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Sex differences in the utilization of essential and non-essential amino acids in a Lepidoptera

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Summary statement: Young adult male moths oxidized greater amounts of larval-derived amino-acids than females, and more nectar derived amino-acids after feeding. Under starvation, adult females exhibited the opposite pattern.

Abstract

The different reproductive strategies of males and females underlie differences in behavior that may also lead to differences in nutrient use between the two sexes. We study sex differences in the utilization of two essential amino acids (EAAs) and one non-essential amino-acid (NEAA) by the Carolina sphinx moth (*Manduca sexta*). On day one post-eclosion from the pupae, adult male moths oxidized greater amounts of larval-derived AAs than females, and more nectar derived AAs after feeding. After four days of starvation the opposite pattern was observed; adult females oxidized more larval-derived AAs than males. Adult males allocated comparatively small amounts of nectar-derived amino acids to their first spermatophore, but this allocation increased substantially in the second and third spermatophores. Males allocated significantly more adult derived AAs to their flight muscle than females. These outcomes indicate that adult male and female moths employ different strategies for allocation and oxidation of dietary AAs.

Introduction

The resource investment of male sperm is considered to be low compared to the large and nutrient-rich oocyte produced by the females (Parker, 1982). This has led to the suggestion that there are differences in behavior and mating strategies between the two sexes (Lauwers and Van Dyck, 2006; Levin et al., 2016). Female lepidopterans typically lay hundreds of protein-rich eggs (Levin et al., 2016; O'Brien et al., 2002) during a narrow reproductive window that begins after eclosion from the pupa, and may last only a few days.

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Therefore, it is reasonable to predict that the amino acids (AAs) accumulated from their larval diets could be a limiting resource for egg production.

It is well established that vertebrates 'spare' protein oxidation until after carbohydrates and lipids have been consumed (Castellini and Rea, 1992; Wang et al. 2006; McCue, 2010, 2012). In contrast, some insects preferentially oxidize proteins even when carbohydrates and lipids are readily available (McCue et al. 2015). It is not clear whether protein oxidation strategies differ between the sexes.

Both male and female lepidopterans often consume floral nectars that can contain significant amounts of AAs (Baker and Baker, 1973; Baker and Baker, 1975), and the consumption of nectar has been shown to increase longevity and reproductive outputs (see (Mevi-Schütz and Erhardt, 2005). O'Brien (2000) originally showed that nonessential amino acids (NEAAs) derived from the nectar-sugar consumed by adult females was vertically transferred to the eggs, but was unable to observe the same outcome for essential amino acids (EAAs, (O'Brien et al., 2000)). Using a more sensitive technique, we recently showed that nectar EAAs are transferred to eggs (Levin et al., 2017b). Because males do not face the high nutrient demands of egg production they may be less dependent on dietary AA inputs during adulthood. The specific mechanisms by which these nectar AAs affect the fitness of lepidopterans is still not well understood.

Research has shown that male and female Lepidoptera prefer flowers with different nectar composition (Alarcon et al., 2010). Males generally prefer sucrose based nectar, whereas females prefer nectar with glucose and AA (Rusterholz and Erhardt, 2000). More recently, Mevi-Schutz and Erhardt (2005) concluded that 1) female butterflies selected amino-acid-rich nectar for egg production, and 2) males, either showed no preference, or selected carbohydrate-rich nectar, presumably to fuel the high costs of flight during mate searches. In addition, we previously found that adult female Lepidoptera oxidize a proportion

of nectar AAs as metabolic fuel during rest, but did not test for sex-specific differences (Levin et al., 2017a). In this study, we examine differences in how male and female Lepidoptera use AAs in their diet. Additionally, we quantify how the timing of the dietary AA inputs, whether consumed as part of the larval diet or in the floral nectars consumed during adulthood, affects how AAs are utilized by the adults.

