Effect of Female Flight Activity on Reproduction in the Navel Orangeworm (*Amyelois transitella*)

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Effect of female flight activity on reproduction in the navel orangeworm (*Amyelois transitella*)

by

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The navel orangeworm *Amyelois transitella* is an economically important pest of almonds and pistachios in California. Successful management at the correct spatial scale requires an understanding of insect dispersal, particularly relative to when mating occurs. A previous study found that unmated navel orangeworms were capable of flying longer distances than previously assumed, but it was not clear how mating affects dispersal in this species, nor how dispersal affects fecundity. To address these critical unknowns, females were allowed to fly on a flight mill on the night either before or after mating, and flight performance was assessed. Following the flight test, the females were set up for oviposition. The eggs produced were collected daily and monitored for fertility and compared to those produced by females that were minimally-handled and by tethered but unflown controls. Timing of flight relative to mating did not affect fecundity, nor did the distance or duration of flight. However, mated females flew significantly longer and farther than unmated moths. In addition, trials were conducted where females were forced to fly for predetermined periods of time up to 2 h. There was no effect of forced flight on fecundity. There was an increase in weight lost with time flown, but the effect was weak. The data from this study revealed no obvious trade-off between flight activity and reproductive output in female navel orangeworm. The results indicate that most females mate in or near their natal habitat, and some may disperse relatively long distances to oviposit elsewhere. It is also possible that the long flights by mated females observed on the flight mills represent appetitive ranging behavior, perhaps searching for oviposition host cues, rather than purposeful dispersal. In that case, net displacement in the field
would be less because of meandering flight patterns. Although it is unclear if the navel orangeworm is a true migrant, mating is associated with different female behavior on the flight mills, including propensity to make longer-distance flights than unmated females. Long-flight capacity without loss of fecundity is an important feature contributing to the colonizing ability of this opportunistic pest.
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DEDICATION

Dedicated to my grandfather, John J. Hudak.
CHAPTER 1

GENERAL INTRODUCTION

Effective management of an insect pest usually requires an understanding of its movement behavior and ecology. Various types of adult traps are often useful for monitoring pest presence. However they do not reveal the ultimate source of the insect, and by themselves usually cannot tell managers whether the insects detected are entering or leaving the field or whether they are engaged in local station-keeping flight. Such information is often needed for correctly interpreting the meaning of trapping data and for making the best possible management decisions.

Dingle and Drake (2007) describe several types of insect movement, most importantly station-keeping, ranging, and migration. Station-keeping includes foraging and escape behaviors, which do not typically take the insect out of its home range. Ranging behavior is appetitive flight marked by active searching for resources, such as food or appropriate habitat, and can be in response to a lack of resources in the home range. Ranging may take an insect permanently out of its home range depending on how far it must search before encountering the needed resource. Migration is a non-appetitive type of movement that is unidirectional and often occurring at high altitude where winds strongly affect speed and direction of flight. Migration always takes the individual out of its home range, and spatial displacement is relatively great. In laboratory experiments, it can be difficult to distinguish between ranging and migratory behaviors.

Due to the energetically-expensive nature of both flight and reproduction, many insect species have evolved strategies to manage trade-offs between them. Often referred
to as reproductive-flight syndromes, some insects time reproductive events to occur outside of times when flight activity will be greatest. Migration in insect species displaying a reproduction-flight syndrome is usually undertaken by individuals who are sexually immature (Fu et al. 2015), and flight stimulates ovarian development or decreases the preoviposition period (Rankin 1991, Papaj 2000, Zhang et al. 2015). The extent and manifestation of trade-offs vary between insect species, and between individuals within a species. How flight impacts reproductive capacity in a particular species is not predictable a priori, but must be determined for each species. Even species within the same genus can differ in their relationship between reproduction and flight.

The navel orangeworm, *Amyelois transitella* (Lepidoptera: Pyralidae), is an economically important pest of pistachios and almonds in California. There is increased concern about insecticide resistance in this species, and widespread interest in using pheromone for mating disruption, and developing the sterile male technique. Pheromones can successfully block mating of sentinel females and males in orchards, and studies have demonstrated between 12% and 37% effectiveness in decreasing damage (Landolt et al. 1981, Curtis et al. 1985, Higbee and Burks 2008). To more effectively implement mating disruption, a better understanding of navel orangeworm adult movement is needed. Sappington and Burks (2014) investigated the flight performance of unmated male and female navel orangeworms using flight mills, and found them to be stronger flyers than previously assumed. It is not clear whether female navel orangeworms entering an orchard after dispersal are unmated, mated or some mixture of both.

The purpose of this study was to investigate the relationship between flight and reproduction in the navel orangeworm. In tethered flight experiments, I tested
the effect of timing of mating on flight activity, and whether flight activity affects reproductive output. Flight performance of mated and unmated navel orangeworms were compared using flight mills, a tool for measuring flight distance, duration, speed and the time of each flight. I then investigated the effect of flight activity on reproductive capacity by monitoring daily egg production until death. The results shed light on the interaction between dispersal flight and reproduction, with implications for effective implementation of mating disruption and sterile male release strategies, and for colonization potential of new habitats, such as previously uninfested orchards.
CHAPTER 2
LITERATURE REVIEW

Flight and Dispersal of Adult Insects

One reason for the success of the insect body plan is its capacity for flight, which allows insects to exploit a variety of ecological resources. Most adult insects possess wings and are capable of flight; indeed, this is a distinctive characteristic of Insecta. Insects fly for a wide variety of reasons including escape, searching for food or mates, or purposeful dispersal.

Types of flight

Dingle and Drake (2007) describe several different types of insect movement, particularly station keeping, migration and ranging. Station keeping is movement within an individual’s home range or temporarily beyond the home range. This includes escape flights, defense, or foraging (Zeil and Wittmann 1989). Movement of this sort is meandering and repetitive. The best-understood form of station-keeping movement involves foraging behaviors. Foraging is primarily mediated by insects following chemical cues (Tumlinson et al. 1993). Station-keeping behavior is heavily associated with the insect’s direct response to stimuli. The insect integrates genetic drives and learned experience to determine a course of action when presented with specific stimuli, such as food, appropriate oviposition sites, or predators (Minkenberg et al. 1992, Vet et al. 1990). Escape behaviors are typically triggered by acoustic or visual stimuli, as opposed to chemical (Hoy et al. 1989).
Ranging movement differs from migration. Although ranging and migration both permanently take an individual out of its home range, the drivers and motivations for each are different. Migration is based on an internal drive to disperse, which may be expressed automatically, or triggered by internal or external cues. Ranging involves searching for a resource such as food, mates, or adequate habitat, which is unavailable in the current home range. In other words, ranging behavior is an active seeking of a required resource, and is more meandering and tends to be slower than migratory flight (Dingle and Drake 2007). Ranging behavior ceases once the resource is encountered. In practice the distinction between migratory behavior and ranging behavior can be subtle when measured in the laboratory, and the cessation of migratory behavior by an individual may be marked by the commencement of ranging behavior (Holland et al. 2006).

Migration in insects refers to long-distance (relative to body size) unidirectional dispersal flights. From a behavioral perspective, migration involves a suite of behaviors marked by unidirectional non-appetitive flight (Hardie 1993). It often takes place above the earth’s atmospheric boundary layer to take advantage of high wind speeds. Migratory behavior is triggered by specific life history events, such as age or mating, or a suite of environmental factors (Reardon et al. 2006, Dorhout et al. 2008). In many migratory species, migration is seasonal. The rice leaf roller migrates during June and July from overwintering sites to agricultural regions in northern China, where resources are more abundant during the summer (Fu et al. 2014). The silver Y (Autographa gamma) migrates from winter breeding grounds in the United Kingdom to summer breeding grounds in the Mediterranean basin (Chapman et al. 2012). Due to their dramatic migratory behaviors,
monarch butterflies are a popular model species for migration in lepidopterans (Reppert et al. 2016). Monarchs use light cues to trigger migratory behavior and for orientation (Guerra and Reppert 2015). Temperature can also trigger behaviors related to migration in this species. Fall migrants exposed to cold temperatures prematurely reverse flight orientation independent of light stimuli (Guerra and Reppert 2013).

*Flight and reproduction in insects*

Flight and reproduction are often linked in insect life histories. Dispersal can provide a reproductive advantage when an insect is able to find a suitable habitat for offspring, increasing their chances of surviving to adulthood. Two general strategies are used by insects to cope with seasonal changes in habitat quality, diapause and migration (Dingle 2006). Many insects use strategies for spreading risk, including dispersal, to deal with less predictable changes in habitat quality (Hopper 1999). Long-distance flight is energetically expensive, and may use resources that could be utilized for reproductive development. Oogenesis and flight activities compete for energy reserves, and extended flight can cause a decrease in fecundity and fertility (Bartholomew et al. 1978, Shirai 1995, Evenden et al. 2015). However, larger individuals or individuals with higher-quality diets may not experience a decrease in reproductive development associated with long flight (Willers et al. 1987, Gunn et al. 1988, Wheeler 1996, Attisano et al. 2013), but the relationship between size and flight capacity is not straightforward (Kaufmann et al. 2013). Several physiological adaptations may contribute towards an individual’s capacity for long-distance flight, involving physiological adaptations for long-distance flight. These adaptations may be controlled by genes regulating hormones such as juvenile
hormone, development of musculature, and lipid mobilization (Van Vielendaele et al. 2013, Jones et al. 2015).

Risks, rewards, and trade-offs

The advantages of long-distance dispersal may outweigh a resulting decrease in reproductive output of energy expenditure during flight. Dispersal and migration present an opportunity to colonize a novel habitat and exploit novel resources (Roff and Fairbairn 2007), but dispersal also presents some risks. Migration particularly presents a risk of dying prior to reaching a new resource-rich environment, either through predation or starvation (Hopper 1999), although some migratory species are able to manage these risks by travelling in aggregate, which reduces predation risk for individuals within the group (Srygley and Lorch 2016). The trade-off between migration and reproduction may be ameliorated if an individual can to take advantage of energy resources in a new habitat. For long-distance dispersers, nectar provides fuel, which can be used for continued flight or reproduction (Rudolph et al. 2006). For example, Gunn et al. (1988) found a negative linear relationship between flight activity and fecundity in the African armyworm, *Spodoptera exempta*, but the reduction in fecundity was amended when the insects were allowed to consume sucrose after flight.

Host suitability and larval experience play an important role in ovarian development in insects. One factor contributing to the evolutionary success of insects is the rapid development of large numbers of eggs allowing for rapid maturation and quick generational turnaround. The ability to regulate ovarian response to environmental conditions including temperature, photoperiod, social context and diet also contributes to
the continued success of insects (Papaj 2000). Insects are able to time reproductive
activities relative to environmental, nutritional, and life-history events or developmental
stage. Some species coordinate life history events quite dramatically, such as migratory
locusts, which molt between a gregarious phase and a reproductive phase (Van
Wielendaele et al. 2013). Others use more subtle timing and display less-dramatic life
history trade-offs.

