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Crop-emptying rate and nectar resource allocation in a nectivorous pollinator

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ABSTRACT

In nectivorous pollinators, timing and pattern of allocation of consumed nectar affects fitness traits and foraging behavior. Differences in male and female behaviors can influence these allocation strategies. These physiological patterns are not well studied in Lepidoptera, despite them being important pollinators. In this study we investigate crop-emptying rate and nectar allocation in *Manduca sexta* (Sphingidae), and how sex and flight influence these physiological patterns. After a single feeding event, moths were dissected at fixed time intervals to measure crop volume and analyze sugar allocation to flight muscle and fat body. Then we compared sedentary and flown moths to test how activity may alter these patterns. Sedentary males and females emptied their crops six hours after a feeding event. Both males and females preferentially allocated these consumed sugars to fat body over flight muscle. Moths began to allocate to the fat body during crop-emptying and retained these nutrients long-term (four and a half days after a feeding event). Males allocated consumed sugar to flight muscles sooner and retained these allocated nutrients in the flight muscle longer than did females. Flight initiated increased crop-emptying in females, but had no effect on males. Flight did not significantly affect allocation to flight muscle or fat body in either sex. This study showed that there are inherent differences in male and female nectar sugar allocation strategies, but that male and female differences in crop-emptying rate are context dependent on flight activity. These differences in physiology may be linked to distinct ways males and females maximize their own fitness.

1. Introduction

Lepidoptera are important pollinators of both agricultural crops (Requier et al., 2023) and native plants (Walton et al., 2020), and their feeding behavior and fitness are therefore linked to yields of agricultural crops and conservation efforts, respectively. Crop-emptying rate and nectar allocation choices of insects affect foraging behavior (Piñero et al., 2021, Boggs, 1992). Crop-emptying refers to the release of consumed nectar from a storage organ, the crop. Nectar allocation is the distribution of consumed nectar nutrients to various functions in the body. Therefore, greater understanding of digestive physiology is the first step in predicting how nectar consumption can affect fitness of pollinators and their efficiency as pollinators. Studies on the internal crop organs of adult Lepidoptera and its effects on nutrient allocation are limited. Such studies have focused on crop-emptying rate effects on diuresis (excretion of excess water) after eclosion and feeding (Bushman, 1996, Hainsworth et al., 1991, Bushman et al., 2002). Rate of nectar release from the crop and nectar nutrient absorption have been calculated as "energy processing" in the butterfly *Vanessa cardui L.* (Hainsworth et al., 1991) and in blowflies (Hainsworth et al., 1990), but as of yet, no study has looked at the timing of when a nectar meal is completely emptied from the crop and allocated into specific body tissues. It is unknown how the crop-emptying rate affects the timing of allocation. This information is necessary for determining how long after the ingestion of nutrients they are used in fitness-increasing traits and behaviors. This study addresses this gap by investigating how a single nectar meal is stored, allocated, and metabolized over time.

Fitness of nectivorous insects is determined in part by when and how they allocate consumed nectar nutrients. Nutrients are allocated to various functions, such as flight, storage, somatic maintenance, growth, and reproduction (Boggs, 2009). Short-term behaviors, such as foraging, can have long-term effects on fitness (Mangel & Clark, 1988). Short-term foraging behaviors may be especially impactful on fitness in systems where food sources are sparsely distributed and feeding events may be rare. We use the model organism *Manduca sexta* hawkmoth (Sphingidae), which is ideal for this study since adults likely encounter bouts of

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starvation due to their preferred nectar plant's variability in availability and sparse distribution (Raguso & Willis, 2003, Riffell et al., 2008, Alarcón et al., 2008, Levin et al., 2016).

There is a lag period between the time of nutrient acquisition and the time of nutrient assimilation, affecting the timing of nectar allocation to fitness-increasing behaviors and traits. The rate limiting step in this process is the emptying of the crop (Treherne, 1967, Bernays & Simpson, 1982), an impermeable organ located in the foregut of insects where food is stored before being digested in the midgut (Maddrell & Gardiner, 1980). Once released from the crop, digested nutrients from consumed nectar can be allocated to fitness-related traits such as producing eggs, developing flight muscles, or fat body storage, which then contribute to reproductive output and lifespan (Murphy et al., 1983, Hill & Pierce 1989, Hill, 1989, Boggs & Ross, 1993, Leahy & Andow, 1994, O'Brien, 1999, Mevi-Schütz & Erhardt, 2005, Niitepõld and Boggs, 2015). Therefore, the timing of nectar release from the crop dictates how quickly it can be allocated to fitness-increasing traits or behaviors.

Both the timing of crop-emptying and allocation decisions could be altered by flight behavior. Locomotor activity in bees increases crop-emptying rate, likely due to its higher metabolic cost (Blatt & Roces 2002a,b). Flight is energetically costly (Bartholomew and Casey, 1978, Casey et al., 1985), but necessary for locating food and mates while avoiding predators. Hawkmoths engage in hovering flight when feeding, one of the most metabolically costly behaviors (Biewener & Patek, 2018). The high metabolic cost of flight could increase the crop-emptying rate, leading to faster assimilation of consumed nutrients. Flight can affect when and how nutrients are allocated throughout the body for functions other than fueling flight itself. Nectar sugars are allocated to flight muscle to repair oxidative damage from flight in moths (Levin et al., 2017a), and flight increases the amount of consumed nectar sugars allocated to eggs in butterflies (Boggs & Niitepõld, 2014, Niitepõld & Boggs, 2015).

The fitness benefits of nutrient allocation decisions can vary by sex. Allocation strategies may be driven by sex-specific differences in behavior, including the types of flowers male and female Lepidoptera visit and their feeding frequency (Smith et al., 2019). In *Manduca sexta*, sex affects body size (Stillwell and Davidowitz, 2010), floral preference (Alarcón et al., 2010), feeding frequency (Ziegler, 1991), and lifespan (Wone et al., 2018). Nectar amino acid utilization is sex specific in *M. sexta*; for example, males allocate more consumed amino acids to fight muscle than do females (Levin et al., 2017b).

In this study we first determined how long it takes for consumed nectar to be released from the crop and allocated to body tissues in sedentary male and female moths over a period of four and a half days, about the estimated lifetime of a wild *M. sexta* moth (Levin et al., 2016). In this sedentary lifetime experiment, we measured the volume of nectar stored in the crop over time and sugar allocation after a single nectar meal in unmated sedentary male and female *M. sexta* hawkmoths. Second, we examined the effect of flight activity on crop-emptying and immediate pattern of sugar allocation to body tissues. In both the sedentary lifetime experiment and activity experiment, consumed sugars were tracked in two tissues, flight muscle and fat body. We used stable carbon isotopes to determine where moths allocate consumed nectar nutrients. Studies on sugar allocation have used natural differences in the ^{13}C fractionation of sugars produced by plants that use the C3 and C4 photosynthetic pathways (O'Brien et al., 2004; Boggs and Niitepõld, 2014). Lepidoptera raised on a larval diet of C3 plant sugar (beet) have a different isotopic signature than that of C4 plant sugar (cane), making it possible to track where fed moths allocate cane sugar from an artificial nectar.

