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Impact of Soft Drink Feeding on Honeybee Mouth Parts Formation and the Effect of Supposed Malformation on the Quality of Gathering Nectar and Pollination

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Abstract: The morphological characters of honeybees were measured using a Scan Photo technique (SPT) that involves the use of a stereo binocular microscope and Photoshop program combined together. The body parts of a honeybee worker were scanned as images after they have been dissected. Photoshop program was used to view the images and their characters were measured using the ruler of the software program. While measuring the chosen morphometric characters, a significant difference was observed when the SPT and using a binocular microscope were compared. The measurements of 7 morphological characters of honeybee workers from nectar-feeding colonies were compared with those of Pepsi cola feeding colonies.

There are more than 24 different subspecies of honeybees (*Apis mellifera*) classified based on their morphological characteristics. As a result, morphological characteristics are usually considered when classifying honeybees. Many authors for various reasons have used body morphological characteristics as well as sets of wings to classify subspecies of honeybees. Such characteristics have been defined overtime and sourced from diverse studies. Of all body morphological characteristics studied so far, wing venation characteristics have been given more attention. Up to now in Saudi Arabia, there are no specific review articles that focus mainly on *Apis mellifera jemenitica* body morphological characteristics. Consequently, information gathered from sampling and measurement methods, and factors affecting these characteristics especially mouthparts is to recognize the possibility of finding any changes in configuration as a result of feeding on soft drink. The morphology and functional anatomy of the mouthparts are examined by dissection, light microscopy, and supplemented by feeding type observations. This manuscript focuses on the differentiation of the proboscis which is formed by the glossa, in context with feeding from natural nectar and soft drink. In the literature, morphological innovations have been described for insects, particularly for bees. These innovations are critical for the production of differentiation in proboscis such as proboscis hair formation and modification. The present study showed that the proboscis of honeybees feeding on Pepsi is wider, thicker and very hairy compared to the natural feeding honeybee's proboscis.

The morphological structures of the head, proboscis, hind leg, and wings were measured for the two groups of bees, the first naturally nourished with nectar, and the second feeding on the drink Pepsi Cola. The results showed significant differences in nectar and Pepsi feeding in different length measurements of head (18.37 ± 0.08 , 17.71 ± 0.07), proboscis length (24.88 ± 0.25 , 26.15 ± 0.01), proboscis width (0.58 ± 0.04 , 1.51 ± 0.05), fore wing length (13.75 ± 0.10 , 12.95 ± 0.23), fore wing width (41.99 ± 0.03 , 40.84 ± 0.23), hind wing length (7.87 ± 0.09 , 8.00 ± 0.06), hind wing width (29.27 ± 0.25 , 29.18 ± 0.04), femur length (12.05 ± 0.11 , 11.35 ± 0.02), tibia length (14.27 ± 0.06 , 13.62 ± 0.04), basitarsi length (8.57 ± 0.15 , 8.43 ± 0.06), and the basitarsus width of the third leg wrist (4.44 ± 0.03 , 4.69 ± 0.05), respectively.

Proboscis elongation in context with nectar and pollen feeding has been discussed. Morphological features were studied using morphometrical methods. Essential conclusions and recommendations for researchers are presented from the results obtained in this study.

Keywords: *Apis mellifera jemenitica*, Morphometry, Morphology, Body characteristics, Honeybees, Sensory, Chemoreceptor, Nectar-feeding, Mouthparts, Proboscis, Glossa, Soft drink, Tibia, Basitarsus

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Introduction

Apart from being widespread in Europe, Africa as well as parts of Asia, *Apis mellifera* L. has a wide diversity of subspecies that can be classified using morphometric tools (Ruttner *et al.*, 1978). Biogeographically, Ruttner *et al.* (1978) reported that *Apis mellifera* can be divided into three different branches; (A) one of South and Central Africa, (B) one of Western Europe and North Africa, and (C) one of the North Mediterranean. Afterwards, another branch (O) was also added by Ruttner (1988). This branch included the Near and Middle Eastern subspecies. All the branches mentioned above are found in the Middle East and Mediterranean and are represented by 16 subspecies that have been identified.

The honeybee, *Apis mellifera* L., is widespread globally with subspecies that are widely diversified and can be classified using morphometric tools (Ruttner *et al.*, 1978). Numerous studies have been conducted on honeybees utilizing morphological characteristics (Garnery *et al.*, 2004; Abou-Shaara *et al.*, 2012). Wing venation characteristics, color and length measurements are the three major groups of morphological characteristics. Previously, Abou-Shaara (2014) reviewed wing venation characteristics intensively. Recommendations about sampling, measuring methods and limitations of body morphological characteristics are presented in the current

study by reviewing studies conducted using morphological characteristics (Abou-Shaara *et al.*, 2013) to discover the possibility of changes in morphological structures in external appendages especially mouthparts due to feeding on soft drinks.