Individuals within a species can have different propensities or capacities for
flight, which affect their life histories. Differences in flight behavior within species can
be caused by either environmental or genetic factors that affect resource allocation to
flight muscles (Saastamoinen and Hanski 2008, Rauhamaki et al. 2014, Evenden et al.
2015). Many hemimetabolous insect species are wing dimorphic, where some individuals
have longer wings and robust flight muscles, whereas others lack well-developed wing
musculature. In these species, the long winged morphs have an increased capacity and
propensity for flight and are generally the migratory forms of the species. Wing
development is associated with several life history traits, and wing development is under
hormonal control. However, not every long-winged insect is migratory in practice (Roff
and Fairbairn 1991). The grass thrips Anaphothrips obscurus exhibits wing polyphenism,
and Jiang et al. (2015) found that the long-winged and short-winged morphs differed in
early fecundity. Flightless females produced more eggs early in their adult lifespan,
although mean lifetime fecundity was not affected by the potential for flight. Along with
flight muscle morphology, wing morphology is important for determining flight capacity
in moths independent of body weight (Torres et al. 2015, Bartholemew and Casey 1978).
For example, within the gypsy moth Lymantria dispar, females of some strains are
capable of flight, and females capable of flight were found to have smaller wing loads than females of flightless strains (Shi et al. 2015).

Migratory behaviors can be an inherent characteristic of the species (obligate) or individuals can display migratory behaviors in response to environmental conditions (facultative), and the relationship between reproduction and flight activity can be affected by those conditions (Showers et al. 1989, Saethre and Hofsvang 2002, Dorhout et al. 2008). Larval experience can affect migratory potential in various ways. In the black cutworm, *Agrotis ipsilon* (Lepidoptera: Noctuidae), crowded conditions for the larvae leads to a decrease in migratory flight capacity (Sappington and Showers 1992). Conversely, high larval density leads to a greater flight potential in the beet webworm *Loxostege sticticalis* (Cheng et al. 2012). In the Oriental armyworm *Mythimna separata*, moderate larval density encourages migratory behavior in adults. However, overcrowding results in adults that have lowered flight performance, reproductive capacity, and smaller adult size (Jiang et al. 2011). In the rice leaf roller *Cnaphalocrocis medinalis*, high larval rearing density decreased time to maturity, but did not affect female reproductive development or adult propensity to migrate (Yang et al. 2014). Migration in the Mormon cricket *Anabrus simplex* is triggered by crowded conditions and the presence of predators (Srygley and Lorch 2016). Crowding alone may not be the cause of migratory behaviors, but related instead to resource availability. In crowded conditions, there are fewer resources to go around, so insects must engage in dispersal to locate more sufficient resources. Mormon crickets with well-balanced diets that included carbohydrates and protein did not move as far as crickets that were starved or were fed only protein, indicating movement associated with nutritional deficiency (Srygley and Lorch 2013).
The extent and manifestation of trade-offs between flight and reproduction varies greatly within and between insect taxa, and is largely species-specific (Guerra 2011), as are the strategies for managing these trade-offs even among insects demonstrating a reproductive-flight syndrome. Individuals with a lower propensity or capacity for flight may lay eggs near the natal habitat whereas individuals with a greater propensity for flight may migrate prior to mating or laying eggs. Some insects are able to modulate their reproductive output to changing environments. In the milkweed bug, *Oncopeltus fasciatus*, females that fly have lower levels of ovarian resorption, indicating a plastic ovarian response (Attisano et al. 2013).

**Reproductive syndromes related to flight**

Physiological syndromes coordinate the timing of development with life history events such as diapause or migration in “here later, there now” strategies (Dingle 2014). Delay of ovarian development until after migratory flight is one way to manage trade-offs between reproduction and flight which is seen in several species (Zhou et al. 2000), a phenomenon referred to as the oogenesis-flight syndrome (Dingle 1972, Rankin et al. 1986, Papaj 2000). Migration in insect species displaying a reproduction-flight syndrome is undertaken by individuals who are sexually immature (Fu et al. 2015), and flight stimulates ovarian development or decreases the preoviposition period (Zhang et al. 2015). Reproduction is then followed by a decrease in long-distance flight activity. In the Old World cotton bollworm, *Heliothis armigera*, for example, reproductive maturity is correlated with a decrease in flight activity (Colvin and Gatehouse 1993). However, sexual maturity does not necessarily terminate migratory behaviors. For instance,
individual oriental armyworms (*Mythimna separata*) sometimes have mature ovaries or spermatophores present during active migration (Colvin and Gatehouse 1993, Zhou et al. 2009, Xiao et al. 2016). The oriental armyworm is able to modulate migratory behavior in response to reproductive status. Calling by females is associated with a decrease in flight propensity (Han and Gatehouse 1993). Another example is found in the spruce budworm *Choristoneura fumiferana* (Lepidoptera: Tortricidae). Rhainds and Kettela (2013) found that the oviposition threshold for flight in females was around 50% of their egg complement; i.e. females had to lay at least some eggs prior to migratory flight. In other species, migratory behaviors may follow reproductive maturity and increase after mating. One such species is the wheat midge *Sitodiplosis mosellana*. After mating, females engage in high-altitude flight, which allows them to take advantage of the wind for dispersal (Miao et al. 2012).

Many orthopteran species display reproductive-flight syndromes, and have been the subject of much study to understand the nature of differential resource allocation to flight and reproduction. Many orthopteran species are wing-dimorphic. The sand cricket *Gryllus firmus* consists of two different morphs: long-winged and short-winged. Compared to the long-winged morphs, the short-winged crickets have less developed flight muscles and less capacity for flight, but greater resistance to disease (Park and Stanley 2015). In wing-dimorphic species, often the short-winged morph has a fitness advantage either from increased reproductive output or a shorter preoviposition period. In the cricket *Velarifictorus asperses*, flight encourages reproductive development (i.e. gonad maturation) in both males and females, although the time to reproductive maturity still lags behind short-winged morphs (Zeng et al. 2014). This is not the case for the
migratory locust *Locusta migratoria*. In this species, the short-winged morph did not differ significantly from the long-winged morph in age at first reproduction, egg production, or offspring body weight (Nishide and Tanaka 2013). In the pygmy grasshopper *Tetrix subulata*, long-winged individuals and short-winged individuals consume substantially different diets, indicating they are not in competition with each other for resources (Karpestam and Forsman 2013).

**Methods for Studying Insect Flight Behavior**

Insect flight and movement can be studied using a variety of laboratory and field methods involving both tethered and untethered flight (Wang 2005). In the field, insect movement can be estimated using population genetics methods, mark-recapture, and radio tagging. In the laboratory, study of insect movement uses a variety of tethered and untethered flight methods.

*Methods for studying flight in the laboratory*

Untethered or “free flight” techniques are useful for studying insect movement and flight behavior in the laboratory. One advantage of an untethered flight technique is that it minimizes handling by researchers. Srygley and Thomas (2002) used a wind tunnel to visualize the specific movements of red admiral butterflies (*Vanessa atalanta*) to generate lift during flight.

Tethered flight is often employed in studying energy use in insects. In a tethered flight study of *Aphis fabae*, Cockbain (1961) investigated the relationship between flight capacity and diet, finding that insects with higher lipid stores were able to fly longer.
Tethered flight techniques are often used to investigate flight biomechanics. Brodsky (1991) used high-speed video of tethered flight of the peacock butterfly, *Inachis io*, to investigate the mechanics of wing movement during flight. Tethered flight can be used to investigate specific behaviors. An interesting example is a specific evasion behavior of the male praying mantis, *Parasphendale agrionia*, displayed during flight. When exposed to ultrasound, the male extends the forelegs, decreases wing beat frequency, flexes the abdomen and rolls the head. Yager and May (1990) suggested that it was an antipredatory response.

One method of testing flight is by use of an actograph consisting of a sensor that detects movement across an infrared beam. For example, Kuusik et al. (2002) used a combination of actograph recording and respirometer to monitor flight activity of bumblebees, *Bombus terrestris*, in a laboratory setting. Hashiyama et al. (2013) investigated the flight activity of mated and unmated female *Autographa nigrisigna* using both an actograph and flight mill. They found that mating increased female flight activity when monitored by actograph, but not as measured by flight mill. Hall et al. (2015) used both tethered and untethered flight techniques to determine the role of halteres in flight in two fly species, *Drosophila melanogaster* and *Sarcophaga bullata*. Tethering was used to investigate the movement of halteres and to take electrophysiological readings of flight muscles. The untethered method used by this group involved a perturbation chamber, where flight was maintained by vibrating surfaces to discourage the flies from landing.
Flight mills

The propensity for flight may vary considerably between individuals in a population (Roff and Fairbairn 2007), and can be measured with flight mills. A flight mill is a tool with which flight parameters – for example, time of flight, duration of flight, and distance flown – can be measured and analyzed for each individual. Flight mill data can provide an estimate of flight capacity in the field. For example, both female and male longhorn beetles, *Monochamus galloprovincialis* (Coleoptera: Cerambycidae), flew about 16 km during their adult lifespan, while mark-recapture data indicated they were capable of flying approximately 15 km during their lifetime. In this case, the flight mill study provided a very close estimate of wild behavior (David et al. 2013), but usually this is not the case. Flight mill data present some limitations when extrapolating to wild populations. A flight mill experiment takes place in an artificial environment, where the advantage is that factors such as temperature, light, and humidity can be controlled, but this environment introduces factors not present in the field. The flight mill adds factors such as friction and the weight of the flight mill arm, and during testing insects are prevented from landing as they may do in the field. In addition, insects must be handled and tethered in preparation for flight mill experimentation. Flight mills also impose unidirectional flight, so distance flown on a flight mill may overestimate total displacement in the field. Many insects, particularly lepidopterans, are able to orient themselves during flight to take advantage of wind movement for long-distance migration (Chapman et al. 2015), so distance travelled on a flight mill by individuals in these species may greatly underestimate total displacement. Moreover, motivation for flight is
difficult to determine in the laboratory. An insect on the flight mill may continue flying whereas an insect in the field would stop once a specific resource is discovered.

On the other hand, flight mill data provide information about minimum flight capacity (Dorhout et al. 2008, Lee and Lesky 2015) and can inform interpretation of field data, such as trapping and mark-recapture data. Although it is difficult to directly extrapolate flight mill data to the field, studies of this kind allow for comparison of relative performance between specific groups of insects or insects under different conditions. Flight mills are useful for comparing insects based on age, sex, mating status, disease, or juvenile experience (Sappington and Showers 1991, 1992a,b, 1993, Sappington et al. 1994, Dorhout et al. 2008, Wiman et al. 2015, McKay et al. 2016). In the black cutworm, high larval density causes a decrease in pupal length and weight and corresponding decrease in adult size (Sappington and Showers 1992b). However, there was no decrease in performance on flight mills between moths reared singly or under crowded conditions. Wiman et al. (2015) used flight mills to examine differential flight performance of the brown marmorated stink bug, *Halyomorpha halys*, based on characteristics such as sex and generation (i.e., whether the individual had overwintered). Castro et al. (2014) found that distance flown on flight mills by *Rhodnius pallescens* increased with increased bacterial load when the insects were infected with *Trypanosoma cruzi*, the causative agent of Chagas disease. The monarch butterfly, *Danaus plexippus*, experiences a pre-migratory reproductive diapause, where the ovaries are undeveloped prior to long-distance migration. Reproductive status did not affect flight propensity on flight mills, but flight efficiency was lower in reproductively active individuals (McKay et al. 2016). Sappington and Burks (2014) found no significant difference in flight
performance between sexes of unmated navel orangeworm, but did find a decrease
associated with age.

