GENOTYPE AND ENVIRONMENT EFFECTS ON SUNFLOWER NECTAR AND THEIR RELATIONSHIPS TO CROP POLLINATION

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Abstract—Whether caused by genotype (G) or environment (E), floral trait variation has consequences for plants and their pollinators. Cultivated sunflower is a model system to explore floral trait variation; though sunflowers are bred to self-pollinate, benefits of pollination by bees remain substantial. To better understand sunflower-pollinator interactions, experiments were conducted to: (i) examine genotype and environment effects on nectar quantity and quality under controlled conditions, and (ii) assess effects of bags used for pollinator exclusion on nectar quantity, quality and bee foraging in a field environment. Contrasting temperature treatments (28°C, 21°C, 28°C/16°C) reveal environment effects or G × E interactions for nectar volume (µl / floret), concentration (°Brix), and sugar composition (% sucrose). Bags used to exclude sunflower pollinators resulted in nectar volumes greater than plants with unrestricted access for bees (= open-pollination), and in ≈ 5-fold increased visitation by wild bees after bags were removed. Differences in bee visits to plants that were previously bagged versus plants never bagged decreased over the 2 h following bag removal. Though genetic variation in sunflower nectar is affected by the environment and G × E interactions, improving pollination via plant breeding still appears feasible. Future research on intraspecific variation in pollen rewards could be helpful, especially because pollen has received little research compared to nectar. For research with nectar or pollen, it seems desirable to measure floral rewards with methods that don't rely on pollinator exclusion (bags or cages), which should provide more realistic data on what pollinators experience while foraging.

Keywords—Floral rewards, plant-pollinator interactions, pollinator exclusion, pollen, nectar, bees, Helianthus annuus L.

INTRODUCTION

Floral nectar traits are heritable in both wild and cultivated plants. In the perennial forb, viper’s bugloss (Echium vulgare L.), almost half of the variation in nectar volume is genetic (i.e., H²=0.48), but heritability estimates of nectar concentration and total sugar per flower are even higher (Klinkhamer & van der Veen-van Wijk 1999). Several other examples of heritable variation in nectar traits for wild plants (or crop wild relatives) have been recently reviewed and summarized (Parachnowitsch et al 2019). Among crops, nectar content of cultivated sunflower (Helianthus annuus L.) appears to have very high heritability (Atlagic et al 2003), and others including alfalfa (Medicago sativa L.; Teuber & Barnes 1979), soybean (Glycine max [L.] Merr.; Erickson 1975), and oilseed rape (Brassica napus L. var. oleifera; Bertazzini & Forlani 2016) show strong genotype effects on per-flower nectar volume.

However, environmental effects sometimes make the study of nectar traits, particularly outside of controlled conditions, prohibitively difficult (Mitchell 2004). Nectar volume may be affected by various factors including temperature (Jakobsen & Kristjánsson 1994), relative humidity (Wyatt et al 1992), soil moisture (Rering et al 2020), and soil nutrients (Burkle & Irwin 2009a). Even when the
environmental effects on floral nectar are generally known, they may be complicated by genotype × environment (G × E) interactions. In rampion bellflower (*Campanula rapunculoides* L.), clones subjected to hot, cool, and crowded environments showed significant G × E interactions for several floral traits, including nectar sucrose (Vogler et al 1999). Genotypes of common viper’s bugloss subjected to high- or low-water treatments showed genotype × environment interactions on per-flower nectar volume (Leiss & Klinkhamer 2005). Clones of white clover (*Trifolium repens* L.) also exhibit G × E effects on per-flower nectar volume when grown in chambers of various temperatures (and constant 65% RH), with some clones exhibiting maximal nectar secretion at low temperatures (Jakobsen & Kristjánsson 1994).

