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## NECTAR SAMPLING FOR PRAIRIE AND OAK SAVANNA BUTTERFLY RESTORATION<sup>1</sup>

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- *Premise of the study:* Understanding floral resources is vital for restoring pollinators in habitats affected by anthropogenic development and climate change. As the primary adult food, nectar can limit butterfly longevity and reproduction. For pollinator restoration, it would therefore be useful to examine nectar resources. However, because many flowers preferred by butterflies are too small for microcapillary sampling and the potential for nectar contamination can make accurate measurement difficult, we developed a modified centrifugation method to extract and separate nectar and pollen.
- *Methods:* We sampled nectar from 19 forbs using a glass wool filter to exclude potentially contaminating pollen during centrifugation. To minimize costs, we measured sugar concentration by refractometry and simple ninhydrin tests for amino acids and improved test accuracy by subsequent image analysis. Artificial nectars were used to verify the new techniques.
- *Results:* This method eliminated pollen from samples, while only slightly increasing sugar concentrations. Some amino acids were lost during centrifugation, but only samples with high concentrations exhibited substantial loss. We found significant differences in nectar quality among species, as well as an unexpected inverse relationship between amino acid and sugar concentrations.
- *Discussion:* This modified centrifugation technique is an efficient, less damaging, inexpensive approach for collecting nectar from small flowers while eliminating pollen contamination, and will facilitate restoration of declining pollinators and thereby the plants they service.

**Key words:** amino acids; butterflies; nectar sampling; pollinator restoration; sugar.

Crop and pastureland now make up roughly 40% or more of the world's land cover (Foley et al., 2005). By the mid-2000s, tallgrass prairie and oak savanna remnants were estimated to comprise less than 1% of vegetation in the Midwestern United States (Summerville et al., 2005). Oak savannas are now considered to be among the rarest natural vegetation types in the world (Anderson and Bowles, 1999). Reduction in these natural habitats has led to a critical decline in the abundance and diversity of native plants and insect pollinators (Swengel and Swengel, 1999; Kocher and Williams, 2000; Meehan et al., 2012; Archer et al., 2014). These population declines have been severe enough to warrant federal endangered listing for the Karner blue butterfly, *Plebejus melissa samuelis* (U.S. Fish and Wildlife Service, 1992), and proposed threatened status for the once common monarch butterfly (U.S. Fish and Wildlife Service, 2014).

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Understanding the key resources for butterfly pollinators is vital for preserving and restoring native habitats and their biodiversity (Vogel et al., 2007). Several studies have found that nectar resources, as indicated by the number of stems or ramets, are strong predictors of overall butterfly richness (Holl, 1995; Matter and Roland, 2002; Pywell et al., 2004; Shepherd and Debinski, 2005). However, most of these studies do not consider the nutritional quality of the available nectar resources, even though other research shows increases in butterfly fitness components in response to nectar quality (Hill and Pierce, 1989; Mevi-Schütz and Erhardt, 2005; Nicolson and Thornburg, 2007; Willmer, 2011; Cahenzli and Erhardt, 2012a).

Nectar is composed of water, as well as nutritionally important compounds such as sugars, lipids, and amino acids (Nicolson and Thornburg, 2007; Willmer, 2011; Cahenzli and Erhardt, 2012b, 2013). The three most common sugars found in nectar are sucrose, fructose, and glucose, which are derived either from sucrose translocated in phloem sap or synthesized within the nectary (Baker and Baker, 1973; Dafni, 1992; Kearns and Inouye, 1993; Nicolson and Thornburg, 2007). Some studies have shown broad correlations between sucrose concentrations and pollinator classes, suggesting that nectars with relatively high sucrose concentration are generally associated with flowers that are pollinated by moths, long-tongued bees, hummingbirds, and butterflies (Nicolson and Thornburg, 2007). Literature also indicates that flowers visited by butterflies are consistently rich in amino acids (Baker and Baker, 1975, 1986; Nicolson and

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Thornburg, 2007; Cahenzli and Erhardt, 2012b, 2013) and that butterflies typically prefer nectar that is high in amino acid content (Alm et al., 1990; Mevi-Schütz and Erhardt, 2003). In Gardner and Gillman's (2001) survey of 30 nectar-producing species, the overall amino acid composition was generally highly conserved, whereas variation in concentration occurred both intra- and interspecifically. Amino acids in nectar influence lifespan, as well as egg production in some butterfly species (Mevi-Schütz and Erhardt, 2005), and have been linked to increased egg and larval sizes or increased survival (Bauerfeind and Fischer, 2009; Cahenzli and Erhardt, 2012a). The contributions of sugars and amino acids to butterfly fitness suggest that their concentrations may be an important consideration for assembling nectar-providing communities for pollinator habitat restoration plans. These findings suggest that assessment of nectar quality is essential to planning habitat restorations for economically and ecologically important pollinators.

Direct collection with microcapillary tubes is usually used for nectar sampling from larger flowers with ample supplies of nectar, but this method requires care not to damage flowers or contaminate the sample with pollen (Kearns and Inouye, 1993). However, plants visited by many pollinators (particularly those visited by butterflies) commonly present inflorescences of very small florets that generally contain less water and produce less nectar (Nicolson and Thornburg, 2007). Alternative methods are needed to sample from such plants because repeated use of microcapillary tubes on small flowers can easily damage tissues and thereby artificially increase amino acid concentrations (Willmer, 1980, 2011). Because of these difficulties, the nectar composition of small, butterfly-preferred flowers has not been extensively researched relative to plants with larger, bee- or bird-pollinated flowers (Willmer, 2011). Wick-based approaches (e.g., Petanidou et al., 2006) avoid damaging floral tissues, but require considerable time and labor in the field to avoid pollen contamination. To assess nectar quality in these plants, we have developed an alternative method for obtaining nectar from small flowers using a modification of a centrifugation technique first proposed by Swanson and Shuel (1950).

The aim of this project was to develop rapid and inexpensive nectar extraction procedures and test them in a preliminary study of the relative sugar and amino acid concentrations of species visited or preferred by butterflies native to the tallgrass prairies and oak savannas of northwestern Ohio. Specifically, we focused on the following questions: (1) How do nectar total sugar and amino acid concentrations vary in common native oak savanna and prairie forb species? and (2) What nectar-producing species might be most beneficial to include in restoration plans targeting the conservation and restoration of pollinators and their habitats?

## METHODS

**Sampling sites and survey species**—We conducted surveys at multiple protected sites throughout the Oak Openings Region of northwestern Ohio, including preserves and parks within Lucas and Wood counties (Table 1). We selected plant taxa flowering from early June until early October 2015 to encompass the majority of the season in which butterflies are present. We focused on 19 common native nectar-producing species reported to be visited or preferred by butterflies (Table 1; Borgmann and Rodewald, 2002) and listed in the Monarch Butterfly North American Monitoring Protocol (Oberhauser et al., 2009). Butterflies of conservation concern found in the Oak Openings Region include the Ohio threatened silver-bordered fritillary (*Boloria selene*), the endangered Persius duskywing (*Erynnis persius*) and frosted elfin (*Callophrys irus*), and the federally endangered Karner blue (*Plebejus melissa samuelis*) (Toledo Metroparks, 2016).