Flight mill experiments are also useful for investigating the relationship between flight behaviors and life history traits in a controlled environment. Khuhro et al. (2014) investigated the relationship between flight activity, longevity, and reproduction in the neuropteran *Chrysoperla sinica*. Female flight duration and distance were measured on a flight mill and fecundity and longevity were monitored. Lacewings that flew longer durations experienced a decrease in reproductive capacity, thought to be the result of a decrease in adult lifespan. In the tortricid *Choristoneura conflictana*, females flown on a flight mill did not experience an effect on mating propensity or number of eggs produced. However, there was a decline in potential fecundity, and the effect of flight was largely dependent upon size (Elliot and Evenden 2012). Schumacher et al. (1997) investigated the effect of mating on flight in the codling moth, *Cydia pomonella*, using flight mills. They found that mated females had a lower propensity for long-distance flight than unmated females, with 17% of unmated females taking long-distance flight (a single flight of 5km or farther) and less than 8% of mated females taking similar flights.

Flight mills are also frequently used to directly investigate the role of flight in reproduction, as in the case of the boll weevil, *Anthonomus grandis* (Rankin et al. 1994). Virgin females were prepared and allowed to fly to exhaustion. After testing, the beetles were killed and dissected to assess ovarian development. They found that flight activity was diminished by maturation of the ovaries. When females were flight tested several times during adult life, the association between ovarian maturation and decreased flight activity was confirmed. Mating also was associated with a decrease in flight activity. In
the beet webworm, *Loxostege sticticalis*, females were flown on flight mills and the consequences for reproduction were investigated (Cheng et al. 2012). Females did not initiate migratory flight until 2-3 days following emergence. Those that engaged in long-distance flight experienced a shortened preoviposition period, although none of the other reproductive parameters studied were affected.

**Navel Orangeworm and Crop System**

The navel orangeworm, *Amelyois transitella* (Walker), is a serious pest of nut crops, particularly almonds and pistachios, in California. Navel orangeworm can cause up to 30% product loss in almonds due to direct consumption, and only 2% product loss is considered the economic threshold for navel orangeworm impact (Higbee and Siegel 2009). Almonds and pistachios are important cash crops for the state of California. The California almond industry generates $21.5 billion in total revenue, with almond farming itself generating $7.6 billion (Sumner et al. 2015). California produces 80% of the world’s almonds, 100% of the almonds in the United States, and they are the state's third largest agricultural product with a reported value of $6.4 billion. Almonds are California's top agricultural export (25% of total; Almond Board of California 2015, Sumner et al. 2015). California produces 98% of the pistachios in the United States, and 20% of the world’s pistachios, behind only Iran. The yearly value of California’s pistachio crop was $518 million in 2007 and is only projected to grow (Kallsen et al. 2009).
**Host plant biology**

In order to understand navel orangeworm movement between orchards, an understanding is needed of host biology as well as navel orangeworm behavior. The navel orangeworm is an opportunist and generalist (Wade 1961). Dispersal is part of the suite of behaviors used by generalists to move from one host to another. In fragmented landscapes, where acceptable hosts may be separated by geographical distance, dispersal is essential for moving between hosts and colonizing new areas (Tsarntke and Brandl 2004). Thus, understanding and predicting navel orangeworm movement in the field requires an understanding of the phenology of its hosts.

The almond, *Prunus dulcis*, is a member of the Rosacea, which includes important cash crops such as apples, pears, peaches, plums, and quince. The almond is native to the Mediterranean and Middle East, in what would currently be considered Syria and Turkey. The almond produces flowers on long shoots and short spurs, which can hold one to several buds. The timing of flower bud initiation differs between varieties of almond, with “Nonpareil”, the most common cultivar in the United States, initiating floral development three weeks after hull split. For other cultivars such as “Butte” and “Caramel”, floral initiation occurs prior to hull split (Lamp et al. 2001). Almond flowers are hermaphroditic, but self-incompatible, requiring pollination by bees and planting schemes typically consist of alternating rows of pollinizers and main cultivars. The tree produces a drupe fruit similar in morphology to a peach. Prior to harvest, the fleshy fruit dries and peels back to expose the kernel (“hull split”) (Rieger n.d.).

The pistachio, *Pistachio vera*, is native to central Asia, where it is an important crop in Iran, Turkey, Syria, and Afghanistan. It is in the family Anacardiaceae, which
includes cashew, mango, poison oak, poison ivy, and sumac. Inflorescence starts during April, and the flowers grow to their ultimate size by late June. In the “Kerman” variety, the most common variety grown in the United States, an inflorescence contains 100-150 individual flowers, and the pistachio bears flowers and fruit on the lateral side of the previous season’s growth. The pistachio is wind-pollinated with different male and female varieties and produces a semi-dry drupe fruit. Fruit maturity is determined by a change in the epicarp, which pulls back to expose the seed, known as a “hull split”. The pistachio is alternate bearing, meaning it produces a heavy crop one year and a light or no crop in alternate years. This is the result of dropping flowers prematurely rather than a lack of bud formation (Crane and Iwakiri 1981).

**Navel orangeworm biology**

The navel orangeworm is a small moth in the family Pyralidae. The adult is gray with black irregular lines on the forewings, and with a wingspan of approximately 20mm. The labial palps form a “snout” at the front of the head. There are no obvious features distinguishing males from females (Harris 2013), although males tend to be smaller in size. Eggs are laid singly or in small clutches on the surface of fruit. The egg is ovoid and slightly flattened, and, if fertile, changes color from cream to a bright reddish orange. The larva is reddish-orange to cream in color, depending on the larval diet, and pupae are reddish-brown (Wade 1961). The larva orients toward any breach in the seed’s protective shell (Hamby and Zelom 2013).

Mating typically occurs during the pre-dawn twilight. Copulation takes approximately an hour and a half, starting in the ultimate hour of scotophase and ending
during the photophase, although about 10% of matings occur during the daylight hours (Parra-Pedrazzoli and Leal 2006). Females perch on a vertical surface and extend the abdomen to release pheromone, which attracts the male. The male fans its wings while approaching the female, then turns to establish a tail-to-tail position (Girling and Carde 2006).

The host range for the navel orangeworm also includes other cash crops such as walnuts, figs, pecans, citrus and pomegranates, and it infests native species such as locust and yucca. The species was first described in the early 20th century, and its geographic range extends from northern California into Mexico (Wade 1961). It has a multivoltine life cycle. The overwintering larvae develop in mummy nuts (i.e., those nuts remaining on the tree postharvest) and emerge in the spring (Sanderson et al. 1989).

The number and length of generations in the navel orangeworm is tied to the seasonality of their food sources. Navel orangeworm adults are attracted to fruit volatiles released by nuts at specific points of maturation. For example, males are preferentially responsive to alkanals, alkanols and alkanones produced by decaying pistachios and almonds (Beck et al. 2014), while female are preferentially attracted to volatiles emitted by the nuts as their hulls split, although both males and females are attracted to the ripe fruits (Beck et al. 2012). Generation number and time is variable due to a variety of environmental factors affecting development time of larvae, including host species and weather. Consequently, different generations increasingly overlap as the summer progresses. In warmer years or in more southern portions of the United States and into Mexico, the navel orangeworm can have 4-5 generations per year (Harris 2013),
although in almonds in most of California it has only 3 generations per year (Sanderson et al. 1989).

Navel orangeworm damages nut crops in multiple ways. In addition to direct consumption of the seed by larvae, both adults and larvae facilitate the spread of *Aspergillus* spp., which produce aflatoxin. Aflatoxin is a serious human and animal food contaminant, causing liver damage including liver cancer, gastrointestinal hemorrhage, spontaneous abortion in livestock and death in livestock and humans (Eaton and Groopman 1994). Navel orangeworm adults are attracted to fungal volatiles (Beck 2013, Beck et al. 2014), and *Aspergillus* infection in almonds is associated with higher infestation with navel orangeworm (Palumbo et al. 2014). In addition, Ampt et al. (2015) found that navel orangeworm larvae preferred almond meal infected with *Aspergillus* spores, and had higher weights and reached adulthood 33% faster than larvae raised on control diet. This may indicate a mutualism between the navel orangeworm and the *Aspergillus* fungus.

**Navel orangeworm monitoring and management**

There is an ongoing need for better monitoring and control techniques for navel orangeworm. Navel orangeworm presence and level of infestation within orchards is monitored with almond meal cakes, which almost exclusively attract females looking for a place to oviposit (Beck et al. 2014). Egg traps work by attracting insects through plant volatiles. They are placed in orchards during the first week of April and increases in egg laying are monitored. This is used to determine when main generational spikes occur in the orchard and to time treatments (Higbee and Burks 2011, University of California
Agriculture and Natural Resources 2016). Egg traps are sensitive to environmental conditions such as weather and orchard environment making them too variable to use for estimating relative population densities between crops and seasons (Kuenen et al. 2010, Higbee and Burks 2011, Nay et al. 2012).

Insecticides, particularly pyrethroids, are applied at the time of hull split to control navel orangeworm, but the most important component of navel orangeworm control is orchard sanitation. This involves removal of mummy nuts, early harvest, and destruction of overwintering pupae prior to the spring generation (Engle and Barnes 1983). Orchard sanitation starts in January when the number of mummies on 20 trees per block is determined. Fewer than two mummy nuts per tree is the goal, and, if needed, mummy removal takes place by the first of February. Sanitation also involves raking all nuts on the orchard floor to the middle of rows and mowing or discing them by the middle of March (Sacramento Valley Orchards 2016). Navel orangeworm is also controlled by various methods in stored products (Tang et al. 2002, Tang et al. 2003), since the presence of larvae and pupae in the commercial product is undesirable.

*Mating disruption*

There is an increased interest in mating disruption as a method of navel orangeworm management. Major components of this species’ sex pheromone were identified in the 1970's (Coffelt et al. 1979), but other essential components were not characterized until more recently (Leal et al. 2005, Kuenen et al. 2010). A more complete pheromone is now available (Thompson et al. 2012). There has been interest in using mating disruption to manage navel orangeworm in orchards. Components of navel
orangeworm pheromone have been investigated for use in mating disruption since the 1980s. Landolt et al. (1981) found that use of a single pheromone component was able to reduce the number of males attracted to caged females in an almond orchard.

Use of mating disruption in almond orchards has had mixed results. Curtis et al. (1985) found that mating was significantly reduced in four experimental plots, with reduction of damage ranging from 12-34%. Similarly, experiments using pheromone puffers in almond and pistachio orchards indicated that mating disruption decreased navel orangeworm damage by 16-37% in “Nonpareil” almonds. There was no change to navel orangeworm damage due to mating disruption in pistachios (Higbee and Burks 2008). Higbee et al. (2014) conducted a successful field trial of a navel orangeworm trap using commercially available pheromone lures for monitoring and mating disruption.

