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Authors

Hoddle, Mark

Hoddle, Christina

Milosavljević, Ivan

et al.

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Assessment of Age, Gender, Mating Status, and Size on Single and Repeat Flight Capabilities of *Heilipus lauri* Boheman (Coleoptera: Curculionidae)

Mark S. Hoddle,^{1,2,4,*} Christina D. Hoddle,¹ Ivan Milosavljević,^{1,*}
Edith G. Estrada-Venegas,³ and Armando Equihua-Martínez³

¹Department of Entomology, University of California, Riverside, CA, 92521, USA, ²Center for Invasive Species Research, University of California, Riverside, CA, 92521, USA, ³Colegio de Postgraduados, Campus Montecillo, Km 36.5 Carretera México-Texcoco, Montecillo, Estado de México, C. P. 56230, México, and ⁴Corresponding author, e-mail: mark.hoddle@ucr.edu

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Abstract

Heilipus lauri Boheman (Coleoptera: Curculionidae) is a specialist pest of avocado fruit and is considered an incursion risk for U.S. avocado producers. At the time work reported here was undertaken the flight capabilities of *H. lauri* were unknown. Consequently, proactive studies were undertaken to quantify aspects of this pest's flight capabilities to inform potential future control efforts. Flight mill studies were conducted in a quarantine laboratory to measure the dispersal capacity of *H. lauri* with respect to gender, mating status, and size on the single and repeat flight capabilities of weevils. Gender, mating status, and size did not significantly affect measured flight parameters. Average total distances flown and flight velocity, and mean maximum flight bout distances and durations significantly declined as weevil age increased and when weevils engaged in repeat flights. Survivorship rates were significantly reduced as the number of successive flights undertaken increased. The distribution of total average flight distances flown and total cumulative flight distances flown was platykurtic. The implications of these findings are discussed in terms of developing incursion management plans.

Graphical Abstract



Photograph of the large avocado seed weevil, *Heilipus lauri*, for possible cover shot for journal.

Key words: avocado, flight mill, invasion, platykurtosis

Invasive insect pests are a perennial global threat to agricultural, urban, and wilderness areas (Dowell et al. 2016). Rates of introduction of nonnative organisms into new areas do not appear to be

abating and may even be accelerating as levels of international trade and movement of goods and humans continues to increase (Seebens et al. 2017). Some conduits for unintentional movement of insect

pests are well known and obvious, such as the trade in live plants, which inadvertently moves pests and hosts in tandem, potentially over long distances, into new areas where associated pests didn't previously exist (Liebhold et al. 2012). Identifying potential invasive species in advance of arrival into new areas vulnerable to incursion is an important step in mitigating establishment likelihood. Such steps are employed in pest risk analyses that aim to facilitate exchanges of goods, such as fresh produce for example, while establishing inspection and infrastructure requirements to reduce the risk of unwanted contaminants, such as insect pests, from being accidentally exported and introduced into receiving regions or countries (FAO 2016).

Avocados, *Persea americana* Miller (Lauraceae), are an iconic specialty crop grown in California, U.S.A. California grown avocados are worth ~US\$412 million per year, the crop is grown by approximately 3,000 growers who farm around 19,000 bearing ha and the 'Hass' variety accounts for >90% of fruit production (CAC 2020). Avocados are native to parts of México, and Central, and South America, where associated native insect biodiversity is high. In contrast, the biodiversity of the arthropod fauna associated with avocados in California is low, consisting primarily of about four invasive pest species of insects and mites that feed on leaves (Lara et al. 2017). Currently, California-grown avocados are free from specialist fruit feeding pests such as seed feeding weevils (e.g., the large avocado seed weevil, *Heilipus lauri* Boheman [Coleoptera: Curculionidae]) and moths (e.g., the avocado seed moth, *Stenomacra catenifer* Walsingham [Lepidoptera: Elachistidae]). Establishment of specialist fruit feeding pests in California would cause significant disruption and threaten the long term economic viability of this industry (Hoddle 2006).

Therefore, undertaking proactive measures to prepare in advance of an anticipated incursion of identifiable avocado pests may help to favorably position an at risk region, such as California, to manage incipient pest populations if they are detected early (Hoddle et al. 2018). With respect to avocado production in California, potential invasive insect pests associated with imports of fresh fruit have been identified and *H. lauri* is a recognized incursion threat associated with imports of fresh fruit into the U.S. from México (USDA-APHIS 2004, Petersen and Ordern 2006) and Colombia (USDA-APHIS 2016a). Importation of avocado fruit from México was banned in 1914 to prevent accidental introductions of specialist fruit feeding pests (USDA-APHIS 2004) and similar restrictions prevented fruit imports from Colombia (USDA-APHIS 2016b). Restrictions on avocado fruit imports into the U.S. were relaxed in 1997 and 2017 for México and Colombia, respectively (USDA-APHIS 2004, CAC 2017).

Heilipus lauri is a destructive avocado pest in its native (i.e., México [Nava-Diaz et al. 2013, Luna et al. 2017]) and introduced range (i.e., Colombia [Caicedo et al. 2010]). In regards to Colombia, *H. lauri* was probably introduced accidentally from México in imported avocado seeds used for root stocks (Castañeda-Vildózola et al. 2017). Female *H. lauri* lay eggs inside holes they drill into fruit using their rostrum and oviposit into an excavated cavity in the pulp. Larvae that hatch from eggs bore through the fruit pulp to the seed where they penetrate the seed to feed and pupate. Internal feeding activity damages fruit, making it unmarketable, and in some instances, heavy damage causes fruit to drop prematurely. Chemical control is difficult because larvae are protected within fruit (Caicedo et al. 2010). Around 60% of Hass fruit have been reported as being damaged by *H. lauri* in unmanaged orchards in Morelos, México (Medina 2005), and in Colombia, damage levels in commercial Hass orchards range ~4–8% (Caicedo et al. 2010). Ecological niche modeling suggests that *H. lauri* has potential for range expansion into

avocado producing regions in México where this pest is not present. One region identified as vulnerable to incursion is Michoacán (Luna et al. 2017), the major Hass producing region in México, from which fruit are exported to California (Nava-Diaz et al. 2013).

A critical first step in managing an incursion is the development of detection and monitoring plans, and in the case of an insect threat, dispersal capabilities by flight need consideration in this initial phase as it may help define survey and quarantine boundaries, deployment distances between traps, and potential rates of spread into new areas (Hoddle et al. 2021). Flight mills are a laboratory-based tool that are used to quantify the flight capabilities of insects (Minter et al. 2018, Naranjo 2019). Flight mill assays are useful for assessing the effects that variables such as gender, mating status, age, insect size, and satiation status, for example, have on the flight capabilities of test insect species (Hoddle et al. 2021, Lopez et al. 2014). Additionally, the underlying distribution of flight distance data (i.e., whether data are mesokurtic, platykurtic, or leptokurtic [Kot et al. 1996]) can be determined which may provide insight into potential rates and patterns of spread, which in turn, may be amenable for use in models investigating different dispersal scenarios and whether proposed management plans are technically feasible and economically justifiable (Barclay et al. 2011).

At the time this study was undertaken, the flight capabilities of *H. lauri* were unknown which is an impediment to the development of detection, monitoring, containment, quarantine, and eradication programs. Consequently, a proactive study in advance of potential incursions by *H. lauri* into uninfested areas, such as California, was undertaken to quantify the single and repeat flight capabilities of male and female weevils of different ages, sizes, and mating status. The results from flight mill studies with *H. lauri* conducted under controlled laboratory conditions are presented here.

