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# Impact of Sex, Age, and Mating Status on Flight Behavior of the Navel Orangeworm (NOW)

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**Project No.:** 13-ENTO2-Burks

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**Objectives:**

1. Examine impact of mating on the age-specific flight capacity of males and females.
2. Examine the association between laboratory flight and the number of eggs laid subsequently.

**Interpretive Summary:**

Previous data from this project show that unmated navel orangeworm adults have a greater flight capacity compared to other pests of the families Pyralidae and Tortricidae. In the current laboratory study, the flight mill performance of mated navel orangeworm adults is compared with previous data for unmated adults. The distance and duration of the longest uninterrupted flight, and of the sum of all flights, was compared between classes of age (2, 3, 5 or 7 day-old adults), sex, and mating status (unmated vs. mated). Flight performance was significantly affected by age and sex for all parameters examined. Mated adults flew less time than their unmated counterparts. There was a trend of stronger flight performance in unmated than in mated males, although interaction of sex and mating status was not significant for any of the parameters examined.

These findings suggest greater dispersal capacity for mated females than males. However, data from other studies of this and other species suggest that oviposition closer to the site of female emergence may be favored over oviposition at the far range of dispersal. Preliminary data are also presented for experiments concerning the association between laboratory flight and the number of eggs. These experiments will provide additional data on the relationship between the flight capacity of navel orangeworm females and the distribution of the damaging larval stage.

## Materials and Methods:

*Insect Culture.* Insects used were obtained as eggs from an almond orchard in western Fresno County in September 2010, and the colony was refreshed with infusion of individuals taken at the same time, place and manner in September 2011. The colony was maintained at 26°C and 14:10 (L:D) on wheat bran diet (Tebbetts et al. 1978). This line had been in culture from 20 to 26 generations (from 2010) at the time of the experiments. Sexes were separated as mature larvae based on the presence or absence of testes (visible through the male dorsal integument). Male and female larvae nearing pupation were sent once a week from Parlier, CA to Ames, IA via overnight express. Pupae were checked daily for adult emergence. Newly-emerged adults were placed in sex-specific holding cages at 26°C and 14:10 (L:D) until they reached the appropriate age for flight tests. The holding cages consisted of a wire-mesh cylinder 13.5 cm in diameter and 15.5 cm tall. Adults were provided free access to water via soaked cotton. To obtain mated adults, unmated males and females were placed together in a holding cage. Under the holding conditions, mating takes place in the hours before the end of scotophase (Landolt and Curtis 1982). Spermatophores were dissected from the bursa copulatrix of females to confirm mating status. This was done with mating partners of males before the flight test, and with females after the flight test was completed.

*Adult Tethering and Flight Mill Procedures.* Tethering and flight mill procedures were based on those described by Dorhout et al. (2008). Adults were attached to flight mills with a tether consisting of a 0.25mm diameter wire, about 5 cm long, and bent at a 90 angle and connected to the flight mill arm by a small sleeve of hollow plastic tubing stripped from copper wire. Moths were sedated by brief cooling in a reach-in freezer at -20°C for <3 min. A 1-mm diameter loop at the end of the 2cm length was attached to the dorsal surface of the junction between the mesothorax and the abdomen with a small amount of Insta-Cure+ glue (Bob Smith Industries, Atascadero, CA). After attachment to the flight arm, a small piece of tissue paper was provided for tarsal contact (Sappington and Showers 1991), which the moths readily grasped. This reduced escape behavior and trivial flight prior to dusk, while allowing spontaneous flight initiation.

Fifteen flight mills were housed in a walk-in environmental chamber maintained at 26°C and 14:10 (L:D). Lights were automatically and gradually dimmed over a 30-min period beginning before lights-off to simulate dusk, and automatically ramped-up beginning 30-min before lights-on to simulate dawn. Each was attached to a Gateway 2000 personal computer by interface hardware and software described by Beerwinkle et al. (1995). The flight mill arm consisted of a triangle-shaped flat piece of aluminum (256 mm long, 156 mm from tip to pivot, 15 mm at the base end). The tether attachment sleeve described in the previous paragraph was slipped over the end of this arm, and the weight of the attached moth was counterbalanced with a moveable clip on the end opposite adult attachment. The setup allowed the adult to fly in a horizontal plane, completing a distance of 1-m per revolution around the central pivot and the pin on which it rested. Revolutions of the flight arm were sensed by an infrared eye below the pin. A vinyl tent surrounded each mill to reduce air movement that might cause the flight arm to drift and generate spurious readings. Males and females were tested separately on given nights to prevent the confounding influence of female sex pheromone.

