

**The Putrescine Puzzle: How do *Manduca sexta* hawkmoths respond to scented nectar in *Datura wrightii* flowers?**

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The unusual large amounts of a polyamine, putrescine, in the nectar of the *Datura wrightii* flower add complexity to the mutualistic interactions between the flower and its pollinator, *Manduca sexta*, acting as a potential modulator in the pollinator's foraging behavior. However, whether this non-sugar metabolite acts as an attractant or a repellent in the floral nectar is not well understood. I performed an innate preference bioassay using the *Datura* nectar and a synthetic sugar solution to analyze the possible effects of putrescine on *Manduca* preference during foraging. Based on the assessment of the animal's first choice, the frequency of visits per flower, and the final volume of nectar consumption measurements, this study failed to find any statistically significant difference in the moth's preference between *Datura* nectar and the synthetic sugar solution. However, while the absence of behavioral changes may suggest the animal's indifference towards the additional polyamines in the nectar, it remains possible that physiological or fecundity implications may arise from a long-term study.

## INTRODUCTION

Outcrossing, or outbreeding, is a breeding system of many flowering plants in which fertilization occurs between two genetically distinct plants. This strategy often leads to a rise in phenotypic variability which has enabled plants to adapt to various environmental conditions, increasing their overall fitness (Simpson, 2019). On the contrary, inbreeding, mating between closely related individuals, could lead to a phenomenon known as inbreeding depression, which results in reduced survival and biological fitness because of the increased frequency of deleterious and often recessive mutations expression from the rise of homozygosity (Charlesworth and Willis, 2009). Due to the benefits of establishing genetic variation, many plants depend heavily on floral visitors to promote outcrossing pollination. The use of floral nectar as a food source is often the primary incentive for pollinator visitation, leading to plant reproductive success. While floral traits such as scent and floral display help direct the pollinators to the plant's desired pollination sites, other secondary floral attractants such as CO<sub>2</sub> emissions and corolla shape also contribute to plant-pollinator dynamics (Goyret et al., 2007, Adler and Bronstein, 2004). However, other characteristics act as possible deterrents, including the secondary metabolites such as amino acids and polyamines in floral nectar.

One extensively studied flower-pollinator relationship is the exchange of ecological services between *Datura wrightii*, the thorn apple plant and *Manduca sexta*, the tobacco hornworm moth. *D. wrightii* produces large, white flowers that open at dusk and emit a sweet-smelling fragrance, containing nectar to attract nocturnal pollinators (Grant, 1983). *M. sexta* hawkmoths gain significant benefits from consuming sucrose-heavy nectar. They obtain energy from the nectar to

perform metabolically taxing activities such as hovering and long-distance flights for mating and finding host plants (Cross and Owen 1970; Janzen 1986, 1987; Powell and Brown 1990). The caloric rich nectar of the *Datura* flower provides enough energy for a hawkmoth for a 3 to 20 min flight (Raguso et al., 2003). In addition, the positive effects of nectar consumption can be seen in the post-mating sugar meals for female *M. sexta* with a greater number of eggs matured, as well as in a subsequent study in female *Hyles lineata* hawkmoths demonstrating greater fecundity (Sasaki et al, 1984, Arx et al, 2012). The *D. wrightii* plant also benefits from the interaction because *Manduca* hawkmoths are highly effective pollinators, facilitating outcrossed pollination and increasing plant reproductive success (Bronstein et al., 2009).

Despite the benefits of the mutualistic relationship, *M. sexta* are also herbivores of the same plant because the ovipositing *M. sexta* females deposit eggs on the *D wrightii* leaves, and the larvae defoliate the plant after emergence, putting a heavy cost of herbivory on the plant in exchange for the pollination services (Bronstein et al., 2007). An individual larva can consume 1400-1900 cm<sup>2</sup> of foliage (Heinrich, 1971, Casey, 1976). Under *ad libitum* conditions, the larva will subsist on its host plant, capable of completely defoliating the entire plant by its last instar (McFadden, 1968) The additional complications of these plant-pollinator interactions share similarity to those of pollinating seed parasite systems such as figs and yuccas (Holland and Fleming, 1999), but *D. wrightii* and *M. sexta* hawkmoths hold a more general mutualistic relationship because neither species is solely dependent on the other. As a result, this interaction provides a good model system for understanding the costs and benefits of mutualism.