Materials and methods

We used the Carolina sphinx moth (*Manduca sexta*: Sphingidae) from a breeding colony maintained for over 140 generations at the University of Arizona (Davidowitz and Nijhout, 2004; Davidowitz et al., 2012). Larvae were raised under a 16:8 light:dark photo-cycle in an environmentally controlled room set at 27°C and 50% RH. Larvae were fed *ad libitum* with a standard laboratory diet (Davidowitz et al., 2003). Pupae that were ready to eclose (19-25 days after pupation and 1 day before eclosion) were removed from the colony daily and isolated. We used this population as the source for the eggs, larvae and adults used in the experiments described below. Individuals from this population were also measured to establish baseline/background ^{13}C-values in the breath and tissues of moths that had no exposure to ^{13}C-tracers (see below).

Oxidation of larval-derived AAs – In order to examine how adult moths use the AAs derived from their larval diets we raised a population of larvae on an engineered diet containing ^{13}C-labeled EAA. About 200 eggs collected from the breeding colony were raised until pupation on the standard diet (see above) supplemented with ^{13}C-leucine (1g 1-^{13}C-leucine to 3 L wet diet). All ^{13}C-tracers were purchased from Cambridge Isotope

Laboratories, Inc. (Tewksbury, MA). After eclosion, adult moths were placed in individual waxed paper bags to minimize activity.

We measured the $^{13}CO_2$ content in the exhaled breath of adult males (n=9) and females (n=10) within one day of eclosion to adulthood. We repeated these measurements four days later on the same moths without access to nectar, thereby simulating food limitation during adulthood. This period of food limitation is ecologically realistic, and it is not uncommon for adults in nature to not have fed on nectar (Levin et al., 2016).

Moths were placed individually into a 200 ml sealed plastic chamber in a temperature controlled room at 27°C. Bottled, dry, CO_2 free air, was then passed through the chamber at a flow rate of 150 ml min^{-1} using a mass flow controller (Alicat, Tucson, AZ, USA). A subsample of 30 ml min^{-1} was pulled from an excurrent manifold directly into a Picarro (Sunnyvale, CA) G2121-i Cavity Ring-Down Spectroscopy (CRDS) $\delta^{13}C$ stable isotope analyzer with an A0502 ambient CO_2 interface. Values were recorded at 0.5 Hz using Picarro software, and we used the mean value collected over a 5-minute period while the moths were at complete rest. All ^{13}C concentrations are expressed in $\delta^{13}C_{VPDB}$ (Slater, 2004; Werner and Brand, 2001). Note that larger (i.e., more postive) $\delta^{13}C$ values indicate higher ^{13}C enrichment.

Use of adult-derived AAs from nectar – To examine how adult moths use the AAs derived from nectar meals we created artificial nectars made of 25% (by weight) beet sugar (beet sugar $\delta^{13}C \approx -26.5‰$) in deionized water. The nectars were isotopically enriched (0.2 g L^{-1}) with one of three ^{13}C-labeled AAs: 1-^{13}C-glycine (Gly, 2.6 mM, non-essential), 1-^{13}C-leucine (Leu, 1.5 mM, essential, branched), or 1-^{13}C phenylalanine (Phe, 1.2 mM, essential, aromatic). These AA concentrations are equal to, or lower than, the concentrations found in natural nectars, and are similar to those used in previously published studies (Gardener et al., 2003; O'Brien et al., 2000).

Tissue allocation in male moths – Male pupae that were ready to eclose were collected randomly from the breeding population and placed into individual cages (30 × 30 × 30 cm, BugDorm, UK). From the day of eclosion, adult males were fed daily with 250 µl of one of the three ^{13}C-labeled nectars. We used five males for each of the three AA treatments (total: n=15 males). The artificial nectar was placed on a piece of Parafilm^{TM} (Pechiney Plastic Packaging Company, USA) in five droplets of 50 µl each. Each moth's proboscis was unfurled using a long needle and its tip placed in a droplet. The moths were observed to consume all of the nectar they were offered.

On the second day, one virgin female was randomly selected from the breeding population and placed inside the cage of a ^{13}C-nectar-fed male. These cages were maintained in the same conditions as those used for the larval rearing with the addition of an artificial 'moon' light during the eight-hour scotophase to ensure mating. At the end of the scotophase these females were removed from the cages and males were fed an additional 250 µl of the ^{13}C-labeled nectar. Afterwards a fresh virgin female was placed in the cage. This cycle was repeated every other day so that each male mated a total of three times.

