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Vitamin C supplementation in honeybee *Apis mellifera* (Hymenoptera: Apidae) colonies to improve drone's quality

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Abstract:

Colony success of honeybee *Apis mellifera* L. (Hymenoptera: Apidae) depends partially on the reproductive success of the queen, which in turn directly impacted by drone's health and fitness. Drones are reared only during the reproductive season and can be affected by many environmental factors. The impact of hive supplements on drone fitness and quality is rarely documented but may improve drone reproductive health and colony survival. In this investigation, feeding sucrose syrup supplemented with vitamin C inserted to colonies in early spring. Drones assessed after emergence in regarding to body weight, total soluble protein, total carbohydrates content, carbohydrate hydrolysing enzymes in addition to sperm number in mature individuals. The mean body weight, total protein and carbohydrates content of newly emerged drones, as well as sperm numbers of matured drones were significantly ($P< 0.05$) higher in vitamin C colonies than control ones. Whereas, insignificant difference was observed in the activities of trehalase, amylase and sucrase enzymes between control and colonies receiving vitamin C as supplement.

Introduction

The survival of a honeybee *Apis mellifera* L. (Hymenoptera: Apidae) colony depends indirectly on rearing of healthy drones. Despite the transitory presence of drones in the hive, they have the important function of mating with virgin queens, transferring their colony's genes to their mates where, drones are responsible for producing semen which transmit to the queen during the mating flight (Czekońska *et al.*, 2013). Honeybee queen can mate with an average of 12 drones and able to hold about 4.3-7.0 million spermatozoa in her spermatheca to fertilize eggs for the remainder of her life (Rhodes, 2002). Adequate numbers of mature age drones, each producing a large volume of semen with high number of sperms result in queen bees with the maximum number of sperms present in their spermathecae after mating. Low numbers of sperm in the queen spermathecae after mating contributes to early supersedure that increase costs to beekeepers due to queen replacement beside weaken in colonies strength and decrease their production during the period of queen replacement (Rhodes, 2008).

Therefore, factors affecting drone reproductive health may directly affect queen fitness and longevity, having great influences at the colony level. Quality and viability of drones particularly their sperm are influenced by several environmental factors and in-hive conditions including nutrition, temperature, age, size of comb cells and season. Pesticide exposure during and after development may also influence drone reproductive quality (Rangel and Fisher, 2019). Drones are also very sensitive to infestation with parasites, e.g. Varroa mites moreover, there is a strong preference for mite reproduction in drone brood over worker brood in a ratio of nine to one when both worker and drone brood are available (Martin, 1995). However, varroa parasitism negatively impacts colony fitness as it decreases drone weight and reduces the number of drones that survive to maturity (Collins and Pettis, 2001). Also, applying miticides to beehives can produce undesirable side effects in honeybees (Johnson *et al*., 2010).

Previous work has demonstrated that the exposure of drones honeybee to miticides can result in decreased sperm viability (Johnson *et al.*, 2013) and has adverse effects on drone survival, drone weight, length and width of wings, and reduces the number of sperm production (Shoukry *et al*., 2013). Excessive oxidative stress is the basis or result of many diseases including parasitosis (Halliwell, 2011). Subsequently, methods reducing the effects of oxidative stress can support the protective forces of an organism. Antioxidant levels and the activity of antioxidative enzymes, which prevent cell damage caused by oxidation, are important factors determining the good health of an organism (Mishra, 2007). One method for decreasing oxidative stress is administration of exogenous compounds with antioxidative characteristics as vitamin C (Ascorbic acid) a natural antioxidant. The objective of this investigation was therefore to evaluate the effects of supplemental vitamin C in sugar syrup in early spring on body weight, sperm numbers in addition to biochemical parameters of honeybee drones .

Materials and methods

1. Field Work:

1.1. Supplemental feeding for colonies:

This study was conducted at the apiary of Plant Protection Research Institute, Agriculture Research Center. Experimental treatments began in early spring where six *A. mellifera* colonies with equivalent adult bee population headed by sister queens were used. Colonies were divided randomly into two experimental groups: Three receiving a sugar solution made up in the ratio of 1 kg sugar dissolved in 1 liter water (1:1) and supplemented with vitamin C at a rate of 1.8 mg per 1 kg syrup. The remaining three colonies received pure syrup as a control. Colonies were fed supplemental food weekly and each colony received 500 ml. Feeding began in early spring nearly 14 days prior the queens begin to lay eggs in drone cells and continued until the time that the experimental drone larvae had been capped.