**Nectar survey**—For each species (Table 1), we sampled populations using line transects that were as long as the distance over which a species was present, with parallel transects placed at least 6 m apart. Along each transect line, sampling points were established every 6 m, with selected plants within a 3-m radius of each sampling point. For uncommon species, each plant was sampled as long as it was at least 6 m from another plant. To prevent contamination or removal by flower visitors, unopened or newly opened flowers and/or inflorescences (depending on the species) were bagged with bridal-veil mesh netting 24 to 48 h before nectar collection. Nectar samples were obtained between 10:30 A.M. and 4:30 P.M. (Eastern Standard Time) and at least 24 h after a rain event to ensure the nectar was not washed out of the flower. Replicates varied between three and 30 depending on species abundance (Table 1).

We collected nectar in one of two ways. For rare taxa for which entire inflorescences could not be collected because of permit restrictions or for species with large flowers (*Lithospermum carolinense* (J. F. Gmel.) MacMill. and *Monarda punctata* L.), we sampled nectar directly from field plants using microcapillary tubes (Drummond Scientific Company, Broomall, Pennsylvania, USA) from 0.5 to 10  $\mu$ L in size (Stubbs et al., 2008). For all other species, there was either very low nectar volume or the microcapillary tube was unable to reach the nectary; for these species, we collected whole inflorescences, positioned stems within a water pic reservoir, and immediately placed them in a cooler containing ice packs to help reduce effects of moisture loss on sugar concentrations (Bertsch, 1983). Samples were transported back to the laboratory, generally within 45 min or less, for further processing.

Because pollen contamination could artificially increase the relative amounts of amino acids in the nectar samples (Gottsberger et al., 1990; Nicolson and Thornburg, 2007), we developed a modified centrifugation technique (adapted from Swanson and Shuel, 1950) to extract nectar, while limiting the effects of pollen on nectar composition. To separate nectar from pollen with this modified technique, we first placed glass wool (~1.5 g) snugly into the bottom of a 1.5-mL microcentrifuge tube, leaving approximately 5 mm for nectar to collect at the bottom. Each sample was placed in the top of a tube with flowers facing downward. The specimen was then centrifuged at 6000 rpm (Tomy HF120 NanoFuge with 6  $\times$  1.5-mL rotor [Tomy Seiko Co., Tokyo, Japan]) for 30–60 s. After nectar collection, we removed the flowers, which remain above the glass wool filter, and counted all individual, open florets. We estimated the volume of nectar obtained with a 10- $\mu$ L micropipettor (to  $\pm$ 0.5- $\mu$ L accuracy).

To determine sugar concentrations, we used a Bellingham + Stanley handheld refractometer (model 45-81, 0–50 Brix, low volume; Bellingham + Stanley, Tunbridge Wells, United Kingdom), which measured the ratio of the mass of dissolved sucrose to the mass of water in the nectar solution. Because samples with high amounts of sugar (>50 Brix) could not be measured accurately and may not spread sufficiently on chromatography paper to allow ninhydrin to penetrate the sample completely (Kearns and Inouye, 1993), such samples were diluted with distilled water by 50% before measuring. We stored the remaining nectar at –20°C for later measurement of amino acids. While costly high-performance liquid chromatography (HPLC) methods are now commonly used to determine the specific amounts of each amino acid found in nectar, we used total amino acids as a simple indicator of floral resource nutritional value across taxa within a site. Therefore, to measure total amino acids, we used inexpensive, rapid chromatography paper ninhydrin tests as described in Kearns and Inouye (1993) and Dafni (1992). After dilution, 2  $\mu$ L of the nectar sample was spotted onto an individual chromatography strip (grade 1 Whatman, 1  $\times$  12 cm) two to three times and 2 cm apart. For each testing day, we prepared a 10-level histidine reference scale using three replicates of fresh amino acid standards with known amounts of histidine (7.58–3900 ng/ $\mu$ L) (Baker and Baker, 1975; Dafni, 1992). Chromatography papers for the amino acid standards were cut into 1  $\times$  20-cm strips and spotted 1.5 cm apart. Samples and standards were allowed to dry completely before applying ninhydrin reagent (0.2% in acetone) directly. All ninhydrin-treated strips were dried for 48 h, after which samples and the standards were scanned (HP Deskjet 3054, 200-dpi resolution; Hewlett-Packard, Palo Alto, California, USA).

Traditional ninhydrin tests estimate total amino acids categorically by visually comparing ninhydrin stain color intensity of a test sample to that of standard histidine solutions. To obtain a less subjective and finer-scaled estimate of the colorimetric change in ninhydrin, we measured the stained areas and intensities using ImageJ (Schneider et al., 2012). We then calculated the integrated density of each spot as the product of the area stained by 2  $\mu$ L of a standard solution and its mean grayscale value. The average integrated density of all the standards was used to create the following conversion equation:  $\text{Log}(\text{concentration of known amino acids from standard histidine scale}) = -5.217 + 1.757 * \text{Log}(\text{average integrated density from image analysis of histidine standards})$ . This equation was applied to the experimental data to generate the estimate of amino acid concentration for each sample (Table 2, Fig. 1).

TABLE 1. Scientific and common names of plant species in the survey, sample sizes for each site, mean sugar and amino acid concentrations, and mean nectar volumes.

