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Physicochemical Characteristics of Honey Produced by Colonies of the Stingless bee *Melipona quadrifasciata*: Seasonal Variations and Influence of Food Supplementation

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Abstract **– Native stingless bees have critical economic and environmental value. During hard seasons (i.e. dry winters, when natural resources are scarce) and when duplicating colonies it is commonplace among meliponiculturists to offer food supplements in order to guarantee colonies' health. However, much of these supplements have not been tested and can promote null results or even jeopardize the colonies' health. Here we tested the influence of four commonly used food supplements on the physicochemical characteristics of honey produced by *Melipona quadrifasciata* colonies: D1: Sucrose solution; D2: Sucrose solution + lemon juice; D3: Sucrose solution + honey; D4: Sucrose solution + honey + Nutritional complex (with amino acids). Experiments were conducted in Muzambinho, Brazil. Honeys collected in the rainy summer had higher levels of acidity, proteins, and moisture than those collected in the dry winter, which surpassed the summer honey in reducing sugars and ash levels ($p<0.05$). Food supplementation including lemon juice was the only that allowed keeping the levels of free acidity comparable to those of the control colonies, mainly during summer. The low protein levels of the winter honey in colonies fed with diets including nitrogen sources had protein levels comparable to that of the summer honey. Regarding sugars, the most apparent result was that diets including sucrose + lemon juice or honeybee honey increased non-reducing sugars only in winter. Moreover, diets with sucrose + lemon juice and sucrose were the only in decreasing ash levels in winter. Finally, all diets offered in winter (but not summer) decreased honey moisture. Our results suggest that food supplementation strategies should consider the influence of seasonal variations on colony's health in order to find the best and more affordable food supplement for each season.**

Keywords **– Mandacaia, Neotropical Bee, Nutrition, Summer, Winter.**

I. INTRODUCTION

With more than 16,000 described species, bees are the dominant pollinators of flowering plants in both natural and agricultural ecosystems, and thus have critical economic and environmental value (Michener, 2000; Danforth, 2007). *Melipona quadrifasciata* Lepeletier, 1836, (Hymenoptera: Apidae: Meliponinae), a Neotropical stingless bee, has been reported from the southern part of Brazil (Mato Grosso do Sul, Minas Gerais, Parana, Rio Grande do Sul, Rio de Janeiro, Santa Catarina, and Sao Paulo states), Misiones Province of Argentina, and Misiones Department of Paraguay (Camargo & Pedro, 2008). This distribution coincides well with the remnants of the southern part of the Atlantic Rain Forest. *M. quadrifasciata* nests in trees and normal colony size ranges from 300 to 400 individuals (Sakagami, 1982; Wille, 1983; Michener, 2000; Antonini & Martins, 2003).

Under natural conditions, bees obtain all their required nutrients from nectar or pseudo nectar (honeydew), pollen, and water (see for example Oliveira-Abreu et al. 2014). Honey precursors (nectar and honeydew) are usually found in the environment surrounding the colony, then collected and transported by the workers (foragers) to the colony where they are concentrated and, then, processed by enzymes in a way that most part of the sucrose is transformed into glucose and fructose (for a review on honey composition and variations, see Wang & Li, 2011). Thus, honey composition mainly depends on plant origin. However, other factors such as bee species, the physiological conditions of the colony, the maturation state of the honey, meteorological condition during the collecting process, and, even, the type of soil, influence honey composition (White, 1957; Alves et al. 2005). On top of that, nectar deficiency can occur after long periods of heavy rainfall and/or low temperatures, when bees are impaired from foraging (Pinheiro et al. 2009) or they lose the competition at floral resources from Africanized *Apis mellifera* (Villanueva-G et al. 2005). Stingless bee colonies, thus, show profound variations in foraging behavior in response to environmental fluctuations, which are transduced into the colonies "health" by the adjustment of physiological outputs, e.g., reducing reproduction efforts (Aidar, 1996; Villanueva-G et al. 2005; Ferreira Junior et al. 2010; Maia-Silva et al. 2015). In addition, much of the natural environment has been devastated by agriculture and other anthropic activities during the last decades (Drummond et al. 2009; Ribeiro et al. 2009; Goulson et al. 2015). In this context, using food supplements can improve colony health and can be useful in efforts to preserve species (Jaffe et al. 2015; Kapheim et al. 2015). Diet supplementations with materials that do not negatively impact on bees and their products (such as honey) help beekeepers maintain a sustainable activity along with preserving this important native stingless bee species (Pires et al. 2009; Ceksteryte & Racys, 2006; Costa & Venturieri, 2009; Farjan et al. 2014). In doing so, meliponiculturists practice different kind of supplementation, much of which have not been tested. Here, we assessed physicochemical characteristics of *M. quadrifasciata* honey produced in two very contrasting seasons and how different food supplementation influence these characteristics.