Whether caused by genotype or environment, floral trait variation has consequences for both plants and their pollinators. Plants in a population of wild mountain laurel (*Kalmia latifolia* L.) that produced greater quantities of nectar per flower received more pollinator visits and had higher reproductive success (= percent fruit set; Real & Ratcke 1991). For production of hybrid onion (*Allium cepa* L.) seed, visitation by bees (= 95% honey bees, *Apis mellifera* L.) increased with nectar per floret, and bee visitation predicted seed set (seeds per umbel; Gillespie et al 2015). Nectar and pollen rewards increase visitation by wild and managed bees to cultivated sunflowers (Mallinger & Prasifka 2017a), and yield increases (compared to pollinator exclusion) are greater for hybrids that attract more bees (Mallinger & Prasifka 2017b). For solitary leaf-cutter bees (Megachilidae), development from egg to adult is supported by provisions left with each egg; the addition of simulated nectar to provisions of several solitary leaf-cutting bees showed that levels of sugar in (unaugmented) provisions limited larval growth (Burkle & Irwin 2009b). Similar supplementation of nectar or pollen to bumble bee colonies can increase numbers of reproductive females (gynes) and males (Pelletier and McNeil 2003; Elliott 2009). Without manipulation but placed along a natural gradient, the longevity and weight of bumble bee colonies increased with the area of flowering crops within 1 km (Gervais et al 2020).

Genetic and environmental variation in floral rewards present opportunities to modify plant-pollinator interactions and the potential of breeding plants to improve pollination has been noted for various crops (Teuber & Barnes 1979; Bertazzini & Forlani 2016; Bailes et al 2018; Prasifka et al 2018). Cultivated sunflower is a model system to explore plant-pollinator interactions; though sunflowers are bred to self-pollinate, pollination by bees remains important to the crop (Mallinger & Prasifka 2017b). Variation in floral nectar is apparent (Mallinger & Prasifka 2017a) and is among the key traits linked to bee preference for particular sunflowers (Mallinger & Prasifka 2017a; Portlas et al 2018). It is also clear that sunflower nectar is influenced by the growing environment (Chabert et al 2020). To better understand factors that influence floral rewards and pollinator visitation in sunflowers, experiments were conducted to: (i) examine genotype and environment effects on nectar quantity and quality under controlled conditions, and (ii) assess effects of bags used for pollinator exclusion on nectar quantity, quality and bee foraging in an uncontrolled (field) environment.

**MATERIALS AND METHODS**

**NECTAR GENOTYPE AND ENVIRONMENT (G × E) EFFECTS UNDER CONTROLLED CONDITIONS**

Seven different germplasms were selected for evaluation; six inbred maintainer lines (HA 323, HA 412HO, HA 434, HA 441, HA 456, and HA 467) and one bulk population (ND-NONOIL B3, hereafter referred to as B3) were selected to represent known variation in cultivated sunflower germplasm for nectar quality (µl / floret) and quality (% sucrose). Seeds from each germplasm were planted singly into small cone-shaped containers (D40 Deepots, Stuewe & Sons Inc., Tangent, OR, U.S.A.) filled with a soil-less medium (Pro-Mix B, Premier Tech Horticulture, Quakertown, PA, U.S.A.). The seeds were germinated and grown in a plant growth chamber (PGC-105, Percival Scientific, Perry, IA, U.S.A.) using a 14:10 light-dark cycle, 18,000 lux illuminance, 28°C temperature, and 65% RH. Shortly after seedling emergence, 2 g of a controlled release granular fertilizer (14-14-16 N-P-K; Haifa North America, Savanna, GA, U.S.A.) was applied to each cone. Once each week, a water-soluble fertilizer (20-20-20 N-P-K; JR Peters, Inc, Allentown, PA, U.S.A.) was applied after mixing to 250 PPM N. Plants were top-watered as-needed.
with municipal tap water early in growth, and generally bottom-watered by partial immersion for up to 8 hours per day during bloom.

Three groups of plants were grown, with each group comprising four replicate plants of each germplasm. The first group, ‘warm,’ was maintained at 28°C though the completion of bloom. Conditions for the second group, ‘mild,’ were changed from 28°C to 21°C a week before the start of bloom and maintained under those conditions. The third group, ‘warm / cool,’ was moved a week before the start of bloom from a constant 28°C to a 28°C day, 16°C night (14:10) cycle.