Liquid-feeding behavior plays an important role in bringing water, sugar, amino acids, organic acids, proteins, fats, vitamins, minerals and other minor components to organisms (Corbet, 2000; Bertazzini *et al.*, 2010). Some studies have investigated the drinking patterns of animals that use tongues or tongue-like devices to load fluid (Kingsolver and Daniel, 1983; Winter and von Helversen, 2003; Krenn, 2010; Karolyi *et al.*, 2014). Most tongues and tongue-like devices are considered just as rigid bodies with permanent surfaces during the drinking process, such as the tongues of butterflies (Lee *et al.*, 2014). The butterfly proboscis is a complex hydrophobic-hydrophilic dichotomous and hierarchical system having nanopores to provide strong capillarity and micropores to facilitate fluid transport, which can form liquid bridges even when the sucking pump is not activated (Abou-Shaara, 2014). Nectar, an aqueous solution of sugar and some chemical ingredients, provides important nutrient sources for honeybees (Abou-Shaara, 2014). The nectar drinking strategy has been studied extensively and the

erection of glossal hairs has been revealed in several researches (Cook, 1880; Snodgrass, 1956; Dade, 2009). The rhythmical erection pattern of honeybees' glossal hairs in nectar-feeding has been reported, but the role of the deformable tongue structure in nectar-feeding has not been investigated yet (Wu *et al.*, 2015). In this work, we focused on the deformation of the proboscis formation of honeybees, especially the morphing of the hair pattern, and its supposed influence on the feeding type and efficiency.

Feeding in honeybees involves the process of nectar coating and nectar uploading (Kim *et al.*, 2011). When the glossa inserts into nectar, the glossal surface of a honeybee is coated by nectar. Analogously to the model established for the uptake of liquid from wet surfaces by a brush-tipped proboscis, the nectar coating of honeybee's glossal surface in a viscous fluid may be mainly caused by wetting and capillary (Lee *et al.*, 2014; Chen *et al.*, 2015). For the nectar uploading process, whether all of the nectar between the proboscis hairs can be in the viscous nectar is really important (Abou-Shaara *et al.*, 2013). The size, space between hairs, and the relative speed between hairs and the fluid determine how fluid flows through the proboscis (Cheer and Koehl, 1987; Loudon *et al.*, 1994; Koehl, 2001). The appendage bearing arrays of hair-like structures functions like a sieve or a paddle judging by the leakiness of a row of hairs on a structure (Koehl, 2001). The leakiness of pairs of hairs is related to the Reynolds number $Re = \rho va / \mu$ (the ratio of inertial to viscous forces) and hair spacing $G/2a$ (G is defined as the width of the gap) (Abou-Shaara *et al.*, 2013). The 'leakiness' of hair gaps can be used to evaluate the uploaded volume in a nectar-feeding cycle. As a preliminary, the proboscis

was modeled as a simple slender cylinder and the fluid was entrained by the outer surface of the proboscis (Kim *et al.*, 2011). Another model was presented in which the proboscis was considered to be a hairy cylinder under the condition of viscous dipping (Yang *et al.*, 2014). The model developed by Yang *et al.* (2014) suggests that the hairs contribute to the nectar uploading but the erection angles in their model remain at 90 deg.

Honeybees and bumble-bees have similar kinds of mouthparts which are the chewing and lapping type. The mouthparts of honeybees are combined so that they can both chew and suck, and this is made possible due to the presence of mandibles and a proboscis.

Mandibles: The mandibles are suspended from the head of the bee as a pair of jaws. They are used by the insect to chew wood and pollen and to work wax when building comb. They are also used for grasping activities. By opening and closing, these paired structures help to get the work done.

Proboscis: The proboscis is formed temporarily from parts of the maxillae and the labium of the honeybee. As a result, it is not a permanent functional organ, it is a distinctive tube used for drawing up liquids such as sweet juices, nectar, water, etc. It is released by the insect when needed for use. When not needed, it is withdrawn and folded back beneath the head.

Labellum: The labellum is hairy, flexible, and spoon-shaped. It is formed as an extension of the glossae. It is used to lick nectar and its palps consist of four segments.

Maxillolabial Structures: The elongated glossa of labium, a pair of galea of maxillae, and two labial palps form the lapping tongue unit.

When in use, the mouthparts are usually unfolded and spread apart. However, they are folded behind the head and held together when not in use.

The glossa is cylindrical in shape and segmented. The diameter of the glossa is 185.0 ± 1.5 and 96.6 ± 0.3 micrometers at the base and in the middle part respectively. Each segment of the glossa is about 23 micrometers in length. Moreover, there are 16-20 hairs on the apical part of each of the segments. Stiff and short hairs (32-63 micrometers long) are found on the basal part of the glossa, whereas those on the middle, as well as the apical parts are longer (171.9 ± 0.3 micrometers long) (Yang *et al.*, 2014). The spoon-like flabellum is found at the apex of glossa. The foreleg tarsi are used to clean the mouthparts (Masuko, 1986; Snodgrass, 2004).