We predicted that males and females differ in both the rate at which they empty their crop as well as in the timing and location in the allocation of consumed nutrients to flight muscle and fat body. As fed *M. sexta* males have larger flight muscles than unfed males (Levin et al., 2016), we expected males to allocate more consumed sugar to flight muscles than females. We expected resting moths to allocate more consumed sugar to fat body, as it has been documented that sedentary flies store excess energy from the crop as lipids (Hainsworth et al., 1990). Additionally, we predicted that flight would increase the rate at which the crop is emptied as well as affect the allocation pattern, with nutrients preferentially sent to flight muscles to fuel flight or repair oxidative damage and to the fat body as lipids are used to fuel flight preferentially after 30 min of flight (Ziegler and Schulz, 1986a).

2. Methods

2.1. Study organism

Manduca sexta (Sphingidae) were raised in a colony maintained at the University of Arizona (Davidowitz & Nijhout, 2004, Davidowitz et al. 2012, 2016). Larvae were reared under a 16:8 light:dark photo cycle in an environmentally controlled room (27 ± 1 °C and 50 % relative humidity). Larvae were fed ad libitum on a standard artificial diet (Davidowitz et al., 2003) with an isotopic signature of $\delta^{13}C \approx -24.08$ ‰ until pupation. Pupae ready to eclose (19–25 days after entering the pupal stage and one day before eclosion) were removed from the colony daily and placed into individual waxed paper bags for eclosion.

2.1.1. Manduca sexta feeding, starvation, and activity level

Sedentary lifetime experiment: Virgin moths were starved for 48 h after eclosion and wing sclerotization. After the 48-hour starvation period, half of the moths were fed 500 µl of 25 % cane sugar ($\delta^{13}C \approx -11$ ‰), the same sugar concentration as the nectar in the preferred nectaring plant of *M. sexta, Datura wrightii* (Riffell et al., 2008). Additionally, this is the average sugar concentration of nectar in butterfly pollinated plants (Heyneman, 1983), and so is likely to be encountered by a wide range of Lepidoptera. This solution was dyed red using food coloring (McCormick & Company, Inc., Hunt Valley, MD, 5 drops/40 ml cane sugar solution) to increase visibility of the nectar meal in the crop and ensure all contents of the crop were removed during dissections, as described below. The other half of the moths remained unfed to serve as a control.

Fed and unfed moths were randomly assigned to one of twelve time intervals in matched-pairs (fed and unfed): 0, 1, 6, 12, 24, 36, 48, 60, 72, 84, 96, and 108 h. Including the 48-hour starvation, the longest time interval encompassed a total 6.5 days The average lifespan is 4.9 days for unfed males and 8.0 days for unfed females in laboratory settings (Wone et al., 2018), with high humidity increasing lifespan (Contreras et al., 2022). However, it is expected that wild *M. sexta* moths likely live for four or five days (Levin et al., 2016); therefore, this experiment encompasses the lifespan of a wild moth.

Fed moths were sacrificed at assigned time intervals after the feeding event, while unfed moths were sacrificed at these time intervals after the initial 48-hour starvation period. Moths were placed in the refrigerator (15 °C) for 5 min at the end of the assigned time interval. Cooling was used to prevent moths from struggling during handling and dissection to reduce further crop emptying from increased activity or increased hydrostatic pressure. Moths were removed from the refrigerator and immediately killed by beheading. Crop dissections were then performed on both fed and unfed moths. Five males and five females were used for the two feeding treatments (fed or unfed) across twelve time intervals, resulting in a total of 240 moths.

As moth flight muscles are not fully developed until the third day after eclosion, and younger moths exhibit lesser flight duration than middle-aged moths (Wone et al., 2018), newly eclosed *M. sexta* adults may not fly very long or far to search for food, and therefore naturally may not feed up to 72 h after eclosion. In fact, in a study of wild-caught *M. sexta* moths, 15 % of moths had not fed at all and 73 % had not fed on their preferred food source (Levin et al., 2016), indicating feeding in adults may be relatively rare. Therefore, this starvation period, starvation experienced by the unfed group, and a single nectar meal in the fed group are ecologically relevant and mimics the experience of some wild

moths (Levin et al., 2016).

Activity experiment: Moths were reared as above and starved for 48 h following eclosion and wing sclerotization. Twenty-four hours after wing sclerotization, scales were removed from a ~ 4 mm^2 area of the dorsal thorax of all moths. Fifteen grit sandpaper was used to remove cuticular wax and a drop of Loctite® Super Glue Gel (Henkel Corporation, Westlake, OH, USA) was used to secure a metal plate (~3x2mm) with a flexible plastic chain (allowing forward-and-back but not side-to-side movement) onto the bare region of the thorax and returned to the waxed paper bags (Wone et al., 2018). After the full 48-hour starvation period, all moths were fed 500 μl of red dyed 25 % cane sugar ($\delta^{13}C \approx -11$ ‰). Immediately following feeding, half of the moths were attached by the chain to the arm of the flight mill via an alligator clip. Moths were allowed to fly without interference until they stopped, after which flight time and distance were recorded. Flight mills can be used for comparative purposes among treatments (Chen et al., 2015), which we used in this experiment, with the caveat that they do not provide accurate representations of free flight performance (Riley et al. 1997). Moths were flown during the dark cycle of the photoperiod when they are most active. When flight concluded, active moths were immediately transferred to a waxed paper bag and placed in the refrigerator (15 °C) for 5 min. The other half of fed moths remained sedentary in a waxed paper bag for the same amount of time as the flight duration of their active counterpart, after which sedentary moths were placed in the refrigerator for 5 min. After 5 min all moths were sacrificed and crop dissections performed as described below. Ten males and 10 females were used for each of the two activity treatments (active and sedentary), for a total of 40 moths.

2.2. Crop dissections

All moths were dissected to reveal the crop inside the abdomen. A sterile 1 ml syringe was used to remove any content left in the crop of fed moths (as unfed moths never had liquid in the crop). Crop contents were weighed. Incremental volumes of the artificial nectar used for feeding were weighed to obtain a regression line. The equation from this regression was used to convert the weight of the removed crop contents into volume (μl). Due to the length of the sedentary lifetime experiment, a new batch of artificial nectar was made halfway through to prevent bacterial growth and change in artificial nectar concentration. The nectar regressions for the two experiments performed are as follows: 1. Sedentary lifetime experiment: $y = 995.28x - 3.2439$, $R^2 = 0.9994$, $p < 0.0001$, $n = 18$; $y = 963.7x - 0.1309$, $R^2 = 0.9997$, $p < 0.0001$, $n = 18$. 2. Activity experiment: $y = 954.78x + 3.0185$, $R^2 = 0.999$, $p < 0.0001$, $n = 17$. In all three regressions, nectar weight was on the x-axis and nectar volume on the y-axis. Moths were then placed in a −20 °C freezer for storage.