Recently, there has been interest in using sterile male technique to manage navel orangeworm. Sterile male technique has been used to control a small number of agricultural pests successfully, but the proper ecological circumstances and logistics of production and deployment are demanding. Sterile male technique has been useful in suppressing populations of codling moth in Canada and pink bollworm (Pectinophora gossypiella) in the United States (Bloem et al. 2005, Proverbs et al. 1996, Tabashnik et al. 2010). Light et al. (2016) sterilized navel orangeworm males in the laboratory, which successfully mated with females who produced no offspring.

An understanding of navel orangeworm movement is needed for its effective management of navel orangeworm. Increased use of insecticides in at-risk crops has lead to an increase of pyrethroid resistance in navel orangeworm populations (Demkovich et al. 2015). Thus, there is a greater need for more effective monitoring and sanitation as
well as alternative methods of management, including mating disruption (Burks et al. 2016) and sterile male technique (Light et al. 2015). Movement relative to mating is an important consideration for the application of mating disruption, because already-mated migrants are not susceptible to mating disruption (Cardé and Minks 1995). Sappington and Burks (2014) investigated flight capacity in unmated navel orangeworm and found that it is capable of covering greater distances than previously thought. Almond orchards 3 miles or less from a potential source of navel orangeworm, such as a pistachio orchard, are considered to be at risk (Higbee and Siegel 2009). However, if moths are capable of flying longer distances, new infestations may originate from orchards even farther away. Results from flight mill studies can inform interpretation of data from trapping studies. Likewise, management can be informed by an understanding of moth behavioral motivations. It is currently not known whether female moths trapped in a field are indicative of an incoming or ongoing infestation, and whether waiting until eggs are detected in traps is too late for insecticide application. In this study, I investigated the relationship between flight activity and reproduction in the navel orangeworm. I used flight mills to compare flight activity between mated and unmated females to determine whether mating enhances or decreases flight activity. I also monitored fecundity and fertility to determine if there is an effect on reproduction caused by increased flight activity.
CHAPTER 3
EXPERIMENTS
Effect of Female Female Navel Orangeworm (Lepidoptera: Pyralidae) Flight Activity on Reproduction

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Abstract

The navel orangeworm \textit{Amyelois transitella} is an economically important of almonds and pistachios in California. Successful management requires an understanding of insect dispersal, particularly relative to when mating occurs. A previous study found that unmated navel orangeworms were capable of flying longer distances than previously assumed, but it was not clear how mating affects dispersal in this species, nor how dispersal affects fecundity. Females were allowed to fly on a flight mill either before or after mating, and flight performance was assessed. Females were then set up for oviposition. The eggs produced were collected daily and monitored for fertility and compared to those produced by females that were minimally-handled or tethered controls. Timing of flight relative to mating did not affect fecundity, nor did flight performance. However, mated females flew significantly longer and farther than unmated moths. Other females were forced to fly for predetermined periods of time. There was no effect of forced flight on fecundity. This study revealed no obvious trade-off between flight activity and reproductive output. The results suggest that most females mate in or near their natal habitat and some may disperse to oviposit elsewhere. Net displacement in the field may be less because of meandering behavior. Long-flight capacity without loss of
fecundity is an important feature contributing to the colonizing ability of this opportunistic pest.

**Introduction**

**Navel Orangeworm**

The navel orangeworm, *Amelyois transitella* (Walker), is a serious pest of nut crops, particularly almonds and pistachios, in California. Navel orangeworm can cause up to 30% product loss in almonds due to direct consumption, and only 2% product loss is considered the economic threshold for navel orangeworm impact (Higbee and Siegel 2009). Almonds and pistachios are important cash crops for the state of California. The California almond industry generates $21.5 billion in total revenue, with almond farming itself generating $7.6 billion (Sumner et al. 2015). California produces 80% of the world’s almonds, 100% of the almonds in the United States, and they are the state's third largest agricultural product with a reported value of $6.4 billion. Almonds are California's top agricultural export (25% of total; Almond Board of California 2015, Sumner et al. 2015). California produces 98% of the pistachios in the United States, and 20% of the world’s pistachios, behind only Iran. The yearly value of California’s pistachio crop was $518 million in 2007 (Kallsen et al. 2009).

The number and length of generations in the navel orangeworm are tied to the seasonality of their food sources. Navel orangeworm adults are attracted to fruit volatiles released by nuts at specific points of maturation (Beck et al. 2012, Beck et al. 2014). Female moths lay their eggs on the surface of the host fruit, typically during hull split, after which the larvae hatch and burrow to consume the seed. In California, the first generation of navel orangeworm emerges in late June to early July. The second
generation develops in the new crop nuts and the adults emerge through late summer and early autumn. The third generation overwinters as pupae inside mummy nuts (i.e. those nuts left on the tree postharvest), and the adults emerge the following Spring or Summer (Sanderson 1989). Increases in oviposition frequency in pistachios are later than in almonds (Rice 1978), due to the difference in the pistachio’s yearly life cycle. Yearly variations in climate also affect the number and timing of generations. In warmer years or in more southern portions of the United States and into Mexico, the navel orangeworm can have 4-5 generations per year (Harris 2013).

An understanding of insect movement is needed for effective management of navel orangeworm. Increased use of insecticide in at-risk crops has lead to an increase of pyrethroid resistance in navel orangeworm populations (Demkovich et al. 2015), leading to a greater need for more effective monitoring and sanitation as well as alternative methods of management, including mating disruption and sterile male technique (Light et al. 2015). Success of mating disruption trials in the navel orangeworm range from 12%-37% reduction in damage and up to a complete elimination of mating activity (Curtis et al. 1985, Burks et al. 2016). Sappington and Burks (2014) investigated flight performance in unmated navel orangeworm and found that the navel orangeworm is capable of covering greater distances than previously thought. Almond orchards 3 miles or less from a potential source of navel orangeworm, such as a pistachio orchard, are considered to be at risk (Higbee and Siegel 2009). However, if moths are capable of flying longer distances, new infestations may not be coming from neighboring orchards. Results from flight mill studies can inform interpretation of data from trapping studies, which estimate
displacement in the field. Likewise, management can be informed by an understanding of why the moth is in the vicinity of the trap.

**Flight and Reproduction in Insects**

Flight and reproduction interact with one another in ways that can fundamentally affect insect life histories. Many species display life history trade-offs between dispersal and reproduction, and the extent and manifestation of these trade-offs is largely species-specific and is not known for many species. Dispersal can provide a reproductive advantage when an insect is able to find a suitable habitat for offspring, increasing their chances of surviving to adulthood. However, oogenesis and flight activities compete for energy reserves, and extended flight can cause a decrease in fecundity and fertility (Shirai 1995, Evenden et al. 2015). Physiological syndromes can coordinate the timing of reproduction and migration (Dingle 2014). Delay of ovarian development until after migratory flight is a way to manage trade-offs between reproduction and flight that is seen in several species (Zhou et al. 2000). Migration in insect species displaying a reproduction-flight syndrome is undertaken by individuals who are sexually immature (Fu et al. 2015), and flight stimulates ovarian development or decreases the preoviposition period (Zhang et al. 2015). Reproduction is then followed by a decrease in flight (Colvin and Gatehouse 1993). However, sexual maturity does not necessarily terminate migratory behaviors (Colvin and Gatehouse 1993, Zhou et al. 2009, Xiao et al. 2016).
Flight mills

The propensity for flight may vary considerably between individuals in a population (Sappington and Showers 1991, Roff and Fairbairn 2007, Dorhout et al. 2008, Sappington and Burks 2014), and can be measured with flight mills. A flight mill is a tool with which flight parameters – for example, time of flight, duration of flight, and distance flown – can be measured and analyzed for each individual. Flight mill data present some limitations when extrapolating to wild populations. The flight mill adds factors such as friction and the weight of the flight mill arm, and during testing insects are prevented from landing as they may do in the field. In addition, insects must be handled and tethered in preparation for flight mill experimentation. Flight mill experimentation also involves unidirectional flight, so distance flown on a flight mill may overestimate total displacement. Wind can affect direction and displacement in the field, which may bring a researcher to over- or underestimate displacement. Moreover, motivation for flight is difficult to determine in the laboratory. An insect on the flight mill may continue flying whereas an insect in the field would stop once a specific resource is discovered.

However, flight mill data provide information on minimum flight capacity that can provide information about behavior in the field (Lee and Lesky 2015) and can inform interpretation of field data, such as trapping and mark-recapture data. Studies of this kind allow for comparison of relative performance between specific groups of insects or insects under different conditions, such as age, sex, mating status, or larval conditions, or pathogen load (Sappington and Showers 1991, 1992a, 1992b, 1993, Sappington et al. 1994, Dorhout et al. 2008, Wiman et al. 2015, McKay et al. 2016). Flight mill experiments are also useful for investigating the relationship between flight behaviors
and life history traits in a controlled environment. Sappington and Burks (2014) tested flight propensity between male and female navel orangeworms and found no significant difference between sexes, but did find a decrease in flight performance associated with age.

In this study, we investigated the relationship between flight activity and reproduction in the navel orangeworm. We used flight mills to compare flight propensity between mated and unmated female navel orangeworm to determine whether mating enhances or decreases flight activity. We also monitored fecundity and fertility to determine if there is an effect on reproduction caused by increased flight activity.

**Materials and Methods**

**Experimental Overview and Design**

The purpose of this study was to investigate the relationship between flight and reproduction in the female navel orangeworm. The study was designed to examine two concepts: the effect of timing of flight relative to mating and the effect of flight on fecundity. To reach this goal, we measured flight performance of female navel orangeworms on a flight mill and then investigated the impact of flight parameters on reproduction by monitoring oviposition and collecting eggs every day and assessing them for fertility until the female’s natural death. Experiments were conducted between May 2014 and April 2016, with no systematic timing of treatments with respect to time of year. Although treatments were not scheduled randomly, the different treatments were spread and intermingled across time based on logistics. An ANOVA did not indicate a significant effect of month on fecundity (unpublished data).
Two different experiments were conducted: “voluntary” flight and “forced” flight. The voluntary flight experiment involved tethering insects for testing on flight mills either prior to mating or after mating. To control for the effect of handling, matched tethered control groups were prepared. Our sample size goal was 50 individuals with usable data per treatment in this experiment based on the level of variability in flight performance experienced with other insect species tested in our laboratory. A forced flight experiment was conducted to separate the effects of flight per se from those arising from an individual’s propensity for flight. Individuals were forced to fly continuously for predetermined amounts of time. The minimum sample size was 30 individuals per treatment for this experiment. For both experiments, individuals were excluded from the data set if they did not meet minimum values of flight performance, fecundity, fertility, and longevity. This was done to prevent inclusion of data from insects that were damaged imperceptibly during handling or were in poor health. Our minimums were arbitrary but conservative. By erring on the side of not including an individual of compromised health, we may have eliminated naturally poor fliers or moths that did not fly for another reason. However, the latter was deemed less of a problem in producing robust results than risking inclusion of an unhealthy individual.