Bees ingest liquid food through the "viscous dipping" mechanism (Abou-Shaara and Al-Ghamdi, 2013). This process starts with the bee extending its tongue and immersing it into nectar. Then, the glossa hairs erect asynchronously (Galic, 1971; Zhao *et al.*, 2015) and the nectar is trapped (Simpson and Riedel, 1964). The ability of a bee to collect nectar can be increased by the erected hairs (Whitehead and Larsen, 1976). Thereafter, the glossa is retracted to a tube formed from the galeae and labial palpi after being coated with nectar. The nectar is then sucked into the pharynx. During drinking, ridges on the inner wall of the galea help to reduce friction (Li *et al.*, 2015, 2016). 1.8 microlitres of diluted nectar can be imbibed by honey bees per second (Roubik and Buchmann, 1984). In the case of higher concentrations of nectar, this rate is lower (Roubik and Buchmann, 1984). The optimal concentration of sugar in nectar, which is

determined from energy content and intake speed has been suggested to be about 52% for bees (Kim *et al.*, 2011). However, sensory organs found on the mouthparts help to facilitate the food intake (Galic, 1971; Whitehead and Larsen, 1976; Masuko, 1986, 1989).

Chewing and Lapping mouthparts: The mouthparts of honeybees are combined and can be used for both chewing and sucking. This is made possible due to the presence of mandibles and a proboscis.

The mandibles are used to chew wood, work wax, clean other bees, and for biting other workers or pests. The main functions of the proboscis include sucking in liquids like nectar, water and honey inside the hive, for exchanging food with other bees, and also for removing water from nectar. By putting a droplet of nectar between the proboscis and the rest of the mouthparts, honeybee workers can increase the surface area, and moving the proboscis back and forth slowly.

Process of feeding: The entire length of the galeae fits against the labial palps forming a roof over the glossae and these create a temporary discharge channel for saliva. When the glossae (tongue) is trusted into flower, it gets smeared with nectar. Afterwards, it is retracted between the galeae and labial palps. Nectar is deposited in the cavity formed by the paraglossae after being squeezed by the galeae. The pharyngeal pump is used to draw accumulated nectar into the esophagus.

Fluid-feeding insects have sucking mouthparts that are unique. Over the past 350 million years, there have been diversifications by fluid feeders to utilize different food sources including nectar, xylem, phloem, etc. The devices used for feeding by insects have

been a common subject of evolutionary biology after Darwin predicted that a sphinx moth with very long proboscis feeds on nectar of the orchid *Angraecum sesquipedale* with an equally long nectar spur. Within the context of the morphology of devices, properties, and functional performance evolution and diversification of insects in relation to their feeding organs are among the most attractive and demanding areas of study. When considering the organism as a hierarchical system having various structural levels and activity, identification of the mechanisms in place for the division of labor and relationship between the structural units, and their adaptability to environmental changes is required. This idea has been successfully evaluated for feeding devices of vertebrates but has not been given attention by those investigating feeding devices of insects.

On the basis of the proboscis, beginning with the works of Bennet-Clark Tawfik, Kingsolver and Daniel, the performance of insects that feed on fluids has been evaluated (Kornev *et al.*, 2017). The sucking pump was largely set aside in insect feeding organs according to structural hierarchy. Kingsolver and Daniel (1983) hypothesized that the muscular energy of the insect is spent on combating viscous friction of fluid moving through the proboscis. As a result, they were able to decouple the pump from the proboscis. However, physiological features of the pump cannot guarantee that the viscous dissipation of moving fluid in the pump is always negligible. X-ray phase-contrast imaging experiments and neurophysiological analysis of the lepidopteran sucking pump revealed complex liquid flow through the pump, adding a degree of doubt to this assumption.

Estimates of pressure generated by the sucking pump of Lepidoptera with long proboscises show that to defeat enormously high viscous dissipation during unidirectional flow of liquid through the food canal requires the insect to invoke other physiological and behavioral mechanisms (Kornev *et al.*, 2017).

Pollination is the most important job performed by bees apart from making honey. One out of every three bites of food we eat depends on animal pollinators. And all kinds of wildlife depend on pollinated plants for food.

Many plants and trees are pollinated by honeybees: apples, apricots, avocados, cherries, strawberries, raspberries, grapes, blueberries, peaches, pears, pumpkins, squash, watermelons, plums, cranberries, cantaloupes, cucumbers, soybeans, and almonds. In fact, almond crops use 1/2 of the honeybees in the country.