Scientific name	Common name	Locations sampled <sup>a</sup> (N Brix sugar, N amino acid, (N <sub>Total</sub> = Brix, AA)	Mean sugar concentration (Brix) <sup>b</sup>	Mean amino acid concentration (ng/μL) <sup>b</sup>	Mean nectar volume per flower (μL) <sup>b</sup>
<i>Asclepias incarnata</i> L.	Swamp milkweed	ERS (2, 2), PR (16, 7), TMG (7, 7), (N <sub>Total</sub> = 25, 16)	58.96 (14.51)	121.28 (32.63)	0.14 (0.037)
<i>Asclepias sullivantii</i> Engelm. ex A. Gray	Sullivant's milkweed	PR (8, 8), ERS (2, 2), (N <sub>Total</sub> = 10, 10)	66 (10.48)	34.67 (7.83)	0.21 (0.007)
<i>Asclepias syriaca</i> L.	Common milkweed	TMJ (1, 1), RS (6, 6), LCC (1, 1), TPK (5, 3), ERS (3, 3), (N <sub>Total</sub> = 16, 14)	37.66 (11.00)	78.80 (17.24)	0.65 (0.075)
<i>Asclepias tuberosa</i> L.	Butterfly milkweed	LCC (5, 3), RS (6, 3), TMJ (6, 3), BC (10, 10), PR (2, 2), SP (1, 1), (N <sub>Total</sub> = 30, 22)	57.23 (17.57)	66.01 (23.86)	0.29 (0.018)
<i>Coreopsis lanceolata</i> L.	Lance-leaved coreopsis	RS (5, 3), (N <sub>Total</sub> = 5, 3)	33.6 (14.88)	146.63 (42.19)	0.07 (0.007)
<i>Coreopsis tripteris</i> L.	Tall coreopsis	LCE (7, 5), SP (5, 4), ERS (4, 3), BC (8, 7), (N <sub>Total</sub> = 24, 19)	53.73 (16.18)	253.87 (70.65)	0.12 (0.0068)
<i>Eupatorium purpureum</i> L.	Joe-Pye weed	LCE (12, 11), (N <sub>Total</sub> = 12, 11)	31.54 (8.86)	55.73 (10.34)	0.30 (0.011)
<i>Helianthus divaricatus</i> L.	Woodland sunflower	TMW (6, 5), LCC (6, 0), LCE (6, 5), SP (1, 1), (N <sub>Total</sub> = 19, 11)	43.61 (17.81)	140.92 (42.31)	0.06 (0.022)
<i>Helianthus giganteus</i> L.	Giant sunflower	LCE (15, 13), BC (3, 3), (N <sub>Total</sub> = 18, 16)	47.23 (10.43)	255.65 (58.93)	4.04 (0.011)
<i>Lespedeza capitata</i> Michx.	Round headed bush clover	SP (12, 9), (N <sub>Total</sub> = 12, 9)	32.67 (13.58)	177.99 (69.52)	1.14 (0.22)
<i>Liatris aspera</i> Michx.	Rough blazing star	SP (9, 9), LCC (7, 6), (N <sub>Total</sub> = 16, 15)	59.75 (13.15)	46.87 (13.04)	0.12 (0.007)
<i>Liatris spicata</i> (L.) Willd.	Dense blazing star	TMG (11, 11), LCE (2, 2), ERS (2, 0), SP (1, 1), BC (11, 10), (N <sub>Total</sub> = 27, 24)	49.52 (9.06)	78.34 (20.53)	0.06 (0.005)
<i>Lithospermum carolinense</i> (J. F. Gmel.) MacMill.	Plains puccoon	LCC (4, 2), JS (5, 0), SP (5, 5), CH (3, 0), (N <sub>Total</sub> = 17, 7)	34.26 (8.74)	37.96 (12.11)	0.45 (0.11)
<i>Monarda fistulosa</i> L.	Wild bergamot	TML (8, 7), ERS (4, 2), SP (1, 1), (N <sub>Total</sub> = 13, 10)	56.12 (13.69)	52.59 (17.04)	0.03 (0.009)
<i>Monarda punctata</i> L.	Dotted horsemint	TMJ (7, 5), RS (9, 8), SP (4, 4), (N <sub>Total</sub> = 20, 17)	61.28 (13.11)	14.00 (1.95)	0.13 (0.014)
<i>Solidago rigida</i> L.	Stiff goldenrod	ERS (5, 4), PR (6, 6), (N <sub>Total</sub> = 11, 10)	57.82 (17.02)	139.21 (52.68)	0.06 (0.032)
<i>Symphoricarichum ericoides</i> (L.) G. L. Nesom	Heath aster	ERS (11, 11), (N <sub>Total</sub> = 11, 11)	63.45 (12.19)	181.61 (67.34)	0.03 (0.012)
<i>Symphoricarichum novae-angliae</i> (L.) G. L. Nesom	New England aster	ERS (11, 11), (N <sub>Total</sub> = 11, 11)	51.81 (14.98)	138.16 (95.26)	0.03 (0.029)
<i>Vernonia gigantea</i> (Walter) Trel.	Tall ironweed	LCE (10, 9), PR (1, 1), BC (7, 5), (N <sub>Total</sub> = 18, 15)	44.84 (13.26)	255.69 (110.54)	0.09 (0.007)

Note: N<sub>Total</sub> = total sample size across all locations.

<sup>a</sup> Sampling sites (and abbreviations) from Lucas Co.: Julia's Savanna (JS), South Piel (SP), and Cactus Hill (CH) from Kitty Todd Nature Conservancy; Jeffers (TMJ), Wabash (TMW), Parkway (TPK), Flying Tigers (TMG), Lark Sparrow Meadow (TML), and Blue Creek Seed Nursery (BC) from the Oak Openings Toledo Metroparks; and Central (LCC) and Entrance (LCE) from Lou Campbell State Nature Preserve; in Wood Co.: Rudolph Savanna (RS) from the Wood County Park District, Poe Prairie (PP) and the Ecological Research Station (ERS) at Bowling Green State University.

<sup>b</sup> Means and standard errors (in parentheses) of sugar (Brix) and amino acid (ng/μL) concentrations and calculated nectar volume (μL) per flower for each taxon.

TABLE 2. Standard least squares regression of integrated density values from amino acid standards centrifuged without a glass wool filter predicting the integrated density values from standards centrifuged with a glass wool filter.<sup>a</sup>

Variable	Source	df	Sum of squares	Mean square	F ratio	Prob > F
Estimated integrated density	Model	1	2.00	2.00	39.45	0.0002
	Error	8	0.406	0.051		
	Total	9	2.41			
Estimated total amino acid concentration	Model	1	7.44	7.44	254.10	<0.0001
	Error	8	0.23	0.029		
	Total	9	7.68			

<sup>a</sup> $R^2 = 0.83$ ;  $\text{Log}(\text{estimated integrated density}) = -0.935 + 1.313 * \text{Log}(\text{integrated density of standards centrifuged through a glass wool filter})$ .

**Method verification**—To test whether our modified centrifugation technique would alter sugar concentrations, we created a standard 20% sucrose solution (to match the composition used for preparation of the histidine standards [Dafni, 1992]) and compared the sugar concentration measured before and after centrifuging with a glass wool filter ( $N_{\text{Total}} = 60$ ). We then compared the measured amino acid concentrations of histidine standards before and after centrifuging through a glass wool filter ( $N = 43$ ). We used ImageJ software, as previously described, to quantify colorimetric stain intensity.

To test the effectiveness of the modified centrifugation technique for separating pollen from the nectar of the flower sampled, nectar was collected from two species: *Symphytotrichum ericoides* (L.) G. L. Nesom ( $N = 3$ ) and *S. novae-angliae* (L.) G. L. Nesom ( $N = 5$ ). For each species, we centrifuged the flowers with and without the glass wool, after which we pipetted nectar onto a Spencer Bright-Line hemocytometer (American Optical Company, Buffalo, New York, USA) and covered the top with a glass cover slip. We then counted pollen grains in 25 of the 0.25-mm<sup>2</sup> grids (five squares in each of the four 1 × 1-mm corners and middle squares) to acquire the average pollen density per microliter as an estimate of the amount of pollen contamination.