1.2. Drones sampling:

Two days before drone's emergence, drone cells were held under protective cages that allowed the collection of emerged drones. Newly emerged drones were investigated for body weight and biochemical assay. Whereas, drones tested for sperms number remained in the colonies for sexual maturation.

2. Laboratory work:

2.1. Body weights:

Newly emerged drones from each group were individually weighed using an electrical balance.

2.2. Sperms number:

Drones remained in the colonies for about 21days to allow full sexual maturation where, emerged drones were held in cages made with queen excluder material and placed into the hive allowing workers to enter and feed the drones **(Collins, 2004)**. Drones of 21-days old, from each group, were chilled to prevent their ejaculation when dissected. The drones were dissected in 0.5% saline solution to remove the seminal vesicle. One seminal vesicle from each drone was placed in 10 ml 0.5% saline solution and punctured with a fine pair of needles. Sperms dispersed by the sucking and expelling action of a pipette. Sperm numbers were counted using a haemocytometer and light microscope (Rinderer *et al*., 1985).

2.3. Biochemical assays:

Samples were homogenized in distilled water using a Teflon homogenizer surrounded with a jacket of crushed ice. The homogenates were centrifuged at 5000 rpm for 10 minutes at 5°C. The supernatants were immediately assayed to determine the following parameters: Total soluble protein and the activity of carbohydrate hydrolysing enzymes whereas, body homogenate used for total carbohydrates analysis.

2.4. Total soluble protein:

Colorimetric determination of total soluble protein in the supernatant of total homogenate was carried out as mentioned by Gornall *et al*. (1949).

2.5. Total carbohydrates:

Total carbohydrates were extracted from the body homogenate of drone's sample and prepared for assay according to Crompton and Birt (1967)

2.6. Carbohydrate hydrolysing enzymes:

The method used to determine the digestion of trehalose, starch and sucrose by trehalase, amylase and sucrase enzymes, respectively carried out the same method mentioned by Ishaaya and Swiriski (1976). The enzymatic activity was expressed as µg glucose released /g body weight/min.

3. Statistical analysis:

All collected data were statistically being analysed and the treatment means were compared using one-way test (ANOVA) by computer statistical COHORT SOFTWARE (2005). Probability value ($p \leq 0.05$) was considered significant.

Results and discussion

1. Body weights:

According to results, drones raised in colonies supplemented with vitamin C in spring weighed significantly more than drones in the control colonies (Table 1).

2. Sperms numbers:

High significant difference was observed between the spermatozoa numbers of drones reared in colonies receiving vitamin C and those reared in colonies fed no supplement ($P<0.05$). Mean sperms number per one seminal vesicle of 5.25×10^6 was recorded for control group compared to 7.75×10^6 for vitamin C group (Table 1).

Table (1): Body weight and sperms number in one seminal vesicle of *Apis mellifera* drones supplemented with vitamin C.

Parameters Experimental Groups	Body weight (g) (Means ± S.E.)	Sperms number ($\times10^6$) (Means ± S.E.)
Vitamin C group	0.2363 ±0.009[a]	7.75 ± 0.373 [a]
Control group	0.1819 ±0.005[b]	5.25 ± 0.167[b]
P	0.0000 ***	0.0004***
LSD 0.05	0.01639	0.865

3. Biochemical assays:

3.1. Total soluble protein:

Data in Table (2) revealed significant higher protein content (33.24± 1.934 mg/gm. b. wt.) in supernatant of freshly emerged drones of the group receiving vitamin C in comparison to the control (27.42± 0.234 mg/gm. b. wt.).

3.2. Total carbohydrates:

An elevation in total carbohydrates content was recorded with vitamin C group compared to control ones. Such increase was statistically significant recorded (3.878 ± 0.024 mg glucose/gm. b. wt.) and (3.577 ± 0.076 mg glucose/gm. b. wt.) for vitamin c and control groups, respectively (Table 2).