Bruyne and Baker (2008) reported that animals have specialized sense organs that detect stimuli from the external environment, including visual, acoustic, tactile and chemical signals. Chemical signals are detected by one of two major chemosensory mechanisms: olfaction and taste. Organisms can recognize volatile cues through the olfactory system that confer the capacity to detect food, predators, and mates (d'Ettorre and Moore, 2008). The discrimination of soluble stimulants that evoke feeding behaviors is usually permitted by the sense of taste, and this can also begin sexual and reproductive responses that are innate in nature. In insects, the early chemoreception steps, that is, those involving primary contact with chemical signals and the activation of signaling pathways occur in porous chemosensory hairs (the sensilla)

(Hildebrand and Shepherd, 1997; Sheppard and Meixner, 2003). According to Robertson *et al.* (2010), these main events include: (i) the uptake of signal molecules from the external environment; (ii) transport (diffusion) through the sensory hair and (iii) interaction with the chemoreceptor, which in turn activates the cascade of events leading to spike activity in sensory neurons. The most important proteins underlying these processes are encoded in moderately sized multigene families. These families encode the odorant-binding proteins (OBPs) and chemosensory proteins (CSPs) involved in peripheral olfactory processing and a chemoreceptor superfamily formed by the olfactory receptors (ORs) and gustatory receptors (GRs). Some authors review several recent comparative evolutionary analyses of the chemosensory multigene families from fully sequenced insect genomes, with a special emphasis on the 12 newly available *Drosophila* genomes. They address fundamental questions concerning the evolutionary dynamics of these gene families, such as the origin and fate of the gene repertoire, the impact of natural selection and the species-specific features of chemoreceptor evolution associated with ecological adaptation (Willmer and Finlayso, 2014).

One of the most intensively studied structures among animals is insect mouthparts. Although reasons for the extensive literature addressing insect mouthpart structures are as varied as insects themselves, three major aspects are main. First, because insects are ubiquitous residents of virtually all terrestrial and freshwater habitats and have elevated taxonomic diversity and ecomorphological disparity, their mouthparts represent a broad spectrum

of feeding modes that are ideal for comparative studies. Second, insect mouthparts represent one of the most externally complex, yet structurally integrated and homologous morphologies known, such that detailed studies can be made of element and multielement innovations in the conversion of one mouthpart type to another or in the convergence toward a mouthpart type among unrelated lineages (Labandeira, 1997). Third, considerable effort in understanding economically related consequences of insect feeding, particularly in agricultural fields such as pest control, crop pollination, and the transmission of insect-vectored diseases, historically has required a fundamental understanding of mouthpart structure and function. Mouth part structure represented in the fossil were recorded even in deposits with well-preserved, soft-part anatomy, detailed preservation of insect mouthparts is uncommon. However, during the past 15 years, particularly Mesozoic deposits (Labandeira, 1997) have yielded sufficient mouthpart details that direct evidence now exists for the occurrence of major mouthpart types in the pre-Cenozoic fossil record. Most of the record of mouthpart structures is driven by exceptionally well-preserved deposits that are spatiotemporally scarce but reveal details of feeding mechanisms for many species (Labandeira, 1997).

d'Ettorre *et al.* (2017) reported that chemical communication is the primordial and possibly most efficient way of transmitting messages between living units (Abdellatif *et al.*, 1977). It has reached its apex in the “superorganisms” (Wheeler, 1911), for example in colonies of eusocial insects, such as honey bees (Sandoz *et al.*, 2007). Colony

survival and reproductive success rely on the chemical communication channel to maintain an advanced social organization characterized by high levels of cooperation and low levels of conflicts (Abdellatif *et al.*, 1977; Boomsma, 2007; Strassmann and Queller, 2010). Eusocial bees and ants are model organisms for understanding social chemical communication; hence, recent research has focused on the identification of chemoreceptors (Pask *et al.*, 2017). A study by Slone *et al.* (Slone *et al.*, 2017) uses the ant *Harpegnathos saltator* to investigate the molecular mechanisms underlying chemoreception of socially relevant semiochemicals. Chemoreceptors can be differentiated into olfactory receptors (ORs), gustatory receptors, and ionotropic receptors, as well as several other receptor classes (Joseph and Carlson, 2015).

ORs are transmembrane proteins representing the interface between animals and their olfactory environment for detecting food sources or, in a social context, nestmates or sexual partners. They are expressed in olfactory receptor neurons (ORNs) in the insect antenna; after binding odors, information is transferred to the brain, eventually inducing behavioral responses. It has long been believed that perception of “general” odors (food and flower scent) is separated from that of “social” odors (e.g., pheromones) and that these are detected by different Ors (Joseph and Carlson, 2015).