Materials and Methods

Source of *Heilipus lauri*

Weevils used for experiments and subsequent colony rearing were collected 11–12 Feb. 2020 from two commercial Hass avocado orchards both in the Municipality of Coatepec-Harinas in the State of México, México. Infested seeds were moved under USDA-APHIS permit number P526P-19-04341 to the Quarantine and Insectary Facility at the University of California, Riverside for rearing. A total of 57 infested seeds were collected from an orchard in Meyuca de Morales, from which 54 weevils emerged with a 48% female sex ratio. From an orchard in Almoloya de Alquisiras, 67 seeds were collected, from which 43 adults emerged with a 49% female sex ratio. Adult *H. lauri* from each collection site were maintained in one of two Bugdorms (model 2120F [MegaView Science Co., Ltd., Taiwan]) labeled by collection locality. Rearing cages were individually kept inside a larger Bugdorm (model 2400F). This double cage containment was implemented to reduce the likelihood of accidental escape. Twice a week adults were fed fresh Hass fruit and bouquets of young branches with immature leaves that were maintained in vials of water. Branches were pushed through perforated vial lids to eliminate drowning risk to weevils. Colonies were maintained in a temperature and humidity-controlled room in the Insectary and Quarantine Facility under 12:12 (L:D), 26°C (± 0.01), and 56% (± 0.31) %RH.

Each time fruit was removed from cages they were examined for oviposition holes. Oviposition chambers containing eggs were excised from fruit using a razor blade. Fruit pulp was cut with the razor blade under a dissecting microscope to open the oviposition chamber. The number of eggs per chamber was recorded and eggs

were removed individually using a fine water-moistened paint brush. Eggs were placed on 23 mm diameter water-moistened filter paper (Whatman 3 1003-323, Cytiva, Little Chalfont, Buckinghamshire, UK) contained within 35 mm Petri dishes (Falcon 351008, Falcon Plastics, Brookings, SD). Filter papers were divided into quadrants, each numbered sequentially, one egg was added per quadrant, and data were kept on orchard source of eggs and days to egg hatch. Eclosed larvae were used to inoculate Hass avocado seeds. Individual larvae were lifted off filter paper using a fine water-moistened paint brush. Larvae attached to damp paint brush bristles were introduced into holes in Hass seeds that were artificially made with a dissecting needle attached to a wooden dowel handle. Once larvae were placed into the tunnel and had crawled inwards, the opening to the hole on the exterior surface of the seed was closed using fine pieces of seed that had been excavated with the needle. Seeds were inoculated with one larva and then maintained individually in clear 163 ml soufflé-cups (Amerifoods Trading Co., Los Angeles, CA) fitted with ventilated lids, and labeled with a unique identification number, date of seed inoculation, and the name of the orchard from which the parents originated. Days to adult emergence were recorded. Upon emergence, weevils were either kept individually (unmated) or in male–female pairs (mated) in clear 470-ml plastic containers (114D × 76H mm; Uline Inc., Pleasant Prairie, WI) with perforated lids (~five 0.3 mm diameter holes per lid to permit air exchange). Weekly, adult weevils were provided with fresh green Hass avocado fruit to feed on.

Age and Gender of Weevils Used for Experiments, Experimental Design, and Flight Mill Set Up

Adults that were used for flight mill experiments fell into the following age categories: (1) 7–10 d of age ($n = 27$ adults; 13 females, 14 males, all unmated), (2) 13–20 d of age ($n = 33$ adults; 15 females, 18 males, all unmated), and (3) 20–47 d of age ($n = 36$ adults; 20 females, 16 males, all mated except for four females). Weevils in age categories 1–3 were reared from seeds inoculated with larvae that hatched from eggs laid in quarantine (see above). (4) Weevils > four months of age ($n = 30$ adults; 15 females, 15 males, mated) used in experiments were reared from seeds collected in México and used for initiating colonies. These 126 weevils were flown once for a 24 hr period. A subset of 32 weevils ($n = 16$ females, 16 males, all mated) from the 30–47 d of age category were used for repeat flight trials. Each flight trial was 24 hr in duration and weevils used in repeated flight trials were flown 12 consecutive times with an average rest interval of $7.04 \text{ d} \pm 0.10$ between flights. During recuperation periods, weevils were maintained in ventilated plastic containers, fed avocado foliage and fruit, and held in the same temperature and humidity-controlled room as described above. For each flight mill trial, four weevils were randomly assigned to flight mills (see below for details). Weevils were tethered (see below for details) to flight mills in the morning, with setup occurring between 8:00 am and 9:30 am.

For every flight mill trial, each flight weevil was paired with a nonflying control weevil. Nonflying control weevils were maintained individually in ventilated plastic containers without access to food or water. Containerized control weevils were held for the duration of the flight mill assays in the same room the flight mill experiments were conducted in. Before the commencement of each flight experiment the weight of each weevil (i.e., flyer and control) was recorded on a digital balance (GF600, A & D Instruments, Elk Grove IL). All weevils were weighed again at the completion of experiments to determine percentage weight change for flight mill and control weevils. Survivorship of flight and control weevils was recorded after each 24 hr assay.

Flight assays were conducted on four flight mills that were set up in the quarantine insectary room that weevil colonies were maintained in (i.e., 12:12 [L:D], 26°C [± 0.01], and 56% [± 0.31] %RH). Each flight mill was enclosed in a Bugdorm (model 2120F) to prevent escape of weevils should they become untethered during flight assays. Details on flight mill construction, laptop computer, and software to run flight mills and record data are described in detail by Lopez et al. (2014). Weevils were attached to flight harnesses using rapid set glue (DAP Rapid Fuse, Fast Curing All Purpose with Plastic Primer [DAP Products Inc., Baltimore MD]). To facilitate attachment the dorsal surface area of the thorax was rubbed for about 45 s with the Rapid Fuse primer stick to remove surface waxes that prevented firm adhesion of the flight harness. To adhere the harness to the weevil thorax, 1 small drop of glue was applied to the primed area on the thorax. The harness was pressed into the glue droplet and held firmly until set occurred (~90 s). Weevils fixed to harnesses were checked to make sure glue didn't interfere with leg or elytra movement and then they were immediately attached to flight mills. The length of the flight mill arm to which weevils were attached was 30.5 cm and modeling clay of the same approximate weight as tethered weevils was attached to the opposite end of the flight mill arm to counterbalance the weight of the adult beetle.

Statistical Analyses

Before statistical analyses, data were checked for normality and, if necessary, research variables were subjected to Box–Cox procedures to determine power transformations to satisfy model assumptions (PROC TRANSREG in SAS [version 9.4; SAS Institute 2013]). The following transformations (where y = research variable) were made (if not indicated, the variable was not transformed before analysis): 1) weight before trial: log transformed, 2) total distance flown within a 24-hr period: square root transformed, 3) velocity and 4) maximum bout distance within a 24-hr period: square root transformed, 5) maximum bout length in 24 hr: $y^{0.25}$, 6) total distance flown across twelve 24-hr flight trials: log transformed, 7) velocity across successive flights: square root transformed, 8) maximum bout distance flown across twelve 24-hr flight trials: square root transformed, and 9) maximum bout length over the course of successive flights: square root transformed.

Initial Starting Weights of Experimental Weevils

The weight of *H. lauri* adults before trials by origin (i.e., weevils collected in seeds from Mexico vs. weevils reared from eggs in quarantine), gender, age (i.e., weevils reared in quarantine that were 7–10, 13–20, and 20–47 d of age, and >4 mo [weevils collected in seeds from Mexico]), flyer status (i.e., flying vs. nonflying control weevils), and mating status (i.e., mated vs. unmated weevils) was analyzed using a five-way analysis of variance (PROC GLM [SAS Institute 2013]) to determine if significant differences in weight across these categories existed for weevils before experiment initiation. Six interaction terms could not be estimated because of lack of multiple responses for each combination of factors and were removed from the analysis. The six removed interaction terms were all for male and female *H. lauri* collected in Mexico (≥ 4 mo of age category) and included origin by age, origin by mating status, origin by flying status, age by mating status, age by flying status, and mating status by flying status.