**Statistical analyses**—All analyses were performed using JMP 12.0 (SAS Institute, Cary, North Carolina, USA) using  $\alpha \leq 0.05$  to determine significance for each analysis. Assumptions of homogeneity of variance were assessed for each test by evaluating residual plots, while normal quantile plots of residuals were analyzed to examine assumptions of normality for each test.

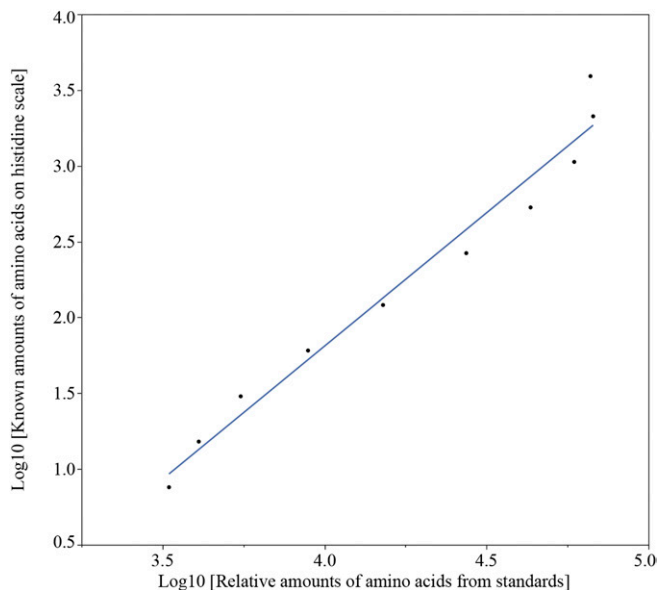


Fig. 1. Standard least squares regression of the known amounts of amino acids from the histidine scale compared to the integrated density of the created standards, acquired from the colorimetric change of ninhydrin tests and analyzed on ImageJ software ( $F_{1,9} = 254.10$ ,  $P < 0.0001$ ,  $R^2 = 0.966$ ).

**Method verification**—To test the effectiveness of our modified centrifugation technique, we used a nonparametric Wilcoxon rank-sum test, as nectar sugar concentration data were nonnormal and transformations did not improve the fit. We compared the amount of pollen contamination (pollen density) for the two species centrifuged with and without the filter using a Wilcoxon test. For total amino acid concentrations, we performed ANOVA using standard least squares (SLS) estimation to evaluate the effects of centrifugation technique (before and after centrifuging with a glass wool filter), histidine concentration, and their interaction.

**Nectar survey**—We tested for differences among species in total sugar and amino acid concentration (log transformed) using SLS estimation ANOVAs with pairwise comparisons among species for each response variable using Tukey's honest significant difference (HSD) test. Finally, using amino acid concentration as a response variable, we investigated the relationship between sugar and plant genus as well as their interaction terms using a SLS regression, as there were insufficient degrees of freedom for analysis among species. We excluded interaction terms from final models when they were nonsignificant.

## RESULTS

**Method verification**—Because nectar is difficult to collect from clusters of small flowers visited by many pollinators, a primary motivation for developing this modified centrifugation technique was to exclude pollen while extracting the nectar samples. We found no pollen grains in the samples centrifuged with the glass wool filter, compared to the average 2187.43 pollen grains found across the two types of nectar samples centrifuged without the glass wool filter (Wilcoxon  $\chi^2$  test = 12.89,  $df = 1$ ,  $P = 0.0004$ ).

The process of centrifugation very slightly increased (by 7%) estimated sugar concentrations, with sucrose concentrations before centrifuging significantly lower in average concentration (mean 16.3 Brix) than after centrifuging with the glass wool filter (mean 17.53 Brix, Wilcoxon  $\chi^2 = 30.35$ ,  $df = 1$ ,  $P < 0.0001$ ; Fig. 2). However, when we compared the total amino acid concentrations of artificial nectar standards, we found a significant difference in amino acid concentration before and after centrifuging through a glass wool filter ( $F = 36.66$ ,  $df = 9$ ,  $P < 0.0001$ ; Fig. 3). The loss of amino acids increased with histidine concentration, with a greater loss for samples corresponding to a 4 or above on the histidine scale ( $\geq 60.49$  ng/ $\mu$ L). At the lower end of the histidine scale, corresponding to a 3 or below ( $\leq 30.17$  ng/ $\mu$ L), the change in concentration was lower, with an average loss of 14.01 ng/ $\mu$ L. At higher concentrations, the loss grew quite large, exceeding 90% for the richest samples.

Because the loss of amino acids was more pronounced for the most concentrated artificial nectar samples, we surmised that more amino acids may have been retained in the glass wool at these higher concentrations under the short centrifugation conditions used (30–60 s). To correct for this problem when estimating concentration of the wild nectars, we used the integrated density value of the histidine samples centrifuged with and without the glass wool to create a correction equation to estimate the concentration loss for wild nectars, where:  $\text{Log}(\text{integrated density of centrifuging without glass wool}) = -0.935 + 1.31 * \text{Log}(\text{integrated density of centrifuging with glass wool})$  ( $F_{1,8} = 39.45$ ,  $P = 0.0002$ ,  $R^2 = 0.83$ ). This correction factor was not used for *Lithospermum carolinense* and *Monarda punctata*, the two species that were collected directly. For all other species surveyed, we then used this corrected density value in the conversion equation (as described above) to acquire an estimate of the total amino acid concentration.

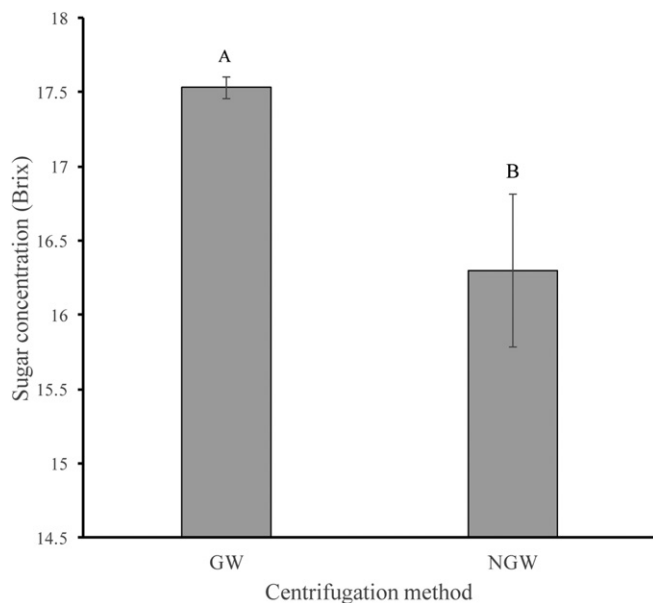


Fig. 2. Differences in sugar concentration between centrifuging with glass wool (GW) and without glass wool (NGW) ( $\chi^2 = 30.35$ ,  $df = 1$ ,  $P < 0.0001$ ; Wilcoxon 2-sample test). Centrifuging with glass wool increased the sugar concentration by 1.23 Brix (by 7%). Bars separated by letters are significantly different from one another. Error bars represent standard error.