II. Materials and Methods

A. Meliponary and Treatments

Experiments were carried out during the summer of December 2011-January 2012; and the winter of July-August 2012, using 20 colonies in rational hives (four colonies/group) in Muzambinho, Minas Gerais State, Brazil (21°22'S, 46°31'W; 1,048 m). The used hives were INPA type (20 x 20 x 20 cm) with an estimated population of 1,300 bees. The region where the meliponary is installed has the following climatic profile: *Summer*: minimum temperature 17.34 ± 0.8 ºC, maximum 24 ± 3.33 ºC (mean 20.16 ºC ± 5.43); 273 mm mean rainfall. *Winter* – minimum temperature 11.67 ± 1.7 ºC, maximum 22.67 ± 4.33 ºC (mean 17.27 ºC ± 10.54); 22.01 mm mean rainfall (Pereira et al. 2009). In this area, which is next to remnants of rainforest, temperature shows low seasonal variability, rainfall is the prominent aspect of climate variation, with dry winters and rainy summers.

We tested the influence of four commonly used food supplements on the physicochemical characteristics of honey produced by *Melipona quadrifasciata* colonies:

D1: Sucrose solution (saturated in water; energetic); D2: Sucrose solution + lemon-juice (*Citrus aurantifolia;* 4 mL: 1mL; suggested to improve bee health, Farjan et al. 2014); D3: Sucrose solution + honey (from *A. mellifera*, 1mL: 1mL; suggested to improve bee health, Mao et al. 2013); D4: Sucrose solution + honey + Nutritional complex (Aminomix Pet^R - with amino acids, vitamins and minerals; http://www.nobico.com/loja/passaros/fabricantes/vetnil/suplemento-aminomix-pet-vetnil.html) (40 mL: 40mL: 1g; suggested to improve bee health, Brodschneider & Crailsheim, 2010); Control: without food supplementation.

These food supplements were chosen because they are easily available and affordable. Colonies were fed three times a week with 150 mL of food supplement.

B. Physicochemical Analyses of Honey Samples

After two months of food supplementation, honey samples (100 mL) were collected using sterile syringes and stored in sterilized glass recipients. We measured at least twice the following physicochemical characteristics: Moisture, ash, reducing and non-reducing sugars, protein concentration, and acidity.

Moisture:

Water content was measured in 5 g of honey at 20 ºC with a digital ABBE WAY-1S refractometer. The refractive index values were converted to moisture contents as in AOAC (2012).

Ash:

Ten grams of honey were ashed in an oven at 600 °C (~5 h) until a constant weight was obtained (Rodrigues et al. 2005).

Sugars:

Reducing sugars were measured by the Lane-Enyon method (Fehling's reaction). This method detects any carbohydrate that is capable of being oxidized and thus causes the reduction of other substances [in this case, copper (II) ions from the Fehling solution]. Generally, all monosaccharaides (e.g. glucose) with free aldehydes or hydroxyl ketonic groups are capable of being oxidized. We used 2 g of each sample dissolved in 250 mL of water. Non-reducing sugars were estimated after applying the Lane-Enyon method on HCl-treated honey samples.