2.3. Tissue collection and $\delta^{13}C$ analysis

Whole-bodied moths were oven-dried for 72 h at 50 °C. The entirety of flight muscle and fat body were dissected from the dried thorax and abdomen, respectively, and then ground and homogenized in a mortar and pestle. Flight muscle makes up the majority of the thorax and is easily identified and extracted. Fat body is a loose tissue, that when dried, forms a white powdery substance that dries along the interior of the exoskeleton. Trachea and other tissues can be extracted from the powder to ensure more pure fat body collection. The homogenized flight muscle and fat body were collected in 0.75–1.0 mg samples and loaded into 6x4mm tin capsules. The $\delta^{13}C$ signature was measured using a Picarro (Santa Clara, CA) G2121-i cavity ring-down spectroscopy (CRDS) ^{13}C stable isotope analyzer with an A0502 ambient CO_2 interface, an A0201 combustion module, and an A0301 gas interface (CM-CRDS, (as described in Levin et al., 2016). All ^{13}C concentrations are expressed in $\delta^{13}C_{VPDB}$ (Werner & Brand, 2001). Data from the CRDS was recorded at 0.5 Hz using Picarro software. A less negative $\delta^{13}C$ value indicate higher amounts of ^{13}C. In fed moths, $\delta^{13}C$ values in a tissue that are less negative than baseline ($\delta^{13}C \approx -24.08$ ‰) indicate nectar allocation to that tissue.

2.4. Statistics

2.4.1. Sedentary lifetime experiment

Given the dearth of literature on crop emptying rates, particularly in Lepidoptera, we had no *a priori* reason to assume a particular shape of the emptying rate. We therefore first plotted the crop emptying rate for each experiment, then used the Akaike information criterion (AIC) to determine the best fit model for the change in crop contents over time in sedentary moths comparing linear and non-linear rate changes depending on the shape of the plot. Due to small sample sizes non-parametric tests were used. A Wilcoxon signed-rank test (μ=0) was used to determine if the crop was empty at each time interval. We tested each time interval sequentially and the crop was considered empty when the measured crop volume was no longer significantly different from 0 μl, therefore, a test for multiple comparisons was not needed. A Wilcoxon rank-sum test was used to determine differences in male and female crop contents. As nutrients can be stored, reabsorbed, and reallocated in insects, we could not assume a linear increase of consumed sugar over time (reviewed in Stjernholm et al., 2005). For regressions of tissue $\delta^{13}C$ of fed and unfed sedentary moths, AICc was used to determine best fit models due to small sample sizes (Hurvich & Tsai, 1989). Baseline (time = 0) of unfed tissue $\delta^{13}C$ values for each tissue type and sex were compared with Wilcoxon rank-sum tests to determine natural differences in amount of ^{13}C to inform future comparison of amount of cane sugar allocation. To determine relative allocation of consumed nutrients to fat body and flight muscle, time intervals were binned into time frames (0–6 h, 12–36, 48–72 h, and 84–108 h), and the percent change in $\delta^{13}C$ of tissue from fed moths and mean $\delta^{13}C$ of tissue from unfed moths was calculated for each time frame. For each time frame the samples sizes were 15 males and 15 females. We used Kruskal Wallis chi-square tests with Dunn-Bonferroni post hoc tests to determine differences in $\delta^{13}C$ across time frames for males and females. Differences in percent change of $\delta^{13}C$ between tissue type and sex were calculated with Wilcoxon rank-sum tests. Allocation of artificial nectar to tissue was measured by whether the percent change of $\delta^{13}C$ was significantly different from 0 % (no change from the mean of the $\delta^{13}C$ of unfed tissue) using a Wilcoxon signed-rank test (μ=0). Metabolism of allocated sugars was indicated if the percent change of $\delta^{13}C$ moved closer to 0 % over time.

2.4.2. Active experiment

AICc was used to determine best fit models for crop-emptying rate and tissue $\delta^{13}C$ for active and sedentary male and female moths. Wilcoxon signed-rank tests were used to determine differences in crop volume and $\delta^{13}C$ values of fat body and flight muscle in active and sedentary males and females.

2.4.3. Programs

Analyses were done in RStudio (R 4.3.1, packages: AICcmodavg (Mazerolle, 2023), cowplot (Wilke, 2023), dplyr (Wickham et al., 2023a), dunn.test (Dinno, 2017), gpubr (Kassambara, 2023b), lme4 (Bates et al., 2015), lsmeans (Lenth, 2016), minpack.lm (Elzhov et al., 2023), plyr (Wickham, 2011), rstatix (Kassambara, 2023a), tidyr (Wickham et al., 2023b), tidyverse (Wickham et al., 2019). We used JMP® Pro 16.2.0 for the Wilcoxon signed-rank test against a mean of zero with the Pratt method for including zeros. Significance was determined with $\alpha = 0.05$.

3. Results

3.1. *Crop-emptying of sedentary moths over lifetime*

Of the 500 μl of artificial nectar consumed, an average of 132.94 ± 45.98 μl of artificial nectar in females and 152.38 ± 56.20 μl of artificial nectar in males immediately bypassed the crop and was sent directly to the midgut (Fig. 1). There was no difference in crop volume in males and females immediately after feeding (time interval = 0) (Wilcoxon rank-sum test, W = 15, p = 0.691). The pattern of crop-emptying was best explained by an exponential decay function (Table S1).

Female and male crops were empty at 6 h after a feeding event (Table 1). Some individuals had artificial nectar left in their crop at 60, 84, and 108 h after feeding (Fig. 1), showing the potential of long-term nectar storage in the crop.

Table 1
Wilcoxon signed-rank test (μ=0) results comparing female and male crop volume of sedentary moths at each time interval to a mean of 0.

	female		male	
Time (hours)	W	p	W	p
0	7.5	0.031*	7.5	0.031*
1	7.5	0.031*	7.5	0.031*
6	7	0.063	7	0.063
12	4.5	0.250	7	0.063
24	0	0.500	2.5	0.500
36	2.5	0.500	0	0.500
48	2.5	0.500	0	0.500
60	2.5	0.500	0	0.500
72	0	0.500	2.5	0.500
84	0	0.500	2.5	0.500
96	0	0.500	0	0.500
108	0	0.500	2.5	0.500

Asterisks (*) indicate crop volume is significantly greater than 0. W = Wilcoxon signed-rank test statistic.

3.2. *Allocation of consumed sugar over sedentary lifetime*

Baseline: First, we determined the $\delta^{13}C$ values of the two tissue types, flight muscle and fat body, in unfed moths at time interval = 0, as this group represents moths that have not been fed and have not undergone experimental starvation. Flight muscle had an average $\delta^{13}C = -23.06 \pm 0.599$ ‰. There was no difference in $\delta^{13}C$ values between baseline male and female flight muscle (Wilcoxon rank-sum test, W = 21, p = 0.095). In the fat body, females had a significantly less negative $\delta^{13}C$ value than males (W = 79, p = 0.029), showing a greater enrichment of ^{13}C in the female fat body: female fat body has an average $\delta^{13}C = -26.036 \pm 0.588$ ‰ and male fat body has an average $\delta^{13}C = -26.575 \pm 0.365$ ‰. The $\delta^{13}C$ of flight muscle is significantly more enriched in ^{13}C than fat body (females: W = 0, p = 0.008; males: W = 0, p = 0.008). For this reason, tissue and sex are plotted separately for all allocation data.