Bloom for each plant was considered to start (= day 1) once all of the outermost florets on a head had begun shedding pollen. Sampling for nectar on each plant took place over three days (days 2–4), between 9:00–10:00 AM, using 1 µl glass microcapillary tubes (Drummond Scientific Company, Broomall, PA, U.S.A.). In general, nectar from three pistillate (= female phase, with receptive stigmas visible) florets was collected into a single microcapillary on each day of sampling, though just one floret was used for plants with the most nectar. After recording the nectar height and number of florets sampled for each microcapillary (to calculate nectar quantity in µl / floret), nectar was dispensed onto a hand-held refractometer (Bellingham + Stanley, Royal Tunbridge Wells, UK) to estimate the sugar concentration (Brix) in the floral nectar. A total of 12 separate nectar collections was made for each germplasm and temperature combination, though observations on nectar volume and sugar concentration were averaged over days 2–4 of bloom for each individual plant.

Sucrose concentration of nectar was measured for each plant after one of the three days of regular nectar collection. After nectar was dispensed, the mostly empty glass microcapillary was rinsed with distilled water from a water bath at 90°C, with the rinsate held in a 1.5 ml microcapillary tube. The tubes were placed in floating rack in the water bath and held at 90°C for at least 10 minutes to denature any enzymes present, after which samples were stored frozen at -80°C until carbohydrate analysis. Glucose, fructose and sucrose concentrations were determined by high performance anion exchange chromatography (HPAEC). Extracts were diluted with deionized water as needed, injected onto a 250 x 2 mm Dionex CarboPac PA 10 column (Sunnyvale, CA, U.S.A.) equipped with a 50 x 2 mm Dionex PA 10 guard column, and eluted isocratically with 60 mM NaOH and 10 mM NaOAc at 0.25 mL / minute. Carbohydrates were detected with an electrochemical detector (Dionex DC) operating in pulsed amperometric mode using the manufacturer’s recommended settings for carbohydrate analysis. Standards were prepared using glucose (G5400), fructose (F0127), and sucrose (SX1075) purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.).

**EFFECTS OF POLLINATOR EXCLUSION ON FLORAL NECTAR AND BEE VISITATION**

In sunflower breeding and research, pollinators are often excluded from heads using bags made from cloth or extruded polypropylene film. Though bags prevent cross-pollination, removal of bags during anthesis may present an unusual surge of floral resources for pollinators. Two experiments were used to examine the effects of pollinator exclusion by bagging on floral traits. Though each experiment was conducted in a single year (2020), they combine to show how bagging effects plants and pollinators under typical summer weather conditions (see Supplementary Table S1 for related weather data).

For the first experiment, plants were grown under controlled conditions and moved outdoors 1–2 d prior to the start of anthesis; this allowed data to be collected earlier in the summer than field-planted sunflowers started to bloom. Sixteen plants of HA 434 were grown at 28°C (soil-less medium, fertilizer and environmental conditions as described for G × E experiment, ‘warm’ group), and moved outside from 17–22 July 2020. Plants were placed adjacent to the Horticulture Gardens on the North Dakota State University campus in Fargo, North Dakota, and bottom-watered by partial immersion. Four plants were randomly assigned to each of four treatments: open (unbagged), bagged (except during nectar sampling), bagged at 11 AM, or bagged at 4 PM. The plants assigned to be bagged at 11 AM or bagged at 4 PM were unbagged (i.e., open to pollinator foraging and ambient conditions) each day between the start of nectar sampling (9 AM) and the indicated time of bag replacement. Nectar volume and concentration were measured on each
plant over three consecutive days as previously described.

