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RESEARCH ARTICLE

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

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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 studied 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 larva-derived AAs than females, and more nectar-derived AAs after feeding. After 4 days of starvation, the opposite pattern was observed: adult females oxidized more larva-derived AAs than males. Adult males allocated comparatively small amounts of nectar-derived AAs 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.

KEY WORDS: *Manduca sexta*, Nutrient use, Metabolism, Stable isotopes, $\delta^{13}C$

INTRODUCTION

The resource investment of male sperm is considered to be low compared with 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. 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, 1975), and the consumption of nectar has been shown to increase longevity and reproductive output (see Mevi-Schütz and Erhardt, 2005). O'Brien et al., 2000 originally showed that non-essential amino acids (NEAAs) derived from the nectar-sugar consumed by adult females was vertically transferred to the eggs, but were 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 input 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 (Alarcón et al., 2010). Males generally prefer sucrose-based nectar, whereas females prefer nectar with glucose and AA (Rusterholz and Erhardt, 2000). More recently, Mevi-Schütz and Erhardt (2005) concluded that (1) female butterflies select AA-rich nectar for egg production, and (2) males either show no preference or select 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 we did not test for sex-specific differences (Levin et al., 2017a). In this study, we examined differences in how male and female Lepidoptera use AAs in their diet. Additionally, we quantified 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* (Linnaeus 1763): 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 h:8 h light:dark photocycle in an environmentally controlled room set at 27°C and 50% relative humidity. 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 of 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 larva-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 EAAs. About 200 eggs collected from the breeding colony were raised until pupation on the standard diet (see above) supplemented with ^{13}C-leucine (1 g 1-^{13}C-leucine to 3 l wet

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diet). All ^{13}C-tracers were purchased from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA, USA). 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 1 day of eclosion to adulthood. We repeated these measurements 4 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 G2121-i Cavity Ring-Down Spectroscopy (CRDS) δ^{13}C stable isotope analyzer (Picarro, Sunnyvale, CA, USA) 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 min period while the moths were at complete rest. All ^{13}C concentrations are expressed in $\delta^{13}C_{VPDB}$ (Slater et al., 2001; Werner and Brand, 2001). Note that larger (i.e. more positive) δ^{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 mass) 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 mmol l^{-1}, non-essential), 1-^{13}C-leucine (Leu, 1.5 mmol l^{-1}, essential, branched) or 1-^{13}C-phenylalanine (Phe, 1.2 mmol l^{-1}, 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, Totnes, 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™ (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 8 h 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 at −20°C for 2 h. They were then thawed and surgical scissors were used to remove the spermatophore from the bursa copulatrix. These spermatophores were then dried at 50°C until they reached a constant mass ±0.01 mg (analytical balance, Mettler-Toledo XS, Columbus, OH, USA). 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, Picarro).

After the third mating (day 5), the males were killed by freezing at −20°C for 2 h. Their flight muscles were dissected using surgical scissors and dried before measuring δ^{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 5 after eclosion (as for the males), their flight muscles were removed and δ^{13}C values were 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 day after eclosion) adults from the breeding population were fed 200 µl of one of the three δ^{13}C-labeled artificial nectars as described above (n=24 moths in total). The δ^{13}C in the exhaled CO_2 was measured for 120–180 min with maximum δ^{13}C values usually observed ∼90 min after feeding. We used the peak δ^{13}C values of each individual for statistical comparisons. All statistics were conducted using JMP software (JMP 11.0, USA) and α=0.05 was used to determine statistical significance.

RESULTS

Oxidization of larva-derived AA

The δ^{13}C of all adults raised on the ^{13}C-leucine diets was 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 δ^{13}C in the exhaled CO_2 was significantly higher in males than in females (Fig. 1, t-test assuming unequal variance, t=5.33, d.f.=11.38, P=0.0002). On day 4 post-eclosion, the opposite trend was observed, whereby females had a

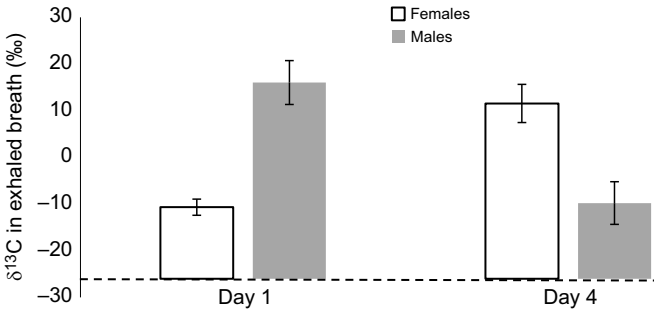

Fig. 1. δ^{13}C in exhaled CO_2 from 1 and 4 day old virgin male and female moths reared as larvae on a diet enriched with ^{13}C-leucine. n=9 males and n=10 females. Error bars are 1 s.e.m. The dashed horizontal line signifies the background δ^{13}C value of control moths fed the standard diet without ^{13}C-tracers. A less-negative number indicates more tracer. One-way t-test between sexes, P<0.001.

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significantly higher $\delta^{13}C$ than males (t=−3.48, d.f.=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 min (Fig. S1).

Males had significantly higher maximal $\delta^{13}C$ values in their breath when fed either of the labeled EAAs (Fig. 2; Kruskal–Wallis rank sums test: Leu, P=0.0339; Phe, P=0.0339), but there was no significant difference between the sexes when fed with the labeled NEAA (Fig. 2; Gly, P=0.5637).