Flight muscle: The relationship between the $\delta^{13}C$ of flight muscle and time in unfed females, unfed males and fed males were best explained by a linear regression (Fig. 2A,C; Table S2). In fed females, the relationship between the $\delta^{13}C$ of flight muscle and time is best fit with a logarithmic decay (Fig. 2A; Table S2). The slopes of these regressions were not significantly different from 0 (Table 2). The logarithmic decay in females occurred over the first hour after a feeding event, indicating rapid allocation of consumed artificial nectar to flight muscle during consumption followed by the metabolism of some of those sugars.

Fat body: The $\delta^{13}C$ of fat body over time in unfed females was best fit with a linear model and that of unfed males was best fit with a cubic model (Fig. 2B,D; Table S2). These significant regressions (Table 2) indicate that over the lifespan of the unfed moths, the $\delta^{13}C$ value of the fat body increased. This increase in the $\delta^{13}C$ is likely due to the preferential use of ^{12}C in metabolism, indicating the high metabolism of fat body in unfed moths. Fed male and fed female $\delta^{13}C$ of fat body both exhibit a logarithmic growth regression over time (Table S2), indicating quick allocation of consumed artificial nectar over the first 6 h following a feeding event before allocation begins to levels off, though these regressions were not significant (Table 2). *Relative Allocation:* In males,

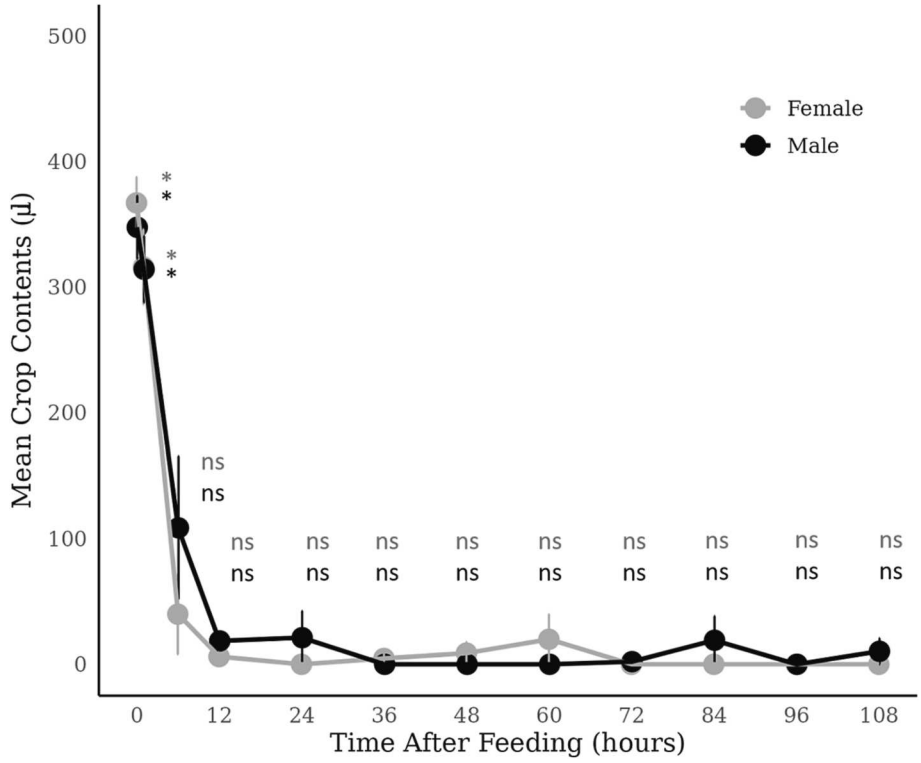

Fig. 1. Average volume of nectar in the crop over time in male (black) and female (grey) moths. Asterixis are significance of male (black) and female (grey) mean crop volume from a mean of 0: * = p < 0.05, ns = not significant. Bars depict standard error.

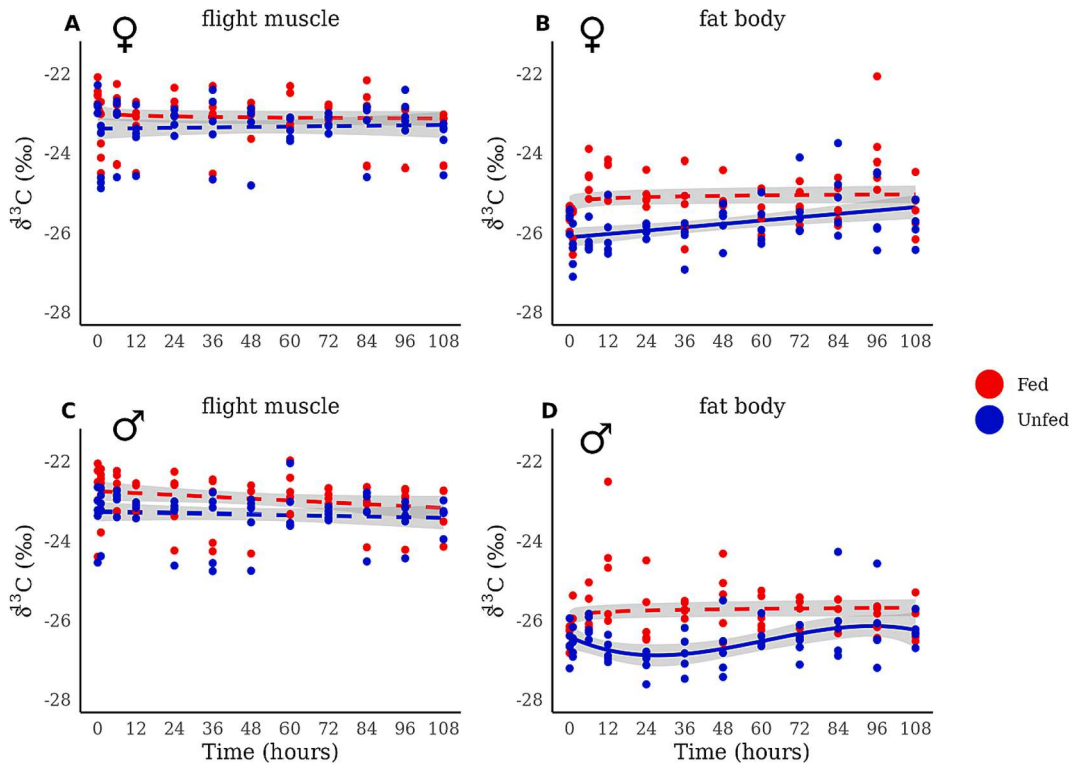

Fig. 2. The $\delta^{13}C$ values of *M. sexta* in A. female flight muscle, B. female fat body, C. male flight muscle, and D. male fat body. Tissue of fed moths in red, tissue of unfed moths in blue. 95% confidence interval is shown in grey. Dotted lines denote non-significance of regressions. Note that less negative $\delta^{13}C$ values indicate higher amounts of ^{13}C.

Table 2
Regressions for best fit models of tissue $\delta^{13}C$ in sedentary moths.

Flight muscle		Function	DV	IV	R^2	p
unfed	female	linear	δ13C	time	−0.015	0.704
	male	linear	δ13C	time	−0.009	0.502
fed	female	logarithmic decay	δ13C	time	0.026	0.113
	male	linear	δ13C	time	0.039	0.071
fat body						
unfed	female	linear	δ13C	time	0.152	0.001*
	male	cubic	δ13C	time	0.127	0.014*
fed	female	logarithmic growth	δ13C	time	0.040	0.068
	male	logarithmic growth	δ13C	time	0.046	0.056

Asterisks (*) indicate significance. DV = dependent variable. IV = independent variable.