Materials and Methods

Experimental study on honeybee colonies:

The experiment was carried out in a farm far from populated areas and noise, ten modern Langstroth hives were used. Each hives had 6 frames with waxy bases, 1000 g of bees and

queens (less than a 4 m width, to 6 m long and 3 m high). Five groups of bees were inserted inside the isolator and were placed on the same platform with an (A) symbol. They were fed with sugary solution (Pepsi) and pollen grains. On the other hand, five colonies of bees were kept as a control group with similar conditions on the same platform and symbolized by letter (B), left to feed naturally in the fields, taking into consideration while last harvesting honey before the start of experiments that the beekeeper leaves a quantity of honey in the cell as a winter feed for bees.

Procedure of nourishment with Pepsi:

In this study, two groups of feeding were tested, one in natural field, the second with Pepsi (soft drink) under laboratory conditions, Feeding Pepsi groups were isolated in specific platform isolator to ensure that bees could not escape. 1.5 liters of Pepsi drink per day, poured in a plastic container for 6 weeks with a piece of floating cork on the feeding fluid to allow the bees to stand during the licking process. Feeding has been done in three months. Adult stage of wild bee workers was chosen to conduct the current study (Edrees *et al.*, 2019).

Collection of bee samples:

During morphological analysis, it is expected that at least 15 honeybee workers should be collected from each colony (Ruttner *et al.*, 2000; Sheppard and Meixner, 2003; Guler, 2010) and also for morphological studies, a minimum of eight colonies in a district can be considered sufficient (Abou-Shaara *et al.*, 2012). Contrarily, Miguel *et al.* (2011) reported using only one honeybee worker per colony for geometric morphometrics but usually, to obtain reliable results, more is

required. Collection of samples can be done in a number of ways (i) directly from brood comb according to Padilla *et al.* (1992), (ii) shaking bees into a jar, (iii) collecting forager bees and (iv) taking one-day-old bees by placing sealed brood combs into incubators. After collection, bees can be preserved in 95% ethanol before dissection (Arias *et al.*, 2006) or in 70% ethanol (Adl *et al.*, 2007) or keeping them in a deep freezer until they are dead and then dissected (Abou-Shaara *et al.*, 2012). Temporary preparation of the samples can also be used (Miladenovic *et al.*, 2011). It is important to note that factors that can impact on morphological characteristics must be taken into account. Comb cell size has an impact on morphological characteristics (Ruttner *et al.*, 2000; Gencer and Firati, 2005; McMullan and Brown, 2006), where workers that emerge from large wax cells have larger morphological characteristics. As a result, the comparisons between different morphological characteristics data could be affected by sample size and time of taking the samples. Generally, during morphological analysis, it is very crucial to collect samples at the same time for all replicates that are being studied and also, under the same feeding condition, samples should be taken from new combs.

Methods of measuring body:

After their collection, Abou-Shaara and Al-Ghamdi (Abou-Shaara and Al-Ghamdi, 2013), Abou-Shaara *et al.* (2013) have described that samples can be mounted on sticky pieces to facilitate the measurements of characteristics. In addition, other mounting methods for body parts such as double glass slides can be used (Abou-Shaara *et al.*, 2012). Several methods have been applied to take body measurements; (i) stereomicroscope with an

ocular micrometer (Ruttner *et al.*, 1978; Mattu and Verma, 1983; Edriss *et al.*, 2002; Stanimirovic *et al.*, 2008; Gencer and Firati, 2005; Mostajeran *et al.*, 2006; Adl *et al.*, 2007; Marghitas *et al.*, 2008), (ii) Photomicroscope (Morris-Olson, 2002), (iii) Projecting mounted slides onto a TV screen (Kandemir *et al.*, 2000), (iv) Using computer-based methods mainly for forewing and some body characteristics (Rinaldi *et al.*, 1970; Ruttner, 1988; Meixner, 1992; Shanbhag *et al.*, 2000; Meixner *et al.*, 2007; Shaibi *et al.*, 2009; Miladenovic *et al.*, 2011; Abou-Shaara and Al-Ghamdi, 2012; Abou-Shaara *et al.*, 2012), and (v) Special programs using image analysis systems and the IMAGO program (Padilla *et al.*, 1992). Also, software programs like Image-Pro Plus version 3.0.1 for Windows 3.1 and Media Cybernetics were used (Andere *et al.*, 2008). A simple method for measuring body and wing morphological characteristics was presented by Abou-Shaara *et al.* (2011) where they used photoshop program; this method was named the Scan Photo method while Miladenovic *et al.* (2011) used AutoCAD program to take the measurements. In general, all the previous methods can be used but it is preferable to use a computer-based method to save time where microscopic methods were found to be time-consuming (Szymula *et al.*, 2010; Abou-Shaara *et al.*, 2013). Furthermore, measurements can be taken using any suitable software in combination with a camera or scanner.

