

**Sweet and salty:
Pollinators and sodium-enriched nectar**

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Project Summary/Abstract

Floral resources shape plant-pollinator interactions, in large part due to animal pollinator's dependence on nectar. Many plants have evolved nutrient rewards to attract pollinators, but the vast majority of research has focused on floral nectar, and the sugar content of this floral resource. Concentrations of sodium, however, a micronutrient in low concentrations in plant nectar, vary significantly in floral nectar within plant communities and are essential for animal metabolism. Yet, the effects of sodium in floral nectar are largely unexplored. Here, we investigate whether experimentally enriching floral nectar with sodium influences the structure, diversity, and abundance of pollinator interactions. In July and August 2022 at the Rocky Mountain Biological Laboratory in Western Colorado, USA, we experimentally enriched sodium concentrations (0.5% w:v NaCl) in plant species in a subalpine meadow. We compared Na-enriched nectar to a control nectar addition treatment to determine sodium's effect on plant-pollinator interactions. We find that experimentally enriched floral nectar with sodium increases visitation rate, number of visitors, and diversity of pollinators. These findings provide evidence consistent with the 'salty nectar hypothesis' and evidence for the importance of sodium limitation in pollinators. Climate and land-use change are also expected to cause broad-scale changes to sodium availability through precipitation, evapotranspiration, salting roads, and the distribution of large herbivores. An understanding of sodium's effect on plant-pollinator interactions could indicate how such changes could impact the structure and dynamics of plant-pollinator communities.

Keywords: micronutrients, sodium, plant-pollinator interactions, *Delphinium barbeyi*, *Bombus* spp.

Introduction

Pollinators depend heavily or exclusively on floral resources to meet their nutritional needs—adult bees foraging on nectar and providing pollen for developing larvae (Michener 2000). Because of their complete dependence on flowers, floral resource availability, including the availability and distribution of critically important macro- and micronutrients, largely influence the quality, distribution, and abundance of plant-pollinator interactions (Roulston & Goodell 2010; Woodard & Jha 2017; Pope & Jha 2018; Finkelstein *et al.* 2022).

To attract pollinators and improve reproductive fitness, some plants increase the quantity and quality of their nectar rewards (Veits *et al.* 2019). Improved rewards from higher sugar concentrations can increase pollinator visitation (Cnaani *et al.* 2006) and visit duration (Manetas & Petropoulou 2000), likely improving pollination services and plant reproductive success (Brandenburg *et al.* 2012). Other components of nectar composition—even those occurring in low-concentrations—are likely adaptive (González-Teuber & Heil 2009). However, there exists a lack of direct evidence showing the relationship between floral resource availability of other macro- and micronutrients and the frequency, structure, and stability of plant-pollinator interaction networks.

Among these nutrients, sodium (Na) may have an underappreciated and critical role in the structure and function ecosystems, especially in shaping the foraging habits of herbivores, including pollinators (Kaspari 2020). Sodium exists in plants at low concentrations and with little metabolic functions; for plant consumers, including pollinators, sodium is essential for metabolism and maintaining Na-K ATPases (Kaspari 2020). Indigenous communities, ranchers, and hunters have historically used sodium-rich patches to attract game and cattle via saltlicks (Kennedy *et al.* 1995). Wild herbivores also use naturally occurring salt deposits to avoid sodium deficiency (Hebert & Cowan 2011). Sodium limitation also effect invertebrates. A large-scale manipulation of micronutrients found that herbivorous prairie insect communities are limited by sodium and track its availability (Prather *et al.* 2018; Welti *et al.* 2019). This limitation is supported by experimental Na-enrichments showing enhanced foraging ant recruitment (Kaspari *et al.* 2008, 2020) and increased termite activity (Kaspari *et al.* 2014). Swallowtail butterflies also exhibit a puddling behavior in sodium-rich solutions to supplement nutrients lacking in nectar (Arms *et al.* 1974). It is also commonly observed for pollinators, such as sweat bees and other solitary bees, to land on humans to lick salt from sweat or tears (Banziger *et al.* 2009).