Table 3
Wilcoxon signed-rank test (μ=0) results comparing the percent change of $\delta^{13}C$ of fed tissue to a mean of 0 % change.

	female			male	
tissue	Time frame (hours)	W	p	W	p
flight muscle	0–6	32	0.060	20	0.011*
	12–36	29	0.042*	18	0.008*
	48–72	60	0.511	15	0.004*
	84–108	39	0.126	44	0.195
fat body	0–6	9	0.001*	24	0.021*
	12–36	0	<0.001*	0	<0.001*
	48–72	11	0.002*	1	<0.001*
	84–108	27	0.319	25	0.024*

Asterisks (*) indicate the percent change is significantly different from 0 (no change from the $\delta^{13}C$ of the unfed tissue). W = Wilcoxon signed-rank test statistic.

consumed cane sugar was allocated to flight muscle 0–6 h after feeding, as the crop is being emptied (Table 3). In males, this allocated cane sugar was completely metabolized by 84–108 h after the feeding event, as the percent change was no longer significantly different from 0 %. However, male allocation to flight muscle did not change significantly across time intervals (Kruskal-Wallis chi-squared = 5.767, df = 3, p = 0.124), indicating they hold onto these allocated sugars for an extended time (Fig. 3B).

In females, the percent change of $\delta^{13}C$ in flight muscle was not significantly different from 0 % until 12–36 h after a feeding event (Table 3), after their crops have already emptied. Allocated cane sugar was then metabolized from flight muscle by 48–72 h after the feeding event (Fig. 3A). Female allocation did differ across time frames (Kruskal-Wallis chi-squared = 8.809, df = 3, p = 0.032), with allocation greater at 0–6 h post feeding than 48–72 h post feeding (Dunn-Bonferroni post hoc: z = -2.864, p = 0.013), again showing that some of the allocated sugars in the flight muscle had been metabolized by this time (Fig. 3A).

The only time frames at which males and females significantly differed in the percent change of $\delta^{13}C$ of flight muscle was 48–72 h post feeding (Wilcoxon rank-sum test, W = 185, p = 0.00196). Males and females did not significantly differ in amount of sugar allocated to flight muscle at any other time frame (Wilcoxon rank sum test, 0–6: W = 122, p = 0.713; 12–36: W = 144, p = 0.202; 84–108: W = 130, p = 0.486).

Male allocation to fat body differed across time frames (Kruskal-Wallis chi-squared = 23.936, df = 3, p=<0.0001) (Fig. 3B). A Dunn-Bonferroni post hoc test showed that the percent change in fat body significantly increases between 0 and 6 h post feeding and 12–36 h post feeding (z = 3.847133, p = 0.0004), indicating continued allocation of consumed cane sugar to fat body. The percent change of $\delta^{13}C$ in 12–36 h

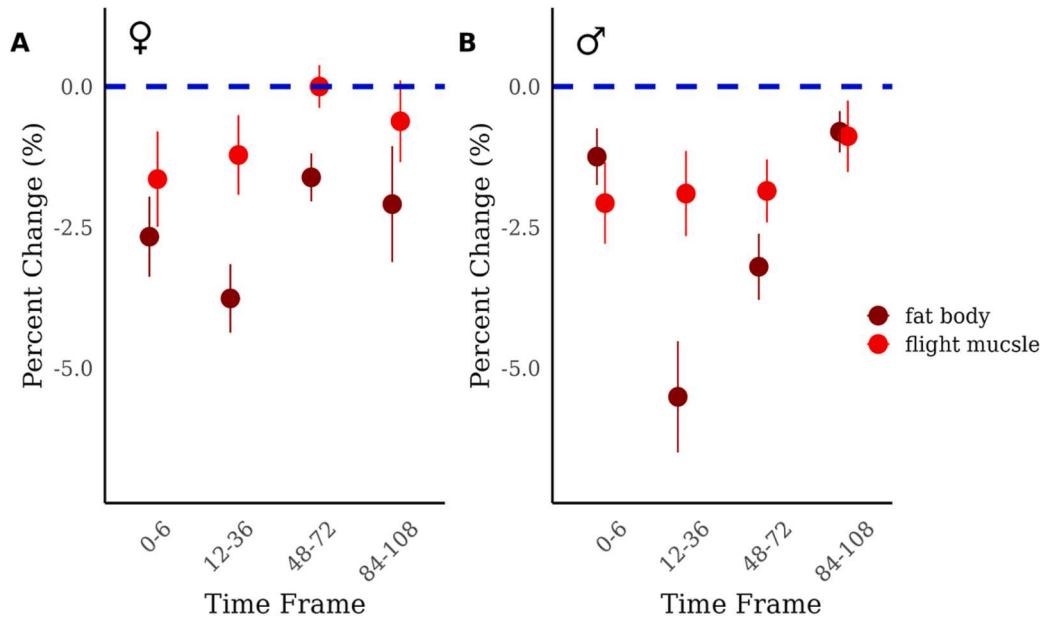

Fig 3. The percent change of $\delta^{13}C$ values of tissue of fed moths relative to the mean of unfed moths, showing the relative allocation of consumed cane sugar, in A. females and B. males. Time intervals are binned into four time frames. Fat body is shown in dark red and flight muscle is shown in light red. Bars are standard error. The blue dotted line indicates 0% change from mean value of unfed tissue. Note the more negative the percent change, the greater the amount of ^{13}C, and thus greater nectar allocation.

and 48–72 h post feeding was significantly greater than 84–108 h post feeding (Dunn-Bonferroni post hoc: 12–36: z = -4.255, p = 0.0001; 48–72: z = 2.676, p = 0.022), indicating the metabolism of some of these allocated sugars. In females, the percent change in $\delta^{13}C$ in fat body also changes across time frames (Kruskal-Wallis chi-squared = 8.277, df = 3, p = 0.041). The percent change of $\delta^{13}C$ of fat body lessened from 12 to 36 h post feeding to 48–72 h post feeding (Dunn-Bonferroni post hoc: z = -2.624, p = 0.026), indicating the sugar in the fat body was metabolized during this time frame.