Proteins:

We used Lund's reaction followed by micro-Kjeldahl's indirect method (AOAC, 2012) on an initial honey sample of 2 g.

Acidity:

Ten grams of honey were dissolved in deionized water and titrated with NaOH 0.1 N until reaching pH 8.3. Results were expressed as meq Kg^{-1} of acid.

C. Statistical Analyses

The obtained values were subjected to analysis of variance (ANOVA). Scott-Knott test was used to compare the mean values. All variables attended the normality assumptions and homogeneity of variance. The statistical analyses were conducted with the statistical software R, version 3.1.1 (R Core Team, 2014), significance level of 5%.

III. Results

A. Honeys Produced in Different Seasons have Different Physicochemical Characteristics

The honey produced by *M. quadrifasciata* colonies in different seasons (rainy summer and dry winter) differed in five out of the six measured parameters (two-way ANOVA test): Moisture (in summer 75.6 %, in winter 27.6 %, p<0.002), Acidity (in summer 40.27 meq/Kg-1, in winter 20.30 meq/Kg-1, p<0.00019), and Protein (in summer 0.31 %, in winter 0.1 %, p<0.004) levels were higher in summer (Non-reducing Sugars in summer 2.23 %, in winter 1.76 %, p<0.1), whereas Reducing Sugars (in summer 37.12 %, in winter 61 %, p<0.02) and Ash (in summer 0.05 %, in winter 0.16 %, p<0.01) levels were higher in winter samples (Figure 1, Tables S1 and S2).

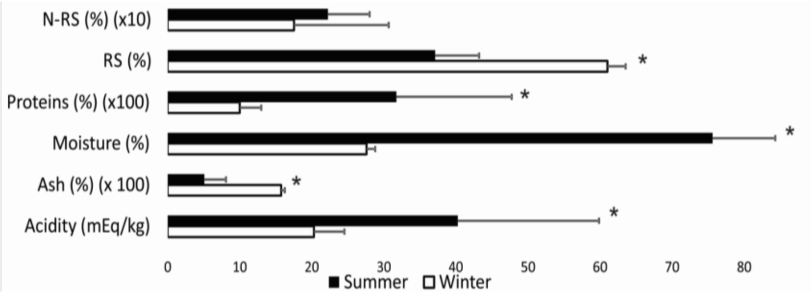

Fig. 1. Physicochemical characteristics of *Melipona quadrifasciata* honey samples (n= 4) collected in two different seasons, summer and winter, without food supplementation. Means and SDs. ANOVA test, Reducing Sugars $p<0.02$; Proteins $p<0.004$; Moisture $p<0.002$; Ash $p<0.01$; Acidity $p<0.00019$.

B. The Influence of Different Food Supplements Varies According to the Season

Acidity:

We found that only D2 diet (Sucrose solution + lemon-juice) kept acidity within the Control levels in summer (70.48 ± 10.23 meq.kg $^{-1}$, p<0.05) (Figures 1 and 2A and Tables S1 and S2). The other diets (D1, D3, and D4) showed in summer a decreasing effect on the natural acidity of honey and the respective honeys' acidity was between 18.67 and 9.91 meq.kg^{-1} (Scott-knott, p<0.05), much the same to that of winter honey. In winter, acidity levels were pretty the same in all treatments, ranging from 33.9 to 15.92 meq.kg $^{-1}$.

Proteins:

Protein levels were always higher in the summer samples, irrespective of the diet (Scott-knott, p<0.05). Interestingly, D4 (and D3 though not significantly) had an increasing effect on protein levels of the winter honey (Figures 1 and 2B and Tables S1 and S2). On the other hand, D1 and D2 had a decreasing effect on the protein levels of the summer honey.

Reducing Sugars:

The amount of reducing sugars was in all cases higher in winter honey. Artificial diets did not alter reducing sugars neither in summer nor in winter (Scott-knott, p<0.05) (Figures 1 and 2C and Tables S1 and S2).