Besides the corolla structure and other floral traits that are integrated in the pollinator's foraging sensory system, the chemical composition of the nectar itself also plays an important role in influencing the pollinators' preferences. The floral nectar is composed of complex compounds including proteins, lipids, amino acids, secondary metabolites such as polyamines (Afik et al., 2006; Kessler et al., 2012). One possible explanation behind the presence of these additional constituents besides the sugar revolves around the evolution of defense mechanisms to combat the antagonistic effects of nectar robbers and thieves using toxic chemical compounds (Kessler and Baldwin, 2006). The presence of these toxins was previously presumed to be an inevitable cost of deterring pathogens and herbivores, but in some cases, it benefits the plant by increasing pollen distribution. Floral nectar in *Nicotiana attenuata* contains nicotine, which is a repellent that decreases the nectar consumption, but the toxin increases outcrossing pollination from the plant's important pollinator, hummingbirds (*Archilochus alexandri*) by reducing the nectar consumption per flower but increasing the number of flowers visited (Kessler et al., 2012). Similarly, the nectar of *Gelsemium sempervirens* flowers contains the alkaloid gelsemine, which decreases the duration of pollinator visitation but increases the number of flowers visited (Irwin and Adler, 2008). The influence of nectaring behavior for a profitable consequence for the plant challenges the previous notion that nectar toxins are an avoidable side product of chemical defense. Specifically, the presence of nitrogenous compounds, including nectar alkaloids and proteins as well as free amino acids is highly prevalent among flowering plants, a recorded observation dating back to the mid-20<sup>th</sup>-century (Adler, 2000; Beutler, 1935; Baker and Baker, 1973; Ziegler, 1956; Lüttge, 1961). This widespread phenomenon raises the question of the function of these amino acids, in which multiple studies have shown has effects on the foraging behavior of organisms from multiple taxa like flies, bees, and butterflies (Alm et al., 1990,

González-Teuber and Heil, 2009, Lanza, 1988, Rathman et al., 1990) as well as *M. sexta*, which exhibited a strong preference for amino acid content in nectar (Broadhead and Raguso, 2021).

Similarly, the nectar chemical composition of *D. wrightii* has been studied and contains concentrations of putrescine and alkaloids, seemingly unfavorable compounds for attracting pollinators (Torres et al., 2013). However, whether the presence of these nectar toxins has a similar effect on the plant with its floral visitors to that between *N. attenuata* and hummingbirds is not well understood. Previous studies (Raguso and Willis 2005, Alarcon et al., 2010, Riffell et al., 2014) have shown that male *M. sexta* demonstrate foraging interest with proboscis extension and feeding in response to visual cues and floral fragrance of the *D. wrightii* plant.

However, it is not clear to what extent non-sugar nectar constituents contribute to the behavior of *M. sexta* moths when pollinating flowers of *D. wrightii*. One specific nitrogenous compound, the polyamine putrescine, is present in high concentrations in the nectar of *D. wrightii* (W. Kandalaft 2021, unpublished honors thesis) and produces a pungent scent, raising the question of whether it has a positive or negative olfactory influence on the hawkmoths' foraging behavior at the final stages of flower visitation. The neurobiological basis of nectar foraging behavior by *M. sexta* has been intensively studied, making it an ideal model to examine the effects of nectar chemistry on its foraging preferences (Guerenstein et al., 2004, Riffell et al., 2008). Examining the impact of these sensory signals on the pollinators' behavior can further develop our understanding of the nuanced mutualistic dynamics between plants and insects.

In my experiment, I investigated the effects of putrescine on *M. sexta* hawkmoth's foraging preference using a bioassay of the *D. wrightii* nectar and a synthetic sugar solution without putrescine or other secondary metabolites and observing the behavioral choices of the moth in a controlled environment. Initially, I will conduct an experiment to test our hypothesis that there is a significant bias between the nectar and an artificial solution consisting only of sucrose and hexose sugars. The first experiment will be used to determine whether the chemical constituents of the nectar besides the sugar have a behavioral effect on the moth's foraging choices. My null hypothesis states that there will be no statistically significant difference between the moth's choices between the *D. wrightii* nectar and the artificial sugar solution. Assuming the data fails to reject the null, I expect to see similar numbers of first choice in feeding and measurements in visitation frequency along with nectar consumption for both choices from the moths.

However, if there is a significant difference between the moth's foraging preference of the nectar and artificial solution, I will expand on that finding and examine whether putrescine is the variable responsible for the moths' selectivity. In such a case, I would perform another bioassay using two artificial sugar solutions, one containing natural dosages of putrescine and the other without. Similar to the previous experiment, I will observe if the moth shows a preference for one sugar solution over the other by comparing the visitation frequency, duration, and nectar consumption from their visits.

If the moths do not demonstrate a strong preference of one sugar solution over another, the data will present no difference in visitation and volume consumption of neither solution, and I will reject the hypothesis that putrescine itself explains the difference in foraging behavior observed

in the first bioassay. However, if this case is not true, in which the moths do show a preference of one sugar solution over another, I propose three possible alternative hypotheses: initially, I propose that putrescine is a deterrent and reduces nectar consumption rates in comparison to the sugar solution, similar to the role of nicotine in *N. attenuata* and hummingbirds (Kessler et al., 2012). Another hypothesis states that the moths employ putrescine as an honest cue for nectar availability, so the presence of this chemical constituent would enhance the nectar consumption rates vs sugar solution. Finally, the constancy hypothesis suggests that putrescine is a memory enhancer and encourages constancy to the *D. wrightii* flowers (this hypothesis is not tested here and will require future investigation beyond this study) (Wright et al., 2013).