Analysis of Mean Distances Flown and Flight Velocities, and Mean Maximum Flight Bout Distances and Times

To be classified as a flight to be included in analyses, flight bouts had to exceed 5 s of activity, any flight bout that terminated for

>5 s was considered to have ended, and total cumulative distance flown in 24 hr had to exceed 25 m to be included in analyses. These qualifying thresholds for flight were necessary as some weevils would engage in numerous sequential bouts of ‘wing buzzing’ (i.e., tethered weevils would open elytra, unfold wings, and vibrate them vigorously for < 5 s without flying). Software would record wing buzzing vibrations as flight activity although no flight occurred. Flight data that satisfied these requirements were used to calculate total cumulative distance and time flown in 24 hr, average cumulative flight distances, and accompanying velocities. For calculation of mean maximum flight bout distances and bout durations, the preceding two discrimination criteria were used and flights had to exceed 2 m.

The average total distance flown 24 hr flight trials ($n = 377$) by origin (i.e., weevils collected in seeds from Mexico vs. weevils reared from eggs in quarantine), gender, age (i.e., 7–10, 13–20, and 20–47 d, and >4 mo), and mating status (i.e., mated vs. unmated weevils) were analyzed using a four-way analysis of variance (PROC GLM [SAS Institute 2013]). The following interaction terms could not be estimated because of the lack of multiple responses for each combination of factors and were removed from the analysis: origin by mating status, origin by age, and age by mating status. For mean total distance flown and velocity per flight, mean maximum flight bout distance and duration, by gender and flight trial, a linear mixed effects model for repeated measures data was used to make comparisons (PROC MIXED [SAS Institute 2013]). Fixed effects in the model included gender and trial number (i.e., one through twelve), and their interaction. Repeated measurements were recordings of distance flown recorded per weevil for each flight trial. Separate models were conducted for each of the four flight variables measured across twelve successive flights (i.e., mean total distance flown and flight velocity, and average maximum flight bout distance and bout duration). Pairwise comparisons for significant main effects were adjusted using the Tukey–Kramer method. Significance for all tests was set at $\alpha < 0.05$.

Proportion of Weevils Flying Different Distances and Cumulative Total Distances Flown

Flight distance data across all flight trials were pooled and were divided across distance flown bins according to Sturges’ formula, where the number of distance bins used per flight trial = $1 + \log_2(n)$ (n = number of observations [Sturges 1926]) and maximum flight distance per trial was used as the upper bin limit. Data were used to calculate the proportion of weevils and mean distance flown per distance bin. These data were also used to calculate total cumulative distances flown across all flights by weevils used in repeat flight experiments.

Weevil Weight and Distance Flown and Weight Loss Following Flight

Pearson’s correlation analysis was performed to determine if a significant relationship existed between the initial weight of weevils tethered to flight mills and total distance flown over the course of the first 24 hr flight. The χ^2 and two-tailed t-tests were performed for each of the four age flight categories (i.e., >4 mo old, 7–10 d of age, 13–20 d of age, and 20–47 d of age), to examine differences in the percentage weight loss over a 24-hr period between a flight cohort and its paired control cohort. Additionally, for the first 24 hr flight across all age categories tested, Pearson’s correlation test was used to examine if significant correlations existed between percentage weight loss of flying weevils and total distance flown.

The procedure GENMOD in SAS (SAS Institute 2013) with binomial distribution and logit link function (Warton and Hui 2011) was used to test if percentage weight loss over the course of 12 successive flights was influenced by gender, flyer status (i.e., flying vs. nonflying control weevils), the 24 hr flight trial (i.e., repeat flight trials 1–12 for weevils in the 20–47 d of age category only), and their interactions. The response variable was the total percentage weight loss recorded per weevil per flight trial. Tukey tests at the 0.05 level of significance were conducted to separate means when significant effects were detected.

Survivorship Analyses

Kaplan–Meier analyses were performed using PROC LIFETEST (SAS Institute 2013) on survival data for weevils. Kaplan–Meier curves, as a function of survival probability and days survived, and survival probability and percentage adult weevils alive over time, were generated for each gender by flyer status (i.e., flying vs. nonflying control weevils). These curves were subjected to a log-rank test in PROC LIFETEST (SAS Institute 2013) at the 0.05 level of significance to determine if significant differences in percentages of alive adult weevils over time, or days survived existed between flying and nonflying control male and female weevils.

Calculation of Dispersal and Redistribution Kernels for *H. lauri* Using Distance Flown Data

No significant differences in flight activity were detected as a function of gender when linear mixed effects regression analyses were completed (see Results). Consequently, all flight data by sex were combined and used to define dispersal curves and corresponding redistribution kernels.

Flight bout data used for these analyses had to exceed 5 s of activity and any flight bout that terminated for >5 s was considered to have ended. Total cumulative distance flown in 24 hr had to exceed 25 m to be included in analyses. Flight data from all 12 repeat flight trials that satisfied these requirements were included in analyses. Flight distance data for individual flights 1 ($n = 32$ weevils flew >25 m), 2 ($n = 32$), 3 ($n = 32$), 4 ($n = 31$), 5 ($n = 31$), 6 ($n = 26$), 7 ($n = 24$), 8 ($n = 23$), and combined flights, flights 9–12 (Flight 9 [$n = 18$], 10 [$n = 16$], 11 [$n = 10$], 12 [$n = 8$] were all combined so sufficient data points were available for analyses), as well as all flights combined (1–12), were divided across distance flown bins according to Sturges’ formula (Sturges 1926) and maximum flight distance per trial was used as the upper bin limit.

Binned flight (number of bins used [$n = 5$]) was consistent across all flight trials) data were used to generate a frequency histogram for each flight trial and all flights combined and the mid-point in each bin was identified. To these midpoints, five different dispersal curves (see Kot et al. [1996] for equations for model curves 1, 2, 3, 4, and 7) with finite integrals were examined for goodness of fit to binned data using sums of squares error (SSE) and coefficient of determination ($R^2 = 1 - \text{SSE}/\text{total sums of squares [SST]}$). Curve equation parameters were determined using PROC NLIN (SAS Institute 2013) and the best (determined by size of SSE (nonlinear model with the smallest value of residual sum of squares (RSS) indicated the best fit to the data) parameterized equation standardized by multiplying by bin width, number of weevils flown per flight trial, and a normalizing constant specific to each individual flight trial and combined flight trials (see Kot et al. [1996] for normalizing constant calculations) were fitted to binned flight data specific to each analysis for each flight trial. Dispersal curve equations were parameterized and normalized to provide an area under the curve of 1 when reflected about the origin, which generated

the redistribution kernel for the distances flown by weevils for each individual flight trial and trial combinations (Kot et al. 1996). The best fitted models to individual and combined flight data sets were tested for kurtosis using the following equation:

$$\text{Excess Kurtosis } (k) = \frac{\int x^4 f(x) dx}{[\int x^2 f(x) dx]^2} - 3$$

which was solved using the option vardef = *n* in PROC MEANS (SAS Institute 2013). Values of $k > 0$, $k = 0$, and $k < 0$ indicate leptokurtosis, mesokurtosis, and platykurtosis, respectively.

Results

Initial Starting Weights

No significant differences in pre-trial weights were detected for control and experimental weevils based on origin, sex, flyer status, mating status, age, origin by sex, flyer status by sex, sex by mating status, and sex by age (Table 1A).

Mean Total Distances Flown and Average Flight Velocities

For the first flight of single and repeat flyers, 123 out of 126 weevils flown (i.e., 98%) flew total cumulative distances >25 m and were included in analyses. No significant differences in average total distance flown and average flight velocity in 24 hr were detected for experimental weevils based on gender, mating status, or gender by mating status (Table 1B and C). Mean flight velocity over the 24 hr flight period did not differ by origin and age (Table 1C; Fig. 1A). Interactions of origin with the gender of experimental weevils were not significant, nor was the interaction between gender and age (Table 1B and C). Mean total distance flown within a 24-hr flight period was significantly different for weevil age and origin (Table 1B), being shortest for weevils >4 mo collected in seeds from Mexico and used to start colonies that flew, on average, less than 3 km in 24 hr with an average velocity of <1 m/s (Fig. 1A).