Mated females (confirmed by the presence of a spermatophore) were killed immediately after the end of the scotophase by freezing in -20°C for 2h. These were thawed and surgical scissors used to remove the spermatophore from the bursa copulatrix. These spermatophores were then dried at 50ºC until they reached a constant mass ± 0.01 mg (analytical balance, Mettler-Toledo XS). The dry spermatophores were ground by mortar and pestle and 1 mg of the homogenate was loaded into a tin capsule. The δ^{13}C of each sample was measured using the Cavity Ring-Down Spectroscopy δ^{13}C stable isotope analyzer described above paired with an A0201 Combustion Module (Picarro) and an A0301 gas interface (CM-CRDS).

After the third mating (day 5), the males were killed by freezing in -20°C for 2h. Their flight muscles dissected using surgical scissors and dried before measuring $\delta^{13}C$ as described above.

Tissue allocation in female moths – Five female pupae were randomly selected from the breeding colony and fed after eclosion one of the ^{13}C-labeled nectars described above (250 µl a day). These females were mated to an unlabeled colony male and allowed to lay eggs on an oviposition platform with host-plant extract (see Levin et al., 2016). Females were killed on the morning of day five after eclosion (as with the males), their flight muscles removed, and $\delta^{13}C$ values measured as described above for the males.

Oxidation of nectar AAs from the adult diets – Males (n=4) and Females (n=4) were used to examine how moths oxidized the AAs in the nectar. Recently eclosed (1 days after eclosion) adults from the breeding population were fed 200 µl of one of the three labeled AA artificial nectars as described above (n=24 moths in total). The $\delta^{13}C$ in the exhaled CO_2 was measured for 120-180 minutes with maximum $\delta^{13}C$ values usually observed ~90 minutes after feeding. We used the peak $\delta^{13}C$ values of each individual for statistical comparisons. All statistics were conducted using JMP software (JMP 11.0, USA), and $\alpha = 0.05$ was used to determine statistical significance.

Results

Oxidization of larval derived AA – The $\delta^{13}C$ of all adults raised on the ^{13}C-leucine diets were significantly higher than background levels of control moths (one-sample t-tests against a mean, $p \leq 0.0062$ in all cases). On the first day after eclosion from the pupae, the $\delta^{13}C$ in the exhaled CO_2 of the males was significantly higher than females (Fig. 1, t-test assuming unequal variances, t=5.33, df=11.38, P=0.0002). On day four post-eclosion, the opposite trend was observed whereby females had a significantly higher $\delta^{13}C$ than the males (t=-3.48, df=15.99, P=0.0031).

Oxidation of adult derived AAs – The $\delta^{13}C$ in the exhaled breath began to increase minutes after feeding and reached maximum values by 150 minutes (Supplementary Figure 1). Males had significantly higher maximal $\delta^{13}C$ values in their breath when fed either of the EAAs (Fig. 2, Kruskal-Wallis Rank Sums test: leucine, P=0.0339; phenylalanine P=0.0339), but there was no significant difference between the sexes when fed with the NEAA glycine (Fig. 2, P=0.5637).

Allocation of nectar AAs to muscle – The $\delta^{13}C$ in the muscles of males and females fed all three AAs were elevated from background levels ($P \leq 0.0251$ in all cases; Fig. 3). In males, the $\delta^{13}C$ values in the muscle only differed between the leucine and glycine treatments (P=0.0216). We found significantly higher $\delta^{13}C$ values in the muscles of males than in the females across all three AA treatments (Kruskal-Wallis rank sums test; glycine, P=0.0122; leucine, P=0.0122; phenylalanine, P= 0.0358; Fig. 3). In female flight muscles, the $\delta^{13}C$ values were higher in the NEAA treatment than the EAA treatments (Wilcoxon multiple comparison test; leucine-glycine, P=0.0216; phenylalanine-glycine, P=0.0119).