Because sodium concentration in nectar varies substantially within plant communities, including subalpine meadows (Hiebert & Calder 1983), it is hypothesized that plants may be selectively spiking their nectar with sodium to attract pollinators. Finkelstein *et al.* (2022) recently found that the number of visits from pollinators and the diversity of pollinators increased on plants with sodium-enriched nectar. The near doubling of diversity and visitation was observed in a potted plant experiment in a semi-natural meadow in near Burlington, Vermont, USA. Here, we explore the effects of sodium-enriched nectar with wild, native plants within an intact subalpine meadow ecosystem in the Colorado Rocky Mountains.

We experimentally enrich floral nectar with Na to test the response of plant-pollinator interaction networks in a natural, subalpine environment. We ask: 1) Are pollinator foraging patterns

(visitation rates, diversity, and handling time) different in a sodium-enriched plant communities?
2) Does sodium-enrichment improve plant fitness and seed set size for *D. barbeyi*?

This research adds clarity to the ‘salty nectar hypothesis’ and evidence for the importance of sodium limitation in pollinators and evolution of nectar attractants. Climate and land-use change are also expected to cause broad-scale changes to sodium availability through precipitation, evapotranspiration, salting roads, and the distribution of large herbivores. An understanding of sodium’s effect on plant-pollinator networks could indicate how such changes could impact the structure and dynamics of plant-pollinator communities.

Methods

Site description

We conducted this work in a subalpine meadow (Marriage Meadow, 38°58’N, 106°59’W, 2900 m above sea level) near the Rocky Mountain Biological Laboratory in Gothic, Colorado, USA between July 15 and August 2, 2022 (Figure 1). The study area is a mosaic of wet and dry meadows, interspersed with aspen and conifer forests (CaraDonna & Waser 2020).

Sampling and treatment design

Within the study meadow, we selected conspecific pairs of each of the 4 focal species, for a total of 4 plant units throughout the meadow. Focal species for this study include *Delphinium barbeyi* (Ranunculaceae), *Erigeron speciosus* (Asteraceae), *Helianthella quinquenervis* (Asteraceae), and *Heliomeris multiflora* (Asteraceae). These plant species have a diverse morphology and community of pollinators (CaraDonna & Waser 2020; Bain *et al.* 2022). All study species are abundant, co-flower in July, and co-occur naturally with sufficient abundance in this subalpine meadow. *D. barbeyi* and *E. speciosus* were selected and labelled at the individual level, while adjacent groupings (2-5 individuals) of *H. quinquenervis* and *H. multiflora* are considered a single unit because each individual has few or one flowering unit compared to the other focal species. Each of these plants form a conspecific pair with another adjacent flower of the same species and relatively consistent size, one receiving the control treatment and the other receiving the sodium-enriched treatment.

We experimentally manipulated the nectar of all flowering heads of our focal species. We created two stock solutions for our control and experimental treatments; the control nectar contains 50% sucrose (weight : volume) and our sodium-enriched (NaCl) experimental nectar will contains 50% sucrose + 0.5% NaCl (weight : volume). To make the artificial nectar solutions, we added 170 g of sugar to 200 ml of water to achieve 0.5 °Bx. For the Na-enriched treatment, we added 1.55 g NaCl. The amount of artificial nectar to be applied to each flowering species depended on the naturally occurring volume of nectar for each species at the Rocky Mountain Biological Laboratory (Kirschke *et al.* 2019; Kirschke & CaraDonna unpublished data). The sugar content of each treatment is consistent with the range of sugar concentration observed for each focal species (Kirschke *et al.* 2019). Observations of sodium concentrations in floral nectar in the Colorado Rocky Mountains range from < 0.001% Na to 0.012% Na (Hiebert & Calder 1983). Therefore, the 0.5% NaCl (0.077% Na) experimental sodium-enriched nectar is

approximately 5x naturally occurring concentrations of sodium in *D. barbeyi* at the RMBL (Table 1) and is also presumably diluted by the ambient nectar.

Sodium-enrichment and pollinator observations

We applied these treatments and observed interactions between flowering plants and insect pollinators from July 15 to August 3 on warm, sunny days. The order of species and treatment application on any given treatment day was random. Treatments were applied by gently inserting the pipette tip into the nectar spur (*D. barbeyi*), into individual florets (*H. quinquenervis*, *H. multiflora*), or spread across the surface of the capitulum (*E. speciosus*). We replaced the pipette tip between each plant individual to avoid cross-pollination and cross contamination of nectar solutions. Plants that were designated as sodium-enriched exclusively received the sodium-enriched nectar treatment for the duration of the experiment.