The following measurements were taken using a micrometer with the help of a stereo binocular microscope:

1. Length and width of proboscis.
2. The hind and fore wings. Length and width

3. Hind legs, femur, tibia, and basitarsus measurements
4. Head width

Body characteristics:

Many authors have measured different body characteristics of honeybees (Meixner *et al.*, 2007; Shaibi *et al.*, 2009; Miladenovic *et al.*, 2011; Abou-Shaara and Al-Ghamdi, 2012; Abou-Shaara *et al.*, 2012). Body characteristics measurements are usually carried out according to the methods of Ruttner *et al.* (1978) and Ruttner (1988), and these characteristics are divided into head, thorax and abdomen characteristics. (i) Head characteristics include; head capsule width (HCW) and length (HCL), antenna length (AL) and number of segments (ANS), compound eyes length (CEL) and width (CEW), and tongue length (TonL). The mandible length (ML) has been studied by several authors apart from other characteristics. (ii) Thorax characteristics; fore wing length (FWL) and width (FWW), hind wing length (HWL) and width (HWW), number of hooks (NH), thorax width (TW), femur length (FL), tibia length (TL), basitarsus length (BL) and width (BW), and pollen basket size (PBS), brush hair rows number (HN). (iii) Abdomen characteristics; lengths of tergite 3 (T3) and 4 (T4), body size (T3+T4), length of hairs on tergite 5 (HLT5), pigmentation of tergite 2-4, length of sternite 3 (LS3), wax mirror length (WML) and transversal (WMT) and sting shaft length (StL). Generally, except for the number of hooks, measurements should be taken in units of millimeters and as the maximum distance. In addition, some indexes such as the forewing index have been revealed from these characteristics (Abou-Shaara *et al.*, 2013). In this study, the differences of head, wings,

proboscis and hind leg between the two groups have been measured.

Factors affecting body measurement variation:

Environmental factors have been reported to have a major impact on morphological characteristics of honeybee workers (Eischen *et al.*, 1982; Marghitas *et al.*, 2008; Stanimirovic *et al.*, 2008). Also, the proboscis of honeybee workers in the mountainous regions of Transylvania for example, was found to be 6.21 mm longer than that in lower regions (5.99 mm). High levels of hybridization within populations may be induced as a result of the importation of honeybee subspecies into different areas (Garnery *et al.*, 1998; Rortais *et al.*, 2004; Alqarni *et al.*, 2011), thereby producing subspecies admixtures (Arias *et al.*, 2006). In addition, migratory beekeeping may play a vital role in forming differences (Marghitas *et al.*, 2008). Low stability over time has been observed in morphological characteristics for uncontrolled honey bee populations (Abou-Shaara *et al.*, 2012). As a result, when characterizing uncontrolled populations, it is highly recommended to take the characteristics mean for two successive years. Abou-Shaara *et al.* (2013) reviewed other factors that may impact on wing and body morphological characteristics.

Results

There was significant increase in the pepsi group versus nectar in Proboscis length ($P < 0.007$), Proboscis width ($P < 0.0001$) (Figs. 2, 3, 6, 7) and Basitarsus width ($P < 0.003$). While, head width (Figs. 4, 5), fore wing length (Figs. 8, 9), fore wing width (Figs. 10, 11), femur length, tibia length (Figs. 12, 13) were significantly shorter in pepsi group compared to nectar group ($P < 0.0001$, $P < 0.022$,

P <0.007, P <0.003, P <0.0001, respectively) (Table 1, Fig. 1).

Importance of morphological measurements:

Measurements of morphological characteristics are carried out for various reasons. It can be used to characterize honeybee races and individuals (Ruttner, 1988; Meixner *et al.*, 2007), determine the degree of hybridization with foreign races (Bienefeld *et al.*, 1996; Radloff *et al.*, 2003) and also for the discrimination between honeybee subspecies (Tofilski, 2004; Abou-Shaara and Al-Ghamdi, 2012). In order to investigate the impacts of imported queens on populations of honeybees (Guler, 2010) and also to check for populations purity (Miladenovic *et al.*, 2011), morphological characteristics were measured. Body characteristics such as wing length, wing width, and tongue length have been used to differentiate between subspecies of honeybee (Bucu *et al.*, 1987; Reindeer *et al.*, 1993; Crewe *et al.*, 1994; Ftayeh *et al.*, 1994; Diniz-Filho, 1995).

