Plant Archives Vol. 25, Supplement 1, 2025 pp.1305-1311 e-ISSN:2581-6063 (online), ISSN:0972-5210

Plant Archives

Journal homepage: http://www.plantarchives.org
DOI Url : https://doi.org/10.51470/PLANTARCHIVES.2025.v25.supplement-1.177

PLANNED PAIRWISE COMPARATIVE STUDY OF SEA BUCKTHORN-BASED POLLEN AND NECTAR SUBSTITUTE FORMULATIONS ON DIET CONSUMPTION OF WESTERN HONEYBEE (*APIS MELLIFERA*)

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(Date of Receiving : 25-08-2024; Date of Acceptance : 26-10-2024)

ABSTRACT

Pollen and nectar is essential for honey bee colonies, and their scarcity can lead to starvation and contribute to colony collapse disorder (CCD). In tropical and subtropical regions, harsh summers can significantly weaken bee populations due to limited forage. A well-formulated artificial diet can enhance nutritional balance and colony health. In India, many beekeepers either don't provide external food or only use sugar syrup for their colonies. Researchers have attempted to create alternative foods for bees during food shortages, but no standard formulation is widely accepted. Addressing the seasonal pollen shortages and improving beekeeping efficiency requires effective substitutes. This study focuses on evaluating the quality of sea buckthorn fruit-based pollen and nectar substitutes by analyzing their physicochemical properties and diet consumption. Hence the present research aims to develop sea buckthorn fruit-based pollen and nectar substitutes to improve diet consumption during the dearth period. The proximate content of particle size distribution, carbohydrates, crude protein, and crude fat for pollen formulation 1 (P_1) was as follows: particle size distribution of 605.3 ± 0.02 nm, carbohydrates at 79.4 ± 4.84%, crude protein at 4.02 ± 0.23%, and crude fat at 5.7 ± 0.45%. In comparison, pollen formulation 2 (P_2) had a particle size distribution of 508.9 ± 0.02 nm, carbohydrates at 79.9 ± 4.39%, crude protein at 1.35 ± 0.08%, and crude fat at 6.3 ± 0.57%. The viscosity, osmolarity, total soluble solids, and total sugars of sea buckthorn fruit-based nectar formulation 1 (N_1) were measured at 0.0011±0.0001 Pa. s and 643±28.94 mmol/L, while nectar formulation 2 (N2) had 0.00089±0.00005 Pa. s viscosity and 773±64.33 mmol/L osmolarity. The evaluation of diet consumption through planned pairwise comparisons showed that both nectar formulation 1 (N1) and nectar formulation 2 (N2) significantly influenced diet consumption in *Apis mellifera*. Among the formulated diets containing ingredients from pollen formulation 2, the highest diet consumption was recorded for nectar formulation 2 (N2). The results indicate that the sea buckthorn-based pollen and nectar formulations, specifically pollen formulation 2 (P2) and nectar formulation 1 (N1), demonstrated promising outcomes in diet consumption.
***Keywords*: Sea buckthorn, bee pollen, nectar, pollen formulation, nectar formulations, diet consumption and *Apis mellifera*

Introduction

The success of beekeeping in a region depends on the prevailing climatic conditions, availability of bee forage, and effective management practices. The availability of bee flora varies by season, area, and locality. It also depends on the type of forest, horticultural crops, and other seasonal crops grown in that region.

Honey bees require protein, carbohydrates, minerals, lipids, vitamins, and water for proper growth and development, which they obtain from pollen, nectar, and water (Shehata, 2016). Pollen and nectar are essential for honey bee colonies, and their scarcity can lead to starvation and contribute to colony collapse disorder (Seitz *et al*., 2015). In tropical and subtropical regions, harsh summers can significantly weaken bee populations due to limited forage. A well-formulated artificial diet can enhance nutritional balance and colony health. Tailoring nutritional strategies to specific regions and colony needs, such as providing pollen supplements, can maintain brood production during lean times, promoting rapid growth when conditions improve (Prakash *et al*., 2007).