The differences between percent change of $\delta^{13}C$ of tissues was examined by sex. In females, percent change in $\delta^{13}C$ is greater in fat body than flight muscle at 12–36 h post feeding (Wilcoxon rank-sum test, W = 46, p = 0.005) and 48–72 h post feeding (W = 47, p = 0.006), indicating a preferential allocation to fat body than flight muscle at these time frames (Fig. 3). The percent change of $\delta^{13}C$ between fat body and flight muscle in females did not differ at the other time frames

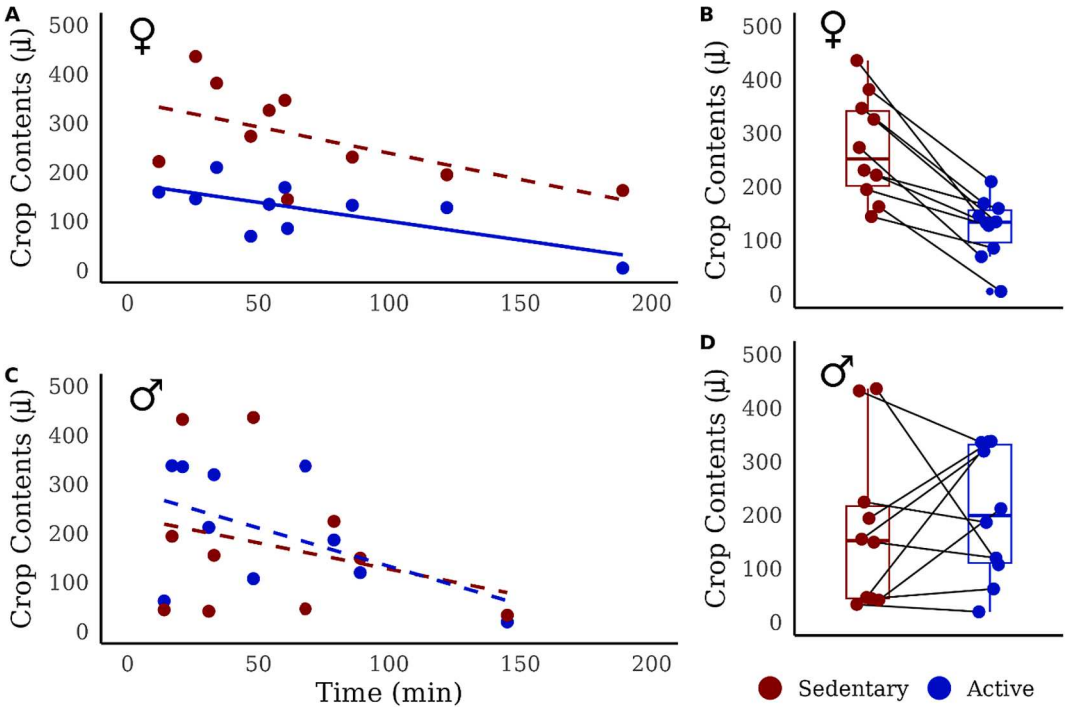

Fig. 4. The volume of artificial nectar in crops of A. females and C. males. Sedentary moths are shown in red and active moths are shown in blue. B. and D. depict the crop volumes of match-paired sedentary and active moths remained sedentary or were flown for the same amount of time, respectively. Dotted lines denote non-significance of regressions.

(0–6: W = 105, p = 0.775; 84–108: W = 91, p = 0.389). The percent change of $\delta^{13}C$ between tissues in males only differed at 12–36 h post feeding, with greater allocation to fat body than flight muscle (W = 52, p = 0.011). At all other time frames, the percent change of $\delta^{13}C$ in flight muscle and fat body did not differ (0–6: W = 149, p = 0.137; 12–36: W = 83, p = 0.233; 84–108: W = 134, p = 0.389).

3.3. Effects of flight on crop-emptying

There was an average flight time of 69.1 ± 60.0 min for females and 54.5 ± 40.2 min in males. Flight time between males and females did not differ (Wilcoxon rank-sum test, W = 58, p = 0.579). The crop-emptying rate for active males, active females, sedentary males, and sedentary females were all best fit with a linear model (Fig. 4A, C; Table S3). Only the regression of active females was significant (Table 4). Flown females had significantly less nectar in their crops than sedentary females (Wilcox signed-rank test, W = 0, p = 0.002) (Fig. 4B), but no difference in crop volume was found between active and sedentary males (W = 32, p = 0) (Fig. 4D).

3.4. Effects of flight on allocation

3.4.1. Flight muscle

In active females, sedentary females, and sedentary males, the relationship between the $\delta^{13}C$ of flight muscle and time was best fit by a linear model (Fig. 5A, C; Table S4).

While these linear regressions were not significant (Table 4), the regression of sedentary males trends upwards, indicating possible allocation of cane sugar to flight muscle over time. In active males, the $\delta^{13}C$ of flight muscle over time was best fit with a logarithmic decay (Table S4), also not significant (Table 4). There was no difference in the $\delta^{13}C$ of flight muscle between sedentary and active moths in either males or females (Wilcoxon signed-rank test, males: W = 13, p = 0.160; females: W = 17, p = 0.322) (Fig. 5B, D). These results show that flight did not affect allocation of nectar sugars to flight muscle.

3.4.2. Fat body

Linear models best explained the change in $\delta^{13}C$ of fat body for active males, sedentary males, and active females (Fig. 6A, C; Table S4). The $\delta^{13}C$ of fat body in sedentary females was best fit with a logarithmic decay (Fig. 6A; Table S4). None of these regressions were significant (Table 4). There was no significant difference in the $\delta^{13}C$ values of fat body of flown and sedentary moths in either males or females (Wilcoxon signed-rank test, males: males: VW = 25, p = 0.846; females: w = 32, p = 0.695) (Fig. 6B, D). These results show that flight did not affect allocation of consumed sugars to fat body.

4. Discussion

In this study, we examined the crop-emptying rate and sugar allocation of a single nectar meal in an insect nectarivore as the timing of nutrient allocation and allocation choices have consequences on organismal fitness and performance. Lepidoptera are important pollinators in wild and agricultural settings (Requier et al., 2023, Walton et al., 2020). An understanding of their foraging and resource allocation behaviors can better inform conservation efforts and agricultural crop yield.

4.1. Crop-emptying rates

In sedentary moths, 500 μl of artificial nectar either bypassed the crop or were released from the crop by 6 h after a feeding event (Fig. 1). While differences in male and female crop-emptying rate were predicted, sex had no effect on crop-emptying rate while the moths were sedentary. The crop-emptying rate of *M. sexta* was faster than those recorded in other insects. Blowflies still had 25 % of a meal in their crop 26 h after a feeding event (Stoffolano, 2019), swallowtail butterflies still contained "large volumes" of nectar in the crop 24 h after a feeding event (Nicolson, 1980), and bees exhibited a crop-emptying rate of 30 μl/hr on 30 % sucrose solution (Roces & Blatt, 1999) while in this study *M. sexta* had an overall rate of ~ 60 μl/hr on the 25 % sugar solution, though the different methodologies among the studies makes direct comparisons difficult.

Sex specific differences in crop-emptying were found with flight activity. Flown females had emptied more nectar from their crops than sedentary females, but no effect of flight was found in males. As crop-emptying rate is increased with increased metabolic output in bees (Blatt & Roces 2002a,b), it was unexpected that flight did not affect crop volume in males.

Flying females may empty their crops faster than males due to differences in reproductive behaviors. First, flight increases allocation of carbon from consumed nectar to eggs in female butterflies (Niitepõld &

Table 4
Regressions for best fit models of tissue $\delta^{13}C$ in active and sedentary moths.

crop-emptying		function	DV	IV	R^2	p
female	sedentary	linear	crop volume	time sedentary	0.245	0.083
	active	linear	crop volume	time flown	0.423	0.0248*
male	sedentary	linear	crop volume	time sedentary	−0.031	0.419
	active	linear	crop volume	time flown	0.181	0.122
flight muscle						
female	sedentary	linear	δ13C	time sedentary	−0.122	0.884
	active	linear	δ13C	time flown	−0.098	0.671
male	sedentary	linear	δ13C	time sedentary	0.253	0.079
	active	logarithmic decay	δ13C	time flown	0.004	0.338
fat body						
female	sedentary	logarithmic decay	δ13C	time sedentary	0.276	0.068
	active	linear	δ13C	time flown	−0.116	0.801
male	sedentary	linear	δ13C	time sedentary	−0.062	0.511
	active	linear	δ13C	time flown	−0.111	0.760

DV = dependent variable, IV = independent variable. Asterisks (*) indicate significance.