In some studies (Morimoto, 1968; Souza *et al.*, 2002; Marghitas *et al.*, 2008), tongue length was reported as an indicator of geographical variation. Between *A.m.mellifera*, *A.m.carnica*, and *A.m.caucasica*, among other characteristics, proboscis length was found to be the most differentiated (Szymula *et al.*, 2010). Correlations exist between body measurements and honey yield. In Carniolan honeybees, Kolmes and Sam (1991) found correlations between honey production and overall size, corbicular area and wing measurements. Colony productivity may be predicted indirectly by body characteristics where it has been known that honeybees with

bigger legs and wings have higher flight power and could be more useful for broodrearing and colony population (Mostajeran *et al.*, 2006). Honey production and corbicular area have been reported to have a positive correlation (Milne and Pries, 1984). Honey production has been reported to have significant and positive correlations with both the fore and hind wing areas by Szabo and Lefovich (1988). Honey production was reported by Mostajeran *et al.* (2002) to be related to tongue length, fore wing length and width, hind wing length, leg length, femur length, tibia length, and metatarsus width. A correlation between body size and colony productivity was found by Waddington (1989). Edriss *et al.* (2002) indicated that through the selection of the forewing width, honey production can be improved. Evidence has shown that body morphological characteristics are very vital and correlated with colony productive characteristics. However, it must be noted that these relations should not be taken as general rules but are only attributed to some specific conditions.

The understanding of honeybee anatomy is essential for much of the work described in the other reports. Similarly, basic dissection techniques are also fundamental to many facets of the study of honeybees. Man has kept honeybees for many thousands of years, and they have long held a fascination for those keen on understanding natural history. The development of our understanding of the anatomy of honeybees has been outlined by Crane (1999). In the modern era, two textbooks have become standard, those by Snodgrass (1956, 2004), and Dade (1962, 2009), and these are still readily available. For the purposes of this paper, we have therefore

Table 1: Comparison of measured parameters between nectar and pepsi groups

Groups Parameters	Nectar (n=5)	Pepsi (n=5)	Significance
Proboscis length	24.88±0.25	26.15±0.01	0.007**
Proboscis width	0.58±0.04	1.51±0.05	0.0001***
Head width	18.37±0.08	17.71±0.07	0.0001***
Fore wing length	13.75±0.10	12.95±0.23	0.022*
Fore wing width	41.99±0.03	40.84±0.23	0.007**
Hind wing length	7.87±0.09	8.00±0.06	0.265
Hind wing width	29.27±0.25	29.18±0.04	0.752
Femur length	12.05±0.11	11.35±0.02	0.003**
Tibia length	14.27±0.06	13.62±0.04	0.0001***
Basetarsus length	8.57±0.15	8.43±0.06	0.410
Basetarsus width	4.44±0.03	4.69±0.05	0.003**

Data expressed as mean ± standard error. Significance between nectar and pepsi groups was made using unpaired student “t” test.

tried to give essential information only, and we suggest that the reader seeking further information consults these.

Features used in the identification of bees:

Like all other insects, bees have three body regions, head, thorax and abdomen. A description of the external morphological features used in the keys is given below.

Head: The head bears a pair of antennae, two compound eyes, three simple eyes and three pairs of mouthparts formed into a proboscis or “tongue”. The difference in the number of

antennal segments is useful in sex determination. The apical segments that form the flagellum of the antenna are 10-segmented in the female and 11-segmented in the male. The relative lengths of these segments, especially the basal segments, is of considerable value as a key character, this point gives a comparative study of the antenna generated by the two types of nutrition, an important value for future study.

A large number of characters used in the higher classification of bees are based on features of the proboscis. The proboscis is

Fig. 1: Comparison of measured parameters between nectar and pepsi groups. Data expressed as mean $\pm$ standard error. Significance between nectar and pepsi groups was made using unpaired student “t” test.

Fig. 2 : Width of proboscis (nectar feeding group) light microscopic capture /0.673 μ m

Fig. 3: Width of proboscis (Pepsi cola feeding group) light microscopic capture /1.593 μ m

Fig. 4: Width of head (nectar feeding group)/18.656 µm

Fig. 5: Width of head (Pepsi cola feeding group)/17.966 µm

Fig. 6: Length of proboscis (nectar feeding group) light microscopic capture /25.522 µm

Fig. 7: Length of proboscis (Pepsi cola feeding group) light microscopic capture /26.111 µm

Fig. 8: Fore wing (nectar feeding group) binocular stereomicroscopic capture/ length 42.096 µm, width 13.440 µm

Fig. 9: Fore wing (Pepsi cola feeding group) binocular stereomicroscopic capture /length 41.415 µm, width 12.366 µm

Fig. 10: Hind wing (nectar feeding group) binocular stereomicroscopic capture /length 30.161 μm , width 7.537 μm

Fig. 11: Hind wing (Pepsi cola feeding group) binocular stereomicroscopic capture /length 30.287 μm , width 8.437 μm

Fig. 12: Foreleg (nectar feeding group) binocular stereomicroscopic capture /femur 12.433 μm , Tibia length 14.473 μm , Basitarsus length 8.049 μm , Basitarsus width 4.480 μm