For the second experiment, two single-row plots (5 m long, 0.76 m between rows) of both HA 434 and HA 456 were grown as part of a larger (0.20 ha) sunflower area planted on 15 June 2020 at North Dakota State University’s Agronomy Seed Farm in Casselton, North Dakota, U.S.A. HA 434 and HA 456 were selected based on previous research that showed them to differ for nectar volume and sugar composition (Mallinger & Prasifka 2017a). Each plot was thinned shortly after emergence to include 14 plants. One day prior to bloom, heads in one row each of HA 434 and HA 456 were covered with cloth bags. During bloom (20–27 August 2020), bags were removed at 10AM and replaced just after 12PM. During this period each day from 24–27 August, nectar was sampled from previously bagged heads at 10 AM and 11 AM (N = 3 per line at each time, using the same individual plants each day) as described for the G × E experiment, except that up to 10 florets per head were sampled, using larger microcapillary tubes (6.66 µl or 2.0 µl) to accommodate greater sample volumes. Nectar sampling included a total of 12 separate collections for each germplasm (HA 434 or HA 456) and time (10 AM or 11 AM) combination. Counts of plants in bloom (= in anthesis) were made in all four plots from 20–26 August, and walking counts of bees foraging (i.e., on heads collecting nectar or pollen) in each plot were made seven times (every 20 minutes starting at 10 AM) per day. Using the number of blooming plants in a plot each day as an adjustment, bee abundance for each walking count was summarized as the number of bees per 100 blooming plants.

DATA ANALYSES

Analyses were performed using SAS (SAS Institute Inc 2014) with specific procedures (‘PROC’) noted for each analysis. For G × E effects on nectar under controlled conditions, dependent variables of interest and units of measurement were nectar volume (µl / floret), concentration (Brix), and sucrose (%; sucrose / [sucrose+fructose+glucose]). A separate analysis (PROC GLM) was conducted for each dependent variable with genotype (= inbred line), environment (= temperature), and genotype × environment included as experimental variables. Because prior information was available on nectar quantity or quality for several of the included genotypes, comparisons of least-squares estimated means were only made across the three environments (28, 21, and 28°C / 16°C) using t-tests.

Nectar data for plants grown in containers, moved outdoors and bagged at various times were averaged (for each plant) over the three consecutive days of nectar sampling. One-way analyses of variance (ANOVA) were used to test for effects of bagging treatments on nectar volume and concentration (PROC GLM), with comparisons of least-squares estimated means made with t-tests. Nectar sampling data from field plots were treated similarly; data for individual plants were averaged over the four consecutive days of nectar sampling. Two-way ANOVA were used to test for effects of inbred line (HA 434 and HA 456), time (10 AM and 11 AM), and a germplasm × time interaction on nectar volume and concentration (PROC GLM). Because of a clear interaction, the effect of time after bag removal on nectar volume was analysed within each line via t-tests.

Bee foraging data from field plots (HA 434 and HA 456, with or without bagging) were averaged for individual observation periods (10:00 AM, 10:20 AM...12:00 PM) across the six days of walking counts. This permitted use of a repeated-measures ANOVA (PROC GLM) to investigate bee foraging after removal of bags. In this analysis, the four plots were subjects, bagging (+/-) was the between-subject variable, and time (measured in 20-minute intervals following removal of bags) was the within-subject variable.

RESULTS

Under controlled conditions, nectar volume (µl / floret) was influenced by genotype (F = 108.88; df = 6, 61; P < 0.001), environment (F = 37.06; df = 2, 61; P < 0.001), and the genotype × environment interaction (F = 7.50; df = 12, 61; P < 0.001) (Fig. 1). Acknowledging the significant G × E interaction, nectar volumes differed in all paired comparisons of environments, with mean values of 0.34 µl / floret at 28°C, 0.29 µl / floret at 21°C, and 0.18 µl / floret at 28°C / 16°C. Nectar concentration was significantly affected by genotype (F = 7.75; df = 6, 61; P < 0.001) and environment (F = 11.52; df = 2, 61;
Comparisons showed similar mean nectar concentrations of 65.6 and 63.8 °Brix at 28°C and 21°C, respectively, both of which were greater than 58.5 °Brix in the 28°C / 16°C environment. Nectar sucrose was not consistent among plants for B3, so this germplasm was excluded from the analysis; the remaining six lines showed significant genotype \((F = 698.72; \text{df} = 5, 52; \ P < 0.001)\), environment \((F = 7.16; \text{df} = 2, 52; \ P = 0.002)\), and genotype × environment interactions \((F = 4.16; \text{df} = 10, 52; \ P < 0.001)\) (Fig. 3). Pairwise comparisons indicate greater nectar sucrose at 21°C compared to 28°C and 28°C / 16°C environments. However, excluding HA 456 and B3, sucrose was nearly absent; of the remaining samples, 49 of 59 (83%) had < 1% sucrose and a nearly equal division of nectar sugars between fructose and glucose.