Allocation of nectar AAs to muscle

The $\delta^{13}C$ in the muscles of males and females fed all three labeled AAs was elevated from background levels (P≤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 those of females across all three labeled AA treatments (Kruskal–Wallis rank sums test; Gly, P=0.0122; Leu, P=0.0122; Phe, P=0.0358; Fig. 3). In female flight muscles, the $\delta^{13}C$ values were lower in the labeled NEAA treatment than in the labeled EAA treatments (Wilcoxon multiple comparison test; Leu–Gly, P=0.0216; Phe–Gly, 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±0.02% of dry body mass, mean±s.d., n=92) than females (19.26±0.015%, n=88). $\delta^{13}C$ values are size independent; however, because male flight muscles are larger than those of females, the absolute amount of AAs allocated to male flight muscle is even greater than indicated solely by the $\delta^{13}C$ shown in Fig. 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≤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 enrichment (Fig. 4). The $\delta^{13}C$ values in the first spermatophores did not differ significantly among the three labeled AA treatments (Kruskal–Wallis rank sums test, P=0.1845). In the second spermatophores, only Phe and Leu values 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 is given elsewhere (Levin et al., 2017a).

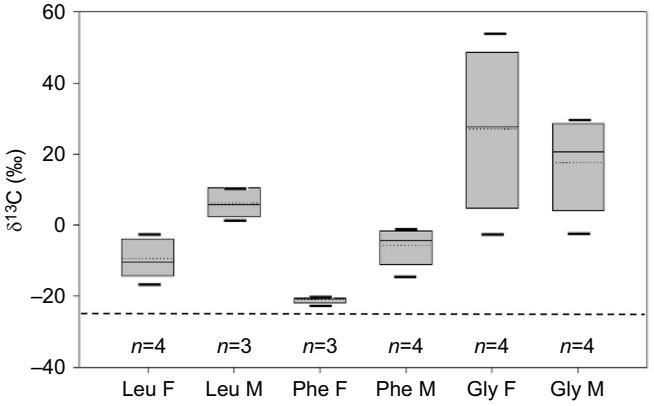

Fig. 2. Average $\delta^{13}C$ in the exhaled breath of male and female adult moths fed nectar enriched with one of three ^{13}C-amino acid tracers. Dotted lines indicate means, solid lines are medians, solid bars are maximum and minimum. The dashed horizontal line signifies the background $\delta^{13}C$ 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 amino acids [EAA; Kruskal–Wallis rank sums test: leucine (Leu), P=0.0339; phenylalanine (Phe), P=0.0339], but not non-essential amino acids [NEAA; glycine (Gly), P=0.5637].

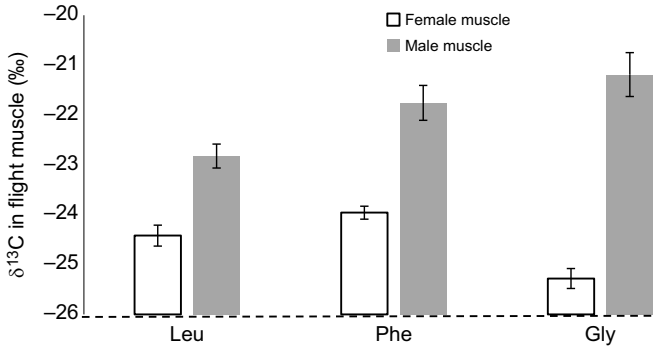

Fig. 3. $\delta^{13}C$ in the flight muscle of male and female moths fed one of three ^{13}C-labeled AAs in nectar. n=5 males and n=5 females for each AA. Error bars are 1 s.e.m. The dashed horizontal line signifies the background $\delta^{13}C$ value of control moths fed the standard diet without ^{13}C-tracers. A less-negative number indicates more tracer. Kruskal–Wallis rank sums test between sexes: Gly, P=0.0122; Leu, P=0.0122; Phe, P= 0.0358.

DISCUSSION

We found fundamental differences in the way adult male and female *M. sexta* use their dietary AAs. These sexual differences exist when the AAs were originally derived from the larval diet as well as when they were derived from the nectar 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 is less well developed for other taxa (McCue, 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 (McCue et al., 2015). For

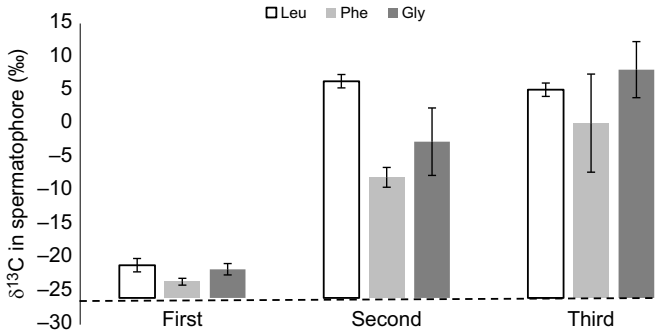

Fig. 4. $\delta^{13}C$ in three sequential spermatophores of male moths fed one of three ^{13}C-labeled AAs in nectar. n=5 for each treatment. Error bars are 1 s.e.m. The dashed horizontal line signifies the background $\delta^{13}C$ value of control moths fed the standard diet without ^{13}C-tracers. 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≤0.0001 in all cases).

example, some insects increase their use of AAs 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 a 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 than 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 6 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 AAs 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, 2003). It has been suggested that this difference is related to the effect of 17-β-estradiol in females, which 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 another 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 their 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 with female flight muscle (Fig. 3) as we expect a higher turnover of proteins in male flight muscles compared those of 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 differences 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
The authors declare no competing or financial interests.

Author contributions
Conceptualization: E.L., M.D.M., G.D.; Methodology: E.L., M.D.M., G.D.; Validation: E.L.; Formal analysis: E.L., G.D.; Resources: G.D.; Writing - original draft: E.L., M.D.M., G.D.; Writing - review & editing: E.L., M.D.M., G.D.; Visualization: E.L., G.D.; Supervision: G.D.; Funding acquisition: G.D.

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Supplementary information
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