## **METHODS**

Prior to the experiment, I performed a bioassay with 75 animals using the artificial sucrose solution for both choices to determine if there is a side-bias in the flight room setup. *M. sexta* moths (Linnaeus 1763) were bred from Robert Raguso Lab's breeding colony (see Broadhead et al., 2017). Prior to eclosion, individual moths were sorted by sex and transferred to separate cages in the greenhouse room 414 in Corson Mudd. On the third to fourth day after emergence, the moths were transferred to the flight cage (61x61x91cm polypropylene mesh cage (BioQuip, Inc.)) to carry out the experiment. Moths of both sexes remained unmated and unfed prior and during the duration of the behavioral trials. The flight cage consists of a metal ring stand covered with black fabric to block the moths' vision of seeing what is under the stand. Two apparatuses with grip ends are placed under the stand to secure two artificial flowers on the stand. The artificial flower is a 3 cm diameter plastic funnel covered with white paper in the interior, and the funnel is inserted into a microcentrifuge tube to hold the 200  $\mu$ L sucrose solution for the moth to



consume. The sucrose solution consists of 337.5 mg glucose, 276.79 mg fructose, and 1589.29 mg sucrose that is filled to 10 mL with distilled water. These ratios are intended to match those identified for nectar from *D. wrightii* (Raguso et al, 2003). To stimulate the moths' olfactory responses to drive foraging behavior, a cotton swab with 5  $\mu$ L of bergamot oil, which has similar chemical properties to many hawkmoth-pollinated flowers, was attached along the side of each artificial flower (Goyret and Raguso, 2006). An addition Erlenmeyer flask filled with water was placed under the metal stand adjacent to each apparatus clamp, and a *D. wrightii* leaf was inserted into the flask and positioned to be visible next to each artificial flower to act as additional contextual attractants and provide vegetative odours.

The experiment began during scotophase due to peak activity of the moths during that time (Raguso and Willis 2005; Broadhead et al. 2017). The two bioassay choices were the artificial sugar solution and *D. wrightii* nectar. The nectar used in the experiment was collected from a recently bloomed *D. wrightii* flower (no more than a day old) by cutting the end tip of the stem, exposing the nectaries, and the nectar was squeezed out from the nectaries into a microcentrifuge tube. Upon release, the moth would be "primed" by the presentation of a different *D. wrightii* flower devoid of nectar to eliminate putative preferences prior to the experiment. To ensure a thorough removal of nectar, a squirt bottle of water was used to rinse the 5 separate nectaries of the flower from the corolla position facing down, basal to the plant. Upon release, the moth was presented two flowers as the bioassay choices (one containing the artificial sucrose solution while the other containing *D. wrightii* nectar), and I analyzed its behavior using both human observation and video recordings. Each moth was given 5 minutes to make its sugar solution preferences, and the following measurements were recorded in a scientific journal during the

experiment: initial approach selection (approach was defined as direct contact with flower and proboscis extension), initial feeding selection (feeding was defined as encompassing the approach description along with the brief termination of physical motion of animal), number of visits per flower, and the overall preference. The overall preference in the bioassay would be determined by the first flower visited upon release and the observed count of visits per flower. After the experiment, the microcentrifuge tubes of the flowers were removed, and the remaining nectar was measured to record the volume of nectar consumed in each trial.

The experiment itself repeated the procedure mentioned above except with the addition of a pre-trial exposure stage and adjustments to the solution content and provided volumes. Before each experimental trial, the moth would be exposed to and taste both the artificial sucrose solution and *D. wrightii* nectar before making a preference. One flower would contain 25  $\mu\text{L}$  of artificial sucrose solution while the other would contain 25  $\mu\text{L}$  of nectar. After the pre-trial exposure, the microcentrifuge tubes would be replenished with 175  $\mu\text{L}$  of the same solution from the previous round (artificial or nectar), and the moth would be given 5 minutes to make a preference and visit the flowers. By maintaining the same conditions beyond the nectar choices, I could observe whether the moths showed a preference for one of the solutions over the other. This specific design allowed me to interpret moth choices as being informed (having tasted both nectars) rather than being an artifact of continuing to feed from the first flower probed. The data collected would be analyzed, and the discrete variables (choice data) would be analyzed via a chi square and g test using R- statistical software, whereas I would perform a t test to determine the average differences for the continuous variables (number of visits and nectar volume consumption). All

statistical tests would be two-tailed because the alternative hypotheses involved either attraction or repulsion of putrescine in sugar solutions.

Assuming the data revealed a preference for one solution over the other, I would then repeat the procedure but employ one synthetic sugar solution to which I would add appropriate amounts of putrescine and another without putrescine as the two available choices to assess the effects of just putrescine on the moth's behavior, excluding the other non-sugar components (e.g. amino acids, salts) of the *D. wrightii* nectar composition. I would perform the same statistical analysis for the data resulting from this experiment.