Males allocate significantly more resources to flight (t-test assuming equal variances, $P<0.0001$, $t_{1,177} = 8.86$) and have comparatively larger flight muscles (21.66%±0.02sd, of dry

body mass, n=92) than females (19.26%±0.015sd, n=88). $\delta^{13}C$ values are size independent; however, because male flight muscles are larger than in females, the absolute amounts of AAs allocated to male flight muscle is even greater than indicated solely by the $\delta^{13}C$ shown in Figure 3 (McCue, 2011).

Allocation of nectar derived AAs to male reproduction – Significant ^{13}C enrichment above background levels was seen in the spermatophores of males consuming the AA tracers at all time points (one-sample t-tests; $P \leq 0.0001$ in all cases). Comparatively small amounts of the AAs were allocated to the first spermatophore, but the subsequent spermatophores contained much higher ^{13}C-enrichments (Fig. 4). The $\delta^{13}C$ values in the first spermatophores did not differ significantly among the three AA treatments (Kruskal-Wallis Rank Sums test, P=0.1845). In the second spermatophore, only phenylalanine and leucine differed significantly (P=0.0122) from one another. We did not detect any significant differences among the three AAs in the third spermatophore (P=0.5527). Allocation of NEAAs and EAAs in the female eggs are given elsewhere (Levin et al., 2017a).

Discussion

We found fundamental differences in the way adult male and female *M. sex*ta use their dietary AAs. These sexual differences exist when the AAs were both originally derived from the larval diet as well as when they were derived from the nectars consumed by adult moths.

The current paradigm for fuel use during fasting and food limitation in animals is that proteins will be oxidized only after carbohydrate and lipid reserves have been exhausted (McCue, 2012). This paradigm is well established for vertebrates (Castellini and Rea, 1992; Wang et al., 2006), but less well developed for other taxa (McCue et al., 2010; Secor and

Carey, 2016). In a recent comparative study across species and life stages, it was shown that insects do not fit into this paradigm, for example, some insects increase their use of amino acids as a metabolic fuel at the onset of food limitation; however, that study did not compare males and females (McCue et al., 2015). In the present study, adult male and female moths initially had dramatically different reliance on AA oxidation and the direction of this difference reversed as fasting progressed. Under positive AA balance (when moths just eclosed from the pupae or when fed nectar with AAs) males will oxidize AAs in higher ratios then females. Under starvation, males will conserve AAs while females will oxidize AAs more than the males.

We suggest that this opposite effect of starvation on fuel use is related to the different reproductive strategies of the two sexes: unmated, starved females will start to mature eggs on the day of eclosion, but may then re-absorb the eggs (Boggs and Ross, 1993; Jervis et al., 2005; Watanabe, 1988) and use these nutrients for maintenance under continued food limitation. In contrast, males eclose with sufficient resources to produce a large, first, spermatophore and should try and conserve their flight muscles as long as possible in order to locate and mate with additional females, which can be up to six times in this species (Levin et al., 2016).

When adults were allowed to nectar feed, both sexes oxidized significant amounts of the dietary AAs even though they had an abundance of carbohydrates as an energy source (the ratio between amino acids and carbohydrates in the nectar was 1:1250). Fed males oxidized more EAAs than fed females (Fig. 2), but no difference was found in the oxidation of the NEAA (Fig. 2). Females can synthesize NEAAs from the carbon skeleton of the sugars in the nectar (O'Brien et al., 2002), which can explain why they preferentially oxidize them as metabolic fuel. EAAs, in contrast, are a limited resource, and nectar EAAs are preferentially

allocated to reproduction (Levin et al., 2017a) so they are conserved by females and apparently to a lesser extent, by males.

The same sexual differences in the metabolism of AAs have been reported for humans under normal dietary conditions: men generally oxidized a greater proportion of dietary AAs than women (Lamont, 2005; Lamont et al., 2001; Lamont et al., 2003). It has been suggested that this difference is related to the effect of 17-beta-estradiol in the female that inhibits AA oxidation (Tarnopolsky, 2008). Even though this is considered a vertebrate hormone, it has been found to cause the development of female characteristics in other moth species, the silkworm (*Bombyx mori*) (Shen et al., 2015). We suggest that the mechanism regulating the level at which AAs are oxidized, could be conserved among taxa, and is related to cholesterol derived estrogen-like hormones – a promising topic for future studies of comparative physiology and endocrinology.