For plants grown in containers and moved outdoors, bagging treatments significantly altered nectar volume per floret \((F = 12.07; \text{df} = 3, 12; \ P < 0.001)\); florets of plants bagged continuously or at
Figure 3. Sucrose content (mean % sucrose ± SE; % = sucrose / sucrose + fructose + glucose) of sunflower nectar from plants held under constant humidity and 14:10 (L:D) lighting with temperatures during bloom considered warm (constant 28°C), mild (constant 21°C), and a warm / cool diurnal cycle (28°C / 16°C). Genotype ‘B3’ was excluded from analysis because of apparent segregation for % sucrose. Analysis showed significant (P < 0.05) effects of genotype (G), environment (E), and G × E interaction.

11 AM contained the most nectar (means of 0.50 µl / floret for both), plants bagged at 4 PM contained an intermediate amount (0.33 µl / floret), and unbagged (open-pollinated) plants contained the least (0.10 µl / floret) nectar. Bagging also significantly affected sugar concentration of nectar (F = 12.43; df = 3, 12; P < 0.001), with more sugar in nectar of plants that were continuously bagged (mean = 34.2 °Bx) or bagged at 11 AM (31.8 °Bx) than plants bagged at 4 PM (22.3 °Bx) or left unbagged (20.0 °Bx).

In field-grown plots of HA 434 and HA 456, nectar per floret was significantly affected by line (F = 11.05; df = 1, 8; P = 0.010), time (F = 47.97; df = 1, 8; P < 0.001), and their interaction (F = 19.65; df = 1, 8; P = 0.002). When examined within each line, plants of HA 434 contained more nectar per floret at the time of bag removal each day (10 AM), but nectar volume declined sharply by 11 AM; for HA 456, an apparent decline in nectar between 10 AM and 11 AM was not significant (Fig. 4). The sugar content of nectar samples was only affected by line (F = 6.65; df = 1, 8; P = 0.033), with nectar from HA 456 (23.2 °Bx) containing slightly more sugar than HA 434 (20.0 °Bx). The repeated-measures analysis of bee foraging in field plots with and without pollinator exclusion showed significantly more bees foraging in plots that had been bagged for the previous 22 hr (F = 873.91; df = 1, 2; P = 0.001), with the number of bees declining over time (F = 10.76; df = 6, 12; P < 0.001). However, the presence of a bag × time interaction (F = 5.37, df = 6, 12, P = 0.007) and visual examination of bee activity in the plots (Fig. 5) suggests the decline in bee visits may only apply to the bagged plots.

**DISCUSSION**

Contrasting temperature treatments reveal environment effects or G × E interactions for components of nectar rewards in cultivated sunflower, including nectar volume (µl / floret), concentration (°Brix), and sugar composition (% sucrose). Effects on nectar volume seem most significant to foraging bees and crop pollination for at least two reasons. First, nectar quantity is positively related to bee visits in sunflower.
(Mallinger & Prasifka 2017a), a relationship also observed in other crops (see Prasifka et al 2018). Second, the magnitude of the interaction was greatest for nectar volume; while some tested lines produced far more nectar under warm (28°C) conditions, other lines did not respond to increased temperature or showed maximum nectar per floret under a mild (21°C) temperature (Fig. 1) when humidity was consistent (65% RH) across temperature treatments. The opportunity presented by G × E interactions for nectar volume has been previously noted (Jakobsen & Kristjánsson 1994); breeding lines that produce high levels of nectar under the conditions prevalent during bloom (which vary by location and time) could ensure crop varieties that are most attractive to pollinators.

