D'Agostino & Pearson omnibus normality test. All data for mean number of eggs and mean number of larvae were normally distributed. All data for percentage larval emergence were non-normally distributed. Female age at mating was treated as an independent variable. For each age group, 20 female flies were tested: 10 each from two different cohorts approximately 1 month apart. The differences in mean number of eggs laid (fecundity) and the mean number of emerging larvae from these eggs were analyzed via one-way analysis of variance. The differences among mean eggs or among mean larvae were then compared via Tukey's multiple comparisons. Differences in fertility (i.e. mean percentage of emerged larvae) among the different experimental age groups were first analyzed via the Kruskall–Wallis test. Then, comparisons of fertility across each of the different age groups were made using Dunn's multiple comparisons test on the mean percentages of emerged larvae.

Results

There was no difference in the number of eggs laid by females whose first mating occurred on day 0 to day 4 (Fig. 1). However, females that had been prevented from mating until the fifth day after emergence (day 5) produced significantly fewer eggs compared with day 0 earlier-mated females ($P = 0.0006$) (Fig. 1). The number of emerging larvae showed a trend toward a gradual decrease in females whose mating had been delayed
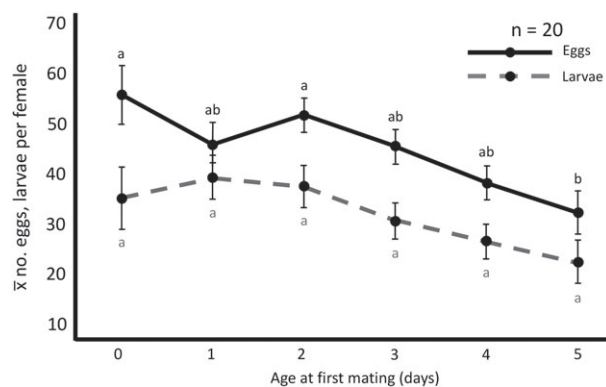
Fig. 1. Black solid line: mean ± SEM number of eggs laid by female *Lycoriella ingenua* flies of different delayed mating groups. Day 0 comprises mating occurring during the first day of emergence (i.e. during the first 24 h of adulthood). Each point shows the mean number of eggs laid by 20 different females of that experimental age group from two different cohorts. Grey dashed line: black points on the grey dashed line represent the mean ± SEM number of larvae that emerged from the eggs laid by these 20 different female flies comprising each experimental age group. All data were normally distributed and differences among mean egg numbers within each age group and the mean numbers of larvae emerging within each age group (d.f. = 5, $P = 0.06$) were first analyzed by analysis of variance. Then, differences among mean egg numbers and among the mean number of larvae emerging for each age group were compared via Tukey's multiple comparison test. Mean numbers of eggs or larvae with no lowercase letters in common are statistically significantly different (d.f. = 5, $P < 0.05$).
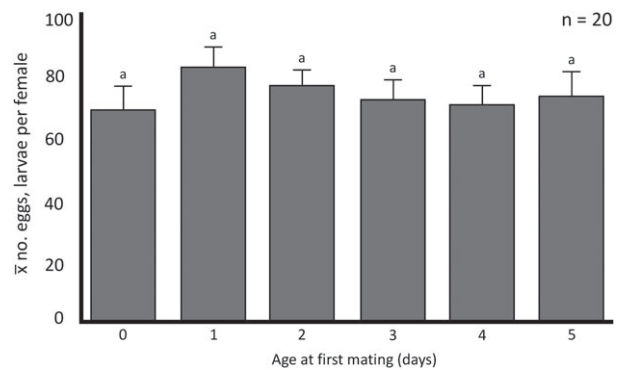
Fig. 2. Differences in fertility (i.e. mean ± SEM percentage of eggs) laid by female *Lycoriella ingenua* that produced emerged larvae among the different delayed mating groups. Day 0 comprises mating occurring during the first day of emergence (i.e. during the first 24 h of adulthood). Each vertical bar shows the mean percentage of emerged larvae emerging from these eggs laid by 20 different female flies from two different cohorts for each age group. All data were non-normally distributed and differences among mean percentage of emerged larvae between each experimental age group were first analyzed via the Kruskall–Wallis test, and then differences between mean percentages of emerged larvae for each experimental age group were compared via Dunn's multiple comparisons test (d.f. = 5, $P = 0.7$). Means with no lowercase letters in common are statistically significantly different (d.f. = 5, $P < 0.05$).

for 3–5 days (day 3 to day 5) (Fig. 1). However, this reduction in number of emerged larvae was not significant (Fig. 1). The fertility of the eggs (i.e. the percentage of eggs that produced larvae) was not significantly different among all female groups with respect to age at first mating ($P = 0.7$) (Fig. 2). Of further note is that none of these lengths in delay of mating appeared to decrease the speed with which *L. ingenua* females accepted males for mating. When males were placed into experimental containers with virgin females of any of the different ages, mating commenced almost immediately.