Insect Culture

Navel orangeworms were obtained from a laboratory colony at the USDA-ARS, San Joaquin Valley Agricultural Sciences Center in Parlier, CA. This colony was established in 2010 from eggs collected in an almond orchard in Fresno County in September 2010 and refreshed by individuals from the same site in September 2011.
Larvae were maintained on a wheat bran-based diet (Finney and Brinkman 1967). Late-instar larvae were segregated by sex based on visibility of the testes through the dorsal integument of males and were shipped weekly via overnight express from Parlier to Ames, IA, and allowed to pupate. Pupae were checked daily for adult eclosion, and adults were moved to sex-specific and date-specific holding cages until ready for tethering or mating. Containers consisted of a 1-quart mason jar sealed with a wire mesh lid. A 2.25-cm strip of filter paper folded accordion-style was affixed inside the jar, to provide a perch for the moths. Water was freely available via soaked cotton inside an inverted jelly cup on top of the wire mesh. At all life stages, insects were held at 26°C with a photoperiod of 14:10 (L:D).

**Flight Performance**

To test the effect of timing of flight relative to mating, moths were either tested on the flight mills the night prior to mating (designated as Fly-Mate) or the night after mating (Mate-Fly) (Table 1). Moths were allotted one full night to fly and one full night to mate.

*Adult tethering and flight mills*

Methods were adapted from Dorhout et al. (2008) and Sappington and Burks (2014). Each moth was attached to a tether made from a ~ 5-cm-long 0.25-mm diameter wire affixed to a short sleeve of insulation tubing stripped from the wire. Before tethering, moths were weighed individually in jelly cups. A small piece of kneaded putty eraser was attached to the ultimate 1 mm of the wire, then flattened on one side. Moths
were prepared for tethering by brushing the scales from the dorsal surface of the abdomen directly posterior to the metathorax. Anesthetization was usually unnecessary, but restive moths were cooled briefly (< 3 min) in a -20°C freezer before handling. While holding the moth, a tiny drop of Sobo fabric glue (Plaid, Atlanta, GA) was placed on the flattened side of the putty, which was lightly pressed to the cuticle to affix the tether.

Fifteen flight mills were housed in an environmental chamber held at 26°C with a photoperiod of 14:10 (L:D). Dusk and dawn were simulated by programmed 30-min ramping of 40W incandescent bulbs as described by Sappington and Burks (2014). The individual mills were housed in vinyl tents to minimize air movement. Each mill was attached to a Gateway 2000 personal computer running flight mill software as described by Beerwinkle et al. (1995). Moths were attached to the flight mill by slipping the tether over the point of the flight mill arm. The weight of the moth was counterbalanced by moving a clip on the opposite end of the flight arm. The flight mill arm was a triangle-shaped flat piece of aluminum (256 mm in length, 156 mm from tip to pivot, 15 mm in width at the base end). Moths flew in a horizontal plane traveling a distance of 1 m per revolution around the central pin of the flight mill. Rotations were registered by an infrared eye mounted on the post below the central pin. At the time of attachment to the flight mill, each moth was given a small piece of tissue paper for tarsal contact. Most moths readily grasped the paper and folded their wings, helping reduce premature flight before dusk.

Female moths could not be set up to mate on the night of eclosion; this meant that the moths that mated prior to flight were one day older than moths that flew prior to mating, and the effect of age on flight behavior cannot be discounted. Designing the
experiment to fly moths in the Mate-Fly and Fly-Mate treatment groups at the same age would have necessitated the females mating two days apart instead of one, which we deemed even less desirable. Sappington and Burks (2014) found a slight but statistically nonsignificant increase in flight performance of 2 day old unmated females over those that were 1 day old.

_Wing measurements_

Wings of moths tested on the flight mills were measured to test for association with flight performance. After natural death, the forewings and hind wings were removed and mounted onto card stock. Lengths and surface areas of both forewings and both hind wings were determined using ImageTool (http://compdent.uthscsa.edu/imagetool.asp) and the largest values for either of the paired forewings and the largest values for either of the paired hindwings were used for analysis.

**Effect of Flight on Reproduction**

The effect of flight on reproduction in the navel orangeworm was investigated by conducting two experiments, a “voluntary flight” study and a “forced flight” study. Moths in the voluntary flight study were attached to a flight mill throughout a full night, and were free to engage in flight activity or rest. A computer recorded the moths’ and flight duration, speed, distance, number of separate flights and time of night of each separate flight. To test the effect of timing of flight relative to mating, moths either flew the night prior to mating (Fly-Mate) or the night after mating (Mate-Fly) (Table 1).
### Table 1: Experimental Treatments

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Treatment Name</th>
<th>Day 1</th>
<th>Day 2</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Voluntary Flight</td>
<td>M Mate</td>
<td>Oviposit</td>
<td></td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>Mate-Tether</td>
<td>Mate</td>
<td>Tether</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>Tether-Mate</td>
<td>Tether</td>
<td>Mate</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>Mate-Fly</td>
<td>Mate</td>
<td>Fly</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>Fly-Mate</td>
<td>Fly</td>
<td>Mate</td>
<td>63</td>
</tr>
<tr>
<td>Forced Flight</td>
<td>T0M Tether+Mate</td>
<td>Oviposit</td>
<td></td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>MF3 Mate</td>
<td>Fly 3 min+Oviposit</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MF30 Mate</td>
<td>Fly 30 min+Oviposit</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MF60 Mate</td>
<td>Fly 60 min+Oviposit</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MF120 Mate</td>
<td>Fly 120 min+Oviposit</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F3M Fly 3 min+ Mate</td>
<td>Oviposit</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F30M Fly 30 min+Mate</td>
<td>Oviposit</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F60M Fly 60 min+Mate</td>
<td>Oviposit</td>
<td>38</td>
<td></td>
</tr>
</tbody>
</table>

On the day of emergence (Day 1), moths were prepared to fly, be tethered, or mate depending on the group to which they were assigned. Beginning on day 2 or 3 following eclosion, depending on treatment, all successfully mated moths were allowed to oviposit until natural death.
Moths were allotted one full night to fly and one full night to mate. To control for the effect of handling, two tethered controls were included a group that was tethered but not flown (Tether-Mate) the night prior to mating and a group was mated prior to being tethered but not flown (Mate-Tether). The tethered control groups were held overnight in the flight mill room and each individual was contained in a 50-mL test tube with a small square of tissue paper as resting substrate. We used the test tube to prevent unnecessary movement while allowing them to experience similar conditions to the flighted groups. A minimally-handled control group that was neither tethered nor flown (M) was also included (Table 1). On the day of eclosion, female moths were assigned to one of the five flight or control groups. Following completion of the prescribed sequence of mating, tethering/flight (Table 1), females were allowed to oviposit until natural death.

A forced flight study was conducted to investigate whether any differences in fecundity associated with level of flight activity are attributable to the individual moths’ propensity for flight, perhaps as part of a physiological syndrome, or to the act of flying itself. Moths were allotted one night to mate the first night after eclosion. One group was forced to fly during the following day for discrete amounts of time: 3, 30, 60, or 120 minutes (treatments MF3-MF120) on the day after mating. A second group was forced to fly 3, 30, or 60 minutes on the day immediately following emergence, prior to mating the following night (treatments F3M-F60M). A F120M treatment was attempted, but the rate of successful mating was too low to allow testing in the time frame available. An additional tethered but unflown group, T0M (Table 1) was included to compare to the F3M-F60M series of forced-flight treatments. The Mate-Tether control group from the
voluntary flight experiment served as the equivalent of an MT0 control, and the same data from the former were used for comparison to the MF3-MF120 series of forced-flight treatments.

*Mating and oviposition trials*

The morning following flight, the moths were prepared for mating or oviposition after being released from the tether by snipping it just above the attachment point. Mating jars were prepared in the same way as the holding containers. Each female was prepared for mating by placing it in an individually marked 1-quart mason jar sealed with a wire mesh led with a 2.25-cm-wide strip of filter paper folded accordion-style attached inside. Each female was presented with at least 1 virgin male, or 2 males when possible, and allowed one night to mate in an environmental chamber held at 26°C with a photoperiod of 14:10 (L:D). Twilight was simulated by a Sunbeam (Boca Raton, FL) baby light 30 minutes prior to full light, which greatly facilitated mating activity. Sunset was not simulated, because navel orangeworms have a greater propensity to mate at dawn rather than dusk (Burks et al. 2011). Water was freely available as described above. Within the first 30 minutes of full light, moths were checked for mating. Those observed *in copula* were prepared for flight or oviposition, while those moths not mating were discarded.

The same type of jar was used to set up moths for oviposition. A #2 white bleached coffee filter (Hy-vee brand, Des Moines, IA) was provided as an oviposition substrate for each moth. Each coffee filter was placed over the lip of the jar to contain the moth. Filters were collected and replaced daily and the eggs counted and assessed for fertility. Fertile eggs became bright orange within 24 hours of oviposition (Parra-
Pedrazzoli and Leal 2006), so freshly-laid eggs were allowed to mature overnight in the environmental chamber.

Forced flight

Moths were tethered as described above. Tethered moths were weighed before and after testing to determine weight loss during the flight. Tethers were attached to wire shelving with masking tape in such a manner that each moth hung free in a horizontal orientation. Moths were continually agitated by air flow from small oscillating fans positioned in front and underneath, both on the lowest setting. Moths were observed during the entire duration of the flight and prevented from taking rests by gently touching the tarsi or tapping on the tether whenever they stopped flying. Moths that could not be induced to resume flight within 10 seconds were discarded. After weighing at the end of the flight, the tether was snipped close to the putty with scissors, and the moths were prepared for mating or oviposition.

To determine whether the weight lost during forced flight was due to flight effort or dehydration, a weight loss control experiment was conducted. Moths were tethered and set up in front of the fans as described above, and weighed prior to being set up near the fans. Wing movement was prevented by stretching a piece of lightweight gauze across the insects. The immobilized insects were held in this arrangement for the same predetermined periods of time as above. After this period of time, the moths were weighed again, assessed for mating (if part of that treatment) and discarded.
Data Analysis

To be eligible for inclusion in all analyses, moths must have lived for at least three days following flight or post-flight mating, and have oviposited at least one fertile egg during that time. In addition, to be included in the voluntary flight analyses, moths must have made at least one continuous flight lasting at least three minutes, and must have survived the flight. For the forced flight analysis, if an individual ceased flying and could not be coaxed to fly within 10 continuous seconds by gently tapping the tarsi or tether, that individual was excluded from analysis. This criterion is conservative, but few were discarded for this reason, and we were not constrained by availability of moths regardless.

The flight mill program measured time of night flown, duration of flight, distance flown, and number of flights by compiling the number of revolutions made by the flight mill arm in 1-min intervals. A single flight was considered terminated if the flight mill arm remained motionless for 1 minute.

Weight data for the forced-flight experiment were not included if an individual moth was altered in some way, for example through loss of a leg, or if the tether had to be reattached.