Seabuckthorn berries from the Ladakh region of Jammu and Kashmir are rich in multivitamins, including 275 mg/100g of vitamin C, 432.4 IU/100g of vitamin A, and minerals such as 647.2 mg/l of potassium, 176.6 mg/l of calcium, and 30.9 mg/l of iron (Stobdan *et al*., 2017). Seabuckthorn has 10 times more vitamin C than kiwi fruit (Nawaz *et al*., 2019). The seeds contain valuable oil with high oleic acid content and a balanced ratio of omega-3 and omega-6 fatty acids. The high vitamin concentration makes seabuckthorn fruit highly suitable for the production of nutritious soft drinks (Choton *et al*., 2023). The pollen substitute diets were found to be better than pollen/pollen supplements for honey bees in terms of acceptability and nutritional value.

In India, most of the beekeepers either do not provide any external food to bee colonies or just supply sugar syrup. Attempts have been made by scientists to formulate food to be fed to bee colonies during dearth period to solve the problems of food shortage and to get better output (Chhuneja *et al*., 1992; Sihag and Gupta, 2011; Kumar *et al*., 2013), but a standard and well-accepted formulation is still not available in our country. Keeping the above facts in view, the shortage/ unavailability of pollen in different seasons, and improving beekeeping efficiency depends on developing effective pollen and nectar substitute diets. Therefore, this study aims to determine the quality of sea buckthorn fruit-based pollen and nectar substitute diets. The objective of this investigation is to analyze the physicochemical properties of the pollen and nectar substitute diets and diet consumption of *Apis mellifera* colonies.

Material and Methods

The experiment was conducted in the P.G Block of the College of Horticulture, Mojerla, Wanaparthy District, during June and July 2024. Various pollen substitute treatments were applied to three colonies per treatment as replicates. All colonies were equalized based on total brood area, honey stores, and bee strength (8 bee-frame strength), with pollen-containing combs removed beforehand.

Two Sea buckthorn fruit-based pollen substitute diets were prepared which include sea buckthorn (30.24%) + Sweet potato starch (70.99%) + Yam starch (25.02%) + Mogroside (6.49%) + polycrystalline rock sugar (6.49%) + yeast (6.49%) + baobab (6.49%) + red banana peel extract (0.152%) + black acacia gum (17.145%) + Nannari (22.99%) + liquorice root extract (5.76%) and red banana syrup (1.74%)and two nectar substitutes included 25ppm thaumatin stock solution (25ml) + solution containing ingredients of Baobab, Yeast, Red banana peel extract, Mogroside, Polycrystalline rock sugar, Liquorice root extract powder, flower extract of Nyctanthes and fire of forest, Black acacia gum and red banana syrup (25µl) + Red banana syrup (25000µl) + monk fruit juice concrete (1ml) + 19% Poly crystalline rock sugar solution (500ml) + Monk fruit sugar (15%) + Sea buckthorn fruit juice (40 ml).

The diet was placed over the brood frames or top bars, with the formulated diet fed using the standard top bar method (Haydak, 1967). A pollen substitute diet was offered on craft sheets within the hives for easy access to the bees, provided 1g at weekly. Additionally, 20 ml of prepared syrup was poured into feeding tubes weekly and placed inside the hive.

Particle size analysis of sea buckthorn fruit-based pollen was performed using Horiba SZ-100 software (Ver 2.40) with dynamic light scattering (DLS). Key parameters included a 90° scattering angle, 25.2°C temperature, and 0.892 mPa·s viscosity for the dispersion medium. Results were presented as intensity distribution, with a transmission intensity of 21971, a monodisperse distribution, and a count rate of 23 kCPS. Mean values for an additional peak were included in the results.

The total carbohydrate content as a percent dry weight basis was determined by mathematically working out the % moisture, % ash content, % crude protein and % crude fat parameters and then deducting them from 100 as mentioned in (Ranganna 2009), crude protein content was determined using the AOAC (2012) method with a KJELTRON instrument, fat content was determined using the AOAC (2012) method with the Automatic Sox Tron system (Model: Sox-2 version 0.1).