Non-Reducing Sugars (NRS):

D3 diet on summer honey imposed the lowest non-reducing sugars levels of all treatments (mean 1.14 ± 0.34%) (Scott-Knott p<0.05). The other treatments values in summer ranged from 1.67 % to 3.26%. In winter, D2 and D3 diets increase NRS (5.62 ± 2.13 and 5.3 ± 2.46, respectively), when compared to the other treatments, whose values ranged from 1.76 to 3.35) (Figures 1 and 2D and Tables S1 and S2).

Ash:

Diets D1 and D2 decreased ash percentage in winter honey when compared to the other treatments. On the other hand, D4 increased ash in summer samples in relation to all the other treatments (Scott-Knott p<0.05) (Figures 1 and 2E and Tables S1 and S2).

Moisture:

Here we found that, in winter, all food supplementations decreased honey's moisture (Scott-Knott p<0.05). No modulatory effect of supplementation was found in summer honey (Figures 1 and 2F and Tables S1 and S2).

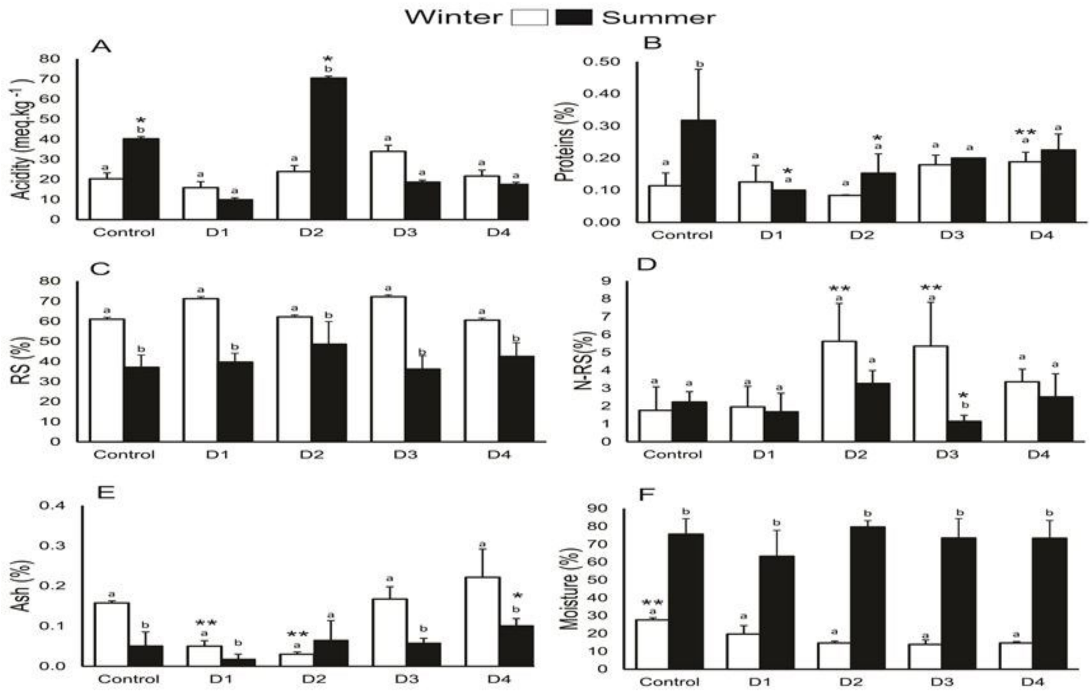

Fig. 2. Physicochemical characteristics of *Melipona quadrifasciata* honey samples (n= 4) collected in two different seasons, summer and winter, after food supplementation. Means and SDs. Differing letters indicate statistical difference between seasons (same treatment). (*): Different from the other treatments in Summer. (**): Different from the other treatments in Winter. Scott-Knott $p<0.05$. Control: no supplement; D1: Sucrose solution; D2: Sucrose solution+lemon juice; D3: Sucrose solution+honey; D4: Sucrose solution+honey+Nutritional complex.