After applying the nectar treatments to a pair of plants, we conducted 20-minute observations of pollinators at each plant, recording the identity of each pollinator, its total number of visits, and the total time of its foraging bout. Plants and pollinators were identified to the finest taxonomic unit possible. If species level identification was not possible, pollinators were recorded as distinct morphospecies (e.g., Bain et al. 2022).

Data analysis

To determine whether Na-enriched changes pollinator foraging patterns, we compared the total visitation rate, number of unique visitors, species richness, and handling time between Na-enriched and control plants during each 20-minute observation period. Because the data for total visits, number of visitors, and species richness are count-based and overdispersed, we examined differences in the number of visitors between treatments and plant species using a generalized linear model with negative binomial error distribution. The models included treatment and plant species as fixed effects. Because of the expected and observed variation of visitation between days and across the meadow, we included sampling date and the plant pair number as random effects. We also examined whether handling time increased with Na-enrichment. Because of the variation in foraging patterns across pollinator species, we estimated the handling time of only *Bombus* spp. (the most common visitor across all species) by dividing the total duration (in seconds) of each individual's foraging bout by the number of flowers it visited. Our linear mixed-effects model included handling time and plant species as response variables, and a treatment x species interaction. We included treatment and plant species as fixed effects and date and pair number as random effects, as with our other models above. The only plant species with sufficient *Bombus* spp. visitation (*D. barbeyi* and *H. quinquenervis*) were included in this model.

We designed another experiment to observe pollinators' response to a range of sodium doses in artificial nectar and compare the seed set of *D. barbeyi* under our different treatments (Appendix A).

Results

Over three weeks and 45 hours of observation for 4 plant species, we recorded 4,429 pollinator visits from approximately 565 unique pollinator visitors. The most common visitors included *Bombus appositus*, *B. flavifrons*, and *B. bifarius*. Other common pollinators included Lepidoptera, Diptera, and Hymenoptera were observed.

Across all species, we observed more than twice as many visits from pollinators on Na-enriched plants ($\bar{x} = 47.7 \pm 7.22$ s.e.; $\tilde{x} = 27$) than on our control plants ($\bar{x} = 17.0 \pm 3.09$ s.e.; $\tilde{x} = 10$; Figure 2). Despite the differences in ambient and treatment visitation between species, the general pattern is shared among all plant species (plant species x treatment interaction term: $\chi^2_4 = 1.7908$, $p = 0.7742$; Table 2). In other words, Na-enrichment increased visitation regardless of the plant species in question (Fig. 2). Total visitation differed among plant species (species effect: $\chi^2_4 = 52.1848$, $p = > 0.0001$; Figure 1), with *D. barbeyi* receiving the most visits ($\bar{x} = 64.7 \pm 11.8$ s.e.), and *E. speciosus* having the fewest ($\bar{x} = 5.53 \pm 1.75$ s.e.) per observation period.

We also observed more unique visitors, estimated as each distinct foraging bout, on Na-enriched plants ($\bar{x} = 5.74 \pm 0.604$ s.e.; $\tilde{x} = 4$) compared to the control plants ($\bar{x} = 2.88 \pm 0.323$ s.e.; $\tilde{x} = 3$; Figure 3). We observed more visitors regardless of plant species (plant species x treatment interaction term: $\chi^2_4 = 0.9619$, $p = 0.9155$; Table 2). The richness of pollinator visitors also increased with the Na-enriched treatment during each observation period ($\bar{x} = 2.69 \pm 0.281$; $\tilde{x} = 1.91 \pm 0.276$ s.e.; Table 2, Figure 4). The evenness of visits, estimated with the Simpson's Index, decreased in the sodium-enriched treatment ($\bar{x} = 0.277 \pm 0.281$ s.e.) compared to the control ($\bar{x} = 0.468 \pm 0.276$ s.e.; $\chi^2_1 = 7.2037$, $p = 0.0072$; Figure 4).