Mean total distances flown and mean flight velocity by experimental weevils for each repeat flight trial did not differ by sex or sex by flight trial (Table 2A and B). Significant differences in average total distance flown and average flight velocity across 12 consecutive flights were observed (Table 2A and B), and a marked reduction in flight capabilities (i.e., total distances flown and flight velocities) was observed after trial four (Fig. 1B).

Mean Maximum Flight Distances and Flight Bout Durations

Mean maximum flight bout distances and durations within a single no repeat 24-hr flight period were significantly different for origin and age (Table 1D and E), being shortest for weevils >4 mo that were collected in seeds from Mexico (Fig. 2A). Mean maximum flight bout distance and mean maximum flight bout duration exhibited by experimental weevils across 12 consecutive flight trials did not differ by sex or sex by flight trial (Table 2C and D). Average maximum flight bout distances and bout duration times decreased significantly after flight one as the number of consecutive 24 hr flight trials increased with a corresponding concomitant increase in weevil age (Table 2C and D; Fig. 2B).

Proportion of Flights in Distance Bins and Distances Flown and Cumulative Total Distances Flown

Approximately 0.54 (i.e., 407 flights out of 754 that provided useable data) of total flight distances were ≤4.05 km with ~90% of

total flight distances being ≤8.11 km. Less than 1% of total flight distances exceeded 10.82 km flown in a 24 hr period (Fig. 3A). As the proportion of flights in bins of increasing distance decreased a corresponding linear increase in average flight distance resulted due to bin distance size (Fig. 3B).

The longest recorded cumulative flight distance in a 24 hr period was by an unmated female in the 13–20 d of age category (i.e., 19 d of age at time of flight initiation). This female was flown once and covered a cumulative total distance of 13.10 km with a total flight duration of 2 hr 50 min and 54 s and a corresponding weight loss of 16.14%. A total of 251 repeat flights were made by the 32 weevils used for repeat flight trials. The average number of repeat flights made was 7.97 (±0.60 [all means are presented ± SE]) (range 1 [$n = 4$ weevils] to 12 [$n = 8$]). The maximum cumulative distance flown across 12 flights was by a mated female, 34 d of age at time of first flight, which flew a total of 79.16 km. In contrast, the minimum cumulative distance flown by a mated male 39 d of age at time of first flight that was subjected to repeat flight assays was ~1 km by time of death after flight 5. The mean cumulative total distance flown by repeat flight weevils was 30.13 km (±3 km) and the median cumulative total distance flown was 27.97 km.

Initial Starting Weights and Weight Loss Relationships with Flight Activity

No correlation was detected between initial weights of weevils tethered to flight mills and the average total distance flown over a single 24 hr flight ($r = 0.02$; $P = 0.81$). Similarly, no correlation was detected between percentage weight loss of flying weevils and total distance flown over 24 hr ($r = 0.02$; $P = 0.81$). Differences in mean percentage weight loss over a 24-hr period within flight age cohorts and their paired control cohorts were not significant for any of the four age categories tested (χ^2 tests: $P > 0.16$; two-tailed t -tests: $P > 0.16$) (Fig. 4A). Weevils that flew on flight mills and nonflying control weevils, on average, tended to exhibit weight loss of around 14% (Fig. 4A).

Mean percentage weight loss of adult weevils across 12 successive flights as a function of weight in the preceding flight did not differ by sex ($\chi^2 = 0.68$, $df = 1$, $P = 0.41$), sex by flyer status ($\chi^2 = 0.05$, $df = 1$, $P = 0.97$), or sex by flight trial ($\chi^2 = 7.27$, $df = 11$, $P = 0.78$) (Fig. 4B). Significant differences in average percentage weight loss were observed for control and experimental weevils based on flyer status ($\chi^2 = 40.43$, $df = 1$, $P < 0.001$), flight trial ($\chi^2 = 64.91$, $df = 11$, $P < 0.001$), and flyer status by flight trial ($\chi^2 = 64.32$, $df = 11$, $P < 0.001$) (Fig. 4B). By the end of repeat flight trials, control weevils exhibited ~4% weight gain compared to initial starting weights (Fig. 4B). In comparison, weevils on flight mills exhibited consistent weight loss over flights 3–10, and surviving weevils used in flights 11 and 12 exhibited increased average weights when compared to preceding flights. This may have been due, in part, to these surviving weevils being heavier (i.e., >0.15 g for flight 11 and >0.19 g for flight 12) than those in preceding flights that had died (i.e., weevils <0.14 g died prior to or after flight 10 and before flight 11) (Fig. 4B).

Weevil Survivorship Probabilities Across Consecutive Flights

The log-rank test assessing the probability of days survived by adult weevils was significant for flyer status ($\chi^2 = 12.29$, $df = 1$, $P < 0.001$) and sex by flyer status ($\chi^2 = 18.52$, $df = 3$, $P < 0.001$), but not for sex ($\chi^2 = 0.14$, $df = 1$, $P = 0.71$) (Fig. 5A). Flying males were significantly shorter lived than nonflying control male and female weevils ($\chi^2 = 21.95$, $df = 2$, $P < 0.001$). Male and female fliers lived on average for 110.41 ± 17.54 d and 118.64 ± 13.76 d, respectively.

Table 1. Results of ANOVA examining the effects of gender, origin (i.e., weevils collected in seeds from Mexico vs. weevils reared from eggs in quarantine), age (i.e., weevils 7–10 d of age and reared in quarantine, weevils 13–20 d of age and reared in quarantine, and weevils 20–47 d of age and reared in quarantine; >4 mo [weevils collected in seeds from Mexico and used to start colonies]), mating status (i.e., mated vs. unmated weevils), and their interactions on (A) initial starting weights of weevils, (B) mean distance flown, (C) average velocity, (D) mean maximum bout distance flown, and (E) mean maximum bout duration recorded across experimental weevils tethered to flight mills within the 24-hr flight period

	Num df	Den df	F	P
(A) Initial starting weights				
Origin (O)	1	238	0.06	0.81
Gender (G)	1	238	1.19	0.28
Flyer status (F)	1	238	1.07	0.31
Mating status (M)	1	238	0.24	0.62
Age (A)	3	238	1.56	0.21
G × O	1	238	0.03	0.86
G × F	1	238	3.10	0.08
G × M	1	238	0.19	0.67
G × A	3	238	1.85	0.14
(B) Distance flown				
Gender (G)	1	114	0.56	0.45
Mating status (M)	1	114	1.16	0.28
Origin (O)	1	114	5.33	0.02 ^a
Age (A)	3	114	8.64	<0.001 ^a
G × O	1	114	0.66	0.42
G × M	1	114	0.01	0.95
G × A	3	114	0.53	0.67
(C) Velocity				
Gender (G)	1	114	0.81	0.37
Mating status (M)	1	114	0.09	0.77
Origin (O)	1	114	3.56	0.06
Age (A)	3	114	0.24	0.79
G × O	1	114	3.38	0.08
G × M	1	114	0.28	0.59
G × A	3	114	1.56	0.21
(D) Maximum bout distance flown				
Gender (G)	1	114	0.26	0.61
Mating status (M)	1	114	0.48	0.49
Origin (O)	1	114	19.58	<0.001 ^a
Age (A)	3	114	4.37	0.01 ^a
G × O	1	114	0.25	0.62
G × M	1	114	0.55	0.46
G × A	3	114	0.27	0.85
(E) Mean maximum bout length				
Gender (G)	1	114	0.95	0.33
Mating status (M)	1	114	1.83	0.18
Origin (O)	1	114	17.58	<0.001 ^a
Age (A)	3	114	3.04	0.03 ^a
G × O	1	114	0.38	0.54
G × M	1	114	0.11	0.75
G × A	3	114	0.19	0.91

^aIndicates significance at the 0.05 level.