IV. Discussion

We found that physicochemical characteristics of *M. quadrifasciata* honey, physiological markers of colonies' health, is affected by season (Figure 1) and that feeding them with artificial food supplements has season-specific effects on those characteristics (Figure 2 and Tables S1 and S2).

Acidity:

The *M. quadrifasciata* colonies produced honey with free acidity comparable to that of other stingless bees. Carvalho et al. (2005) showed that the range of acidity variation among the stingless bees is between 8.88 and 112.80 meq.kg $^{-1}$.; see also Vit et al. 2004; Souza et al. 2006; Almeida-Muradian et al. 2007; Souza et al. 2009; Lage et al. 2012). The acidity levels we found are also comparable to that of honeybee (*A. mellifera*) honey and within the range suggested for the commercialization of stingless bee honey (50 meq.kg.; Carvalho et al. 2013; Codex Alimentarius; for a review on *A. mellifera* honey composition, see Wang & Li 2011 and Silva et al. 2016). Summer honey had almost double the free acidity found in winter honey (in control colonies), which is a relevant finding that should be taken into account when analyzing the physicochemical characteristics of honeys produced in geographical regions with disparate seasons. Remnants of native forests and flowering crops, such as those surrounding our meliponary (Pereira et al. 2009), during rainy seasons are thus associated to increased acidity of *M. quadrifasciata* honey. Variations in acidity were also found in *A. mellifera* honey in a region 300 km South of our meliponaries. Bendini et al (2010) found winter honey with 24 meq.kg^{-1} and summer honey with 16 meq.kg^{-1}, and suggested this variation is due to differences in the flowering species in each season. Honey acidity can be chemically influenced by variations in organic acids concentration in nectar sources, by the levels of glucose oxidase (central enzyme in the pathway leading to gluconic acid), and by the kind of bacteria and the minerals present in the honey (White 1957). In our experiments, not unexpectedly, supplementation with a solution that included lemon juice allowed keeping the levels of free acidity comparable to those of the control colonies, mainly during summer (70 meq.kg^{-1}). However, this makes honey less palatable and puts it above the limits suggested for commercialization. High acidity levels in the diet may also be deterrent and can even cause physiological disturbances such as premature aging, alteration in flight capacity and foraging, etc. (Brighenti et al. 2011). Thus, our results suggest that food supplements including lemon juice (commonplace among some meliponiculturists) for *M. quadrifasciata* colonies should be used with restriction. Additionally, all the other diets failed to maintain honey's acidity levels, clearly confirming the influence of the flower sources on honey characteristics and suggesting bees avoid foraging when food is artificially available.

Proteins:

Our collected summer honey was strikingly richer in protein levels than the winter honey (see White 1957; Souza et al. 2009; Almeida-Muradian et al. 2013), maybe because of a higher pollen availability and foraging activity during this season (Ferreira Junior et al. 2010; Maia-Silva et al. 2015. Diets with sucrose and sucrose + lemon juice (D1 and D2, without any source of proteins) had a decreasing effect on the protein levels of the summer honey, while diets including some amino acids/ protein like those with honeybee honey and nutritional complex did not alter the protein levels of the collected honey. Interestingly, the protein levels of the winter honey in colonies fed with diets including nitrogen sources (mainly D4) were higher than the honey of all the other colonies and comparable to that of the summer honey. This demonstrates that this kind of food supplementation is an alternative for maintaining protein levels in *M. quadrifasciata* honey during the dry season. Moreover, we found that D3 (though not significantly) was as effective as D4 for this purpose and it is cheaper. Since constant feeding of bees with carbohydrate rich diets promotes alterations in gene expression patterns and is associated with immune system and developmental impairment (Brodschneider & Crailsheim 2010; Wheeler & Robinson 2014), including protein in the bee diet might be a good way to improve honey quality and, consequently, colony health. In addition, protein intake is a prerequisite of reproduction, and maintaining protein levels compatible with oogenesis and development would translate into lesser colony member diminutions during winter.