**Nectar survey**—Sugar concentrations varied, ranging from 31.5 Brix to 66 Brix. Sugar concentration differed among species ( $F_{18, 297} = 9.16$ ,  $P < 0.0001$ ,  $R^2 = 0.36$ ; Table 3, Fig. 4). Of the 19 species surveyed, *Asclepias sullivantii* Engelm. ex A. Gray and *Symphotrichum ericoides* had the highest sugar

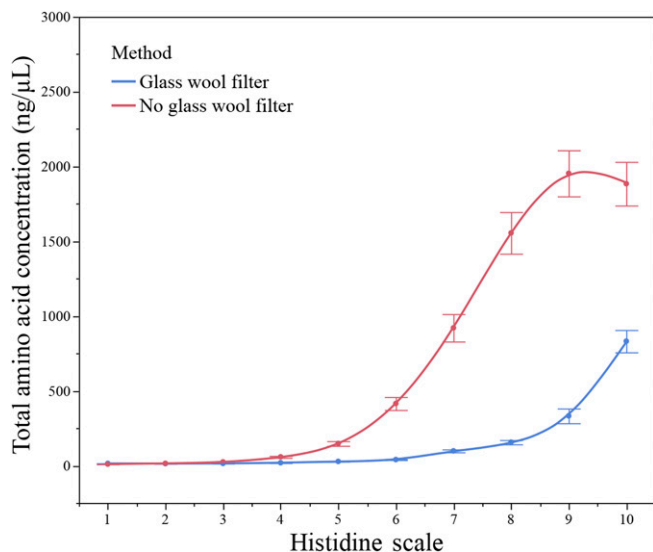


Fig. 3. Analysis of amino acid concentrations (AA) of artificial nectars representing different levels on the histidine scale (1–10, 7.58–3900 ng/μL) before and after centrifugation with a glass wool filter (standard least squares regression; AA = histidine scale × centrifugation method:  $F = 36.66$ ,  $df = 9$ ,  $P < 0.0001$ ). The loss of amino acids was more pronounced in nectar samples from the higher end of the histidine scale ( $\geq 4$  [ $\geq 60.49$  ng/μL]), suggesting that more amino acids may have been retained in the glass wool at these higher concentrations.

concentrations (66 and 63.5 Brix, respectively), which were roughly twice the concentration found in species with the lowest levels, *Eupatorium purpureum* L. and *Lespedeza capitata* Michx. Species such as *A. sullivantii*, *S. ericoides*, and *Monarda punctata* had significantly higher sugar concentrations than *E. purpureum*, *L. capitata*, *Coreopsis lanceolata* L., *Lithospermum carolinense*, *Asclepias syriaca* L., *Helianthus divaricatus* L., and *Vernonia gigantea* (Walter) Trel.

Total amino acid concentrations (corrected for predicted loss) ranged from 14 to 255.69 ng/μL. The species with the highest concentration of amino acids was *Vernonia gigantea*, which measured over four times higher than *Monarda punctata* and *Asclepias sullivantii*, the two species with the lowest amino acid concentrations. *Helianthus giganteus* L. and *Coreopsis tripteris* L. also had high relative amounts of amino acids at 255.65 and 253.87 ng/μL, respectively. More specifically, *H. giganteus* had significantly higher amino acid concentrations than *M. punctata*, *A. sullivantii*, *Liatris aspera* Michx., *L. spicata* (L.) Willd., and *A. tuberosa* L. ( $F_{18, 233} = 3.89$ ,  $P \leq 0.0001$ ,  $R^2 = 0.23$ ; Table 3, Fig. 5).

Interestingly, the two species with the highest sugar concentrations, *Asclepias sullivantii* (66 Brix) and *Monarda punctata* (61.28 Brix), also had the lowest total amino acid concentrations at 34.67 and 14.00 ng/μL, respectively. When we further examined this apparent tradeoff, we initially tested for an interaction between sugar concentration and genus, which was not significant ( $P = 0.50$ ) and was not pursued further. Our final model detected significant differences in total amino acid concentration among genera as well as an inverse relationship between total sugar and amino acid concentrations ( $F_{11, 235} = 6.11$ ,  $P < 0.0001$ ,  $R^2 = 0.22$ ; Table 3, Fig. 6), suggesting that genera that have high amounts of amino acids tend to have lower amounts of sugars and vice versa.

## DISCUSSION

**Method verification**—This modified centrifugation technique is an efficient, less damaging, inexpensive alternative approach for collecting nectar from small flowers or inflorescences across a wide range of taxa. Our method is especially useful for excluding pollen from nectar extractions for examining sugar concentrations while eliminating the potential for pollen contamination. Although sugar concentrations increased slightly, the discrepancy between collection methods is unlikely to be detected by or influence flower visitors, as sugar concentrations increased by an average of only 1.23 Brix. Furthermore, considering the much larger influence of environment on sugar concentrations (Corbet et al., 1979; Herrera et al., 2006; Nicolson and Thornburg, 2007), this is small compared to the levels of variation that may be experienced by pollinators while foraging.

Several limitations of this centrifugation method need further study, particularly for samples with relatively high amounts of amino acids, which showed considerable reduction in estimated amino acid concentration after using the glass wool filter. Because the differences between artificial nectar samples with and without the glass wool filter appeared to increase as amino acid concentration increased, we speculate that this problem was due to an insufficient time (30–60 s) allotted for centrifugation. Future studies will evaluate if longer spinning times or greater initial dilution reduce amounts retained in the flowers or glass wool filter. Because all our field samples were estimated with average

TABLE 3. Method verification and nectar survey data analyses for this study.

Variable	Source	df	Sum of squares	Mean square	F ratio	Prob > F
<b>Method verification<sup>a</sup></b>						
Total amino acid concentration (ng/μL)	Method	1	0.74		11.48	0.0007
	Histidine scale	9	450.40		776.93	<0.0001
	Method × scale	9	36.66		63.23	<0.0001
	Model	19	565.51	29.76	462.07	<0.0001
	Error	860	55.40	0.06		
	Total	879	620.91			
<b>Nectar survey<sup>b</sup></b>						
Sugar concentration (Brix)	Model	18	30759.09	1708.84	9.16	<0.0001
	Error	297	55436.81	186.66		
	Total	315	86195.90			
Total amino acid concentration (ng/μL)	Model	18	19.82	1.10	3.89	<0.0001
	Error	233	65.89	0.28		
	Total	251	85.72			
Trade-off analysis: Total amino acid concentration (ng/μL)	Genus	10	13.69		4.37	<0.0001
	Brix	1	3.86		13.64	0.0003
	Model	11	18.82	1.71	6.11	<0.0001
	Error	235	65.86	0.28		
	Total	246	84.69			

<sup>a</sup>ANOVA analysis, using standard least squares estimation, of centrifuging with and without a glass wool filter on the total concentration of amino acids, as well as the difference found between each level of the histidine scale (ranging from 1–10), and their interaction.