Table (2): Effect of vitamin C on total soluble proteins (mg/g.b.wt.) and total carbohydrates (mg glucose/g.b.wt.) of *Apis mellifera* drones.

Parameters Experimental Groups	**Total soluble proteins (mg/g.b.wt.)**	**Total carbohydrates (mg glucose/g.b.wt.)**
Vitamin C group	33.24^{a} ± 1.934	3.878 ± 0.024^{a}
Control group	27.42^{b} ± 0.234	3.577 ± 0.076^{b}
P	0.0404*	0.0193*
LSD 0.05	5.408	0.220

3.3. Carbohydrate hydrolyzing enzymes:

The enrichment of bee diet with vitamin C induced insignificant increase on the enzyme activity (Amylase, trehalase and sucrase) of newly emerged drones relative to the control group p > 0.05 (Figure 1).

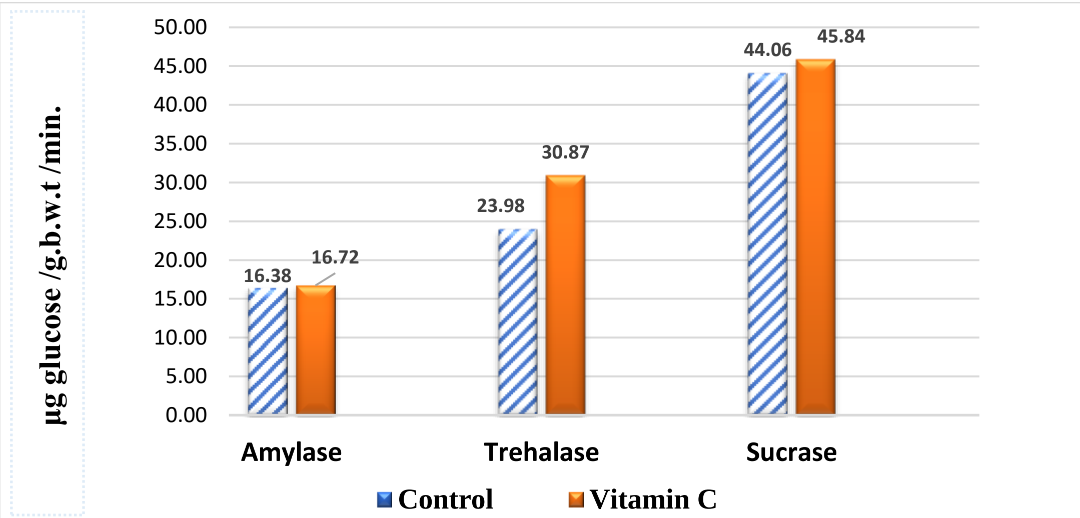

Figure (1): Effect of vitamin C on amylase, trehalase and sucrase specific activity (µg glucose /g. b. wt./min) of *Apis mellifera* drones.