*Impact of mating on age-specific flight capacity.* Mated males and females were flown at ages 2, 3, 5 and 7 days post eclosion. Adults that emerged the previous evening or night were classified as 1 d old when mated on the following evening (i.e., first evening after emergence). In the laboratory mortality is minimal prior to 7 days post eclosion but, in females mated once on the day after eclosion, most of total lifetime fecundity has been realized by this age (Burks, unpublished data). Each individual was flown only once, so data for each age category represent independent trials of moths held in cages without flying until the night of attachment.

*Association between laboratory flight and eggs laid.* Association between laboratory flight and fecundity was examined by compared three experimental treatments representing different scenarios concerning the sequence of mating, dispersal, and oviposition. A similar approach was previously used to study flight and reproduction in the beet armyworm (Jiang et al. 2010). Three additional control treatments were included for comparison.

The three treatment scenarios were: 1) mate-disperse-oviposit; 2) mate-oviposit-disperse-oviposit; and 3) disperse-mate-oviposit. For treatment 1, moths were mated on night 1 (i.e., the night following eclosion), flown on night 2, and then allowed to oviposit until natural death. For treatment 2, moths were mated on night 1, allowed to oviposit on night 2, flown on night 3, and then allowed to oviposit on subsequent nights. For treatment 3, moths were flown on night 1, mated on night 2, and then allowed to oviposit on subsequent nights. The three controls were: 1) mate then oviposit (no flight on flight mill), 2) mate-tether-oviposit (a sham-treated control for treat #1), and 3) tether-mate-oviposit (a sham-treated control for treatment #3). Females were allowed 1 night to mate with 1 or 2 males (per availability) in a quart jar containing a strip of paper for perching and access to water-soaked cotton. Females were removed from the jars and either tethered for flight or placed in a wire-mesh cage with circular wax sheets, 11 cm in diameter, for oviposition. After flying, the tether was gently removed with scissors and the female returned to an oviposition cage. Mating jars and oviposition cages were maintained in an environmental chamber at 26°C and 14:10 (L:D), and approximately 75% RH. The oviposition sheets were changed daily, and evaluated within 48 hours. Fertile eggs were recognizable by the characteristic red color that develops within the first 24 hours after laying.

*Data Analysis.* The number of revolutions was compiled in 1-minute intervals for each flight mill. A flight was considered terminated if there was not a single revolution of the flight arm in a minute. Adults that flew  $\leq 2$  minutes were excluded from analysis to avoid including adults that were artifactually unable or indisposed to fly because of handling associated with the flight mill procedures. Flight characteristics examined for each moth included the duration of the longest uninterrupted flight, the duration of all flights, the distance of the longest uninterrupted flight, and the total distance of all flights. Flight activity before the onset of dusk or after lights-on was excluded from analysis, resulting in a 10.5-hour window of possible flight activity.

Data associated with duration and distance of flight was transformed as the square root of the observation to stabilize variation and improve symmetry of the frequency distribution. Analysis of variance was used for these variables because Levene's test did not find significant departure from homogeneity of variance, and because residual plots indicated that the assumption of random distribution of the error was not violated. A mixed-model ANOVA was used to examine duration of longest flight, total flight duration, distance of longest flight, and total distance flown. Dependent variables included mating status, age, sex, and all interactions.

Date of the flight test, nested in the three-way interaction of mating status × age × sex was used as a random factor. Containment degrees of freedom were used. The numerator degrees of freedom were thus based on the number of separate nights of flight mill testing rather than the total number of moths tested.

The Tukey adjustment for multiple comparisons was used when comparing age classes. Statistical analysis was performed using the SAS System (SAS Institute Inc. 2008), and box plots were generated using SigmaPlot (Systat Software Inc. 2008). Data from the study of association of flight and fecundity were summarized using Statistix 7.0 (Analytical Software 2000). Untransformed means and errors are presented in the tables and figures.