Finally, the handling time of *Bombus* visitors varied significantly among plants, but the Na-enriched treatment had no significant effect ($\chi^2_1 = 2.1398$, $p = 0.1435$). Within *Delphinium barbeyi*, however, handling time increased ($\chi^2_1 = 5.1329$, $p = 0.02348$) in the Na-enriched treatment compared to the control (Figure 5).

Discussion

Pollinators depend on floral resources for their nutrient demands, allowing plants to develop adaptive strategies to attract pollinators to promote their own reproduction (van der Kooi *et al.* 2021). Sugars, amino acids, and VOCs are primary nutrients and attractants with evidence of their ability to lure pollinators (Baker & Baker 1986; Irwin *et al.* 2004; González-Teuber & Heil 2009). Because of nectars primary function as a pollinator reward, other micronutrients found in nectar are likely adaptive, including sodium (Khan *et al.* 2021). Here, we find that Na-enrichment in floral nectar increases the frequency and duration of plant-pollinator interactions in the wild plant community of a subalpine meadow. We observe a two-fold increase in pollinator visitation rates from Na-enrichment, followed by concurrent increases in the number of unique pollinator visitors. We also found support for longer handling time in some plant species and shifts in the diversity of pollinator visitors. These findings are consistent with those of Finkelstein *et al.* (2022) that took place in experimental meadow in a different ecosystem.

Evidence for sodium limitation is present in multiple groups of herbivores, including insect herbivores in prairies (Welti *et al.* 2019), ants (Kaspari *et al.* 2008), bees that drink human sweat and tears (Banziger *et al.* 2009; Molleman 2010), and puddling swallowtail butterflies (Arms *et al.* 1974). Observations that bumblebees, the group that dominates the observations in this study, favor nectar with sodium are not surprising given their dependence on nectar for nutrient demands and the absence of other strategies to supplement sodium. The importance of sodium intake is likely linked to maintaining the digestive, excretory, and neuromuscular systems in these insects (Molleman 2010). Although our work suggests it is important for pollinators, further work is needed to determine whether aspects pollinator demography is sodium limited, or if pollinators just prefer more salty solutions.

The ability for sodium in nectar to effectively lure more pollinators and increase their visitation is evident, but how pollinators are detecting, learning, or responding to Na-enriched nectar remains an open question. Three explanations are possible: pollinators are learning and remembering Na-enriched plants, they are detecting enriched nectar from a distance, or foraging efficiently. Evidence for learning high-quality nectar rewards in bees has been documented on multiple occasions (Dukas & Visscher 1994; Wright *et al.* 2013). While we were not able to spike all plants every day and pollinators were often exposed to ambient nectar on plants observed with Na-enriched treatments, plants received the same treatment for the duration of the study. Depending on the fidelity of pollinator learning, they may have learned Na-enriched plants and continued to return despite the variation in reward. If pollinators learn of the favorable nectar and return quickly within a 20-minute observation, this could also explain the increase in visitors and visitation. It is unknown if or how pollinators might detect Na-enriched nectar. Bees can perceive nectar volatiles with their antennae and increase visitation on high reward flowers (Howell & Alarcón 2007). It could be that salt in nectar modifies the VOCs and floral scents or alters nectar yeast communities and their emitted VOCs (Klaps *et al.* 2020; Mostafa *et al.* 2022). Optimal foraging may also explain the increase in visitation rates of pollinators, with visitors finding a plant particularly rewarding and visiting more flowers on plants with Na-enriched nectar than on control plants (Pyke & Ren 2022). This, however, does not explain the increase in the number of unique visitors and the increase in the richness of pollinators on Na-enriched plants, which at a minimum suggests pollinators must be able to detect something in Na-enriched nectar.

Presumably, more pollinator visitors, increased pollinator visitation, and longer handling time might confer a fitness advantage for sodium-enriched plants, driving the evolution of sodium in nectar. For natural selection to drive the evolution of sodium in nectar, there must be heritable variation in the nectar trait and a fitness difference. Floral nectars in subalpine plant communities exhibit more than a 24-fold variation in the concentration of sodium both among and within plant species (Hiebert & Calder 1983). The heritability of this trait and the extent to which the variation occurs because of genetic or environmental influences remains an open question (Mitchell 2004). Despite the plasticity of nectar traits and their sensitivity to environmental variation (Enkegaard & Boelt 2016; Waser & Price 2016), a metaanalysis of some nectar traits (production rate and 5 different sugar concentrations) found significant heritability ($H=0.31$) (Parachnowitsch *et al.* 2019). No study has reported the heritability of other nectar traits like sodium concentration, leaving this an open question.