To assess viscosity's effect on crop load, a feeder setup was used with unheated solutions. A 20% w/w sucrose solution's viscosity was adjusted by adding varying amounts of Tylose H 10000 P2 (SE Tylose GmbH & Co. KG, Wiesbaden, Germany), based on Josens & Farina's regression equation.

Osmolality was measured using a digital osmometer (TRIDENT MED., Warsaw, Poland) via freezing point measurement with single-point calibration. A test sample was pipetted into the device's cooling chamber, and the measurement started automatically when the head was gripped by the electromagnet. Each determination was done in duplicate, with results displayed in mOsm/kg H_2O.

Results and Discussion

Particle size distribution (µm)

The particle size distribution of pollen formulation 1 (P_1) and pollen formulation 2 (P_2) was measured and the results are shown in Table 1 and graphically represented in Figure-1 and 2. The mean particle diameters of P_1 and P_2 are 605.3±0.02 nm and 508.9±0.02 nm, respectively. P_1 had a wider particle size distribution than P_2. The particle size distribution of formulated sea buckthorn fruit-based pollen substitutes attained in nano size due to the adoption of the granulating technique method for preparation of substitute diet, making the product more stable and less likely to separate, leading to a longer shelf life and less waste while feeding to honey bees.

Carbohydrates (%)

The experimental findings about carbohydrate content are presented in Table 1. From the results, it was found that pollen formulation 2 (P_2) contains 79.91±4.39% carbohydrates, whereas pollen formulation 1 (P_1) contains 79.4 ±4.84%. Thakur and Nanda (2020), found that bee pollen typically contains an average of 54.22% carbohydrates, with levels ranging from 18.50% to 84.25% across different floral sources. According to Paoli *et al.* (2014), maintaining a balance of carbohydrates with essential amino acids in our diets is crucial. Diets with a high ratio of carbohydrates to amino acids can extend lifespan, while diets high in amino acids may reduce longevity. These findings emphasize the importance of carbohydrate variability in formulating pollen substitute diets for honeybees, influencing protein utilization and colony health.

Crude protein (%)

The crude protein content in different pollen formulations was measured and the results are shown in Table 1. The highest crude protein was found in pollen formulation 1 (P_1) at 4.025±0.23%, while the lowest was found in pollen formulation 2 (P_2) at 1.354±0.08%. Danihlik *et al.* (2018) found that the protein content in pollen substitute diets affects antimicrobial peptide production and gene expression in honey bees, showing the importance of protein nutrition for bee immunity. This aligns with Roulston et. al. (2000), who noted that pollen protein content varies widely among plant species and regions, influencing the nutritive values of bees. Therefore, crude protein levels significantly impact the effectiveness of pollen substitute diets for honey bees.

Crude fat (%)

The data shows in Table 1 that the crude fat content was highest in pollen formulation 2 (P_2) at 6.3±0.57 % and lowest in pollen formulation 1 (P_1) at 5.7±0.45 %. The fat content in pollen substitutes affects larval growth. Diets with higher fat content, like those with oils, enhance bee consumption. Using Feedbee® as a pollen substitute increases feed consumption and brood rearing compared to other diets, suggesting that fat content enhances palatability and nutritional value (Manning 2016).

Viscosity (Pa.s)

The data for the viscosity of nectar formulation 1 (N_1) and nectar formulation 2 (N_2) is shown in Table 2. N_1 has the highest viscosity at 0.0011±0.0001 Pa. s, while N_2 has 0.00089±0.00005 Pa. s. Venjakob *et al.* (2022) found that nectar's carbohydrate and amino acid compositions differ among plant species, affecting its viscosity and the purity of honey. Bees prefer less viscous nectar, with an optimal viscosity of 4.2 mPa·s for feeding (Liao et. al. 2022). The viscosity of nectar affects consumption; bees avoid higher viscosity solutions, preferring nectar that is easier to ingest (Nicolson *et al.*, 2013).