<sup>b</sup>Analysis of the differences in sugar concentration (Brix) found among species ( $R^2 = 0.318$ ), as well as for the log transformed total amino acid concentration (ng/μL) ( $R^2 = 0.23$ ), by ANOVA using standard least squares estimation, with Tukey's honest significant difference test. Trade-off analysis examined the change in amino acid concentration with variation in sugar concentrations among genera (using standard least squares estimation,  $R^2 = 0.22$ ). Interactions were nonsignificant and dropped from this model.

concentrations <70 ng/μL before the correction factor, which was near the upper limit of the range of concentrations over which the glass wool technique was performing adequately, these data are likely underestimates of total amounts of amino acids, but will be quite useful for comparing the nutritional value among plants within diverse communities of nectar sources in natural habitats.

In addition, because we used artificial nectar containing only histidine, once centrifugation times that optimize extraction are determined, more studies could determine whether other amino acids are affected similarly and how other nectar constituents, such as lipids (Galletto and Bernardello, 2004), are influenced. Although nonsugar constituents, including amino acids, can contribute to inflation of the refractive index and thereby increase the apparent overall sugar concentration (Inouye et al., 1980), this method provides an inexpensive alternative for acquiring initial total amino acid concentration data before progressing to more expensive methods such as HPLC for pollinator physiological ecology research. This method does provide baseline data on nectar sugar and amino acid resources from flowers of taxa that were otherwise difficult or impossible to obtain and have never before been surveyed. With refinements, this modified centrifugation method will be a valuable addition to the suite of existing nectar collection techniques.

**Nectar survey**—While the species in this survey are visited or preferred by butterfly species, many are also common pollinator food resources within tallgrass prairie and oak savanna habitats. Because we included samples from multiple sites for each species, the considerable variation we found in sugar concentrations was expected and is most likely due to environmental influences such as temperature and humidity that can alter sugar concentrations (Nicolson and Thornburg, 2007). The sugar concentrations data reported here (ranging on average between 31.54 and 66 Brix) are somewhat high for butterfly-pollinated flowers, which are generally around 20–25% Brix (Nicolson and

Thornburg, 2007; Willmer, 2011). However, our survey included few taxa specialized for butterflies (e.g., *Phlox* L.), and their rarity in our study sites prevented inclusion in our permits. Although butterflies were reported to prefer or commonly visit most of these flowers, they are not exclusively butterfly pollinated. For example, species in the genus *Asclepias* L. (average 54.98 Brix) are visited by a range of pollinators, including bees, as well as butterflies (Kephart, 1983), and flowers that are generally pollinated by long-tongued bees tend to have higher sugar concentrations, closer to what we observed, in the 50–60% range (Nicolson and Thornburg, 2007; Willmer, 2011). Alternatively, existing literature for sugar concentrations of butterfly-pollinated flowers may be a biased sample from species with larger flowers with more dilute nectar that are more readily sampled with microcapillary tubes (Inouye et al., 1980).

Although multiple components of nectar composition influence flower–pollinator interactions, the literature suggests that it is important to consider the mouthparts and mode of intake of the pollinator in relation to the sugar concentration and optimal ingestion through them. There are generally three modes of nectar feeding: active suction (in butterflies), capillary suction (in birds), and viscous dipping (in ants, bees, and bats) (Kingsolver and Daniel, 1979; Pivnick and McNeil, 1985; Kim et al., 2011). In flowers commonly visited by butterflies, sugar concentration ranges from about 20–25% (Pyke and Waser, 1981), whereas the optimal concentration for active or capillary suction feeding ranges from 30–40% (May, 1985), and the concentration for viscous dipping is often higher, ranging from 50–60% (Willmer, 2011). Effects of nectar sugar concentrations on uptake through mouthparts may also indirectly influence foraging behaviors or time spent on a flower, which could in turn influence predation risk.

Because floral nectar resources are considered important to increasing butterfly species abundance (Holl, 1995; Matter and Roland, 2002; Pywell et al., 2004; Shepherd and Debinski, 2005), understanding the specific nutritional value of nectars is