## RESULTS

All statistical analyses were conducted in R (<https://www.r-project.org/>).

### 1. Side Bias Analysis

Prior to the experiment, a bioassay with artificial sugar solution on both sides was conducted to test for side bias. 75 moths were used in this round, and before each trial, the animal was primed with a *D. wrightii* flower and allowed to feed from both artificial flowers in the flight cage.

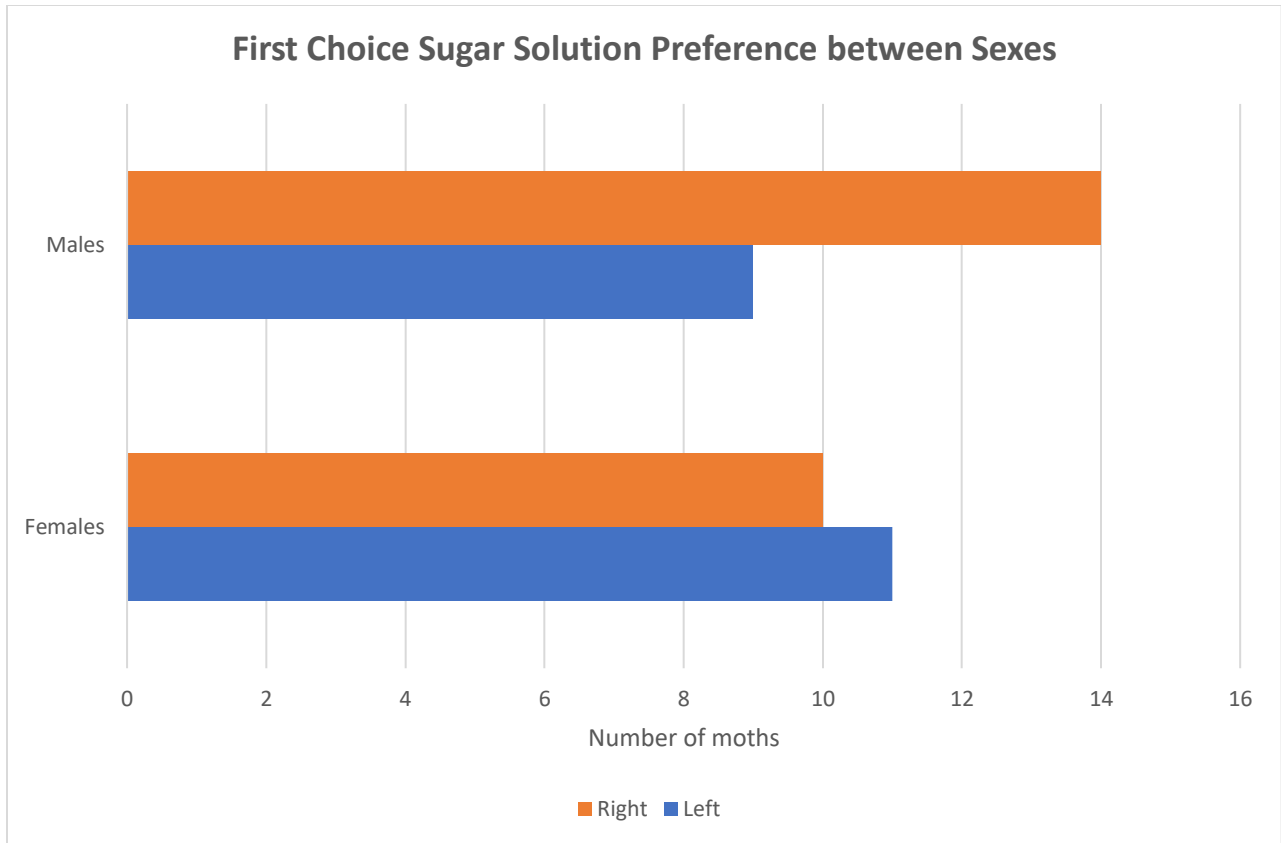
During the bioassay, the animal was given 5 minutes to make a "choice" by displaying interest (hovering near the flower with proboscis extension) or feeding from the artificial flowers, and the observations of the animal's preference were recorded.

**Table 1. Results of First Choice Preference in Approach and Feeding**

	Left	Right	No choice	Success Rate
Approach (number of moths)	22	26	27	64%
Feeding (number of moths)	23	24	25	63%

The success rates between the approach and feeding events for left and right sides were similar, with 64% and 63%, respectively. The values of these success rates indicate that an appropriate number of moths were responding to the bioassay, which removes the possibility of other external factors that may skew the results such as the individual's nutritional state or motivation (Cohen and Voet, 2002). The choices in the bioassay can be appropriately attributed to the experimental treatment and presented sugar solutions.