All statistical analyses were conducted using the R (http://www.R-project.org) statistical package. For the voluntary flight experiment, Welch’s t-test was used to compare most response variables, and a modified Kolomogorov-Smirnov test was used to compare density curves. The forced flight experiment was analyzed using ANOVA followed by Tukey’s Honest Significant Differences (HSD) test to separate treatment means. Fecundity and longevity data from all experiments were analyzed by ANOVA,
with Tukey’s HSD test and Welch’s t-test used for individual comparisons. To compare start and end times on the flight mill, the nonparametric Wilcoxon Rank-Sum test was used because we assumed ordinality, i.e., that some individuals started flying before others (Lowry n.d.). A Poisson Generalized Linear Regression was used to analyze the effect of treatment over time on fecundity. Linear regression and Pearson’s product-moment correlation were used to analyze longevity, fecundity, and flight data. Regressions were used when a causal relationship was suspected.

**Results**

**Flight Performance**

*Effect of mating on flight behavior*

The timing of flight relative to mating affected both distance and duration of flight. Over the entire night, moths that had mated prior to flying performed significantly better than those that had not mated in measures of both total flight distance (Figure 1a) and duration (Figure 1b). There was no difference in mean (± SE) flight speed between treatments Mate-Fly (37.7 ±2.1 m/min) and Fly-Mate (39.1±2.1 m/min) (Welch’s t-test, p= 0.75), nor was there a difference in the total number of flights taken during the night between Mate-Fly (12.5 ±1.3) and Fly-Mate (12.5 ±1.3) (Welch’s t-test, p= 0.99). The general distribution pattern of flight distance was weighted toward shorter flights for both mated and unmated moths, but there was a more even distribution of individuals flying < 30 km among moths that mated prior to flight (Figure 2a). Mated moths showed an overall propensity to make longer duration flights with a distribution of individuals weighted toward the right compared to those moths that flew prior to mating (Figure 2b).
Timing of flight relative to mating also affected the longest single flight of the night in a similar fashion. Moths that mated prior to flight flew farther (Figure 3a) and for a longer time during the longest uninterrupted flight (Figure 3b) than those that flew prior to mating. Speed of flight was not affected by mating (Fly-Mate = 45.6±2.4 m/min, Mate-Fly = 41.7±2.4 m/min, Welch’s t-test, t = 0.31, p = 0.76). While distribution of individual flight distances and durations differed significantly between mated and unmated moths (Figure 4), the density curves of flight durations were weighted to the left for both. Mated moths also started their longest single flight approximately 200 minutes earlier in the night than those that had not mated (Figure 5), but the time of the evening when the longest flight ended did not differ significantly between these groups.

**Figure 1**: Box-and-whisker plot demonstrating the effect of flight timing relative to mating on total flight distance (a) and total flight duration (b). Solid line within the box represents the median, the dot inside the box represents the mean. The whisker lines indicate the 90th percentile, and dots outside the whisker lines indicate individual values outside the 90th percentile. (a) Means: Fly-Mate: 7.0 km; Mate-Fly 14.9 km, t = -3.48, df = 94.67, p < 0.001, (b) Means: Fly-Mate, 165 min; Mate-Fly, 365 min; t = -5.26, df = 96.61, p < 0.001
Figure 2: Density curves comparing the total distance (a) and total duration (b) of flight between individuals of treatments Fly-Mate (FM) and Mate-Fly (MF). Density represents the proportion of individuals that flew at each value. Curves were compared to one another using a modified Kolmogorov-Smirnov goodness-of-fit test (Distance: $D=0.99, p<0.001$, Duration: $D=0.9, p<0.001$).

Moth size was investigated for a role in flight performance (Table 2). No measures of flight performance were significantly correlated with any measures of wing dimensions or weight, except that the hindwing area was negatively correlated with the duration of the longest single flight.

Forced flight

When moths were forced to fly for predetermined periods of time, weight loss was positively related to duration of flight (Figure 6). However, the effect was not very pronounced ($r^2=0.06$) and may not be an accurate measure of flight effort. There was an effect of flight duration on weight loss (Figure 7), but this was driven by the difference between two individual treatments (F3M and MF60, Tukey’s HSD $p=0.007$) and is not necessarily related to mating. Only treatment MF60 demonstrated weight loss that was
statistically different from 0 (p = 0.002), and when an individual that had lost substantially more weight than any other moth was removed from analysis, the effect of treatment on weight loss was no longer significant (ANOVA, $F = 1.54$, d.f.(group) = 7, d.f.(residuals) = 267, p = 0.15). When compared to moths that had been tethered and held stationary for discrete units of time, moths that flew lost significantly more weight than

**Figure 3**: Box-and-whisker plot demonstrating the effect of treatment on distance of the longest single flight (a) and duration of the longest single flight (b). Solid line within the box represents the median, the dot inside the box represents the mean. The whisker lines indicate the 90th percentile, and dots outside the whisker lines indicate individual values outside the 90th percentile. Means are reported within the figure, as well as a Welch’s t-test. (a) Means: Fly-Mate, 5 km; Mate-Fly, 11km; $t = -3.12$, df = 93.32, p = 0.002, (b) Fly-Mate, 96min; Mate-Fly, 251 min; $t = -4.40$, df = 91.37, p < 0.001
Figure 4: Density curves comparing the distance (a) and duration (b) of the longest single flight between individuals of treatments Fly-Mate (FM) and Mate-Fly (MF). Density represents the proportion of individuals that flew at each value. Curves were compared to one another using a modified Kolmogorov-Smirnov goodness-of-fit test (Distance: D= 1, p<0.001, Duration: D=0.98, p<0.001).
Figure 5: Start and end times of the longest single flight of the evening. Numerals indicate the number of minutes after beginning of dusk. Data analyzed represent minutes after dusk of the flight activity event. Solid line within the box represents the median, the dot inside the box represents the mean. The whisker lines indicate the 90th percentile, and dots outside the whisker lines indicate individual values outside the 90th percentile.

***, p < 0.001. Data analyzed using Wilcoxon Rank-Sum test (Time Start: W=1974.5, p <0.001, Time End: W= 1438, p =0.36, n =102).
**Table 2:** Pearson’s product-moment correlation coefficients between dimensions of size and flight performance.

<table>
<thead>
<tr>
<th>Dimension</th>
<th>Total Duration of Flight</th>
<th>Total Distance Flown</th>
<th>Average Speed of Flight</th>
<th>Longest Flight Duration</th>
<th>Longest Flight Distance</th>
<th>Longest Flight Speed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forewing Area</td>
<td>-0.06 n.s.</td>
<td>0.05 n.s.</td>
<td>0.15 n.s.</td>
<td>-0.05 n.s.</td>
<td>0.04 n.s.</td>
<td>0.15 n.s.</td>
</tr>
<tr>
<td>Hindwing Area</td>
<td>-0.15 n.s.</td>
<td>-0.16 n.s.</td>
<td>-0.03 n.s.</td>
<td>-0.24 *</td>
<td>-0.19 n.s.</td>
<td>0.02 n.s.</td>
</tr>
<tr>
<td>Forewing Length</td>
<td>-0.67 n.s.</td>
<td>0.08 n.s.</td>
<td>0.15 n.s.</td>
<td>0.02 n.s.</td>
<td>0.07 n.s.</td>
<td>0.13 n.s.</td>
</tr>
<tr>
<td>Weight</td>
<td>-0.12 n.s.</td>
<td>0.03 n.s.</td>
<td>0.08 n.s.</td>
<td>-0.10 n.s.</td>
<td>0.03 n.s.</td>
<td>0.07 n.s.</td>
</tr>
</tbody>
</table>

n = 102. n.s., p > 0.05; *, p < 0.05

moths that did not fly (Table 3) except for the moths that flew for only 3 minutes (p > 0.1).

Analyses of temporal patterns of flight were examined to provide insight into behavior of mated versus unmated moths. Total flight activity began earlier in the evening for mated moths, but this difference was not significant. These moths must have taken individual flights of longer duration, because direct comparison of total flight duration reveals that mated moths flew for a longer duration than unmated moths (Figure 1b), but there was no difference in the total number of flights. Mated moths also flew longer and farther during their longest continuous flight, which may indicate a greater tendency for a purposeful long-distance dispersal or migratory flight. A propensity for making a greater long-duration flight, which does not occur until after mating, and which begins early in the night compared to flights by unmated moths, is consistent with a programmed long-distance dispersal or migratory flight as a life history strategy for this species.
Fecundity

*Effect of treatment on fecundity and longevity*

There was no difference in egg production or production of fertile eggs between moths that mated prior to flying on the flight mills or moths that flew prior to mating. Moths that were tethered produced fewer total eggs (Figure 8a) and fewer fertile eggs (Figure 8b) than minimally handled control moths.

Temporal patterns of egg-laying revealed that most oviposition occurs early in adult life.

A Poisson regression model was used to estimate the effect of treatment over

**Table 3:** Mean differences for selected treatment comparisons in weight lost by female navel orangeworms during forced flight (F) or tethered but unflown controls (T) for indicated times. Tukey’s HSD was used for individual comparisons. ANOVA df(Groups): 14, df(Residuals): 326, F = 19.937, p < 0.001

<table>
<thead>
<tr>
<th>Treatment Comparison</th>
<th>Time Tethered/Flown (min)</th>
<th>Difference in Weight Loss (mg)</th>
<th>Significance (p) (HSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M3T-M3F</td>
<td>3</td>
<td>7.19x10^-1</td>
<td>0.99</td>
</tr>
<tr>
<td>T3M-F3M</td>
<td>3</td>
<td>1.47x10^-4</td>
<td>1.00</td>
</tr>
<tr>
<td>MT30-MF30</td>
<td>30</td>
<td>9.93x10^-1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>T30M-F30M</td>
<td>30</td>
<td>1.04</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MT60-MF60</td>
<td>60</td>
<td>2.08</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>T60M-F60M</td>
<td>60</td>
<td>1.20</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MT120-MF120</td>
<td>120</td>
<td>1.51</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
Figure 6: Regression of total weight lost on duration of forced flight. Data from all forced flight groups are pooled for analysis. One outlier is not shown for illustrative purposes but was included in analysis. $y = 0.009x + 0.79, r^2 = 0.06, p = 0.005$
Figure 7: Weight lost by female moths forced to fly for predetermined amounts of time. For treatment abbreviations, see Table 1. A “zero” value for treatment group F0M (control group tethered and immediately set up to mate) was included for illustrative purposes. One outlier is not shown but was included in analysis. Treatments with the same letter are not statistically different. ANOVA df(Group): 6, df(Residuals): 101, F = 3.1, p = 0.007
Figure 8: Fecundity of moths by treatment group. Fecundity for the purposes of this experiment is indicated by the total number of eggs produced over a female’s adult lifespan and the number of those eggs found to be fertile. (a) ANOVA df(Group): 4, df(Residuals): 317, F=30.357, p<0.001, (b) ANOVA df(Group): 4, df(Residuals): 317, F=31.062, p<0.001

time on total and fertile eggs produced. Moths laid the greatest number of eggs on the first day observed, and produced fewer eggs each subsequent day until natural death (Table 4). The pattern of egg-laying was not significantly affected by flight behavior, beyond the limitations of the experiment (i.e. we could not measure fecundity during the night when moths were flight-tested). Moths that were mated prior to being tethered produced fewer eggs than moths from any other treatment consistently through time (Figure 9). All treatment groups were analyzed using a logarithmic regression model, which described the decline in egg laying over time fairly well although there was a considerable amount of variation within groups ($r^2>0.2$) for all treatment groups except Mate-Tether ($r^2<0.1$).
Treatment had an effect on longevity (ANOVA, $F = 12.124$, $p < 0.001$), but the pattern observed across treatments was hard to interpret because one would expect the Mate-Tether control to live at least as long as the Mate-Fly females (Figure 10). Production of total eggs and total fertile eggs were positively related to longevity (Figure 11). Longevity was not correlated with total flight distance ($r = -0.049$, $p = 0.62$), distance of the longest single flight ($r = -0.032$, $p = 0.75$), total flight duration ($r = -0.030$, $p = 0.77$) or duration of the longest single flight ($r = -0.022$, $p = 0.82$).