Consequently, no significant differences in average days survived were observed for flying male and female weevils ($\chi^2 = 2.42$, $df = 1$, $P = 0.12$). No significant differences in days survived were detected for flying females, control males and control females ($\chi^2 = 4.43$, $df = 2$, $P = 0.11$) (Fig. 5A).

The log-rank test assessing the proportion of adult weevils surviving was significant for flyer status ($\chi^2 = 50.95$, $df = 1$, $P < 0.001$) and sex by flyer status ($\chi^2 = 57.96$, $df = 3$, $P < 0.001$), but not for sex ($\chi^2 = 2.44$, $df = 1$, $P = 0.12$) across flights (Fig. 5B). Flying males and females died more readily compared to nonflying control male and female weevils (flyer status effect Fig. 5B). No significant differences in proportion of adult weevils surviving were detected for male and female flyers ($\chi^2 = 0.32$, $df = 1$, $P = 0.57$). Control male and female

weevils lived on average for 206.19 ± 18.76 d and 183.07 ± 18.36 d, respectively. No control weevils died during the time period that the 12 sequential flights were conducted (Fig. 5B). Consequently, no significant differences in average days survived were observed for control male and female weevils (the two datasets had a correlation coefficient of $P = 1$).

Dispersal and Redistribution Kernels for *H. lauri* Using Distance Flown Data

No significant differences were detected as a function of gender when linear mixed effects regression analyses were completed (see above). Therefore, all flight data by gender were combined and the best fitting of the five curves analyzed from Kot et al. (1996) was

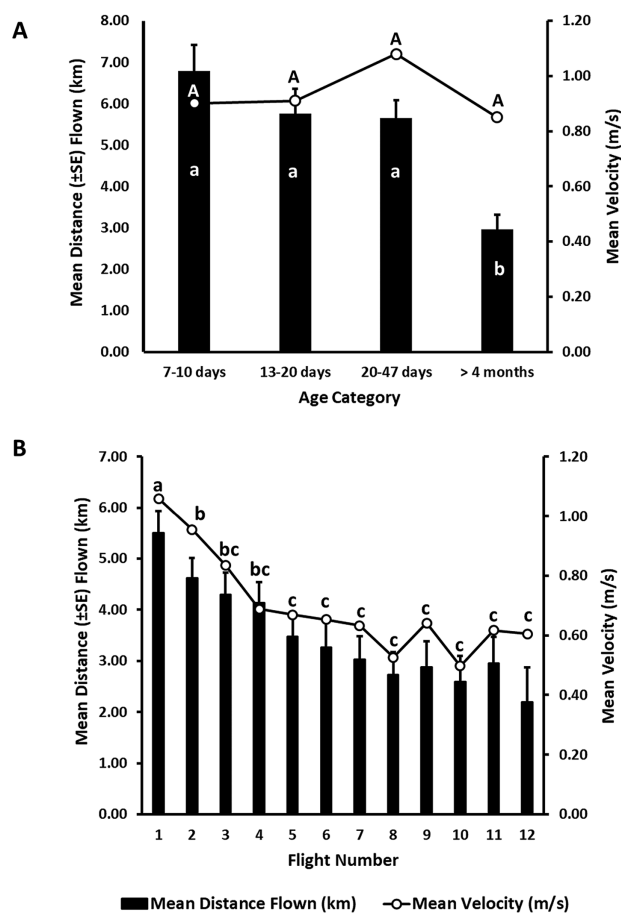


Fig. 1. Mean (\pm SE) cumulative flight distances and flight velocities flown by adult male and female *Heilipus lauri* in four different age categories (A) and (B) in the 30–47 d of age category across 12 consecutive flights. Different letters represent significant statistical differences at the 0.05 level for the distance flown data sets. The one set of letters in (B) apply to distance flown and velocity data.

determined to be curve 3 for flights 1 through 8, 9–12 combined, and 1–12 combined (Table 3). This function was used to generate the corresponding redistribution kernel for each flight. The excess kurtosis measures, k , were <1 , indicating that the equations describing the curves that were produced were all platykurtic (Table 3) (Fig. 6 A–D). The corresponding redistribution kernel plots for flights 1 through 8, 9–12 combined, and 1–12 combined produced graphs with bimodal peaks centered at the origin (Fig. 6 A–D). The redistribution kernel plots provide estimates for the proportion of weevils dispersing various distances from the origin. For all flights combined (i.e., cumulative distances by individual weevils across repeat flights), the model estimated that ~7% of weevils will disperse ~1 km about the origin, ~20, ~33, ~45, and ~56% of dispersing weevils will be within 2, 3, 4, and 5 km of the origin, respectively after 12 consecutive flights (i.e., over ~12 week period) (Fig. 6D). A significant percentage, ~15%, of weevils in the all flights combined model, have the capability to disperse >10 km before either dying or upon completing the final twelfth flight trial (Fig. 6D).

Discussion

Heilipus lauri is a significant avocado pest in native and invaded ranges and it has been identified as an incursion threat to U.S. avocado industries (USDA-APHIS 2004, 2016a). Flight distance data are

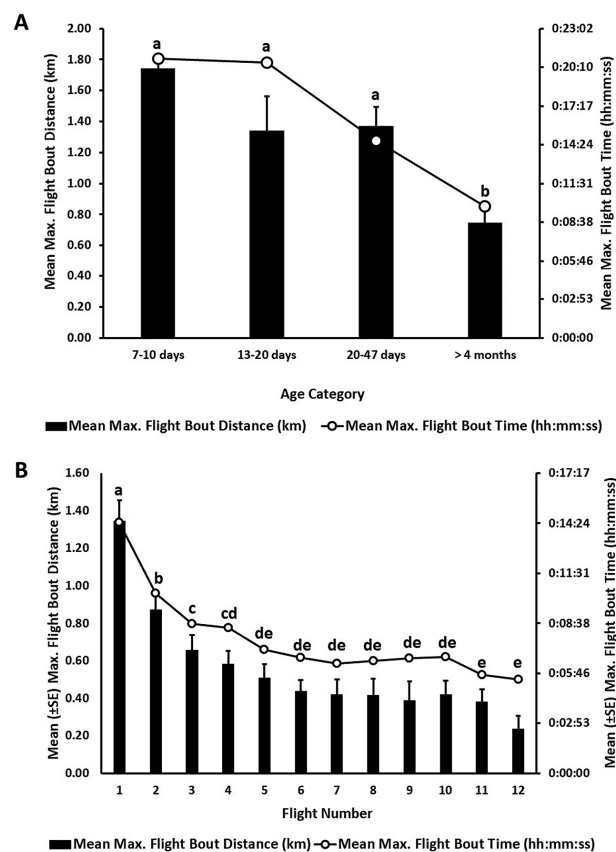


Fig. 2. Mean (\pm SE) maximum flight bout distance flown and flight bout time for adult male and female *Heilipus lauri* in four different age categories (A) and (B) weevils in the 30–47 d of age category across 12 consecutive flights. Different letters represent significant statistical differences at the 0.05 level for both sets of data per graph.

useful for the development of monitoring protocols (Seo et al. 2017) and during the early stages of incursions into new areas an understanding of self-dispersal capabilities via flight would be valuable for the development of monitoring, containment, quarantine, and eradication boundaries. In a proactive approach for preparedness, flight mill studies were undertaken to quantify the flight capabilities of *H. lauri* in advance of an anticipated incursion into California or other avocado production areas in the U.S. (e.g. Florida, Texas, and Hawaii) by this pest.

Flight mill trials conducted in a quarantine laboratory indicated that male and female *H. lauri* readily fly and are capable of repeat flights over their lifetimes. For single or first flights for repeat flyers, 98% of weevils (i.e., 123 weevils out of 126 flown) flew a total cumulative distance >25 m and these distance data were used in analyses. Total flight distances by weevils that flew once in a 24 hr period were significantly affected by age, with weevils >4 mo of age (i.e., >120 d old had an average total flight distance of ~3 km in a 24 hr period) flying total distances that were significantly less than weevils \leq 47 d of age (average total flight distance ~6–7 km over 24 hr).