A Chi-squared test and g test were performed to determine if there was a statistically significant difference between sexes for side preferences. Based on the p-values from the chi-squared test and g test (0.8957 and 0.6711, respectively), there is no significant difference between the first-choice sugar solution preference for the left and right side. This concluded that there is no difference between male and female *M. sexta* hawkmoths in choosing between sides.



**Fig 1. First-choice preference of sugar solution between *M. sexta* sexes.** Preferences for the right side are represented by orange bars, and blue bars represent preferences for the left side. The statistical analysis indicates no significant difference between sexes in side preferences.

Figure 1 presents a bar plot of the number of moths that visited either the left or right side during the bioassay. For the males, 9 moths showed a first choice preference for the left while 14 showed interest for the right while for the females, 11 made a first choice preference for the left while 10 chose the right side in their initial preference.

## 2. First Choice Sugar Solution Preference

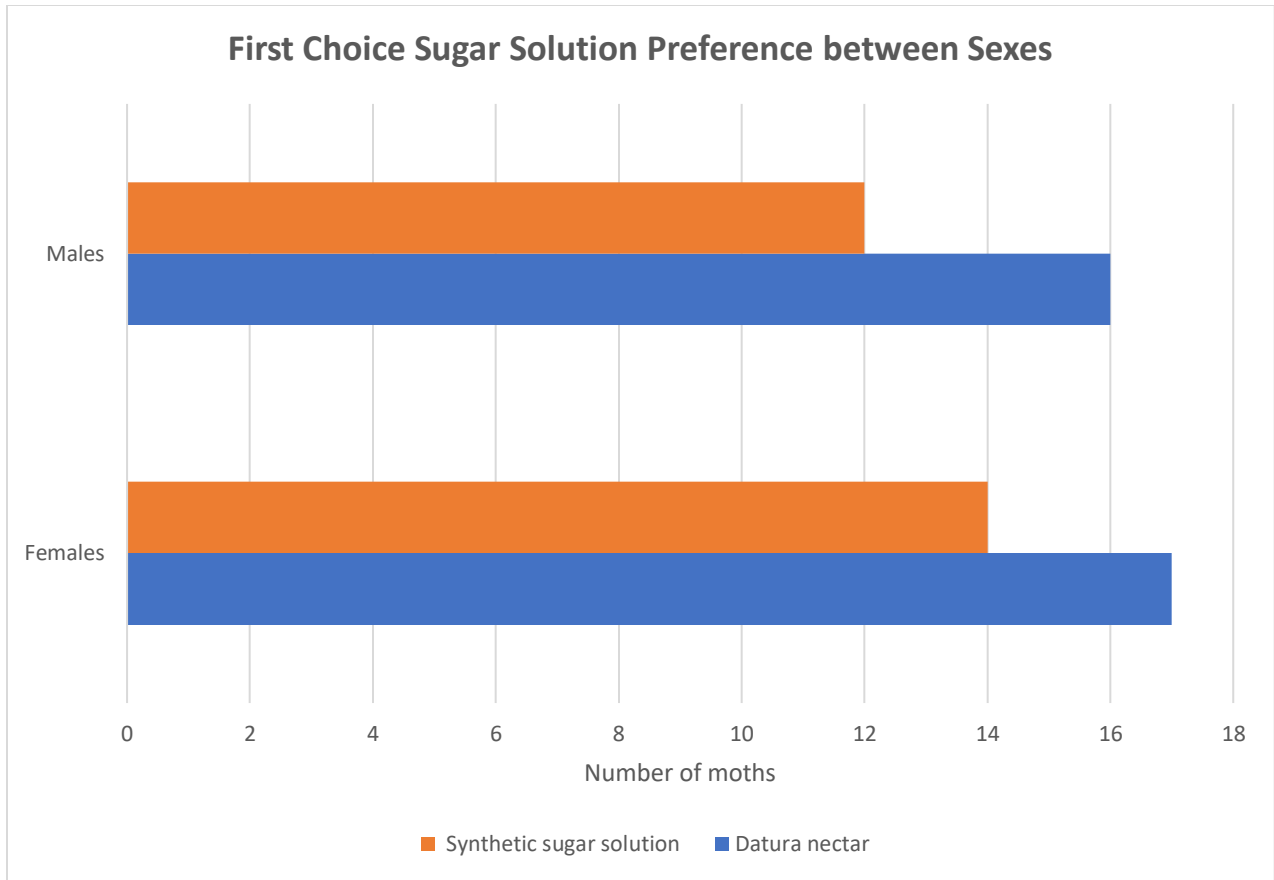
**Table 2. Results of First Choice Preference in Approach and Feeding**

	<i>D. wrightii</i> nectar	Artificial sugar solution	No choice	Success Rate
Approach (number of moths)	33	27	32	65%
Feeding (number of moths)	33	26	33	64%

In the bioassay for artificial sugar solution and *D. wrightii* nectar (sample size = 92 moths), the analyzes done in the previous bioassay were repeated on this data. Similar to the previous bioassay, the success rates for both approach and feeding are similar, 65% and 64% respectively, corresponding to the appropriate success rates for normal healthy hawkmoths.