Based on previous literatures, it seems that vitamin C has antioxidative characteristics where, Farjan *et al.* (2012) found that colonies supplemented with vitamin C have higher contents of antioxidative enzymes. Besides, its positive impact on brood rearing of honey bees and hypopharyngeal glands' development (Zahra and Talal, 2008). In addition, vitamin C was used safely in the feeding sugar solution in honey bee colonies where, Harz *et al*. (2010)stated oral application of vitamin C has no lethal effect on *A. mellifera* carnica worker bees. Also, it constitutes a natural, safe and highly available dietary supplement for wintering and spring worker bees (Farjan *et al*., 2012)**.** Previous studies focused on studying the effect of supplemented feeding on workers' health and only few studies have examined their effects on drones specially vitamin C. Subsequently, the current work focused on improving honey bee drones health and quality where, sufficient rearing of healthy drones is the prime step for successful queen mating. Results showed that colonies supplemented with vitamin **C** significantly increased the weight of newly emerged drones in comparison to the control colonies. In the same context, body weight is one of the main indicators for determining the physiological state of honey bee. Larger drones are considered to have a competitive advantage over smaller ones when fighting for access to the queen during their mating flight (Couvillon *et al.,* 2010). In addition, body size might affect other traits as semen production where, bigger drones produce more sperms (Rousseau and Giovenazzo, 2016). This confirmed by the result in the current study where, drones reared in colonies receiving vitamin C had significantly higher spermatozoa numbers relative drones in control colonies. Thus, indicating that drones with a greater body weight produce more semen. Getting protein profiles can provide worth information about health, development and reproduction of an organism where, it can affect growth rate, body size and fecundity (Fagan *et al*., 2002). In addition, the importance of body protein in honey bee, as storage body proteins affect wintering of honey bees and life span (Otis *et al*., 2004). Moreover, decrease in protein content is an important indicator for disturbance of physiological condition of an organism (Duay *et al*., 2003). In the current experiment, a positive significant effect of vitamin C on body protein was detected in freshly emerged drones in comparison to control drones. Vitamin C is needed for protein synthesis as it is entering into the fat body, haemolymph and intestinal cells initiates the protein elevations depending upon the insect tissue (Sathesh and Kerenhap, 2016). Previous studies of worker bees showed an increase in the brood area, colony population, body weight and protein in colonies supplemented with vitamin C to spring nutrition (Andi and Ahmadi , 2014).

Carbohydrates are the main source of energy for bees where, the diet of honey bee is very rich in carbohydrates (Brodschneider and Crailsheim, 2010). Saccharides are the major source of energy needed for pupation, and monosaccharides are involved in the synthesis of new carbohydrates such as chitin (Draczynski, 2008). And according to Kunieda *et al.* (2006) honey bee genome contains 174 genes responsible for carbohydrate metabolism, but only 28 genes responsible for lipid metabolism. In this study, vitamin C induced a significant increase of carbohydrates content of newly emerged drones. This finding is consistent with the results reported by Farjan *et al.* (2015) who recorded an increase in carbohydrates content in newly emerged workers supplemented with vitamin C where they attributed such increase to the reduction in the activity levels of α-glycosidases (Enzymes decomposing carbohydrates) in the last pupal stage which generated suitable conditions for the accumulation of carbohydrates in emerging honey bees. This also confirmed by Żółtowska *et al.* (2012) the activity of α-glycosidases in developing drone brood of *A. mellifera* carnica. was negatively correlated with sugar levels. In the same context, insects become active immediately after hatching and reserved carbohydrates are the main source of energy till newly emerged bees begin to feed (Winston, 1987).

Generally, honeybee get carbohydrates form nectar or honey. Enzymes as sucrase and glycosidases break nectar sugars into glucose and fructose where, they used directly or transformed into fat body and glycogen. Trehalase is the enzyme that catalyzes the hydrolysis of disaccharide trehalose into two glucose molecules for internal supply for chitin synthesis, muscular activity during flight and other metabolic process (Rajitha and Savithri, 2014). Bee also gain carbohydrates from pollen in small amount and hydrolyses of starch contained in pollen into glucose depend mainly on amylase enzyme. Therefore, the energetic metabolism is based generally on carbohydrates. Studies of carbohydrate hydrolysing enzymes revealed that Ascorbic acid induced a minor increase in trehalase, amylase and sucrase activities in newly emerged drones but such increase was insignificant. The above observations indicate that supplement vitamin C can promote carbohydrate degradation in the enzymatic system of bees (Ohashi *et al*., 1999).

The current study demonstrates that drones emerged in honey bee colonies feeding in spring with sucrose syrup supplemented with vitamin C were characterized by significantly higher body weights, sperm number, protein and carbohydrates contents which indicates that they were better health than control group which in turn plays an important role in colony health.

The present results suggest the importance of adding an antioxidant agent as vitamin C to spring nutrition for drone rearing in honey bee colonies that can improve their quality and reproductive health which in turn essential for colony build up. The diet supplemented with vitamin C positively enhanced drones body weight, spermatozoa number, total soluble protein and carbohydrate content in addition to its minor effect on the activity of carbohydrate hydrolysis enzymes. Consequently, vitamin C can be used as a natural, safe, and relatively cheap diet supplement that can enhance resistance to stress factors including exposure to elevated temperature, diseases and miticides.

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