Similarly, for repeat flights, as weevils simultaneously aged and flew more, average total flight distances showed a steady and significant decline from ~5 km for flight one to ~2 km for flight 12 by surviving adults. Negative relationships between weevil age and increasing repeat flight frequency were observed with average flight velocity and mean maximum flight bout distances and flight bout durations.

Table 2. Results of repeated measures analyses examining the effects of gender, the 24-hr flight trial (i.e., one through twelve), and their interactions on (A) total distance flown, (B) velocity, (C) mean maximum bout distance flown, and (D) mean maximum bout time length recorded across experimental weevils tethered to flight mills across twelve 24-hr flight trials

	Num df	Den df	F	P
(A) Distance flown				
Gender (G)	1	227	2.61	0.11
Flight (F)	11	227	7.63	<0.001 ^a
G × F	11	227	0.82	0.62
(B) Velocity				
Gender (G)	1	227	1.62	0.21
Flight (F)	11	227	21.16	<0.001 ^a
G × F	11	227	1.47	0.15
(C) Maximum bout distance flown				
Gender (G)	1	227	2.02	0.16
Flight (F)	11	227	9.92	<0.001 ^a
G × F	11	227	1.51	0.13
(D) Maximum bout length				
Gender (G)	1	227	0.26	0.61
Flight (F)	11	227	23.03	<0.001 ^a
G × F	11	227	1.05	0.41

^aIndicates significance at the 0.05 level.

When all flight distance data for single and repeat flyers were pooled, the highest proportion (~0.21) of weevils occupied a distance flown bin of 1.37–2.70 km with an average flight distance of ~2km. These pooled data may reflect the ‘average’ flight potential of field collected weevils which would consist of individuals of different ages and flight experiences. Running flight mill studies with field collected weevils of unknown age and flight history could be conducted to test this ‘average distance’ hypothesis.

The longest single flight distance recorded was ~13 km on the first and only flight by an unmated 19 d old female. An additional 10 weevils, all unmated males and females, flew ~10–12 km on their first flights, with a further 25 mated and unmated male and female weevils flying ~7–10 km. These ‘long distance’ flights represent ~10% of the 377 total distance flown flights produced in this study. A steady decay in total flight distances flown per 24 hr assay was not universally observed for repeat flight weevils when individual flight activity was examined. For example, one mated female, 34 d of age at time of first flight, flew ~9 km for the first flight, dropping to ~2–6 km for flights 2–5, then peaking at ~9–11 km for flights 6–8 before declining to ~5 km for flights 9–12. Over the course of 12 flights this female flew a cumulative total distance of ~79 km which was the highest total distance recorded. Individuals, especially mated females, with high dispersal capabilities in comparison to the rest of the population, may be responsible for unexpected and rare rapid long distance spread that results in populations establishing in areas further than anticipated (Lockwood et al. 2013, Jones et al. 2019).

Repeat flight activity had a significant impact on weevil weight loss and subsequent weight recovery. Generally, *H. lauri* were unable to fully recover weight that was lost during flight activity despite resting for ~7 d between flights and being fed with avocado fruit and immature foliage attached to green twigs, all of which appeared to be acceptable food. Additionally, the negative effects of repeat flights were also observed on survivorship rates with 72% (i.e., 23 weevils out of 32 died) of weevils dying by flight 12. In contrast, no mortality was observed for nonflying control weevils that were maintained in an identical manner (i.e., same sized containers, diet, temperature, humidity, and light cycle) over the ~12 wk trials were run for repeat flight assays. For some species of curculionids, body mass may have important impacts on flight capacity and weight loss due to flight

maybe indicative of metabolism of lipids, carbohydrates, or proteins to power flight (Jones et al. 2019, Wijerathna and Evenden 2019). Although no relationship between flight capacity and body mass of *H. lauri* was detected in this study, repeat flights did have negative effect on *H. lauri* survivorship rates. Energy use and depletion of ‘fuel’ (e.g., carbohydrates) by flying *H. lauri* is undetermined and could be investigated with flight mill studies similar to those reported here (Wijerathna and Evenden 2019).

The dispersal distance data for *H. lauri* across all flight and age categories exhibited a bimodal platykurtic distribution which is characterized by ‘light’ or ‘thin’ tails because relatively few extreme flight distance values occur in curve tails. Therefore, a platykurtic distribution of dispersal data is skewed towards distances that are less than would be predicted by a mesokurtic distribution (i.e., normal distribution) (Kot et al. 1996, Lockwood et al. 2013). With respect to *H. lauri* flight data, platykurtic distributions suggest that movement from natal areas may be limited (Jones et al. 2019). An understanding of the distribution pattern underlying dispersal data has important implications for predicting rates of spread which may be particularly useful for modeling different invasion scenarios and designing delimitation boundaries. For example, identification of varying flight distances within a population that result from age, sex, or size differences, may result in dispersal patterns that conform to different kurtoses (e.g., platykurtic and leptokurtic) and could therefore require examination with mixed kernel models (Petrovskii et al. 2008, Jones et al. 2019). If the underlying distribution, or kurtosis, of distance flown data is not determined, flight mill data may be subjected to analyses that could be inappropriate for estimating the frequency or proportion of dispersal distances by a pest of interest and this could affect the accuracy of model predictions (e.g., use of Monte Carlo simulations that assume flight data have a normal distribution [Kees et al. 2017]).

The dispersal model (Fig. 6D) for cumulative flight distances for *H. lauri* that underwent repeat flights indicated that ~56% of dispersing weevils would be within 5 km of the origin after 12 consecutive flights that are interspersed by ~7 d intervals to rest and feed (i.e., a period of ~12 wk). There are a few caveats that need consideration when interpreting these model predictions. First, dispersal data from flight mill experiments are derived under highly

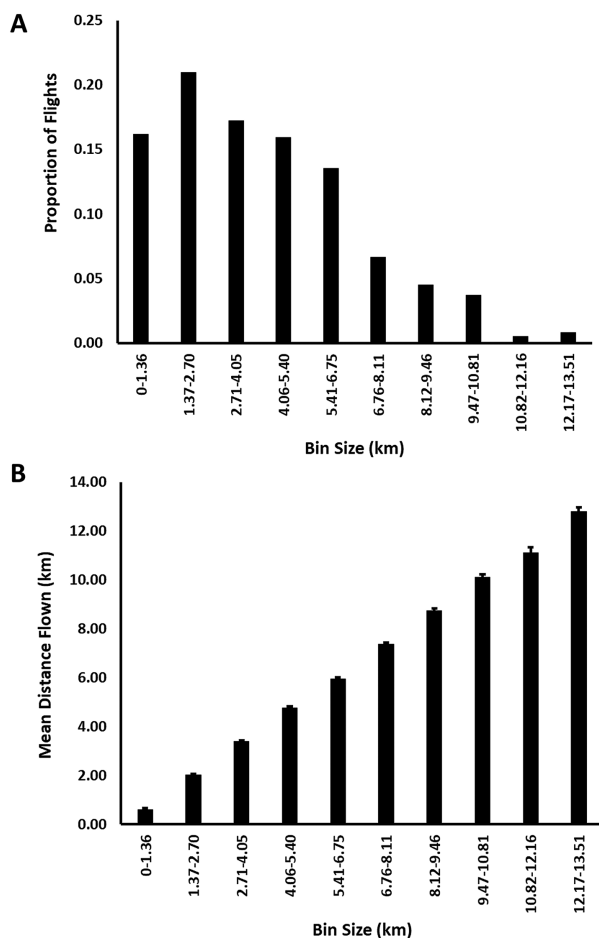


Fig. 3. Proportion of male and female *Heilipus lauri* in flight distance bins (A) and (B) the mean (\pm SE) distances flown by weevils in distance bins for all flights and age categories combined (i.e., all single 24 hr flights and consecutive flights that provided useable flight data).

artificial conditions. For example, insects fly in circles under optimal environmental conditions which may over estimate flight capabilities. The artificiality and shortcomings of flight mill studies are well acknowledged (Taylor et al. 2010, Naranjo 2019). However, it is also recognized that flight mills are useful tools for investigating covariates affecting flight capabilities (e.g., sex, mating status, age, or size) (Minter et al. 2018, Naranjo 2019) and in comparison to alternative technologies for assessing insect dispersal by flight (Kissling et al. 2014), flight mills are currently the best and most cost effective option for studying insect flight.