## 3. First Choice Sugar Solution Preference in Feeding between Sexes

A Chi-squared test and g test were performed to determine if there was a statistically significant difference between sexes for sugar solution preferences. Based on the p-values from the chi-squared test and g test (1 and 0.8587, respectively), there is no significant difference between the first choice sugar solution preference for *D. wrightii* nectar and artificial sugar solution. This confirms no difference between male and female *M. sexta* hawkmoths in choosing between *D. wrightii* nectar or the synthetic sugar solution.



**Fig 2. First-choice preference of sugar solution between *M. sexta* sexes.** Preferences for synthetic sugar solution are represented by orange bars, and blue bars represent preferences for *Datura* (*D. wrightii*) nectar. The statistical analysis indicates no significant difference between sexes in sugar solution preferences.

Fig 2 presents the same information as Fig 1 except with the two bioassay choices of synthetic sugar solution and *D. wrightii* nectar. For the males, 12 moths showed a first choice preference for synthetic sugar solution while 16 showed interest for *D. wrightii* nectar while for the females, 14 made a first choice preference for synthetic sugar solution while 17 chose *D. wrightii* nectar in their initial preference.

#### 4. Frequency of visitation for Sugar Solution

A Shapiro-Wilk test was performed to test the normality of the groups, and due to the small p-value from the artificial sugar solution group ( $p = 0.0004111$ ), the data was not normally distributed, so a Mann-Whitney test was conducted.

**Table 3. Results of Mann-Whitney Test for Visitation Frequency**

Wilcoxon statistic	p-value
1899.5	0.3922

The number of visits for each artificial flower during every trial was recorded via visual observation and tallying from a scientific journal. A visit was defined as a full body immersion into a flower and proboscis extension within the flower. The average values for the visitation frequency to *D. wrightii* nectar and to synthetic sugar solution were  $7.9 \pm 0.52$  and  $7.2 \pm 0.46$  visits, respectively. The p value ( $p = 0.3922$ ) from table 3 suggested there is no statistical difference between the visitation frequency for *D. wrightii* nectar and synthetic sugar solution, further conveying the absence of sugar solution preferences for the moths in their foraging.

#### 5. Total Volume Consumption

Besides the first choice preference and visitation frequency, after the experiment, the remaining volumes of the microcentrifuge tubes were measured to find the total volume consumption for both *D. wrightii* nectar and synthetic sugar solution. The original volume in each tube was 175  $\mu\text{L}$  of either *D. wrightii* nectar or sugar solution. A Shapiro-Wilk test was performed to test the