Table 1 : Physicochemical characteristics of sea buckthorn fruit-based pollen formulation 1 (P_1) and pollen formulation 2 (P_2)

Treatments	Particle size distribution (nm)	Carbohydrate (%)	Crude protein (%)	Crude fat (%)
Pollen Formulation 1 (P_1)	605.8 ± 0.02	79.4±4.84	4.02±0.23	5.7±0.45
Pollen Formulation 2 (P_2)	503.9 ± 0.02	79.9±4.39	1.35±0.08	6.3±0.57

*Values are mean ± standard deviation (n=3)

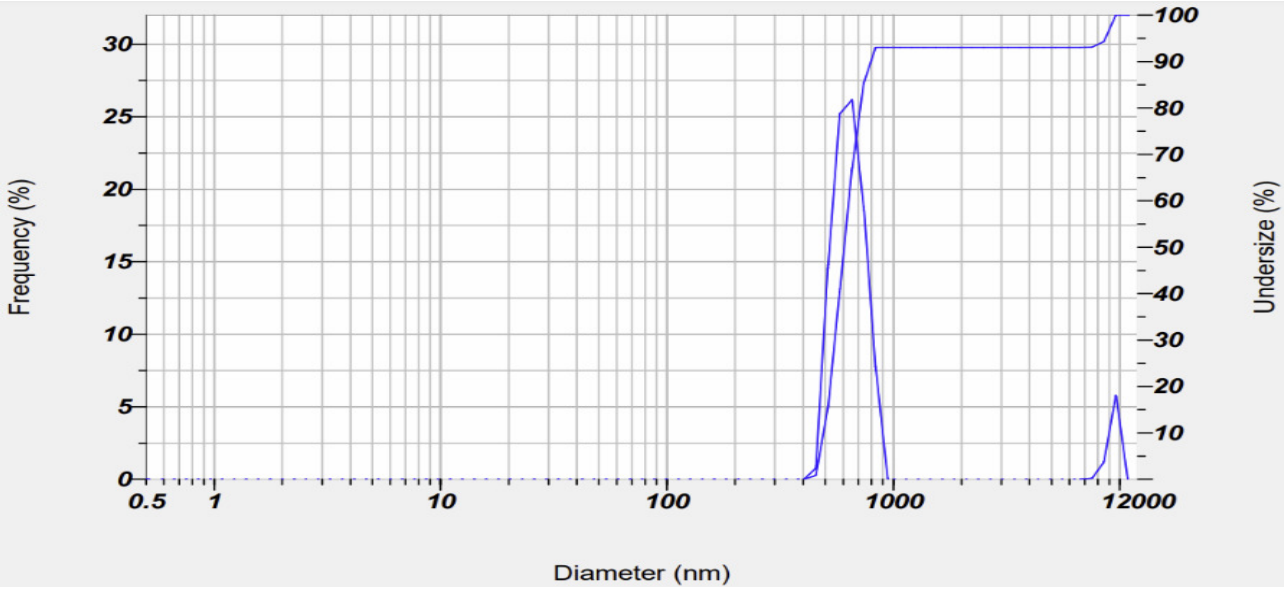

Fig. 1 : Particle size distribution of pollen formulation (P_1)