Second, with respect to the interpretation of dispersal distance potential for *H. lauri*, consideration of its lifestyle is warranted. *Heilipus lauri* adults feed on the skin of avocado fruit, fruit petioles, soft green twigs and stems, and immature leaves. Females oviposit in holes chewed into fruit. These feeding, and to a lesser extent oviposition substrates, are likely present year round in varying abundance on avocado trees and feeding and oviposition behavior generally does not kill host trees. If these assumptions are correct, there may be little motivation for *H. lauri* to disperse over relatively long distances (i.e., hundreds of meters via multiple consecutive flights) when food, mates, and oviposition sites are highly localized and host trees are long-lived. Consequently, under these resource-abundant scenarios, dispersal distances for *H. lauri* could be overestimated by flight mill studies as weevils may not normally engage in single or multiple 'long' distance flights as they are unnecessary and may have

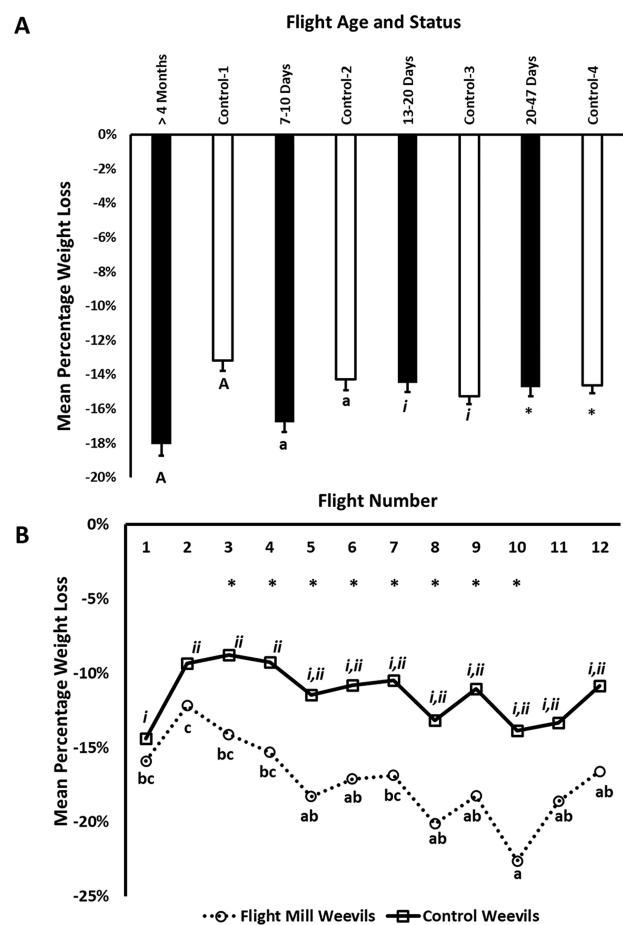


Fig. 4. (A) Mean percentage weight loss for flown male and female *Heilipus lauri* flown in four different age categories and paired control weevils. No significant differences in weight loss were observed between flown and control weevils within age categories. (B) Mean percentage weight loss for control and flown male and female *H. lauri* over the course of 12 consecutive flight assays. Different letters across flight assays indicate significant differences in mean percentage weight loss for flown weevils. Different Roman numerals (italics) across flight assays indicate significant differences in mean percentage weight loss for control weevils. Asterisks indicate significant differences in mean percentage weight loss between flown and control weevils across flight assays. All significant statistical differences were detected at the 0.05 level.

high levels of risk associated with them (i.e., inability to locate new host plants or increased risk of predation). Consequently, the likelihood of repeat flights may increase should dispersing *H. lauri* arrive in areas lacking food. Flight mill trials could assess the effects of suboptimal resources on the propensity for repeat flights by *H. lauri* and the subsequent distances flown which may provide an improved understanding of the dispersal capabilities of this pest under resource limited conditions.

Third, fitted dispersal models (Kot et al. 1996) used in this work indicate that dispersion of flight data is platykurtic suggesting that adult *H. lauri* may not disperse far from areas within which they emerge. Consequently, models with platykurtic kernels may have greater power at predicting *H. lauri* dispersal patterns as the probability of long distance outliers would be low (Jones et al. 2019). Additionally, it is likely that flight activity is not linear as predicted by models and under field conditions, *H. lauri* may exhibit irregular flight patterns as they move within and between trees to find food and mates. This may be especially true if host trees are in close

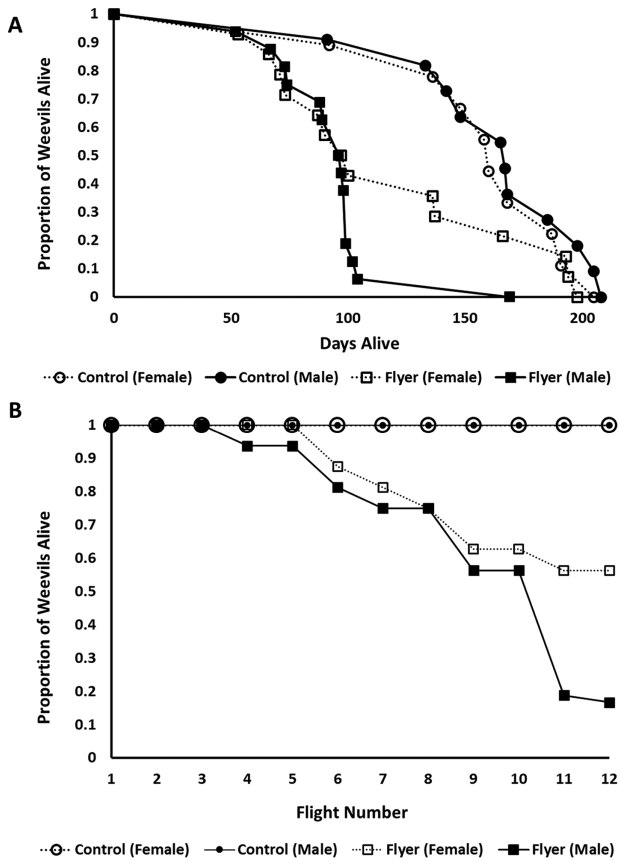


Fig. 5. (A) Proportion of adult male and female *Heilipus lauri* surviving over time that were either flown once on flight mills or used as nonflying control weevils and (B) survivorships rates for weevils that were subjected to repeat flights on flight mills or served as nonflying controls over the same time period.

proximity to each other as would be typical of an avocado orchard. Therefore, characterization and confirmation of dispersal kernels derived from laboratory experiments reported here require field validation to translate estimates of flight mill distances to actual dispersal distances observed in nature. Mark-recapture experiments have been used to validate parameters modeling insect dispersal (Jones et al. 2019) and this approach could have an application to quantifying *H. lauri* movement in avocado orchards.