## Results and Discussion:

*Impact of mating on age-specific flight capacity.* The mixed-model ANOVA revealed consistently significant ( $P < 0.05$ ) differences due to age and sex in the flight performance for all four parameters tested (**Table 1**). In contrast, there were lower levels of significance associated with effects due to mating status and the interaction of sex with mating status. The  $P$  value associated with mating status was significant ( $P < 0.05$ ) for the total duration of all flights. There was also a  $P$  value of  $<0.1$  associated with mating status for the duration and distance of the longest single flight (**Table 1**). The interaction of sex with mating status was not significant at the level of  $P < 0.05$  for any of the four parameters examined, but met the reduced significance threshold of  $P < 0.1$  for the duration of the longest single flight and the distance of all flights combined (**Table 1**). The  $P$  values for interactions not shown in **Table 1** were, in all cases,  $> 0.1$ .

Comparison of flight performance by age reveals a consistent decrease in each of the four parameters between days 2 and 7. The values of all four parameters were significantly less for 7-day-old adults compared to 2-day-old adults (**Table 2**). For total distance of all flights, the distances flown by 5-day-old adults was also significantly less than that flown by 2-day-old adults. For all classes of age and mating status, the maximum and total duration flown by females was respectively 23 and 26% greater than for males. For maximum and total distance, females respectively flew 44 and 48% farther than males. Examination of flight performance by sex and mating status (i.e., a sex-mating status interaction) reveals much more similar performance for mated and unmated females compared to males (**Table 3**). The difference between the performance of mated and unmated females ranged from 0 to 7% for the four parameters tested, whereas unmated males performed 24-37% better than mated males in each of these parameters (**Table 3**). The variability and complexity associated with the combined effects of sex, age, and mating status are depicted with a box plot of the distance of the longest single flight (**Figure 1**). The effect of mating on distance of the longest single flight varied with age with females, whereas unmated males consistently flew longer than mated males on days 2-5.

These data provide evidence that mating status had little effect on the flight capacity of navel orangeworm females, but that mated navel orangeworm males generally flew less time and distance than their unmated counterparts. It is possible that the decreased flight performance of males represents a type of fitness cost associated with the “nuptial gift” associated with the energetic reserves passed in the spermatophore (McNamara et al. 2009, Walker and Allen

2010). Decreased take-off velocity following mating has been documented in free-flight butterflies *Pieris napi* (Lepidoptera: Pieridae) (Almbro and Kullberg 2012). A study of the large aspen tortrix, *Choristoneura conflictana* (Walker) (Lepidoptera: Tortricidae) found consistently stronger flight performance in mated females than in unmated females, while such relationships were not found in males (Elliott and Evenden 2009). The aspen tortrix has cyclical changes in population density; i.e., “outbreak dynamics”. In contrast, less difference was seen between distances flown by mated and unmated females in earlier studies of a variety of species without such cyclical population dynamics (Elliott and Evenden 2009).

The data from this study do not suggest obligate flight behavior prior to mating, as occurs in the European corn borer (Reardon and Sappington 2007, Dorhout et al. 2008). The majority of navel orangeworm females captured in black light traps in a previous study were mated (Landolt and Curtis 1991), suggesting that navel orangeworm females optimize their fitness by minimizing dispersal prior to first mating. In contrast, the pattern of greater flight capacity in females than in males found in the present study is more reminiscent of the relationship between female and male flight performance in the Oriental fruit moth (Hughes and Dorn 2002).

The concern brought up by the authors of this previous study of the Oriental fruit moth; i.e., that some mated females might travel farther than the males monitored with pheromone traps (Hughes and Dorn 2002), also seems to apply for the navel orangeworm. In the case of the navel orangeworm, this concern is exacerbated by the reduced flight capacity of mated navel orangeworm males. Presumably many of the males captured in pheromone traps in the field are mated. This concern is mitigated, however, by evidence from a field study of a non-linear relationship between navel orangeworm damage and distance from source locations, with most damage occurring nearer the source location (Higbee and Siegel 2009). This non-linear pattern could be due to dilution of insect density with distance (Showers 1997), or it could be because distributing more eggs closer to the site of parental development but a few farther away often optimizes evolutionary fitness (Hughes and Dorn 2002, Gu et al. 2006).