Previously, we demonstrated that female moths allocated nectar NEAAs and EAAs into reproduction (Levin et al., 2017a). Here we show that males also allocate NEAAs and EAAs to reproduction through its allocation to spermatophores and flight muscles. We previously highlighted the importance of male hawk moth flight muscles for reproduction relative to the spermatophore (Levin et al., 2016). In *M. sexta*, the spermatophore is relatively small and "inexpensive" comprising <2% of the male body mass. In contrast, investment into flight (thorax dry mass) comprises about 20% of total dry body mass, with males investing significantly more than females (see above). Male hawk moths fly more than females (Ziegler, 1991), and their flight muscles are functionally linked to mating success (Levin et al., 2016).

Flight also causes measurable oxidative damage to flight muscle proteins (Levin et al., 2017b). Because these damaged proteins cannot be repaired, they would need to be replaced to maintain the functionality of the flight muscle. We suggest that the higher flight activity in

male moths is linked to higher oxidative damage and that this could explain the higher allocation of nectar-derived AAs in male compared to female flight muscle (Figure 3) as we expect a higher turnover of proteins in male flight muscles compared to females.

Considering these experimental findings in the context of what we know about the life histories of these moths we conclude that the different reproductive strategies of males and females have selected for differences in metabolic fuel use by the two sexes. We suggest that sex-specific physiological differences in fuel use in animals may have evolved as a product of selection to meet the significantly different costs associated with reproduction of the two sexes, as has previously been shown in plants (Dawson and Ehleringer, 1993). Ultimately, future studies may show that these difference in allocation and fuel use by the two sexes may reflect sex differences in function and behavior in many species of different taxa.

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Competing interests

No competing interests declared

Author contribution

EL, MM and GD designed the experiments, EL performed the experiments. EL, MM and GD analyzed the data and wrote the manuscript.

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Figures

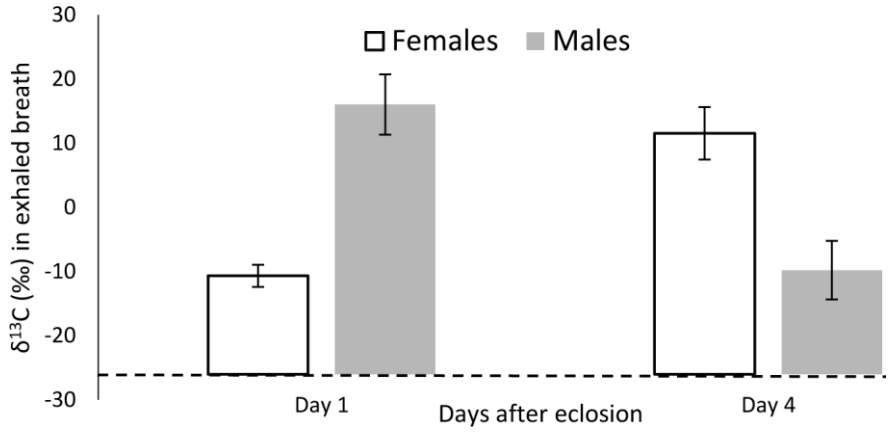


Figure 1: The $\delta^{13}C$ (‰) in exhaled CO_2 from one day- and four day-old virgin male (n=9) and female (n=10) moths reared as larvae on a diet enriched with ^{13}C-leucine. Error bars are 1 SEM. The dashed horizontal line signifies the background $\delta^{13}C$ value of control moths fed the standard diet without ^{13}C-tracers. A less negative number indicates more tracer. One way t-test between sexes $P < 0.001$.