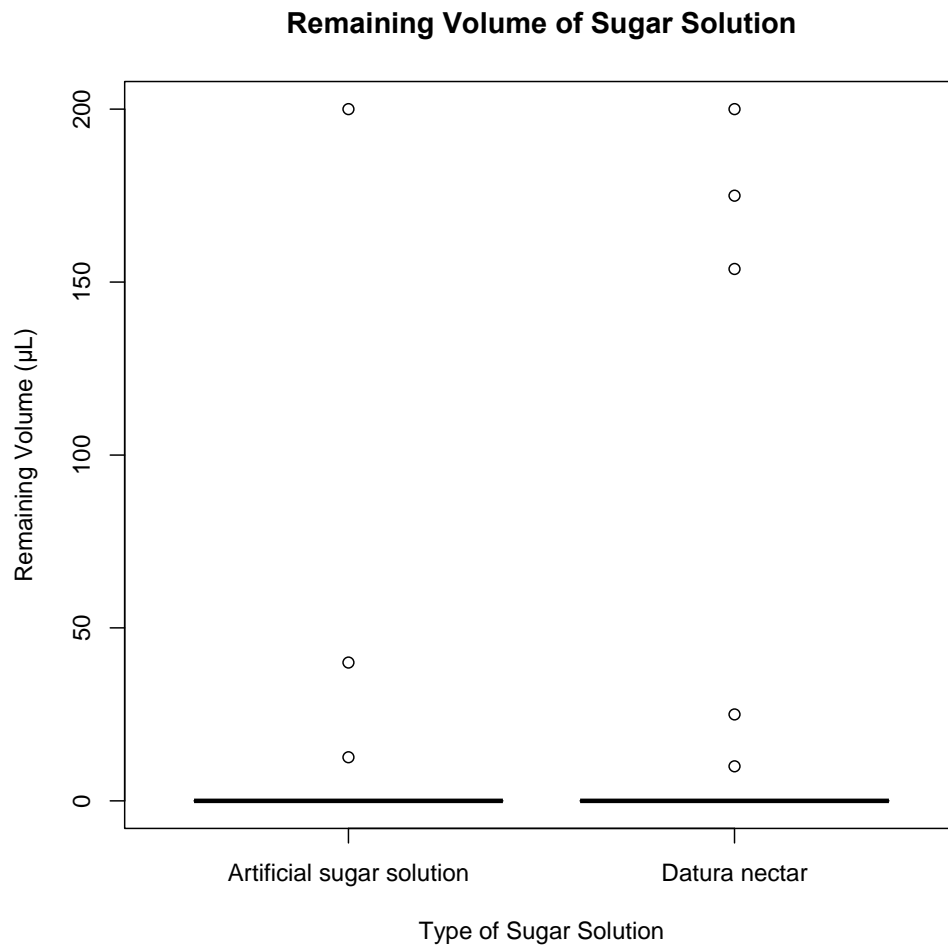


normality of the groups, and due to the small p-value from the *D. wrightii* nectar group ( $p = 1.056e-15$ ), the data was not normally distributed, so a Mann-Whitney test was conducted.

**Table 4. Results of Mann-Whitney Test for Total Volume Consumption**

Wilcoxon statistic	p-value
1799.5	0.47

As depicted in Table 4 ( $p\text{-value} = 0.47$ ), no statistical difference was found between the sugar solution groups for volume consumption. This data supported that the moths do not consume a significantly different volume of sugar solution between the *D. wrightii* nectar and the synthetic sugar solution.



**Fig 3. Remaining volume of sugar solutions for artificial sugar solution and *Datura* nectar.**

Open circles indicate outliers. No statistically significant difference was found between the *Datura* nectar and artificial sugar solution remaining volume consumption.

The averages of the remaining volume for *D. wrightii* nectar and synthetic sugar solution were  $9.6 \pm 4.10$  and  $4.3 \pm 2.76$   $\mu\text{L}$ , respectively. While these values may suggest a slight preference for the synthetic sugar solution, the averages may not accurately depict most of the dataset due to multiple outlier values, as shown in Fig 3. The medians for both groups were 0  $\mu\text{L}$  (or a total volume consumption of 175  $\mu\text{L}$ ), and first and third quartiles for both groups were both 0 visits.

These values correspond with boxplot in Fig 2 and indicate that the average moth consumed the entire volume of both sugar solutions during the bioassay.

#### 6. Second Side bias Analysis

**Table 5. Second Side Bias Analysis**

	Left bias	Right bias
<i>D. wrightii</i> nectar placement	30 times	29 times
First choice preference (number of moths)	32 moths	27 moths
Side bias %	54.2%	45.8%

**Table 6. Side Bias Analysis between Sexes**

	Left bias	Right bias
Male	55%	45%
Females	54%	46%

The *D. wrightii* nectar solution placement was alternated between each trial to eliminate a possible side bias in running the bioassays. As mentioned in table 5, the nectar was placed on the left side 30 times and 29 times on the right. Assuming there is no side bias present in the experimental setup, the moths should visit both sides equally, and the probability for visitation

on both sides should be 50%. As hypothesized, the calculated values based on the first choice preference for side bias for the left and right were 54.2% and 45.8%, respectively, verifying the data from this study were not influenced by side preference. In addition, both males and females do not show a side preference as shown in their similar percentages between the left and right side as illustrated in table 6.

## **DISCUSSION**

Non-sucrose compounds in floral nectar have demonstrated vast regulating effects on different flower visitors, including their pollinators. While nectar toxins may serve as a defense mechanism from the plant to prevent herbivory, recent studies suggest there may be additional ecological functions in both facilitating or inhibiting visitation. Some toxic compounds may function more than a deterrent for nectar thieves but promote outcrossing as seen in the nicotine in the *Nicotiana attenuata* flower's nectar which increased the number of flowers visited by the plant's pollinator, hummingbirds (Kessler et al, 2012). The authors implemented polymorphic microsatellite markers to compare outcrossing in plants with a silenced biosynthetic gene for nicotine production and in control empty vector plants. They found that seeds from flowers that had nicotine showed a greater variability in genetic diversity relative to those from nicotine-free flowers. The manipulative behavior of nicotine proves to be advantageous for the plant due to the increased genetic outcrossing without having to produce more nectar which can be energetically unfavorable and costly. The randomly distributed nicotine concentrations present in the floral nectar among the plants also increase flower visits from the hummingbirds in the search of low nicotine concentrations, further aiding the plant in its overall fitness. In addition, minute

concentrations of caffeine in the floral nectar of *Coffea* species have been shown to improve honey bee's memory through a reward system and enhancing pollinator fidelity, benefiting the plant in its mutualistic interactions (Wright et al, 2012). The odor from the chemical constituent drives associative learning for the animal, making it more likely to visit a flower with the same scent signals to indicate nutrient value.

However, in some cases, other nectar compounds do behave as repellents to the pollinators as studied in the presence of salts, specifically potassium and phosphorus, in avocado nectar, which have an aversive effect on honey bees (Afik et al. 2006). The presence of these mineral salts remains understudied, but one possible explanation relates to the varying responses to these compounds from different pollinators while another theory suggests the increased potassium concentration originates from its high levels in the soil. Nevertheless, the abundance of volatiles and other chemical constituents in nectar continues to be an exciting field in understand their mediating and nuanced roles with flower visitors' interactions.