*Association between laboratory flight and eggs laid.* Preliminary data are reported, based on small numbers and used to guide methods development for this aspect of the project. The comparison in **Table 4** examined the effect of timing of mating, oviposition and dispersal on total female fertility. The control treatments are used to examine whether manipulation associated with the flight mill (rather than flight itself) affected fertility or longevity. Lifetime fertility is low compared to other studies, in which females laid a lifetime total of >100 fertile eggs (Kellan and Hoffmann 1983, Burks et al. 2011). We have requested an extension of performance, and this low total fertility is currently being addressed by revising methods for keeping adults in culture.

The extent to which insects must trade off energy between use for reproduction and use for other purposes (e.g., metabolic needs and flight fuel) have been conceptualized by classification of insects along a continuum between capital breeders (those which reproduce primarily or entirely using resources stored from larval feeding) vs. income breeders (those which obtain much energy for reproduction from adult feeding) (e.g., Tammaru and Haukioja 1996, Jervis et al. 2005). In Lepidoptera, these life history differences mediated in part by differences in the physiology of ovarian maturation (Ramaswamy et al. 1997). Generally,

Pyralidae (like the navel orangeworm) emerge with much of their egg complement, breed soon after emergence, and feed minimally as adults (Ramaswamy et al. 1997). In contrast, in Noctuidae (armyworms and cutworms) eggs tend to develop after adult emergence, feed as adults, and additional mating stimulates additional egg production (Ramaswamy et al. 1997). In the Tortricidae (which includes the codling moth and the Oriental fruit moth), egg development generally occurs after adult emergence, and mating increases egg production (Benz, 1991). In the beet armyworm, which tends toward income breeding, interruption of oviposition with dispersal flights has little impact on lifetime fecundity (Jiang et al. 2010). But in the codling moth, which tends less towards income breeding compared to the beet armyworm, lifetime fecundity is favored by later rather than earlier dispersal (Schumacher et al. 1997). For the Oriental fruit moth, females flown on day 6 had significantly greater lifetime fecundity than those flown on day 1, and flight activity increased significantly on day 3, after approximately 30% of the total egg load had been deposited (Hughes et al. 2004). Results for these two tortricid species suggest physiological, behavioral, and genetic mechanisms that result in a majority of eggs being laid relatively near the site of female development, and some being laid farther away (Schumacher et al. 1997, Hughes and Dorn 2002, Gu et al. 2006).

Compared to oriental fruit moth or codling moth, the navel orangeworm is more of a capital breeder with a greater flight capacity. This suggests that there should be greater competition between resources for either egg production or flight fuel, and that a similar reproductive strategy of favoring oviposition near the natal field is likely, relative to the dispersal range of the navel orangeworm. In experiments for Objective 2, impact of oviposition is examined, whereas in the previous phases only the effect of being mated was examined. The trends in **Table 5** are consistent with the hypothesis that oviposition negatively affects subsequent flight capacity, but methodological refinement and much larger sample sizes are necessary before inference can be made from these data.

*Summary.* The data from this study reveal that the navel orangeworm has a strong flight capacity, and that this capacity is consistently affected by sex and age. Females are stronger fliers than males. There are weaker trends of reduced flight capacity of mated males, but not mated females. While field experience is gained with pheromone lures for the navel orangeworm, practitioners should consider the potential for greater dispersal of mated females compared to males. However, field data from navel orangeworm and from other species suggest that oviposition is biased toward sites closer to the natal field. The present ongoing laboratory study of the association between flight and reproductive performance will provide a better understanding of the trade-offs between dispersal distance and oviposition in the navel orangeworm.

#### **Research Effort Recent Publications:**

Sappington, T. W., and C. S. Burks. 2014. Patterns of flight behavior and capacity of unmated navel orangeworm adults (Lepidoptera: Pyralidae) related to age, gender, and wing size. *Environ. Entomol.* 43: 696-705.

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**Table 1.** *F* and *P* values for selected predictors from mixed-model ANOVA of distance and duration of the longest single flight, and of total flight.