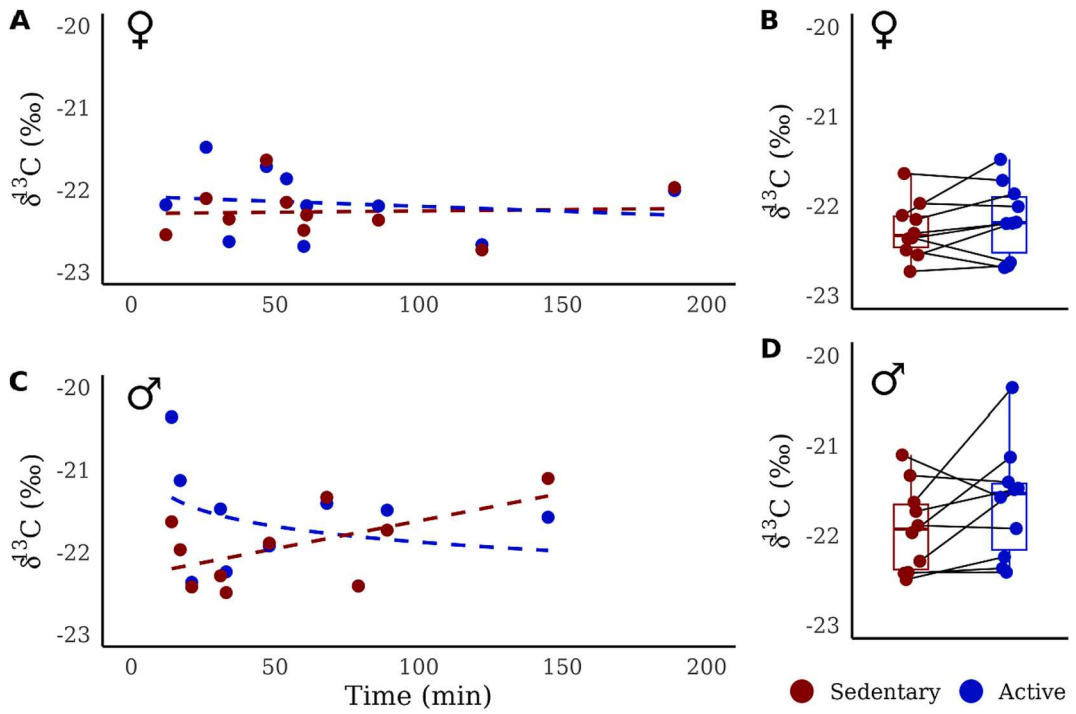

Fig. 5. The $\delta^{13}C$ value of flight muscle in A. females and C. males. B. and D. depict the $\delta^{13}C$ values of flight muscle of paired sedentary and active moths in females and males remained sedentary or were flown for the same amount of time, respectively. Dotted lines denote non-significance of regressions. Sedentary moths are shown in red and active (flown) moths are shown in blue.

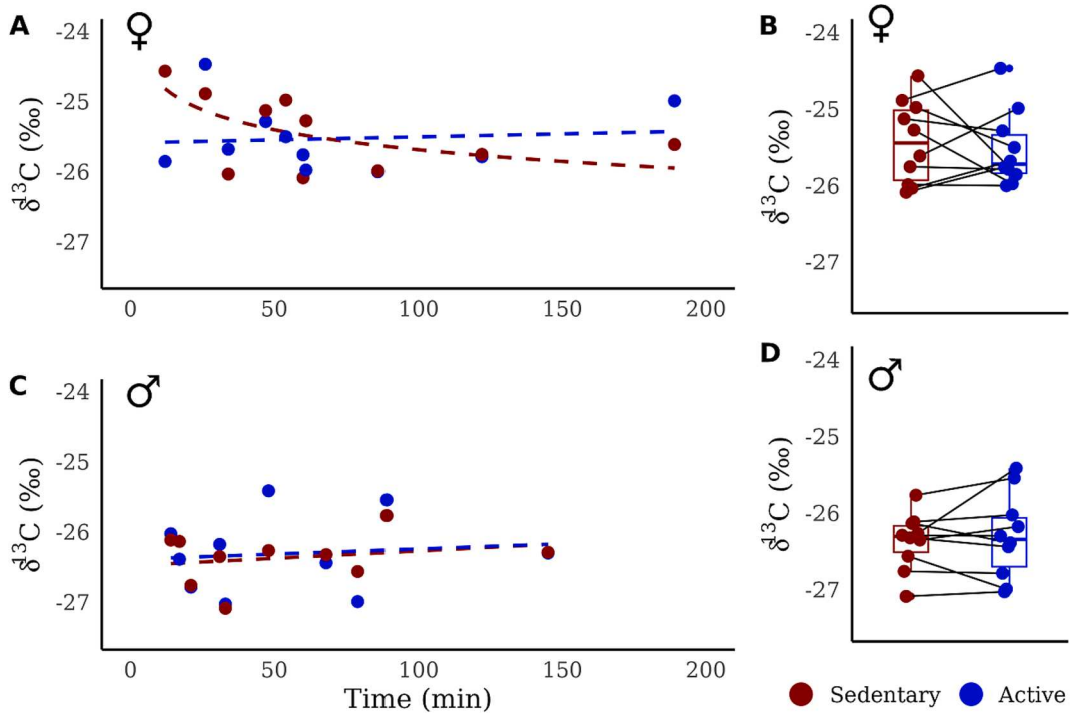

Fig. 6. The $\delta^{13}C$ value of fat body in A. females and C. males. B. and D. depict the $\delta^{13}C$ values of fat body of match-paired sedentary and active moths remained sedentary or were flown for the same amount of time, respectively. Dotted lines denote non-significance of regression. Sedentary moths are shown in red and active (flown) moths are shown in blue.

Boggs, 2022). Increased mobilization of consumed nutrients for reproduction may be underlying the increased crop-emptying. Second, females' reproductive behavior allows them to encounter floral resources more often than males. Female reproductive output relies on laying eggs on the leaves of *D. wrightii*, and females will often feed from the flowers before and after ovipositing (Raguso et al., 2003). If females are likely to encounter a new food source, they may empty their crop faster to fuel flight and allow for further storage of subsequent nectar meals. On the other hand, male *M. sexta* reproductive success may be driven by scramble competition, where males that are more successful at locating

females have higher fitness (Andersson & Iwasa, 1996, Levin et al., 2016). Compared to female *M. sexta*, males are more sensitive to pheromones (Christensen et al., 1989), less sensitive to *D. wrightii* volatiles (Fraser et al., 2003), and less likely to feed (Ziegler, 1991). If males are more driven to find mates than food, males may store nectar in their crops longer than females as they are less likely to encounter another floral food source. Additionally, the slower crop-emptying rate of males may inhibit their subsequent feeding behavior. A slower crop-emptying rate is correlated with increased perching and decreased frequency of foraging bouts in hummingbirds (López-Calleja et al., 1997, Diamond et al., 1986, Karasov et al., 1986) as well as increased feeding inhibition and decreased activity in flies (Dethier, 1976, Browne and Evans, 1960, Choi et al., 2017, Piñero et al., 2021). The slower crop-emptying rate found in *M. sexta* males may inhibit feeding behaviors in flight, leading to an increase in mate-searching behaviors.