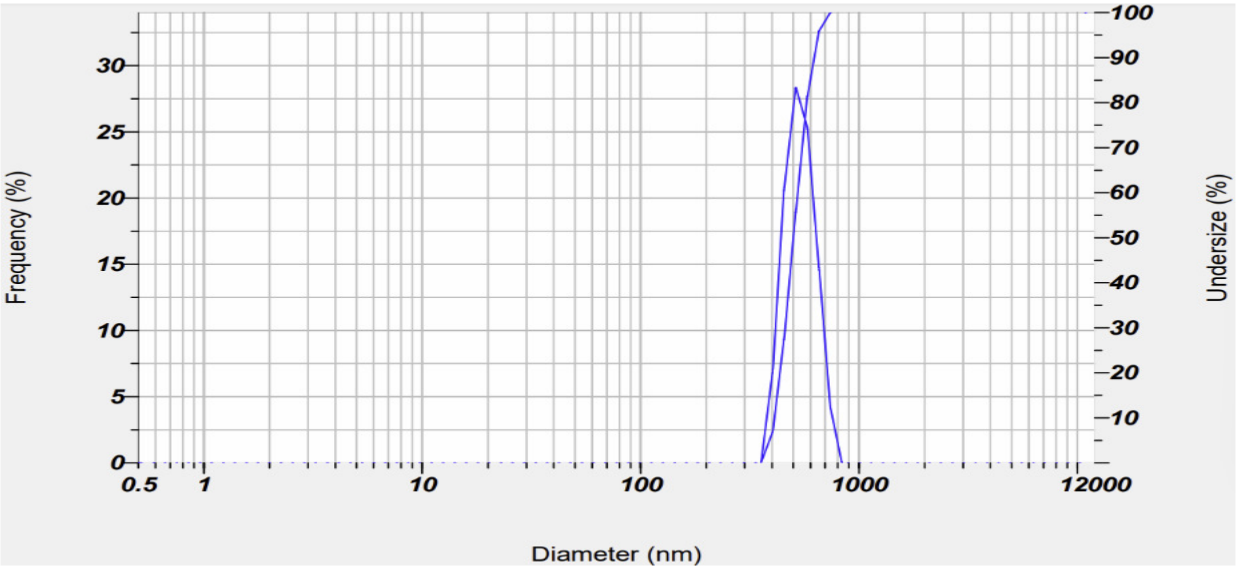

Fig. 2 : Particle size distribution of pollen formulation (P_2)

Table 2 : Chemical composition of sea buckthorn fruit-based nectar formulation 1 (N_1) and nectar formulation (N_2)

Treatments	Viscosity (Pa. s)	Osmolarity (mmol/L)
Nectar formulation (N_1)	0.0011±0.0001	643±28.94
Nectar formulation (N_2)	0.00089±0.00005	773±64.93

*Values are mean ± standard deviation (n=3)

Osmolarity (mmol/L)

The osmolarity of nectar formulation 1 (N_1) is 643±28.94 mmol/L, and nectar formulation 2 (N2) is 773±64.93 mmol/L (Table 2). According to Shi *et al.* (2020) osmolarity of nectar is also affected by temperature and viscosity, as honey bees adapt their foraging behaviour to optimise nectar intake, favouring warmer and less viscous solutions. The osmolarity of nectar collected by honeybees varies significantly, with concentrations reported between 2% and 62% (g sucrose/100 g solution) depending on environmental conditions and floral species.

Diet consumption

The diet consumption of pollen and nectar substitutes by *A. mellifera* was measured, with results summarized in Table 3. In June, colonies fed with T3 recorded the highest mean consumption at 3.66g, while T2 had the lowest at 2.85g. The mean nectar intake was highest in T4 (691.67ml) and lowest in T5 (680.67ml). For pollen and nectar combinations, T8 had the highest consumption at 3.43g and T7 the lowest at 2.8g. The rankings were $T_3 > T_8 > T_9 > T_6 > T_2 > T_7$, with respective consumptions of 3.66g, 3.43g, 3.34g, 3.02g, 2.85g, and 2.8g.

In July 2024, the consumption of pollen and nectar substitute diets by *A. mellifera* colonies was measured every six days. Results showed total diet consumption during the dearth period as follows: T_2 (3.54g), T_3 (3.74g), T_4 (683ml), T_5 (674.34ml), T_6 (3.03g), T_7 (3.11g), T_8 (3.11g), and T_9 (3.44g/colony). The highest consumption was in T_3 (3.74g), with T4 showing the most nectar substitute at 683 ml. The control group (T_1) had free access to foraging without artificial diets (Table 6).