Predictor	Duration				Distance			
	Longest flight		Total of flights		Longest flight		Total of flights	
	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
Mated <sup>a,b</sup>	3.63	0.0599	5.49	0.0212	3.24	0.0752	2.54	0.1144
Age <sup>c</sup>	6.06	0.0008	6.27	0.0006	7.41	0.0002	8.73	<0.0001
Sex <sup>b</sup>	14.16	0.0437	14.16	0.0003	8.62	0.0042	18.48	<0.0001
Mated×Sex <sup>b</sup>	3.04	0.0844	1.31	0.2566	2.63	0.1084	3.45	0.0663

<sup>a</sup>Mating status; i.e., mated or unmated. <sup>b</sup>Degrees of freedom = 1,93 (numerator, denominator). <sup>c</sup>Degrees of freedom = 3,93.

**Table 2.** Effect of age on the duration in minutes (mean ± SE) and distance in miles of the longest single flight and sum of flights of navel orangeworm adults.

Day	Number of adults flown	Duration, longest single flight	Duration, total of all flights	Distance, longest single flight	Distance, total of all flights
2	199	238 ± 14a	331 ± 14a	6.4 ± 0.48a	8.0 ± 0.49a
3	246	203 ± 12ab	314 ± 12ab	5.1 ± 0.36ab	6.8 ± 0.39ab
5	209	199 ± 13ab	294 ± 14ab	4.6 ± 0.40ab	6.0 ± 0.42bc
7	201	150 ± 12b	244 ± 13c	3.7 ± 0.40b	5.0 ± 0.42c

Means within columns followed by different letters are significantly different ( $P < 0.05$ ).

**Table 3.** Effect of sex and mating status on the duration in minutes (mean ± SE) and distance in miles of the longest single flight and sum of flights of mating females (FM), unmated females (FU), mated males (MM), and unmated males (MU).

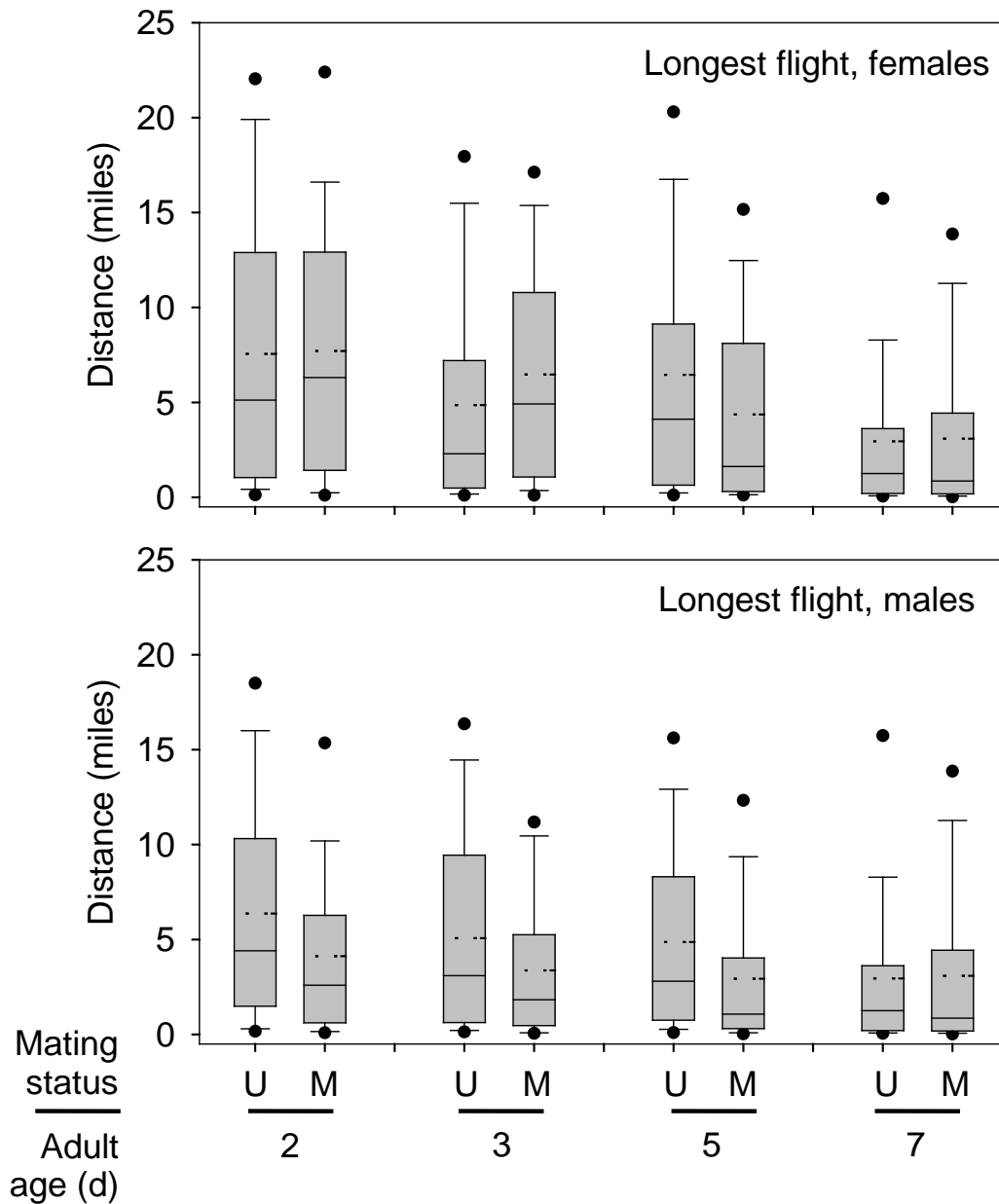
Category	Number of adults flown	Duration, longest single flight	Duration, total of all flights	Distance, longest single flight	Distance, total of all flights
FM	207	224 ± 14	330 ± 14	5.9 ± 0.45	8.0 ± 0.49
FU	221	215 ± 14	331 ± 13	5.8 ± 0.50	7.5 ± 0.39
MM	218	150 ± 11	235 ± 11	3.3 ± 0.40	4.5 ± 0.42
MU	209	205 ± 13	291 ± 13	4.8 ± 0.40	5.9 ± 0.42