We observe increased visitation among all plant species in our study and some evidence that handling time changes depending on the morphology of the flower. Few studies have linked pollinator preference for nectar traits to changes in plant fitness (Parachnowitsch *et al.* 2019). Increased handling time and visits on plants with more nectar have been shown to increase seed set (Jennersten 1988; Brandenburg *et al.* 2012), but other evidence suggests that this variation has no effect on plant reproduction (Adler & Irwin 2005). Future studies should attempt to link pollinator response to nectar traits, like sodium-enriched nectar, to seed set and reproduction.

Future studies could expand on our findings by disentangling the learning or sensing of sodium-enriched nectar and the plant's fitness response to increased visitation and handling time. Our dose-response experiment outside of the GRC was designed to explore these questions, but the timing of *D. barbeyi* flowering and low seed set across all our plants may present challenges (Appendix 1). Our knowledge of sodium in plant nectar is also limited to the findings of Hiebert and Calder (1983) in only a few species at the Rocky Mountain Biological Laboratory. Further investigation should determine the variation of sodium concentrations in nectar across the landscape, across plant communities, and the variation within individual species. Biogeographical studies linking different levels of Na-limitation from proximity to the coast may also reveal a changing effect-size of Na-enrichment and further influence plant's strategies of attracting pollinators (ie. (Kaspari *et al.* 2008)).

Furthermore, we should investigate how individual plants distribute sodium throughout their foliage and nectar. Na-enriched foliage may increase foraging from herbivores (Kaspari *et al.* 2014; Welte *et al.* 2019). Plants may subvert this tradeoff by selectively siphoning sodium to nectar resources thus attracting pollinators, while limiting the amount of sodium in the foliage that would otherwise attract herbivores. The ultimate test for the salty-nectar hypothesis would be finding an enhanced plant fitness in sodium-fertilized plants pollinated by animals and insects (Kaspari 2020).

Our work suggests that sodium in nectar may be an important lure for pollinators in wild ecosystems. By replicating the experiment and findings from Finkelstein *et al.* (2020) in a wild, subalpine meadow with a diversity of native pollinators and using treatments that more closely represent ambient nectar values, we provide further evidence for the importance of sodium and the need to pursue questions about the evolution, strategies, and sensing of Na-enriched nectar.

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Tables and Figures



Figure 1. Research site at Marriage Meadow near the Rocky Mountain Biological Laboratory, Gothic, Colorado, USA ($38^{\circ}58'N$, $106^{\circ}59'W$, 2900 m above sea level).

Table 1. Ambient nectar and experimental nectar traits for the focal species included in this experiment. Volume and sugar concentrations from Kirschke *et al.* 2019 and previous work by Paul CaraDonna. Na concentrations from Hiebert and Calder (1983). (*Should I include the ranges of Na in nectar across the entire community and the **range** of volume and nectar values? My sugar concentrations and volumes fell within the range of Q1 and Q3, even though they do not match the median.*)

Species	Ambient			Experimental Treatment			
	Volume	Sugar (mg/ul)	Na% (w:v)	Volume	Sugar (mg/ul)	NaCl % (w:v)	Na% (w:v)
<i>Delphinium barbeyi</i>	1.2	0.668	0.006 ± 0.001	2.5	0.50	0.5	0.07
<i>Erigeron speciosus</i>	0.16	0.767	-	1.2	0.50	0.5	0.07
<i>Helianthella quinquenervis</i>	0.38	0.727	-	1.5	0.50	0.5	0.07
<i>Helioeris multiflora</i>	1.45	0.685	-	1.2	0.50	0.05	0.07