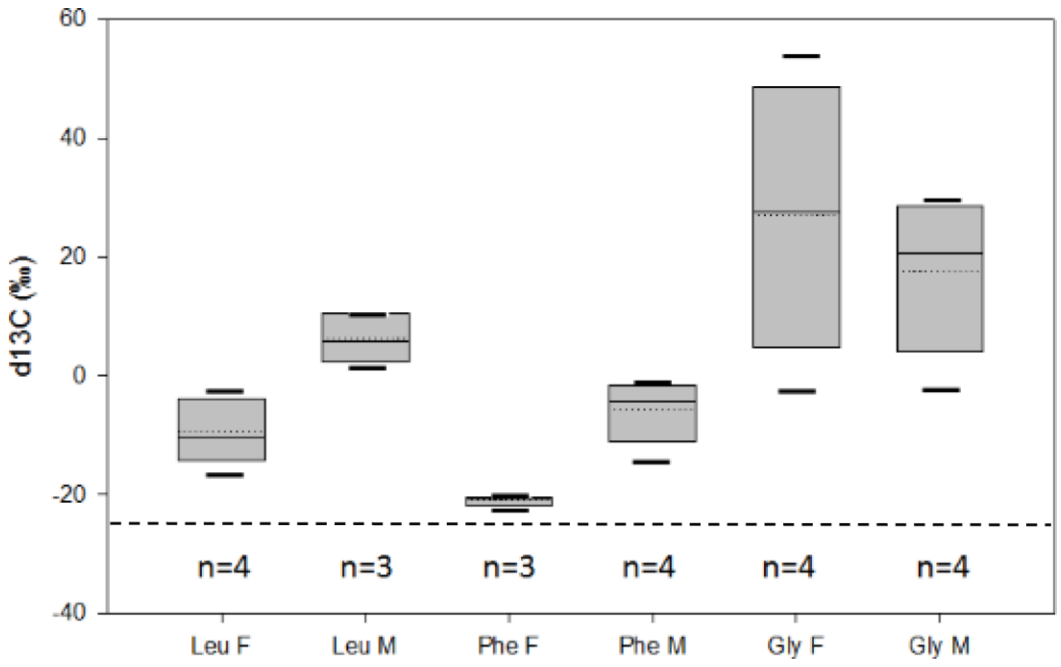


Figure 2: Average $\delta^{13}C$ in the exhaled breath of male and female (n=4 except leucine M and phenylalanine F where n=3) adult moths fed nectars enriched with one of three ^{13}C-amino acid tracers. Means are in dashed lines, medians in solid lines, solid bars are maximum and minimum. The dashed horizontal line signifies the background $\delta^{13}C$ value of control moths fed the standard diet without ^{13}C-tracers. A less negative number indicates more tracer. Males metabolized higher rates of essential AA (Kruskal-Wallis Rank Sums test: leucine, P=0.0339; phenylalanine P=0.0339), but not non-essential AA (glycine, P=0.5637). Abbreviations: Leu- leucine; Phe- phenylalanine; Gly- glycine.