Reducing Sugars (RS):

There was significant difference in RS concentration between both seasons honey, 37 % in summer honey and 61 % in winter honey. For the latter, this is within the range found for *Melipona* honey from Venezuela and for *A. mellifera* honey (Souza et al. 2006). Summer honey, on the other hand, showed to be lower in RS than most reports for Brazilian *Melipona* honey (Souza et al. 2006; Holanda et al. 2012), and lower than reported for *A. mellifera*. None of the food supplements, neither in summer nor in winter, altered RS levels in the sampled honey, suggesting these stingless bees, regarding RS, are not influenced by an eventual artificial availability of RS (eg, from the honeybee's honey, D3 and D4) in their food.

Non-Reducing Sugars (NRS):

The honey we collected had NRS levels ranging from 1.14 to 5.62 %. These are similar to the levels reported for honey from many stingless bees species, including several *Melipona* species (Souza et al. 2006, 2009) and slightly above the level suggested in Codex Alimantarius. As shown by Souza et al. (2006), the levels of NRS vary greatly among different stingless bees and seem to be associated with geographical location (see also Carvalho et al. 2005; Almeida-Muradian et al. 2007; Holanda et al. 2012). Treating colonies with artificial diets promoted interesting though hard to explain results. Among them, during summer, D3 diet (Sucrose solution + honey) was associated to the lowest non-reducing sugars levels of all treatments. Moreover, in winter, D2 (Sucrose solution + lemon-juice) and D3 diets (but not D1 and D4, which also include sucrose) had an increasing effect on NRS. Though worth taking into account when considering supplementing bees food, these findings deserve further investigation in order to highlight their physiological underpinnings, which might reside in an eventual interplay among sugars, citric acid and honeybee honey components (sucrose, etc.).

Ash:

As the reducing sugar levels, the amount of ash was higher in winter honey samples. In both cases, this bias to higher chemical concentration of winter honey might be related to the lower amount of water of this honey (see below) together with the higher levels of environmental pollution typical of this season (winters are dry and the region suffers from episodic human-set vegetation burnings). In the latter context, bees are forced to leave the nest to find water sources. During this flying period they may increase the chances of being exposed to mineral contaminants in the air (Porrini et al. 2003; Contrera et al. 2004; see also Bogdanov et al. 2007), thus maintaining high ash levels in their honey. In any case, the found ash levels (0.05-0.16 %) were within the range previously found for Melipona honeys (the highest ash levels were reported for *Plebeia droryana,* 1.18 %; Souza et al. 2006; see also White, 1957) and honeybee honeys (see, for example, Nanda et al. 2003; Cantarelli et al. 2008). In summer, D4 significantly increased ash levels, what was expected since this diet includes minerals, important fraction of the honey ash (we found the same for the winter honey though it was not statistically significant). On the other hand, in winter, D1 and D2 decreased ash levels in the collected honey, finding that deserves further investigation, though it might be due to a dilution effect of the ingested diet.

Moisture:

As expected, we found remarkable differences in moisture percentage between summer and winter honeys, which in all cases favored summer samples. Our winter samples were well within the range reported for other stingless bees (Souza et al. 2006; Lage et al. 2012). Our summer samples, however, had -as far as we know- higher levels of water than any honey reported so far, even than that reported by Paulo Nogueira-Neto and his students for *M. quadrifasciata anthidioides* in 1964 (almost 42%, see in Souza et al. 2006). The latter also reported moisture of 45% for *M. quadrifasciata* honey. This result is very likely reflecting the general amount of water in the environment (and region) where the experimental meliponaries are installed. Summer, rainy season, is characterized by about 10 times more rainfall than winter (see M & M). Thus, in winter, bees seem to be unable to collect enough water to maintain honey moisture levels in addition to maintaining the general nest homeostasis. On top of that, moisture, which influences viscosity (Yanniotis et al. 2006), is usually higher in stingless bee honey than in *A. mellifera* honey (MERCOSUL/GMC/RES. Nº 89/99; Terrab et al. 2002; Almeida-Muradian et al. 2013; Silva et al. 2016). Since glucose oxidase efficiency increases in honeys with higher levels of water, the resulting honey may have higher acidity thus making it less palatable, correlation we found in our samples. On the other hand, all diets offered in winter decreased honey moisture, maybe by concentrating even more the already water-scarce food resources.