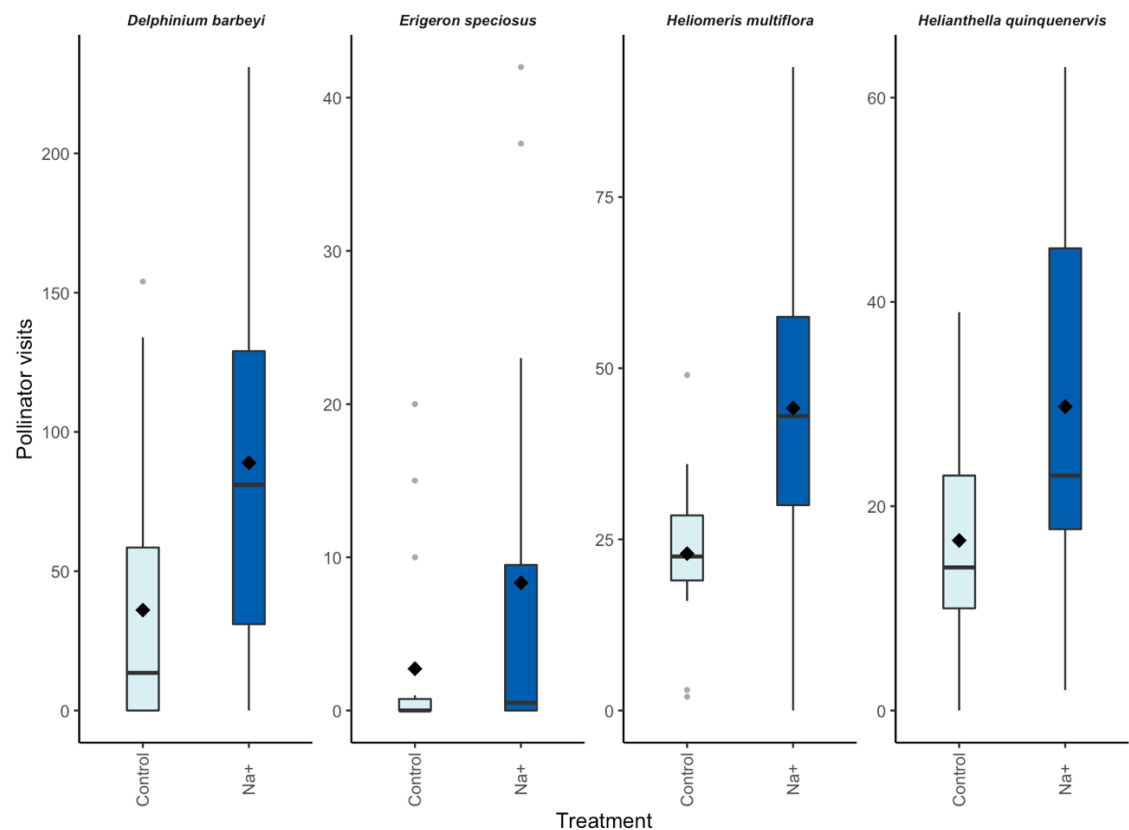


Figure 2. Pollinator visitation with control and nectar treatments for all 4 plant species included in this study. Visitation is significantly different between treatments for all species.

Table 2. Summary statistics for the models describing visitation rate, visitors, and diversity. Significant effects are indicated with an asterisk (*).

Response	Fixed-effect	χ^2	df	p	
<i>Generalized linear mixed-effects model</i> (family: negative binomial)					
Visitation					
	treatment	18.893	1	< 0.001	*
	species	54.383	4	< 0.001	*
	treatment x species	1.431	4	0.83	
Unique visitors					
	treatment	28.6574	1	< 0.001	*
	species	75.3722	4	< 0.001	*
	treatment x species	2.1594	4	0.71	
Species richness					
	treatment	4.6815	1	0.030	*
<i>Linear mixed-effects model</i>					
Simpson's index					
	treatment	74.5416	1	< 0.001	*