There are other reasons to believe the environment or genotype × environment effects on nectar sugar composition (% sucrose) and concentration (°Brix) may be less biologically
Genotype and environment effects on sunflower nectar

important in sunflowers than nectar volume. For example, research using artificial feeders found honey bees preferred nectars composed of 30–50% sucrose over those with more or less sucrose (Waller 1972). However, prior work suggests sunflower nectars often have little sucrose (Mallinger & Prasifka, 2017a) and in growth chambers most of the sunflowers tested had consistently low (< 10%) sucrose nectar in all three temperature treatments (Fig. 3). With regard to sugar concentration, bees often prefer nectars with more sugar to maximize calories ingested (Cnaani et al 2006; Konzmann and Lunau 2014), but under controlled conditions (i.e., plant growth chambers), the nectar of tested lines was generally a high-quality reward that appears close to optimal for social bee species (50–60 °Brix; Kim et al 2011). However, because various field observations show sunflower nectar to be substantially more dilute than nectar samples from growth chambers (Pamminger et al 2019; Chabert et al 2020), environmental effects on nectar concentration still could be important under natural (i.e., field) growing conditions.

Bags used to exclude sunflower pollinators resulted in nectar volumes greater than plants with unrestricted access for bees (= open-pollination), and produced more concentrated nectar in one of two experiments. Of course, without removal of nectar by bees (and other nectar-feeders), it is logical to find more nectar in florets concealed by bags. However, there are reasons to expect other causes of these differences. When bagged and unbagged plants are sampled for nectar prior to observed bee activity (e.g., following a cool evening), greater nectar volumes are still found in bagged plants (Prasifka personal observation). A recent study with sunflowers found relative humidity (expressed as vapour pressure deficit; Chabert et al 2020) influenced both nectar quantity and concentration for nectar samples over a four-year period. Because microclimate effects of increased temperature and humidity are known from other studies that use bags to limit pollination (see McGoey et al 2017), this second, unintended effect, is also a plausible explanation for increased nectar in bagged sunflowers.

Bagging sunflower plants in field conditions also resulted in greatly (≈5-fold) increased visitation by wild bees after bags were removed, though differences between plants that were previously bagged and never bagged decreased over the 2 h following bag removal. This implies increased rewards (greater amounts of nectar [quantified] and pollen [observed but not measured]) induced individual bees to return to the unbagged plots after an initial visit. However, other mechanisms may produce the same pattern. For example, pollinators can perceive various cues prior to sampling nectar or pollen that influence the chances of finding a plant with suitable rewards and foraging on that plant. These cues include electric fields around flowers (influenced by other visiting bees; Clark et al 2013), humidity gradients (which signal the nectar reward; von Arx et al 2012), and the odour or colour of pollen (Nicholls & Hempel de Ibarra 2017). Of course, bags would also trap plant volatiles that are not directly related to nectar or pollen but could influence bee visitation.

In summary, though genetic variation in sunflower nectar rewards is affected by the environment and G × E interactions, improving crop pollination via plant breeding still appears feasible. However, floral rewards in a single crop are complex (combining quality, quantity and accessibility of nectar and pollen) and competition for pollinator visits with other surrounding wild and cultivated plants adds another element to plant-pollinator interactions in sunflowers. Future research on (quantitative and qualitative) variation in pollen could be helpful, especially because of how little attention pollen rewards have received compared to nectar. For work with both nectar and pollen, it also seems desirable to measure floral rewards with methods that don’t rely on pollinator exclusion (bags or cages), which should provide more realistic data on what pollinators experience while foraging.

ACKNOWLEDGEMENTS

We appreciate support in nectar sucrose measurements by John Eide (USDA-ARS). Brian Olson (North Dakota State University) and Chris Misar (formerly USDA-ARS) provided essential help in the preparation, planting, and maintenance of field plots.

APPENDICES

Additional supporting information may be found in the online version of this article:
Table St. Weather observations from NDawn (North Dakota Agricultural Weather Network) at https://ndawn.ndsu.nodak.edu/

REFERENCES


Genotype and environment effects on sunflower nectar

February 2023


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