In the present study, the diet consumption of bee colonies was studied by all possible planned pairwise comparisons which is mentioned in Table 4. On June 4th, colonies exclusively fed with nectar formulation (N_1 vs N_2) consumed significantly more compared to other comparisons. Among the treatments, nectar formulation 1 (N_1) is the most preferred overall by bee hive colonies.

Present data on diet consumption by bee colonies was analysed by all possible pairwise comparisons and depicted in Table 5 & 6. This revealed that on 28th July bee colonies exclusively fed with nectar formulations (N_1 vs N_2) showed a significant increase in consumption compared to other comparisons. Among all possible pairwise comparisons, nectar formulation 1 (N_1) is the most preferred overall by bee hive colonies.

Honeybees exhibit a clear preference for pollen substitute diets that are rich in amino acids, carbohydrates, crude protein, and total polyphenol content. This diet had high levels of protein and polyphenols, resulting in enhanced brood rearing. Carbohydrates play a crucial role in shaping honeybees' diet preferences, influencing their health, foraging behaviour, and overall colony performance. Similarly, pollen and nectar substitute diets containing high polyphenol content, and carbohydrates is consumed higher as compared to other formulated diets.

Conclusion

The use of sea buckthorn fruit-based pollen and nectar substitute diets has shown promising effects on honeybee nutrition. Research shows that specially formulated diets provide essential nutrients, which increase consumption. As a result, during times when natural food sources become scarce, these diets could play a crucial role in helping to sustain and protect bee populations, ensuring their survival through challenging periods of resource depletion.

Table 3 : Diet consumption (g/colony) in the month of June, 2024

Treatment	Diet consumption (grams)					Total consumption
	4 June	10 June	16 June	22 June	28 June	
T_2 (P_1)	0.49 ± 0.02	0.66 ± 0.02	0.71 ± 0.03	0.27 ± 0.01	0.72 ± 0	2.85
T_3 (P_2)	0.58 ± 0.01	0.78 ± 0.04	0.75 ± 0.03	0.69 ± 0.04	0.86 ± 0.03	3.66
T_4 (ml) (N_1)	140 ± 4.25	137 ± 5.47	137.33 ± 8.47	138.67 ± 6.84	138.67 ± 4	691.67
T_5 (ml) (N_2)	136.33 ± 1.65	136.67 ± 4.86	134 ± 6.31	136.67 ± 4.35	137 ± 8.07	680.67
T_6 (P_1N_1)	0.5 ± 0.02	0.61 ± 0.01	0.54 ± 0.01	0.63 ± 0.02	0.74 ± 0.01	3.02
T_7 (P_1N_2)	0.53 ± 0.01	0.66 ± 0.01	0.51 ± 0.02	0.57 ± 0.02	0.53 ± 0.02	2.8
T_8 (P_2N_1)	0.52 ± 0.01	0.7 ± 0.03	0.76 ± 0.03	0.68 ± 0.03	0.77 ± 0.03	3.43
T_9 (P_2N_2)	0.51 ± 0.03	0.74 ± 0.03	0.67 ± 0	0.67 ± 0.02	0.75 ± 0.01	3.34

*Values are mean ± standard deviation (n=3)

Table 4 : Planned pairwise comparison of pollen and nectar substitute diets by using custom contrast method on diet consumption June, 2024

Contrast Method	Diet consumption (grams)														
	4 June			10 June			16 June			22 June			28 June		
Planned Comparison	Estimate	t	p	Estimate	t	p	Estimate	t	p	Estimate	t	p	Estimate	t	p
1 (P1 VS P2)	0.082	0.066	0.948	0.117	0.059	0.954	0.04	0.014	0.989	0.423	0.192	0.85	0.14	0.057	0.955
2 (N1 VS N2)	-3.667	-2.955	0.008	-0.333	-0.167	0.869	-3.333	-1.16	0.261	-2	-0.907	0.376	-1.667	-0.68	0.505
3 (P1N1 VS P1N2, P2N1, P2N2)	-0.06	-0.02	0.984	-0.261	-0.054	0.958	-0.31	-0.044	0.965	-0.04	-0.007	0.994	0.16	0.027	0.979
4 (P1N2 VS P1N1, P2N1, P2N2)	0.06	0.02	0.984	-0.073	-0.015	0.988	-0.443	-0.063	0.95	-0.267	-0.049	0.961	-0.653	-0.109	0.915
5 (P2N1 VS P1N1, P1N2, P2N2)	0.013	0.004	0.997	0.074	0.015	0.988	0.557	0.079	0.938	0.187	0.035	0.973	0.28	0.047	0.963
6 (P2N2 VS P1N1, P1N2, P2N1)	-0.013	-0.004	0.997	0.26	0.053	0.958	0.197	0.028	0.978	0.12	0.022	0.983	0.213	0.036	0.972