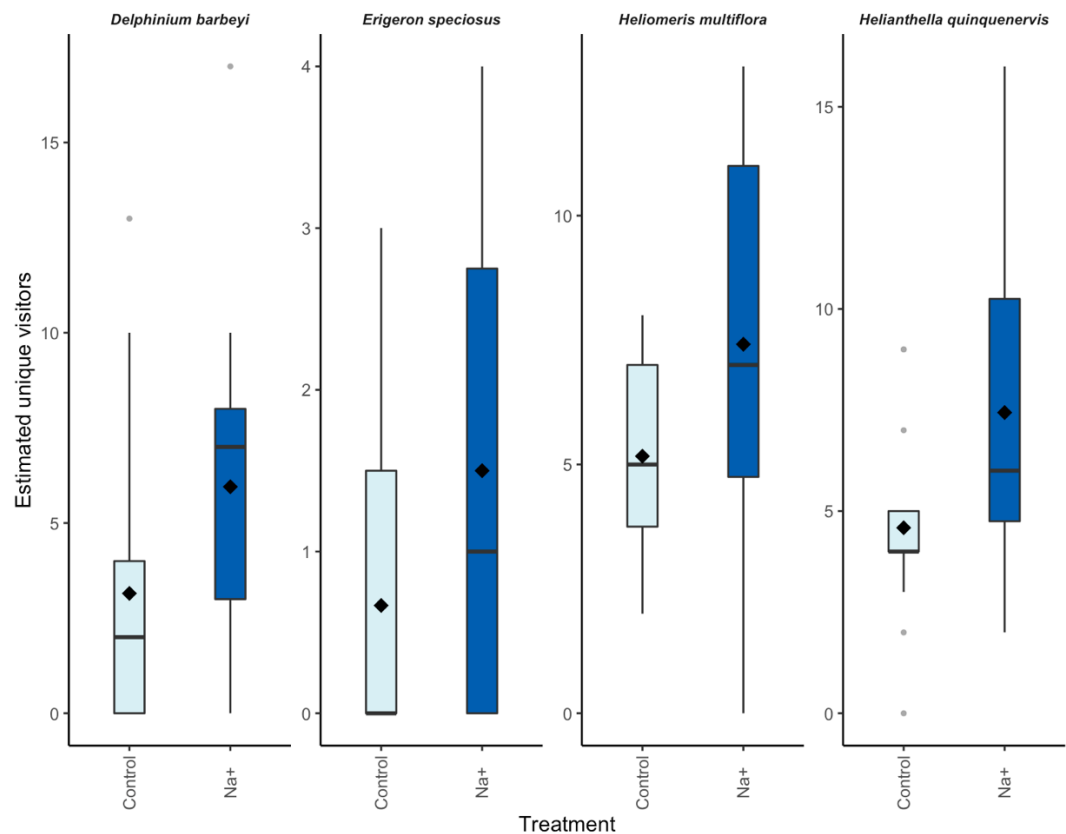


Figure 3. The number of unique visitors with control and nectar treatments for all 4 plant species included in this study.

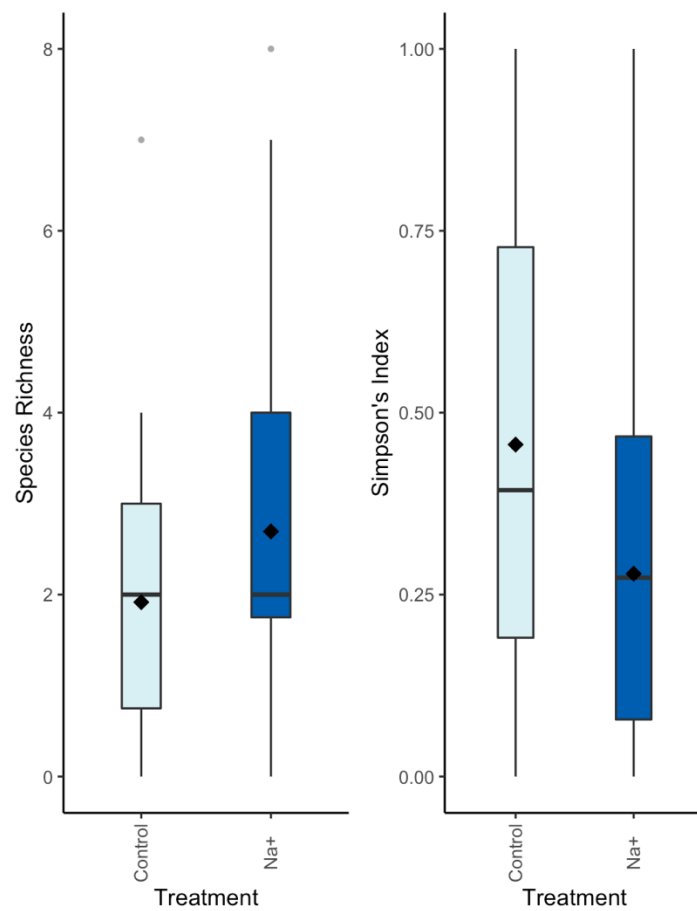


Figure 5. The species richness and Simpson's index of visitors on control and Na-enriched plants. The number of species and Simpson's index was calculated for each 20-minute observation period.