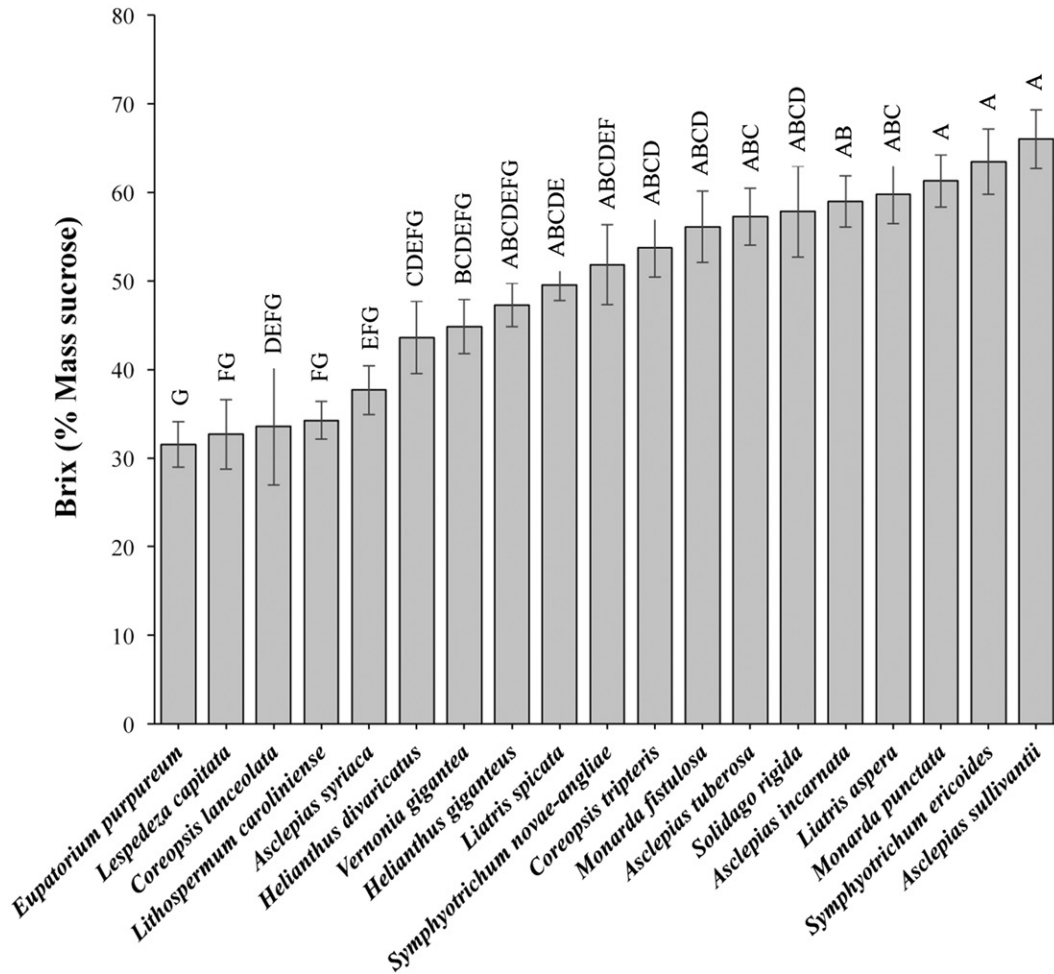


Fig. 4. Variation in sugar concentration among species. Significant differences were found among species by least squares regression ( $F_{18,297} = 9.16$ ,  $P < 0.0001$ ,  $R^2 = 0.36$ ). Species not connected by the same letter were significantly different from one another using Tukey's honest significant difference test. Error bars represent standard error.

essential for recommending which species are potentially more important for their conservation. For example, in a study of pollinator partitioning of three species of *Asclepias* (milkweed) (*A. incarnata* L., *A. syriaca*, and *A. verticillata* L.), Kephart (1983) found that, overall, Hymenoptera (bees and wasps) represented the greatest proportion of insects visiting these species, but Lepidoptera (butterflies and moths) constituted a much smaller proportion of visitors, ranging from 8% to 16% on *A. incarnata* and *A. syriaca*, respectively. Results from Kephart's study are consistent with observations in our survey, where the average for *A. syriaca* fell within the predicted optimal intake range for capillary feeding butterflies (30–40%; May, 1985; Kim et al., 2011) at 37.65 Brix, which also had the lowest sugar concentrations of the four milkweed species surveyed. The three other *Asclepias* species we sampled (*A. incarnata*, *A. tuberosa*, and *A. sullivantii*) had higher nectar sugar concentrations than *A. syriaca*. Whereas milkweeds are essential food sources for larval monarch butterflies (*Danaus plexippus*), this survey suggests that some are also particularly high-quality adult food sources, such as *A. incarnata*, which had relatively high concentrations of sugar in our survey (mean sugar concentrations 58.96 Brix), and also had the highest amino acid concentration

(121.28 ng/ $\mu$ L). Pollinator conservation programs seeking to improve success of this iconic species should consider the importance of milkweed diversity and the preservation of habitats that may be more likely to harbor milkweeds providing higher-quality nectar resources. Additionally, our data suggest that other species, such as *Coreopsis lanceolata*, *Eupatorium purpureum*, *Lespedeza capitata*, and *Lithospermum carolinense*, will be similarly beneficial to include in restoration plans geared toward butterfly conservation, as their nectars also fell within the optimal sugar concentration for feeding by butterflies.

The sugar concentration of nectar may also have important consequences for butterfly longevity and reproduction. Common imperial blue butterflies (*Jalmenus evagoras*) fed on a high sugar concentration diet (50%) did not live as long as those on the medium sugar concentration diet (25%), while the medium and high sugar concentration diets nearly doubled the fecundity compared to a diet of little or no sugar concentration (1% and 0%, respectively; Hill and Pierce, 1989). Cahenzli and Erhardt (2012a) found that in male small heath butterflies (*Coenonympha pamphilus*) moderate amounts of nectar sugars (20%) are primarily used to increase reproduction, whereas high sugar



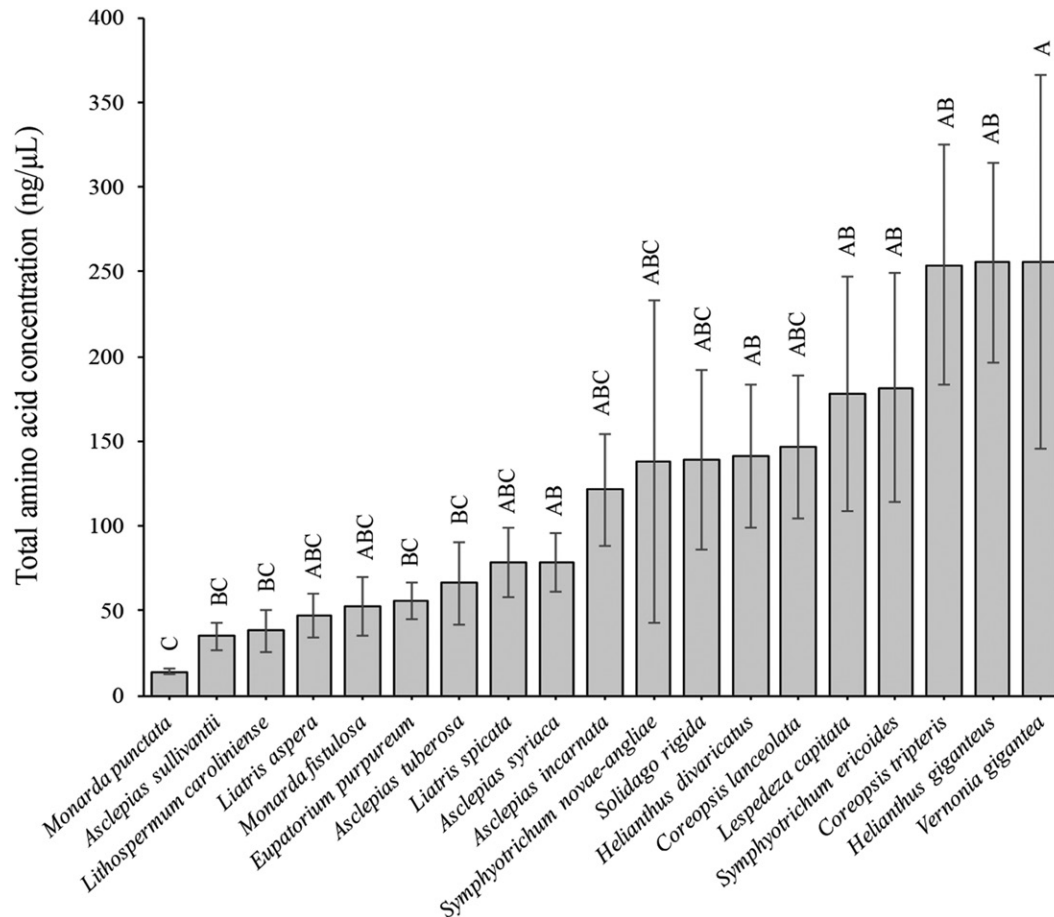


Fig. 5. Variation in total amino acid concentration among species. Data shown are untransformed. Significant differences were found between species following standard least squares regression ( $F_{18,233} = 3.89$ ,  $P \leq 0.0001$ ,  $R^2 = 0.23$ ). Species not connected by the same letter were significantly different from one another using Tukey's honest significant difference test. Error bars represent standard error.

concentrations (30%) benefit longevity, suggesting a tradeoff between reproduction and longevity.