*Effect of flight performance on fecundity*

Data from both Fly-Mate and Mate-Fly were pooled for analysis, because there was no difference in fecundity between those two treatment groups. Flight did not affect reproduction in the navel orangeworm. Total flight distance did not affect the total number of eggs produced (Figure 12a) nor the total number of fertile eggs (Figure 12b). Reproduction was similarly unaffected by total duration of all flights (Figure 13). There was no difference among forced flight treatments on the total number of eggs (Figure 14a) or total number of fertile eggs (Figure 14b).
**Figure 9**: Logarithmic regressions describing Total (a) and Fertile (b) egg production over the course of the first week of egg-laying for the indicated treatments.
Table 4: Average number (± SE) of eggs produced per day by navel orangeworm females

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>63</td>
<td>109.4±9.7</td>
<td>45.5±4.8</td>
<td>44.8±4.5</td>
<td>32.1±2.7</td>
<td>44.3±7</td>
<td>25.7±2.9</td>
<td>16.7±2</td>
</tr>
<tr>
<td>Mate-Tether</td>
<td>84</td>
<td>43.6±5.5</td>
<td>33.8±4.6</td>
<td>25.5±3.6</td>
<td>18.8±2.7</td>
<td>23.9±3.4</td>
<td>13.8±2</td>
<td>13.9±2</td>
</tr>
<tr>
<td>Tether-Mate</td>
<td>60</td>
<td>95.5±13.4</td>
<td>42.3±5.9</td>
<td>30.9±4.3</td>
<td>25.3±3.5</td>
<td>21.4±3</td>
<td>17.6±2.5</td>
<td>11.9±1.7</td>
</tr>
<tr>
<td>Mate-Fly</td>
<td>52</td>
<td>82.8±11.7</td>
<td>36.6±5.2</td>
<td>31.5±4.5</td>
<td>20±2.8</td>
<td>19±2.7</td>
<td>15.5±2.2</td>
<td>13.9±2</td>
</tr>
<tr>
<td>Fly-Mate</td>
<td>63</td>
<td>88±12.6</td>
<td>38.5±5.5</td>
<td>39.5±5.6</td>
<td>21.1±3</td>
<td>22.1±3.2</td>
<td>19.2±2.7</td>
<td>16.6±2.4</td>
</tr>
</tbody>
</table>

Only the first week is included due to low numbers of eggs produced after the first week. Adults lived about 2 weeks on average.

Table 5: Average number (± SE) of fertile eggs produced per day by navel orangeworm females

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>63</td>
<td>98.4±9.6</td>
<td>42.9±4.6</td>
<td>40.7±4.3</td>
<td>27.2±2.6</td>
<td>38.4±6.4</td>
<td>20.8±2.3</td>
<td>13.4±1.7</td>
</tr>
<tr>
<td>Mate-Tether</td>
<td>84</td>
<td>36.4±4.6</td>
<td>26.0±3.7</td>
<td>21.9±3.1</td>
<td>14.5±2.0</td>
<td>20.7±2.9</td>
<td>12.3±1.7</td>
<td>10.9±1.5</td>
</tr>
<tr>
<td>Tether-Mate</td>
<td>60</td>
<td>81.9±11.5</td>
<td>37±5.2</td>
<td>26±3.6</td>
<td>21.2±3</td>
<td>19.9±2.8</td>
<td>14.9±2.1</td>
<td>10.2±1.4</td>
</tr>
<tr>
<td>Mate-Fly</td>
<td>52</td>
<td>73.5±10.4</td>
<td>30.6±4.3</td>
<td>26.8±3.8</td>
<td>23.3±3.3</td>
<td>15.6±2.2</td>
<td>13.7±1.9</td>
<td>10.8±1.5</td>
</tr>
<tr>
<td>Fly-Mate</td>
<td>63</td>
<td>79.9±11.4</td>
<td>33.4±4.8</td>
<td>34.4±4.9</td>
<td>18.9±2.7</td>
<td>19.6±2.8</td>
<td>15.8±2.3</td>
<td>14±2</td>
</tr>
</tbody>
</table>

Only the first week is included due to low numbers of eggs produced after the first week. Adults lived about 2 weeks on average.
Figure 10 Effect of handling and flight on longevity, here indicated by the number of days a female moth survived post-eclosion until natural death. Treatments with the same letter are not statistically different from one another. ANOVA df(Group): 4, df(Residuals): 317, F = 12.124, p < 0.001
Figure 1: Regression of total lifetime eggs produced (a) and total fertile eggs produced (b) by female moths based on adult lifespan. Data from all treatment and control groups in the “voluntary flight” experiment were pooled together for this analysis. (a) $y = 17.16x + 18.36$, $r^2 = 0.37$, $p<0.001$, (b) $y = 14.17x + 14.7$, $r^2 = 0.31$, $p < 0.001$

Figure 2: Regression of total eggs produced on total flight distance (a) and total flight duration (b). (a) $y = 5.5 \times 10^{-4}x + 2.4 \times 10^2$, $r^2 = 0.007$, $p = 0.57$, (b) $y = 5.9 \times 10^{-4}x + 2.1 \times 10^2$, $r^2 = 0.006$, $p = 0.51$
Figure 13: Regression of total number of eggs produced on distance (a) and duration (b) of the longest single flight. (a) \( y = 2.2 \times 10^{-3} x + 2.1 \times 10^2 \), \( r^2 = 0.01 \), \( p = 0.97 \), (b) \( y = 5.9 \times 10^{-4} x + 2.1 \times 10^2 \), \( r^2 = 0.006 \), \( p = 0.51 \).

Figure 14: Fecundity of female moths forced to fly for predetermined discrete units of time. See Table 1 for treatment abbreviations. (a) ANOVA df(Group): 7, df(Residuals): 267, \( F = 1.54 \), \( p = 0.15 \), (b) ANOVA df(Group): 7 df(Residuals): 246, \( F = 1.77 \), \( p = 0.10 \).
Discussion

In insects displaying a reproductive-flight syndrome, migratory flight occurs during the preoviposition period and its termination triggers oogenesis or increased reproductive capacity. Our results show that navel orangeworm behavior is consistent with a coordinated association between flight and reproduction, but not in this classical sense. Increased flight activity neither increased nor reduced lifetime reproductive output.

In navel orangeworm females, mating increased total flight activity, and increased the distance and duration flown during the longest single flight of the night. Unmated females took the same number of flights as mated females, but the flights taken by unmated females tended to be shorter in duration and distance. Unmated females may have different motivations for flight.

Although one cannot directly translate flight mill performance to movement in the field, our data suggest that mated females are capable of traveling across spans of desert between orchards. Our observations of flight performance on flight mills also suggest that prevalent assumptions about adult navel orangeworm dispersal distances and risk to uninfested orchards due to distance from infested hosts may be underestimated. Further research including mark-recapture experiments will help accurately characterize adult movement in nature.

The impact of mating status is species-dependent. Studies of some other moths, such as the beet webworm (Cheng et al. 2012), have not found any effect of mating status on flight performance. Some Lepidoptera, such as *Helicoverpa armigera* (Armes and Cooter 1991), exhibit a decline in flight performance after mating. Studies of other
orchard pests have yielded contrasting outcomes (Schumacher et al. 1997, Hughes and Dorn 2002). A mechanism for an increase in flight performance of mated females compared to unmated females may be related to a transfer of nutrients during mating via the spermatophore. Females of some lepidopteran species are able to utilize nutrients from the spermatophore preferentially for reproductive purposes (Wedell and Karlsson 2003, Ducatez et al. 2012, Levin et al. 2015), potentially freeing a greater amount of larval resources by the female for flight and longevity. Increased resource availability may allow for greater survival of those making the longest flights.

Mated females started their longest single flight earlier in the evening than unmated females (Figure 5). This is consistent with mated and unmated females having different motivations for flight. It may be that mated moths cease flight activity following a long-distance dispersal flight. However, most oviposition in the navel orangeworm occurs in the first few hours of darkness (Andrews et al. 1980), so the earlier initiation of flight activity by mated females could represent ranging behavior in search of an oviposition site. In contrast, most mating occurs closer to dawn (Landolt and Curtis 1982), which is consistent with the later initiation of flight behavior observed for females that had not yet mated. This likely represents ranging behavior as well, perhaps in search of suitable habitat or a perch for calling.

The navel orangeworm displays a "front loaded" reproductive strategy, where most eggs are laid in the first few days after initiation of oviposition (Figure 9; Andrews et al. 1980, Burks and Higbee 2006). Females in all treatment groups consistently laid a greater proportion of their total eggs in the first days of oviposition with a steep decline on subsequent days. Thus, an increase in number of days lived should have little direct
effect on lifetime fecundity. However, longevity could be seen as an indicator of overall health of the individual, and healthier moths may produce more eggs. This would be consistent with the relatively high longevity of moths in the minimally-handled control group. Longevity was not correlated with any aspect of flight performance, indicating no flight-related trade-off between energy expenditure and longevity.

**Management**

Understanding the relationship between flight behavior and mating can assist growers in managing navel orangeworm in their orchards. Sampling in the field is essential for pest management, since managers can assess pest presence or absence and levels of pest presence. This allows for prediction of outbreaks or assessment of current pest management strategies. Traps are a common way to obtain pest samples and monitor pest presence, but captures tend to be highly sensitive to environmental conditions (Dent 2000). Pest behavior in the field may affect how a manager should interpret trap data. For example, traps do not typically provide information about the ultimate source of a captured insect. An understanding of the insect's current suite of activities that brought it into the vicinity of the trap is therefore useful for interpreting trap data and using it to make effective management decisions.

A mark-recapture study by Andrews et al. (1980) found that marked navel orangeworm females laid eggs within a 273 m radius from the site of eclosion. Burks and Higbee (2006) and Higbee and Siegel (2009) present evidence from mark-recapture and colonization studies that suggest most females oviposit near the source while a smaller proportion disperse longer distances. Higbee and Siegel (2009) conclude that most
damage from an established population will occur within a 5-km radius. Our results suggest a much wider radius is possible for oviposition if the mated females fly in a straight line, as would be the case if the long flights observed on the flight mills represent migratory flight. However, the realized displacement of insects in the field may be much less than the individual’s capacity for flight. This would be the case if the long flights we observed on the flight mills represent ranging behavior, because meandering movement associated with search behavior results in less displacement than unidirectional movement (Miller et al. 2015). Although we observed a greater tendency for long-distance flight in mated females, there was still a substantial number of females that did not fly far. The relationship between long-distance dispersal and mating in the navel orangeworm remains difficult to fully characterize, but field and laboratory evidence so far are consistent with Higbee and Siegel's (2009) conclusion that most navel orangeworm oviposition occurs within 5 km of the natal site.