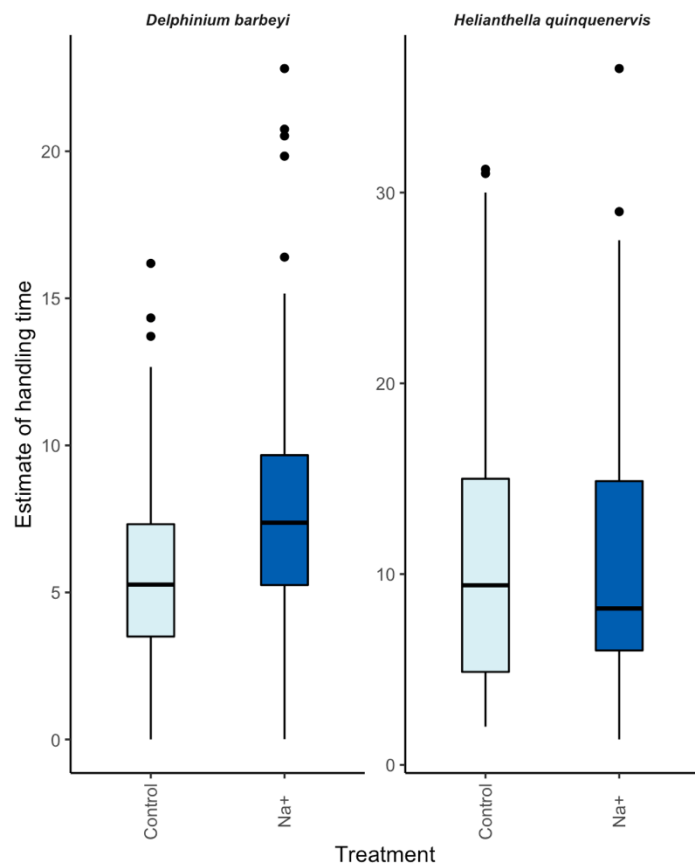


Figure 5. Handling of *Bombus* spp. on *D. barbeyi* and *H. quinquenervis*. The difference is significant between treatments for *D. barbeyi*, but not *H. quinquenervis*.

Appendix 1

Plant reproductive reward from sodium-enriched nectar

Methods

We conducted this experiment in the Gothic Townsite outside of the Gothic Research Center. The experiment occurred for the duration of *D. barbeyi* flowering in July 2022. On the adjacent hill, we selected 8 plants with at least 5 flowering inflorescences to participate in the experiment.

On every plant selected, each of 5 inflorescences will receive a distinct treatment. Treatments include: (1) 50% sucrose + 1% NaCl, (2) 50% sucrose + 0.5% NaCl, (3) 50% sucrose + 0.1% NaCl, (4) 50% sucrose + 0.05% NaCl, (5) 50% sucrose (control 1), and (6) no treatment (control 2). By comparing the no treatment control to the 50% sucrose control, we can determine if our control nectar addition is influencing pollinator visitation or if nectar addition affects plant reproduction.

Before flowering, we erected bridal-veil exclusion nets to limit herbivory, parasitism, and pollination. During peak flowering, we removed the nets and applied these treatments to each plant daily. Each flower on the inflorescences of *D. barbeyi* will received 3 μ l of artificial nectar consistent with nectar volumes from Kirschke et al. (2019). Daily nectar applications were consistent with the flowering time and daily nectar regeneration rates of *D. barbeyi* (Luo et al. 2014). At the end of flowering, the selected inflorescences were bagged and observed for seed set.

Seed set and visitation rates were compared between each treatment (levels of sodium enrichment and controls) to identify: (1) at which sodium concentration is visitation highest? And (2) does sodium-enriched nectar lead to greater seed set?