Discussion

In the present study, we report the first investigation of the effects of delayed mating on the fecundity and fertility of female *L. ingenua* fungus gnats. We focus solely on varying female age at mating at the same time as keeping male age constant. Delaying both male and female age at first mating may result in a combined effect in reducing the fecundity and fertility of females. For example, Wu *et al.* (2018) report that delaying mating of both male and female tobacco cutworm moths *Spodoptera litura* reduces the fecundity and fertility of females significantly more than if either sex is delayed alone. Our current finding for *L. ingenua* of little to no effect resulting from delayed mating should not be surprising because, to our knowledge, no comparable experiments involving forced delays in mating by females are reported for other species of sciarid flies or for other dipterans in general.

The dearth of experimentally delayed mating studies in Diptera may be the result of a scarcity of sex pheromone mating disruption experiments involving flies (Cross & Hall, 2007;

Cross, 2010; Samietz *et al.*, 2012). The scarcity of fly sex pheromone mating disruption studies in turn may be a result of the relatively small number of sex pheromones identified in the Diptera thus far compared with other orders, such as Lepidoptera or Coleoptera. Most of the dipteran pheromones are identified in the Cecidomyiidae (Hall *et al.*, 2012) and Tephritidae (Landolt & Averill, 1999).

By contrast, the extensive use of mating disruption with respect to suppressing populations of lepidopteran pest species (Witzgall *et al.*, 2010) is a stimulus for studies investigating the effects of delayed mating in this large insect order. Several studies show that pheromone mating disruption does not completely prevent female moths from mating during their lifetimes. Rather, disruption only makes it more difficult for females to obtain their first or second matings as fast as females in untreated check plots are able to do (Rice & Kirsch, 1990; Knight, 1997; Fadamiro *et al.*, 1998, 1999). In the laboratory, the mating disruption delays in these species are shown to significantly reduce female fecundity and fertility (Knight, 1997; Fadamiro & Baker, 1999). It is generally accepted that mating-delayed reduced fecundity and/or fertility in moths (Mori & Evenden, 2013) is an important contributing factor to population suppression in most, if not all, successful commercial lepidopteran sex pheromone mating disruption applications (Stelinski & Gut, 2009).

Despite the lack of mating disruption experimentation thus far with *L. ingenua*, we consider that a pre-emptive examination of the potential effects of mating disruption in delaying mating in *L. ingenua* is warranted. We also consider it important to assess, ahead of time, whether delaying mating might increase the chances that successful mating disruption can occur and suppress populations of this pest, whenever the germacradienol pheromone component (Andreadis *et al.*, 2015) is finally completely characterized and synthesized. Our data suggest that, if the synthetic *L. ingenua* germacradienol sex pheromone component is able to be deployed for mating disruption in the future, it would not significantly reduce female fecundity or fertility if matings by females were delayed for 5 days or less by the pheromone. Successful mating disruption for suppressing populations of *L. ingenua* would probably need to keep most of the females in an unmated state for the first 5 days (days 0 to day 4) of their adulthood.

The *L. ingenua* sex pheromone could be useful in other ways, such as for use in monitoring traps, as well as potentially in mass trapping or in attract-and-kill baits, comprising techniques that are shown to be effective for other pest insect species (Witzgall *et al.*, 2010). Male *L. ingenua* develop faster than females and emerge a few days before female flies in a mushroom growing house (Lewandowski *et al.*, 2004). The male flies congregate on the beds of the mushroom compost and appear to wait for emerging female flies and copulate almost immediately with them when they emerge (K. Cloonan & S. Andreadis, unpublished data). A synthetic pheromone as a bait combined with a killing agent and an effective trap could target this first generation of emerging *L. ingenua* males and kill them before female flies emerge. This reduction in male flies could reduce the overall mating success and subsequent population size, reducing the pest pressure within a mushroom growing house.

In our experiments with *L. ingenua*, there is little indication that the delays in female mating create any of these effects because there is no significant effect on fertility from any length of mating delay and a significant decline in fecundity only occurs when mating is delayed to day 5. It would be good to carry out comparable studies on other dipteran species to allow comparison with the results of the present study and thus determine whether mating delays are similarly ineffectual in those species with respect to reducing female fly fertility and fecundity.

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