Table 5 : Diet consumption (g/colony) in the month of July, 2024

Treatment	Diet consumption (grams)					Total consumption
	4 July	10 July	16 July	22 July	28 July	
T_2 (P_1)	0.58 ± 0.03	0.86 ± 0.04	0.64 ± 0.02	0.79 ± 0.03	0.67 ± 0.02	3.54
T_3 (P_2)	0.61 ± 0.01	0.87 ± 0.03	0.61 ± 0.01	0.77 ± 0.01	0.88 ± 0.02	3.74
T_4 (ml) (N_1)	137.33 ± 5.22	135.33 ± 2.08	136.67 ± 3.08	136.67 ± 5.57	137 ± 3.5	683
T_5 (ml) (N_2)	134.67 ± 1.99	135.67 ± 2.71	135.33 ± 3.4	136 ± 3.27	132.67 ± 2.71	674.34
T_6 (P_1N_1)	0.54 ± 0.02	0.7 ± 0.02	0.43 ± 0.01	0.7 ± 0.01	0.66 ± 0.03	3.03
T_7 (P_1N_2)	0.5 ± 0.01	0.77 ± 0.03	0.48 ± 0.01	0.71 ± 0.01	0.65 ± 0.02	3.11
T_8 (P_2N_1)	0.51 ± 0.02	0.57 ± 0	0.55 ± 0.02	0.72 ± 0.03	0.76 ± 0	3.11
T_9 (P_2N_2)	0.5 ± 0.02	0.79 ± 0.02	0.68 ± 0.03	0.72 ± 0.01	0.75 ± 0.02	3.44

*Values are mean ± standard deviation (n=3)

Table 6 : Planned pairwise comparison of pollen and nectar substitute diets by using custom contrast method on diet consumption July, 2024.

Contrast Method	Diet consumption (grams)														
	4 July			10 July			16 July			22 July			28 July		
Planned Comparison	Estimate	t	p	Estimate	t	p	Estimate	t	p	Estimate	t	p	Estimate	t	p
1 (P1 VS P2)	0.033	0.022	0.983	0.003	0.004	0.997	-0.033	-0.027	0.979	-0.013	-0.008	0.994	0.203	0.169	0.868
2 (N1 VS N2)	-2.667	-1.754	0.096	0.333	0.358	0.724	-1.333	-1.067	0.3	-0.667	-0.379	0.709	-4.333	-3.6	0.002
3 (P1N1 VS P1N2, P2N1, P2N2)	0.107	0.029	0.977	-0.017	-0.007	0.994	-0.42	-0.137	0.892	-0.063	-0.015	0.988	-0.167	-0.057	0.956
4 (P1N2 VS P1N1, P2N1, P2N2)	-0.04	-0.011	0.992	0.237	0.104	0.918	-0.22	-0.072	0.943	0.003	7.743×10^{-4}	0.999	-0.233	-0.079	0.938
5 (P2N1 VS P1N1, P1N2, P2N2)	-53.08	-24.5	1	-0.55	-0.241	0.812	0.06	0.02	0.985	0.043	0.01	0.992	0.233	0.079	0.938
6 (P2N2 VS P1N1, P1N2, P2N1)	-0.067	-0.018	0.986	0.33	0.145	0.886	0.58	0.19	0.852	0.017	0.004	0.997	0.167	0.057	0.956

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