V. Conclusion

The honey produced by *M. quadrifasciata* colonies has seasonal variations in physicochemical characteristics and food supplementation promotes season-specific alterations in these characteristics. Taken together, our results lead us to suggest that food supplementation should consider the influence of seasonal variations on colony's health, in order to find the best and more affordable food supplementation strategy for each season. Having colonies producing during the hard seasons a honey that closely resembles that produced without supplementation would mean to have enough resources to maintain colony's health, meaning, the possibility of, for example, not reducing the number of colony members by keeping the reproduction efforts, etc. This would be reflected during the next season since a strong colony entering the following favorable season would start producing marketable products (e.g., honey) earlier, better and more.

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SUPPLEMENTARY MATERIAL

Table S1: Physicochemical characteristics of *Melipona quadrifasciata* honey samples (n= 4) collected in *summer*. Control: no supplement; D1: Sucrose solution; D2: Sucrose solution+lemon juice; D3: Sucrose solution+honey; D4: Sucrose solution+honey+Nutritional complex. Different letters in a line indicate statistic differences (Scott-Knott p<0.05).

Parameter	Control	D1	D2	D3	D4
Free Acidity (meq/Kg)	40.27 ± 19.61a	9.90 ± 9.19b	70.4 ± 10.23a	18.67 ± 7.87b	17.55 ± 8.45b
Proteins (%)	0.3175 ± 0.16a	0.1 ± 0.00b	0.15 ± 0.06b	0.2 ± 0.00a	0.225 ± 0.05a
Reducing Sugars	37.12 ± 6.10a	39.69 ± 4.43a	48.5 ± 11.27a	36.21 ± 6.72a	42.51 ± 6.83a
Non-Reducing S.	2.23 ± 0.58a	1.67 ± 1.05a	3.26 ± 0.72a	1.14 ± 0.34b	2.51 ± 1.29a
Ash (%)	0.05 ± 0.29a	0.02 ± 0.01a	0.06 ± 0.05a	0.06 ± 0.01a	0.10 ± 0.01b
Moisture	75.61 ± 8.75a	63.28 ± 10.76a	79.67 ± 9.16a	73.49 ± 7.67a	73.40 ± 7.4a

Table S2: Physicochemical characteristics of *Melipona quadrifasciata* honey samples (n= 4) collected in *winter*. Control: no supplement; D1: Sucrose solution; D2: Sucrose solution+lemon juice; D3: Sucrose solution+honey; D4: Sucrose solution+honey+Nutritional complex. Different letters in a line indicate statistic differences (Scott-Knott p<0.05).

Parameter	Control	D1	D2	D3	D4
Free Acidity (meq/Kg)	20.30 ± 4.24a	15.92 ± 1.48a	23.93 ± 0.98a	33.9 ± 11.76a	21.66 ± 3.67a
Proteins (%)	0.10 ± 0.03a	0.14 ± 0.05a	0.08 ± 0.00a	0.18 ± 0.04b	0.19 ± 0.04b
Reducing Sugars	61.02 ± 2.54a	71.27 ± 7.63a	62.19 ± 4.90a	72.2 ± 17.51a	60.59 ± 2.51a
Non-Reducing S.	1.76 ± 1.31a	1.95 ±1.16a	5.62 ± 2.13b	5.3 ± 2.46b	3.35 ± 0.72a
Ash (%)	0.16 ± 0.01a	0.05 ± 0.01b	0.03 ± 0.01b	0.17 ± 0.03a	0.22 ± 0.07a
Moisture	27.60 ± 1.21a	19.67 ± 4.79b	14.70 ± 1.05b	13.86 ± 2.53b	14.65 ± 0.83b