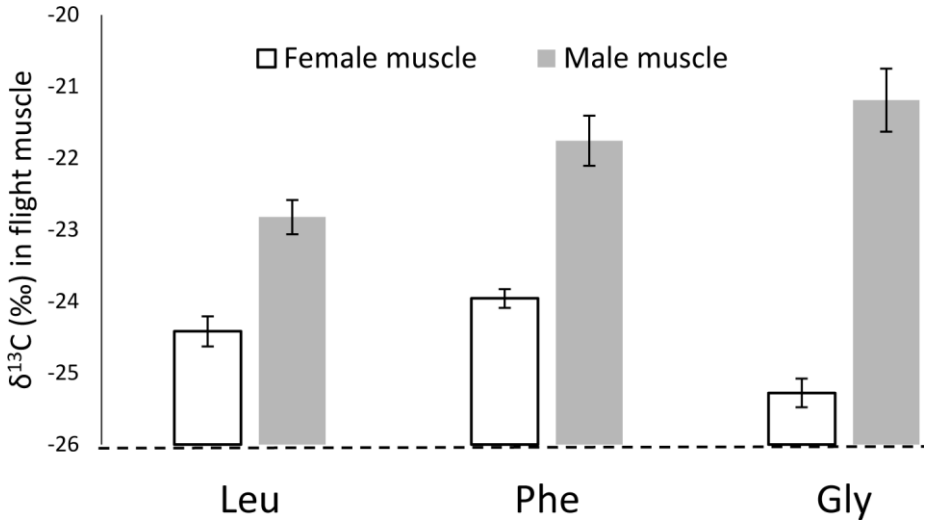


Figure 3: The $\delta^{13}C$ in the flight muscle of male (n=5 for each AA) and female moths (n=5 for each AA) fed one of three ^{13}C labeled amino acids in nectar. Error bars are one SEM. The dashed horizontal line signifies the background $\delta^{13}C$ value of control moths fed the standard diet without ^{13}C-tracers. A less negative number indicates more tracer. Kruskal-Wallis rank sums test between sexes; glycine, P=0.0122; leucine, P=0.0122; phenylalanine, P= 0.0358. Abbreviations: Leu- leucine; Phe- phenylalanine; Gly- glycine.

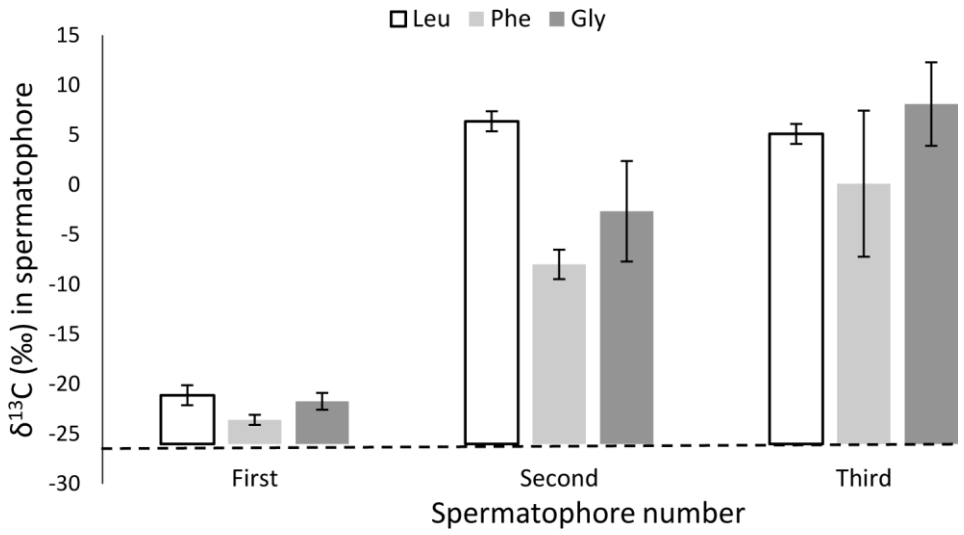
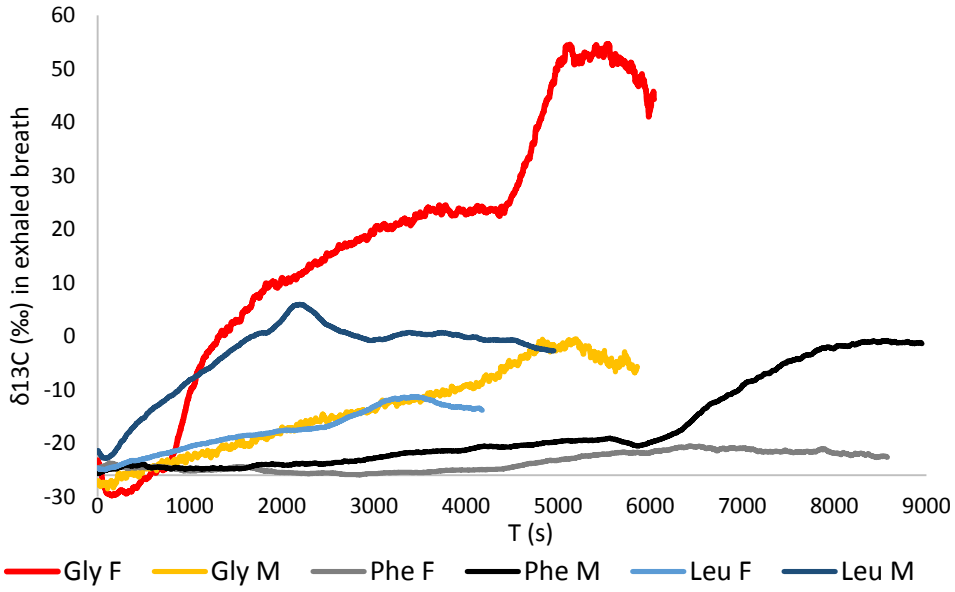