**Table 4.** Effects of flight treatment order on fecundity, fertility, and longevity (mean  $\pm$  SE).

<b>Flight Treatments</b>	<b>n</b>	<b>Lifetime fertile eggs</b>	<b>Percent fertility</b>	<b>Longevity (days)</b>
1) Mate-fly-oviposit	2	27.5 $\pm$ 16.5	100	8.5 $\pm$ 2.34
2) Mate-oviposit-fly-oviposit	16	32.7 $\pm$ 11.0	91.6 $\pm$ 5.44	6.5 $\pm$ 1.02
3) Fly-mate-oviposit	11	15.9 $\pm$ 5.03	85.7 $\pm$ 8.17	10.3 $\pm$ 1.45
<b>Control treatments</b>				
1) No flight	21	38.8 $\pm$ 7.14	92.4 $\pm$ 1.34	14.4 $\pm$ 1.15
2) Mate-sham flight-oviposit	7	15.0 $\pm$ 9.23	87 $\pm$ 6.87	10.7 $\pm$ 2.34
3) Sham flight-mate-oviposit	10	46.0 $\pm$ 16.0	81.3 $\pm$ 9.14	-

**Table 5.** Effects of flight treatment order on distance and duration (mean  $\pm$  SE) of longest single flight and total of all flights.

<b>Flight Treatments</b>	<b>n</b>	<b>Maximum distance (miles)</b>	<b>Total distance (miles)</b>	<b>Maximum duration (minutes)</b>	<b>Total duration (minutes)</b>
1) Mate-fly-oviposit	2	13.4 $\pm$ 2.2	13.4 $\pm$ 2.2	536 $\pm$ 5	545 $\pm$ 4.5
2) Mate-oviposit-fly-oviposit	16	5.3 $\pm$ 1.6	6.3 $\pm$ 1.6	219 $\pm$ 59	296 $\pm$ 60
3) Fly-mate-oviposit	11	2.6 $\pm$ 1.4	3.6 $\pm$ 1.8	95 $\pm$ 46	127 $\pm$ 60



**Figure 1.** Boxplot depicting the distance of the longest single flight of navel orangeworm adults by sex, age, and mating status (U = unmated, M = mated). A total of 855 adults were tested. The box represents the 25<sup>th</sup> and 75<sup>th</sup> percentiles, the whiskers the 10<sup>th</sup> and 90<sup>th</sup> percentiles, and dots indicate the 5<sup>th</sup> percentile and 95<sup>th</sup> percentiles. The effect of mating on distance of the longest single flight varied with age with females, whereas unmated males consistently flew longer than mated males on days 2-5.