In conclusion, this study is the first to document the potential flight capabilities of a *H. lauri*, a destructive pest of avocado fruit, and an incursion risk recognized by U.S. regulatory authorities (e.g., USDA-APHIS). Although potential flight distances have been quantified through flight mill studies conducted under optimal conditions in a quarantine laboratory, interpretation of these dispersal data should be viewed cautiously, especially in the absence of field validation. However, these flight mill data do provide preliminary information that could be used to parameterize dispersal models and guide decision-making processes with respect to monitoring programs in infested areas. For example, initial placement distances between traps could be determined using average flight bout distance estimates derived from flight mill experiments. Field experiments could then be designed to optimize trap placement distances based on initial separation distance estimates from flight mill studies. Similarly, as a starting point, the establishment of containment, quarantine, or eradication boundaries in newly invaded areas could be formulated using flight mill data. For example, mean daily

Table 3. Lowest calculated RSS values fitted to equations for curves 1, 2, 3, 4, and 7 from Kot et al. (1996), parameter estimates, normalizing constants, and measures of excess kurtosis for individual flights 1–8, combined flights 9–12, and all flights combined (1–12)

	1	2	3	4	5	6	7	8	9 to 12	All
Flight number										
Curve 1	11.75	58.12	19.96	22.56	4.87	44.39	31.99	5.39	12.98	64.05
Curve 2	19.49	69.2	34.66	45.79	20.42	54.00	55.75	13.96	47.29	86.76
Curve 3	5.38	28.92	1.23	0.57	0.25	0.98	12.72	5.12	6.24	9.08
Curve 4	18.16	68.42	32.17	43.69	20.24	53.14	57.36	17.03	45.35	83.40
Curve 7	16.04	65.93	27.59	35.56	13.70	50.78	47.60	11.70	31.20	77.23
Curve 3 parameter estimates										
<i>a</i>	25.34	36.60	35.99	41.88	32.25	39.71	42.81	24.22	43.00	90.17
<i>b</i>	8.72	13.43	13.74	16.90	12.90	15.45	17.47	10.32	18.67	19.56
<i>c</i>	-19.47	-32.89	-29.96	-34.84	-23.28	-37.73	-37.38	-15.17	-26.58	-364.40
<i>s</i>	0.67	0.84	0.75	0.74	0.59	0.92	0.80	0.47	0.45	6.99
<i>t</i>	15.88	12.57	11.33	9.62	10.22	10.31	9.19	8.85	9.01	79.45
Normalizing constant	142.38	137.28	127.90	121.58	116.02	107.90	110.66	84.17	160.60	1063.80
Excess kurtosis estimates										
<i>k</i>	-1.22	-1.29	-1.23	-1.25	-1.41	-1.43	-1.23	-1.52	-1.45	-1.35

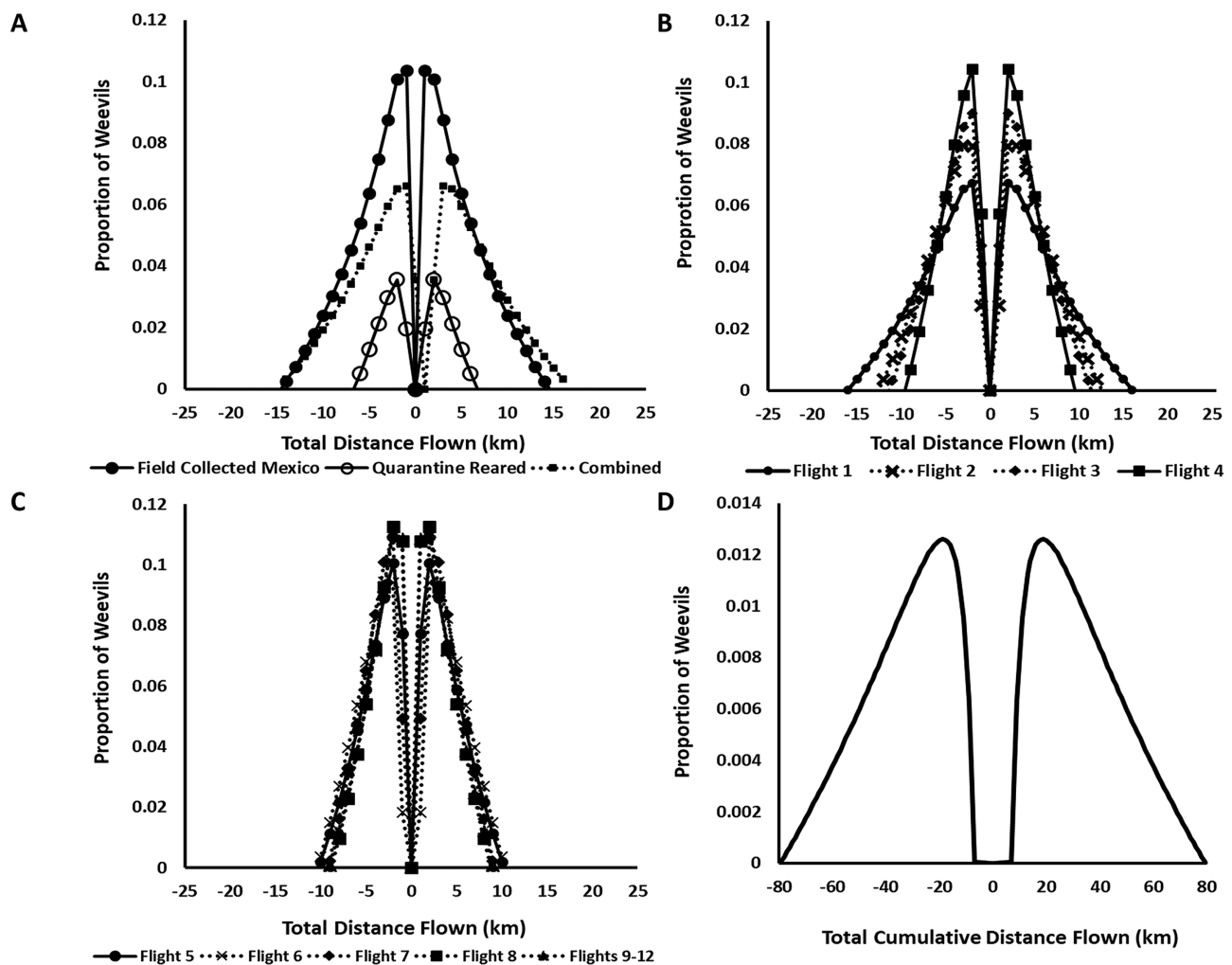


Fig. 6. Redistribution kernel curves for adult male and female *Heilipus lauri* weevils that were flown (A) once on flight mills with weevils that were field collected in Mexico (>4 mo of age when flown), quarantine reared progeny produced by field collected weevils that were 7–10 d of age, 13–20 d of age, and (4) 20–47 d of age that were flown once for a 24 hr period. (B–C) Redistribution kernel curves for repeat flights for 32 weevils ($n = 16$ females, 16 males, all mated) from the 30–47 d of age category that were used for repeat flights, and (D) all repeat flights (i.e., cumulative distances for individual weevils across flights 1–12) combined.

flight distances, average total cumulative distances flown from repeat flights, or estimates of the proportion of weevils capable of varying dispersal distances with the appropriate underlying dispersal distribution (e.g., a platykurtic kernel) from a point source (i.e., origin) over time could be used to set initial boundaries. In some instances, when there is a lack of field or laboratory derived data on flight capabilities, implementation of boundary distances (e.g., quarantine boundaries) for incipient management programs have been based on ‘best guesses’ for newly detected incursions (Hoddle and Hoddle 2016). Information provided here on *H. lauri* flight capabilities will help with informed decision-making should development of incursion management plans be necessary.

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Author Contributions

MSH: conceptualization, funding acquisition, report preparation and submission, project management, specimen collection, data collection and management, preliminary analyses, writing original draft, editing, and processing revisions. CDH: visualization, data collection, data management, preliminary data analyses, review and editing of draft manuscripts. IM: visualization, formal data analyses, review and editing of draft manuscripts. EGEV: specimen collection, assistance with field work logistics, project management in México, editing and review of draft manuscripts. AEM: specimen collection, assistance with field work logistics, project management in México, and review of draft manuscripts.

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