In general, nectar amino acid concentrations tend to be lower than sugar concentrations (Willmer, 2011), with plants specifically pollinated by butterflies averaging amino acid concentrations of  $1.50 \mu\text{mol}\cdot\text{mL}^{-1}$ . Plants in our survey had total amino acid concentrations ranging on average from  $14.00$  to  $255.69 \text{ ng}\cdot\mu\text{L}^{-1}$  (assuming all 20 amino acids were present, this corresponds to  $0.010$ – $1.87 \mu\text{mol}\cdot\text{mL}^{-1}$ ). These results clearly suggest that including or increasing the abundance of nectar sources with abundant total amino acids (such as *Helianthus giganteus*, *Coreopsis tripteris*, *Vernonia gigantea*, and *Symphyotrichum ericoides*) may be particularly beneficial for butterfly conservation.

Although we found differences in amino acid concentrations among genera, we also observed an overall inverse relationship between sugar and amino acid concentrations, contrary to work by Gottsberger et al. (1984), which found no relationship between sugar and amino acid concentrations. Because our survey was not designed specifically to test any hypothesis regarding the generality of this type of relationship nor to sample across all possible nectar sources for pollinators, this result must be viewed as preliminary, at best. Nevertheless, this surprising relationship does suggest a tradeoff between high sugar and high amino acid concentrations across species averaged within genera. This could indicate differences in resource allocation or perhaps

differences in physiological processes, which has been noted with nectar production previously (Torres and Galetto, 1998), and warrants further study.

As recently advocated by Pyke (2016), plant nectars could be viewed as a way to manipulate pollinators. The apparent tradeoff between high sugar and high amino acid concentrations could therefore reflect different strategies for visitor manipulation. For example, nectar concentrations and compositions that are less than ideal for a visitor could encourage that potential pollinator to move to other nearby conspecific plants, thereby increasing outcrossing events (Nicolson and Thornburg, 2007; Pyke, 2016). While many consider nectar primarily as a caloric reward from the use of sugars as carbohydrates, nectar can also offer other rewards to visitors and pollinators such as vitamins, minerals, lipids, and water (Nicolson and Thornburg, 2007; Willmer, 2011), along with secondary chemicals that may enable self-medication (Richardson et al., 2016) or act as deterrents (Adler and Irwin, 2005). Alternatively, pollinators may also take more dilute nectars to meet other needs, such as water intake (Willmer, 1988; Nicolson, 1998). In areas where climate change increases pollinator needs for hydration (Chown et al., 2011), species with more dilute nectars, such as *Eupatorium purpureum*, *Lespedeza capitata*, and *Coreopsis lanceolata*, could become increasingly important (assuming they maintain nectar production under anticipated climate changes).

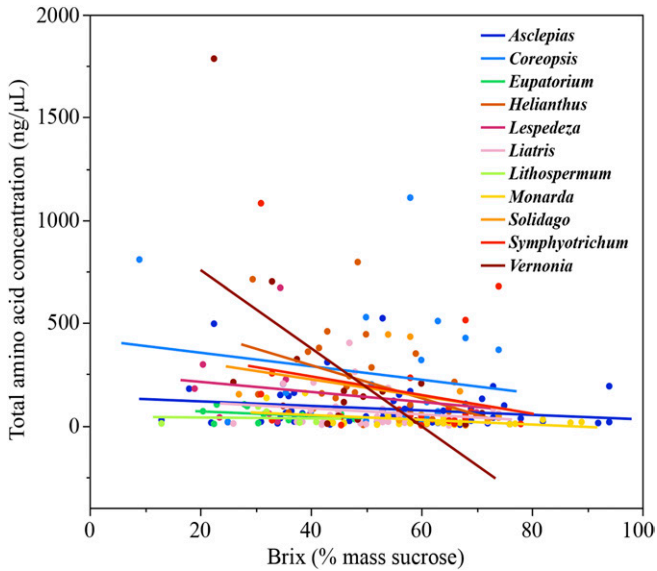


Fig. 6. Relationship of total amino acids (AA) and sugar concentration (Brix) among genera. Standard least squares regression revealed a significant inverse relationship between amino acid and sugar concentrations (model: AA = genus + Brix;  $F_{11, 235} = 6.11$ ,  $P < 0.0001$ ,  $R^2 = 0.22$ ; genus  $\times$  Brix interaction was nonsignificant and removed from the model). Amino acid concentrations displayed are untransformed data.

Understanding requirements for specific butterflies can better help determine appropriate choices for land managers when developing more effective restoration plans (Baz, 2002; Zaman et al., 2015; Thomas and Schultz, 2016). For example, Lepidopteran distributions in Minnesota oak savannas are associated with nectar and forb cover, with Karner blue butterfly occurrence also related to indicators of bison activity (Hess et al., 2014). Although Karner blue nectar plant selection suggests opportunism (Grundel et al., 2000), one of the high amino acid species in our study, *Coreopsis lanceolata*, was selected 84% of the time when present. This plant is common in Michigan during the first brood of adults, but relatively rare in Ohio's Oak Openings Region (Michaels, pers. obs.) and absent from sites where this federally endangered species has been reintroduced. Although species recovery is complex and there are no "magic bullets," feeding studies of monarch butterflies that were provided artificial nectar with an amino acid composition mimicking *C. lanceolata*'s nectar had increased egg production compared to those fed sugar-only nectar (Arnold, 2016).

Furthermore, because various pollinators can forage for nectar that meets specific needs, providing a diverse selection of forb nectar species that provide complementary types of nectar nutritional resources will be beneficial for restoring habitats for pollinators. For example, some taxa will offer high sugar concentrations (such as *Asclepias sullivantii*, *Monarda punctata*, and *Symphyotrichum ericoides*), while others (*Helianthus giganteus*, *Coreopsis tripteris*, or *Vernonia gigantea*) could provide high amino acid concentrations. This array of species would help to increase not only the biodiversity of the plant community, but will also enhance the biodiversity of floral and other resources for the pollinators, which could be essential for regional residential pollinator survival, as well as for supporting migratory species as changes in species distributions subsequent to habitat loss or climatic shifts may reduce critical resources. Future research should examine whether sites with species

containing high sugar and high amino acid concentrations attract a greater abundance or diversity of butterflies and other pollinators.

While this work provides information about nectar quality for many species that have not been sampled previously, a more detailed study of sugar and amino acid composition is needed to understand specific nectar components and importance of each for pollinators. Subsequent surveys, coupled with pollinator observations and studies of their fitness responses, will be essential for evaluating the benefits of foraging on the surveyed species. Together, the findings from this survey along with future studies will assist land managers in determining the appropriate path for developing more effective plans geared for pollinator restoration.

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