In this study, putrescine in *D. wrightii* nectar presents a puzzling case of its role in the plant's interactions with one of its essential pollinators, the *M. sexta* hawkmoth. Upon analysis of the first choice preference, number of visits, and volume consumption between *D. wrightii* nectar and the artificial sugar solution, my experiment presented no statistically significant difference in the *M. sexta*'s foraging behavior between *D. wrightii* nectar and the artificial sugar solution. Thus, the follow-up experiment with a synthetic sugar solution with just putrescine was not conducted due to the lack of statistical difference in the previous bioassay. However, certain limitations in the experimental design may explain the lack of preference in the animals' behavior. One notable observation was the sugar solution volume used in the bioassay which was

175  $\mu\text{L}$  for both *D. wrightii* nectar and the artificial sugar solution, which is more than twice the volume present in a *D. wrightii* flower, which produces about 65  $\mu\text{L}$  upon opening (Bronstein et al., 2009). As a result, the copious amounts of nectar can be an artifact to the moth's behavior than how it might have behaved under its native environment, possibly due to the abundance of sucrose regardless of sugar solution. However, the increased volumes may not attribute to the moths' lack of preference due to their strong preference for higher sucrose solutions as supported by a similar study done on the effect of amino acids in *D. wrightii* nectar on *M. sexta* (Broadhead and Raguso, 2021). In a similar 2-choice innate preference test, the moths showed a behavioral preference for nectar with amino acid composition present in natural floral nectar when the sucrose concentrations were equal through experience and associative learning. Thus, the increased volume in my experiment may not be of concern due to the same sucrose concentrations for both sugar solutions. Rather, another possible consideration may be the timeframe; the moths used in this experiment were naïve and expected to recognize nectar quality and caloric value within the first few minutes while being exposed to the flower for the first time, leading to possible errors during this exploratory phase (Lavery, 1994) Thus, a future study examining the hawkmoths during a longer duration can elucidate more realistic outcomes and examine if the polyamines in the nectar are still considered through foraging experience and learning odorant cues.

The process leading up to the final experimental design has yielded many pilot experiment lessons through various adjustments in method optimization and location of the experimental setup. During the summer 2021, the flight cage was previously located in a different room with the presence of vegetation and other *D. wrightii* plant in the green house, and one prominent issue was the side bias in the flight cage, with a strong preference for the right side. One possible

explanation was an unequal distribution of lighting or a shadow effect which may lead to better illumination on one side over another. Also, the humidity levels were low in the flight cage, which could explain the low success rates of the moths' responses as ambient humidity has been shown to regulate the moths' osmotic state (Contreras et al., 2013). Both problems were alleviated through the transition of the flight cage to the greenhouse room 414 in Corson Mudd. Another adjustment in the experimental design was the removal of the camera used for recording to measure visit duration. Although the camera was intended to record the moth behavior, the light from the lens led to reduced success rates due to the extended time the moths spent probing at the lens. More importantly, one crucial addition to the experiment was the "priming" stage prior to release into the flight room; the moth was allowed to probe at a real *D. wrightii* flower lacking nectar, to acquire associative learning of associating the white color and structure of the flower to food source. The floral odorants from the natural flower also may have helped prime the moths' feeding behavior, as previous studies have shown the importance of odorants in activating foraging behavior (Goyret et al., 2007) and the neural system in the antennal lobe in *M. sexta* (Bisch-Knaden et al., 2018).

While 92 moths were used in this study, a total of 280 moths was employed in the pilot experiment, which tested for side bias, and the final experiment itself. After the modifications, the success rates of the moths' responses increased substantially, going from 28% response rate during the summer 2021 to 64% response rate in the final study. As a result, I gained insightful information throughout the process of designing and adjusting the experiment, learning how to effectively manipulate my experimental design.

Although this study did not show statistical difference in the *M. sexta* hawkmoth's behavioral preference between *D. wrightii* nectar and synthetic sugar solution, putrescine could have other effects on this species such as their physiology or fecundity. Amino acids have been shown to play a role in female moths' reproductive state, suggesting that putrescine, an organic compound, could have a potential effect as well (O'Brien et al., 2002). Other possible effects may correspond to the animal's physiology, and an examination of the muscle tissue or other physiological roles can elucidate the specific roles of putrescine (Levin et al., 2017). Besides putrescine, the nectar composition of the *D. wrightii* plant consists of many other alkaloids and secondary metabolites that have not been extensively examined in their ecological significance, urging us to further research.

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## SUPPLEMENTARY MATERIALS



*Experimental setup.*



*M. sexta* hawkmoths foraging *D. wrightii* flower. Photo taken by Robert Raguso, 2022.



*Double click to see video. Experimental design and *M. sexta* hawkmoth (female) foraging behavior.*



*M. setxa* hawkmoth demonstrating feeding behavior on the right side.