Figure 4: $\delta^{13}C$ in three sequential spermatophores of male moths fed one of three ^{13}C labeled amino acids in nectar (n=5 for each treatment). Error bars are one SEM. The dashed horizontal line signifies the background $\delta^{13}C$ value of control moths fed the standard diet without ^{13}C-tracers. A less negative number indicates more tracer. Significant ^{13}C enrichment above background levels was seen in the spermatophores of males consuming the AA tracers at all time points (one-sample t-tests; $P \leq 0.0001$ in all cases). Abbreviations: Leu- leucine; Phe- phenylalanine; Gly- glycine.



Supplementary Figure 1. Example of $\delta^{13}C$ in the exhaled breath of one male (M) and one female (F) adult moths fed nectars enriched with one of three ^{13}C-amino acid tracers (six individuals total). The horizontal axis indicates the background $\delta^{13}C$ value of control moths fed the standard diet without ^{13}C-tracers. A less negative number indicates more tracer. Abbreviations: Leu- leucine; Phe- phenylalanine; Gly- glycine.

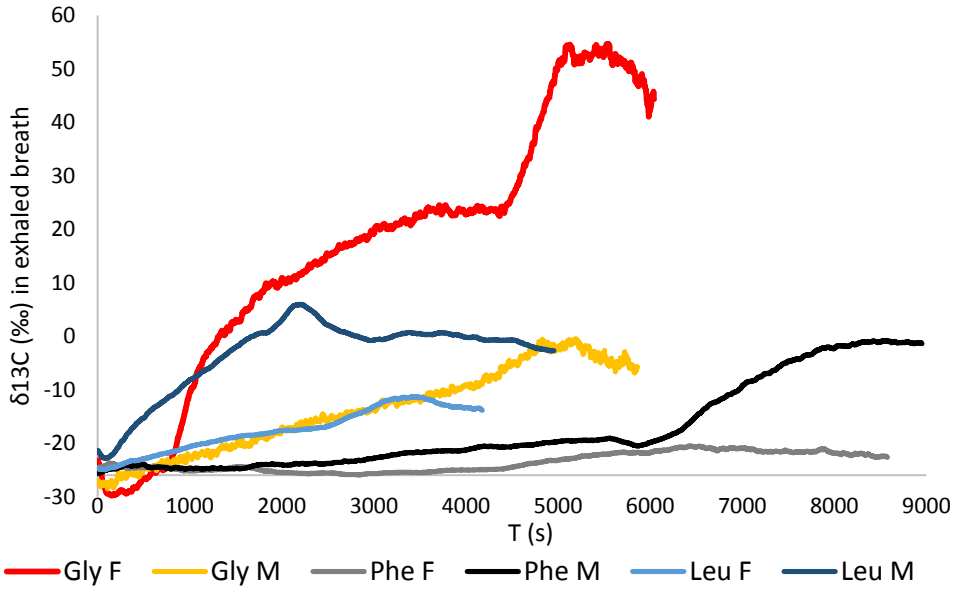


Fig. S1. Example of $\delta^{13}C$ in the exhaled breath of one male (M) and one female (F) adult moths fed nectars enriched with one of three ^{13}C-amino acid tracers (six individuals total). The horizontal axis indicates the background $\delta^{13}C$ value of control moths fed the standard diet without ^{13}C-tracers. A less negative number indicates more tracer. Abbreviations: Leu- leucine; Phe- phenylalanine; Gly- glycine.