4.2. Nectar allocation

4.2.1. Sedentary lifetime

While there was no significant difference between male and female allocation to flight muscle in the first 6 h after the feeding event, male allocation to flight muscle was significantly greater than zero, while female allocation was not (Fig. 3). As predicted, males exhibited greater investment into flight muscles than females, with consumed sugar allocated to flight muscles sooner and stored for longer than in females (Fig. 3). As flight is important for males in finding mates, sedentary males may retain nectar-derived sugars to power future flight. As allocation to flight muscle in females is not significant until after their crop has emptied, nectar sugars are primarily allocated to functions other than flight muscles initially, possibly to metabolism, fat body, or egg production. We may expect to find these allocation pattern differences between male and female flight muscle across Lepidoptera or other taxa where males use flight to find females or defend territories. In both males and females there was a decrease in the percent change in the $\delta^{13}C$ of fed moths in later time frames (Fig. 3), indicating sugars allocated to flight muscle were metabolized. While none of the regressions of the $\delta^{13}C$ of flight muscle over time were statistically significant (Fig. 2A, C), fed females did exhibit a logarithmic decay and fed males had a negative linear slope, indicating that allocated ^{13}C in flight muscles are being metabolized over time which is consistent with Fig. 3.

As predicted, resting moths preferentially stored nectar sugars in the fat body over flight muscles. Within the first 12–36 h of a feeding event, allocation in both males and females was significantly greater to fat body than to flight muscle (Fig. 3). This trend is evident for males and females at each time frame, apart from 0 to 6 h post feeding in males, which trend toward initially allocating more to flight muscle than fat body, though these trends were not significantly different.

As discussed above, the $\delta^{13}C$ of unfed male and female fat body increases over time due to the depletion of ^{12}C in the fat body. Therefore, the decreases in the percent change of $\delta^{13}C$ of fat body in the later time frames do not give an accurate representation of the amount of allocated sugar being metabolized. However, as fat body percent change of $\delta^{13}C$ in males and females were significantly greater than the mean $\delta^{13}C$ of the unfed fat body, we can conclude that some cane sugar allocated to fat body remained in the fat body for the entire length of the experiment.

4.2.2. Effects of flight

We expected flight to increase the crop-emptying rate and allocation of consumed sugar to flight muscles and fat body in both males and females. However, flight only affected the crop-emptying rate of females, increased initial allocation to flight muscles in males, and did not affect allocation into the fat body in either sex.

It is likely that the increased amount of nectar sugars released from the crop in females were metabolized immediately to fuel flight, as allocation to flight muscle and fat body were unaffected in flying females. This agrees with previous studies that have shown that fed moths preferentially fuel flight with carbohydrates before switching to lipids (Ziegler & Schulz 1986b, O'Brien, 1999).

While there was not an overall difference in the $\delta^{13}C$ of flight muscle in flown and sedentary males, moths that had shorter flights (14–33 min) had greater amounts of ^{13}C in flight muscle than their sedentary matched-pairs (Fig. 5), with the shortest flight having the greatest amount of ^{13}C. This pattern was not present in males which underwent longer flights. Males may have allocated a pulse of consumed sugars to flight muscle at the onset of flight, and then metabolized those sugars over the first 30 min of flight. As crop volume was not different between active and sedentary moths, flight onset could have triggered the redirection of consumed sugars already released from the crop. Larger sample sizes would be necessary to fully elucidate this potential trend.

Flight did not affect the allocation of consumed sugar to fat body. There were weak, non-significant upward trends in the $\delta^{13}C$ of fat body in active males and females. However, we see this trend in sedentary males as well, indicating this small increase in the $\delta^{13}C$ of fat body in active moths is likely not due to increased flight activity. While carbohydrates are used to fuel flight initially, lipids are the main fuel source for flight in *M. sexta* (Ziegler & Schulz1986a,b). As such, we expected flight to increase allocation of nectar to the fat body to increase lipid stores to be used throughout the duration of flight. However, in Ziegler and Schulz's (1986a,b) studies on nutrient use in flight, moths were unfed. It is possible *M. sexta* utilize carbohydrates as opposed to lipids to fuel flight for longer durations than previously observed if they have sugar-rich nectar stored in their crops. As *M. sexta* can fly an average of 5 km, and as many as 18 km, in a night (Davidowitz, unpublished), longer flights and multiple bouts of flights could lead to differences in sugar allocation to flight muscle and fat body.

Allocation strategies of pollinators can affect foraging behavior. Crop-emptying rate is the rate limiting step from the acquisition of nutrients to the allocation of those acquired nutrients (Treherne, 1967). Both crop-emptying rate and allocation strategies affect future foraging behavior (Piñero et al., 2021, Boggs, 1992), which in turn affect the fitness of plants visited. For instance, visiting time and number of visits often determines the amount of pollen transferred to and picked up from the plant (Zimmerman 1983, Mitchell 1993, Kiatoko et al. 2023), and the number of and distance among plants visited will increase the genetic diversity of pollen transferred (Brunet and Sweet 2006, Brunet and Homquist 2009). Understanding the timing of crop-emptying and the allocation decisions of pollinators will garner greater insights into pollinator-plant interactions and their fitnesses.

Overall, we found males and females exhibit sex specific digestive physiology and allocation strategies; males had slower crop-emptying rates than females and exhibited greater allocation of consumed sugar to flight muscle than females. These differences align with strategies that may help increase reproductive fitness for each sex as well as the observed differences in male and female *M. sexta* foraging behavior. This highlights the potential of distinct male and female digestive physiology (due to differences in fitness strategies) to result in differences in male and female effectiveness as pollinators.

In this study, we examined how a single, large nectar meal is stored, allocated, and used over time by both active and sedentary moths. A deeper understanding of digestive physiology, such as nectar storage in the crop and how and when nectar nutrients are used, will help us predict how nectar consumption affects fitness of both pollinators and their host plants. With habitat loss and fragmentation increasing, bouts of starvation may become more common and increased flight durations may be needed to access food sources in pollinators (Lebeau et al., 2016). Change in food source availability and increase in flight may alter pollinator resource pools and their allocation of consumed resources. For example, increased habitat fragmentation has led different populations of fritillary butterflies to change investment in flight (Hanski et al., 2002, Schtickzelle et al., 2006). Continued research on underlying physiology in insect pollinators, especially the relatively understudied Lepidoptera, will provide a better understanding of how nectar feeding

behavior links both insect fitness and plant fitness.

CRediT authorship contribution statement

Noah DeFino: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Visualization, Writing – original draft, Writing – review & editing. **Goggy Davidowitz:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Noah DeFino reports financial support was provided by National Science Foundation.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jinsphys.2024.104617.

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