There is a strong ongoing interest in using mating disruption to manage navel orangeworm in orchards, and it is being applied already by many farmers using commercially-available pheromone puffers. Individual pheromone components can disrupt mating in orchards infested with navel orangeworm (Burks et al. 2015). Mating is significantly reduced in the presence of mating disruption, and damage is reduced substantially as well, although perhaps not as dramatically. Trials have demonstrated a significant reduction in damage to almonds (12 to 37%) related to use of mating disruption, which is economically important albeit not always statistically significant because of environmental variability (Curtis et al. 1985, Higbee and Burks 2008, Burks et al. 2016). The behaviors seen in the current study suggest some damage in orchards could
be committed by offspring of immigrant females. However, field experiments suggest most navel orangeworms oviposit within a three-mile radius of their natal site (Higbee and Siegel 2009). If some mated females are ovipositing over a larger area, it would support the view that an area-wide application of mating disruption may enhance efficacy by reducing the number of already mated females arriving in an otherwise protected orchard (Cardé and Minks 1995). Nevertheless, the most important practical consequence of dispersal behavior in this pest is probably in the context of colonizing uninfested orchards.

Navel orangeworm is also considered a candidate for management by the sterile male technique. This strategy would involve sterilizing males with gamma radiation and releasing them en masse into an infested field to mate with wild females, which in turn would oviposit infertile eggs. Light et al. (2016) found that irradiated males successfully mated with females in the laboratory, and that the females produced sterile eggs. Our study suggests that females are capable of flying relatively long distances after mating in or near the natal habitat, and oviposit about 100 eggs within a day thereafter. Thus, it would be best to release sterile males immediately prior to female eclosion. For the same reasons described for pheromone-mediated mating disruption, the use of the sterile male technique will be most effective when applied over large areas, rather than at the level of an orchard or small cluster of orchards. The current study can provide information for estimating a minimum radius for an effective area-wide program of sterile male release.

The difference between long-distance ranging and true migratory behavior is not always easy to distinguish experimentally. It is possible that the long-distance dispersal observed on flight mills represents ranging behavior that is triggered by mating, and that
females stay in the natal orchard if the crop is at an appropriate seasonal stage to serve as a larval host. If the navel orangeworm is a true migrant, ranging for a suitable host would follow the cessation of migratory flight.

References


CHAPTER 4
GENERAL CONCLUSIONS

Implications for Management

Understanding the relationship between flight behavior and mating can assist growers in managing navel orangeworm in their orchards. Sampling in the field is essential for pest management, since managers can assess pest presence or absence. This allows for prediction of outbreaks or assessment of current pest management strategies. Traps are a common way to obtain pest samples and monitor pest presence, but captures tend to be highly sensitive to environmental conditions (Dent 2000). Pest behavior in the field may affect how a manager should interpret trap data. For example, traps do not typically provide information about the ultimate source of a captured insect. An understanding of the insect's current suite of activities that brought it into the vicinity of the trap is therefore useful for interpreting trap data and using it to make effective management decisions.

This study has shown that mated moths fly for greater distances and durations than unmated moths, which could have implications for interpreting trap data. If female navel orangeworms are programmed to take a long-distance dispersal flight after mating, as our data suggest is possible, at least some females do not oviposit near their natal site. Navel orangeworm has been monitored using traps since the 1970’s (Rice 1975, Rice et al. 1976), but it can be important to know whether the data from these traps include migrant females or only infestation by resident females. For example, it is possible that immigrants are coming from a population resistant to insecticides (Demkovitch et al. 2015). Egg traps cannot provide information about the origin of the females laying the
This study shows an increase in flight propensity of females following mating. The picture that is emerging is that female navel orangeworms tend to mate in the natal habitat. Adult females emerge from decaying nuts and typically mate within a day of emergence (Parra-Pedrazzoli and Leal 2006). Males are attracted to volatiles released by decaying fruits and nuts, which may indicate that males are awaiting the females' emergence (Beck et al. 2016). After mating, some females may take a long-distance dispersal flight and oviposit in a new habitat. However, it is also possible that the long flights we observed on the flight mills were appetitive flights, in which the female is searching for host cues for oviposition. In the field such a flight would be meandering in character, so the same distance flown will result in less net displacement compared to a straight-line dispersal flight.

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The difference between long-distance ranging and true migratory behavior is not always easy to distinguish experimentally. It is possible that the long-distance dispersal observed on flight mills represents ranging behavior that is triggered by mating, and females would stay in the natal orchard if at the host is at the appropriate seasonal stage
to serve as a larval host. If the navel orangeworm is a true migrant, ranging for a suitable host would follow the cessation of migratory flight.

The likelihood that an orchard will be infested by immigrant females is related to seasonality of its potential hosts, mainly fruit maturity which occurs at different times for the different hosts. For example, number and timing of generations is slightly different in figs than in almonds due to their differences in seasonality (Burks and Brandl 2004). Fruit maturation is not always synchronous among host species, because different hosts respond differently to climate factors such as heat or rainfall. Under certain conditions, the distance from a pistachio orchard may be a larger risk factor for almond orchards than under other conditions. Almonds are at greatest risk of navel orangeworm infestation in mid-summer, when the fruity hull protecting the almond kernel splits, exposing the kernel. There is greater variability in seasonal risk for pistachios. Pistachios are alternate bearing, meaning that a pistachio tree alternates between large and small yields (University of California Natural Resources 1999). Almonds are also alternate bearing, but the effect is not as strong and varies by region and variety (Dorfman et al. 1988).

In addition, pistachios are at risk of navel orangeworm infestation earlier in the season. Hull split exposes the kernel, allowing navel orangeworm and other pests direct access. Although hull split of pistachios typically occurs in late summer, the former can undergo an early hull split under dry conditions. Often this occurs approximately two weeks before the usual time of hull split although it can occur earlier in the summer. The risk of an early hull split is increased when irrigation schedules are miscalculated or disrupted (Doster and Michailides 1995).
Impact of flight on reproductive success

In insects displaying a reproductive-flight syndrome, migratory flight occurs during the preoviposition period and its termination triggers oogenesis or increased reproductive capacity. Our results show that navel orangeworm behavior is consistent with a coordinated association between flight and reproduction, but not in this classical sense. Increased flight activity neither increased nor reduced lifetime reproductive output. Female navel orangeworms have no decrease in reproductive capability associated with flight, but neither do they display an increase in reproduction following flight. In addition, there is not an association between increased flight and reproduction, neither increasing reproductive output nor reducing reproductive performance. From a management perspective, this indicates that female moths entering an orchard following long-distance flight have the same reproductive capacity as moths that have not flown.

The mechanism by which mating could trigger a purposeful dispersal flight in the navel orangeworm, if such behavior exists, is unknown. Activation of stretch receptors in the bursa copulatrix in lepidopterans is known to trigger specific behavioral responses, including cessation of calling and searching for oviposition sites (Sugawara 1978, Ryne et al. 2001, Al-Wathiqi et al. 2016). In addition, chemicals released by the male can affect female behavior. In the tobacco cutworm Spodoptera litura, exposure to compounds secreted by the males’ accessory glands caused females to become resistant to mating. In addition, exposure to these secretions even in the absence of sperm or mechanical stretching of the bursa copulatrix triggered ovarian maturation and oviposition (Yu et al. 2013). It is not unreasonable to hypothesize that exposure to male
secretions or a stretching of the *bursa* could trigger a cascade of effects culminating in migratory behaviors.

Further research might analyze how egg size or chemistry is affected by flight. Niitepold and Boggs (2015) found that in the butterfly *Speyeria mormonia*, increased flight duration was associated with a decline in egg dry mass, although relative amounts of carbohydrates, protein, and lipids remained unchanged. A decrease in egg dry mass could contribute to less robust offspring, even when the number of offspring remains unchanged.

*Effect of mating on flight*

Flight mill studies are useful for investigating the relationship between mating and flight behaviors. In the navel orangeworm, mating increases flight performance, and increases the distance and duration flown during the longest single flight of the evening. The duration flown by unmated moths was consistent with Sappington and Burks (2014), who investigated flight activity in unmated navel orangeworms. Unmated females took the same number of flights as mated females, but the flights taken by unmated females tended to be shorter in duration and distance. Unmated females may have different motivations for flight. They may be searching for food, mates, or attempting to escape predators such as bats, which would be consistent with the shorter flights observed in the current study.

For estimating adult movement in the field, duration may be a more relevant measure of flight than distance flown. This is because insect movement is affected by weather and wind patterns (Dorhout et al. 2008), and distance measured on the flight mill
may either over- or underestimate displacement in the field. Mated females fly for a longer duration than unmated females, but due to the limitations of flight mill work, it is difficult to directly correlate to movement in the fields. The current data suggest that mated females are capable of traveling across spans of desert between orchards, and current estimations of adult displacement and risk due to distance from alternate hosts are underestimated. In addition, actograph studies would provide more information as to whether the increase in flight activity is unidirectional, as would be expected in migratory flight, or erratic, which would be more consistent with ranging behavior.

The navel orangeworm’s native range includes southern California and northern Mexico (Wade 1961), an environment characterized by patches of vegetation separated by desert. Migration is advantageous in an environment where habitat is fragmented (Heino and Hanski 2001), so the evolution of long-distance dispersal capacity in this species is not surprising. Since reproduction and flight are both energetically-expensive processes, delaying flight until after mating may allow the navel orangeworm to avoid some of the drawbacks of flight. Mated females could use nuptial gifts to fuel ovarian development (Boggs 1990), as opposed to cannibalizing resources from wing musculature. In the migratory two-spotted cricket *Gryllus bimaculatus*, ovarian maturation is associated with a decrease in flight activity. This is due to wing muscle histolysis and utilization of the nutrients for reproduction (Lorenz 2007).

Further research would investigate the effect of flight and larval nutrition on male fitness. Flight activity is associated with decreased reproductive success in the pitcherplant mosquito *Wyeomyia smithii* (Benjamin and Bradshaw 1994), so increased flight activity could cause a decrease in reproductive success. However, given the lack of
reproductive trade-off in female navel orangeworms, there may not be a reproductive trade-off associated with flight for male navel orangeworms.

The navel orangeworm is an important pest of almonds and pistachios in California. The preceding study shows that it displays some flight behaviors consistent with migratory behavior, and the female takes a long-distance flight following successful mating. Migration is timed to certain life history events, such as age, timing of mating, or habitat. In the navel orangeworm, long-distance dispersal flight appears to be triggered by successful mating. In insects where long-distance dispersal is part of their normal repertoire of behavior, one would expect that an individual would not experience a penalty reproductively related to flight. Reproductive output in the navel orangeworm is not affected by flight effort or timing of flight relative to mating. Further research would investigate the effect of larval experience and habitat on migratory propensity in the navel orangeworm.
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