Fig. 13: Foreleg (Pepsi cola feeding group) binocular stereomicroscopic capture /femur 11.411 μm , Tibia length 13.477 μm , Basitarsus length 8.632 μm , Basitarsus width 4.851 μm

formed from the maxillae and the labium. Each maxilla consists of the cardo, stipes, maxillary palp and galea. The labium consists of the prementum, glossa, paraglossa, and the labial palps. In structurally more specialized bees, the glossa becomes acute and more or less elongated, the lacinia becomes reduced in size, the number of segments in the maxillary palps is reduced and various other modifications may be found. The proboscis is extended to probe flowers for nectar. Bees are grouped into either short-tongued or long-tongued species based on the modifications in the galea, labial palps, and glossa. The labrum (upper lip) and mandibles are relatively

simple generalized structures, not markedly different from most insects. However, they show a wide range in form and in relative size in different taxa.

Wings: The wings of bees exhibit a considerable range of variation between genera and the other higher taxa but are commonly of little help in the separation of species within a genus.

Legs: The three pairs of legs bear five segments; coxa, trochanter, femur, tibia, and tarsus. The legs in most female bees differ from those of the other Hymenoptera chiefly in the broadening of the tibiae and basitarsi of

the hindleg and to some degree, the basitarsi of the middle legs. Along with the expansion of these parts, there has been a development of pollen-collecting hairs, which are borne on hind legs in all groups except in the family Megachilidae. Because male bees do not carry pollen, these leg features are lacking, with all segments usually quite slender. In parasitic bees, both sexes are wasp-like in appearance, as the females also have lost their pollen carrying structures. The tibial spurs are the movable inferior apical spurs on the tibiae; there is only one spur on the front tibia, one on the middle tibia and, in nearly all bees, two on the hind tibia. The inner hind tibial spur is especially important taxonomically. This spur usually has two toothed margins; the inner one that is commonly elaborated in various ways and an outer margin. The spurs are described as pectinate when its inner margin is produced into several long, coarse, often blunt projections, even though the number of such projections is in some cases reduced to only one or two. These spurs provide a hold against their nest burrow walls. Bees pivot on these spurs, somersaulting to change the direction of their head. The tibial spines are immovable, sharp superior apical projections, usually small in size, often blunt or minute, found in some bees. There are none, 1 or 2, or rarely 3 spines per tibia.

The Abitibian plate is on the upper or outer side of the base of the hind tibia of many bees. It is best developed in females and presumably is important for support as bees move up or down with legs bent within their burrows in the soil. On the inner surface of the hind tibia of most bees is an area of variable size, covered with hairs of uniform length, usually blunt, truncate, or briefly bifid. These

hairs, the keirotrichia, appear to serve for cleaning the wing. In some bees, they are replaced by longer, more ordinary hairs that may function as part of the scopa in females. On the hind basitarsus of many female bees is a process that extends beyond the base of the second tarsal segment. Sometimes, this process bears on its apex a small brush, the penicillus. Between the tarsal claws, there is often a protruding pad-like arolium.

Scopa: Two forms of pollen-collecting organs are found in bees. In all of the pollen bees (except in parasitic bees), they are composed of localized brushes of hairs, and the resulting organ is known as the scopa. Usually, these scopal hairs are more elongated and spine-like than the other body hairs, and they may be either simple and unbranched or more or less densely plumose.

The honeybees of the family Apidae have a more specialized structure, called the corbicula or "pollen basket". In such bees, the outer surface of the hind tibia is bare and highly polished and is surrounded by a marginal fringe of hairs, forming the basket. Female bees have scopae for holding and transporting pollen while the males lack such structures. Exceptions are the bees of Hylaeinae, Euryglossinae and Colletidae, parasitic and robber bees in various families, and queens of highly eusocial bees (Apidae), all of which lack scopae. The scopa consists of pollen carrying hairs in which pollen is carried back to the nest. In most bees, the scopal hairs are on the hind legs, but in non-parasitic *Megachilidae*, they are on the metasomal sterna; in some Collectids and Halictids, they are on both the underside of the metasoma and on the hind legs.

Discussion

Statistical analysis illustrated that significant differences were found only between workers of nectar-feeding colonies and Pepsi cola feeding colonies mean in tongue length, fore wing length, and basitarsus length. Significant differences were found between them in 7 characters that include fore wing length, fore wing width, hind wing length, hind wing width, femur length, tibia length, basitarsus length, and basitarsus width. In tibia length, basitarsus length and basitarsus width. In a similar study about the effect of the open mating system of different sites in Egypt on the morphological characters of the first single cousins *A. m. carnica*, Eshbah *et al.* (Eshbah *et al.*, 2003) found significant increases in the number of hooks, forewing width, proboscis length, and mandible width. Also, Quezada-Euán and Paxton (1999) showed that individual colonies dramatically changed their worker morphometrics and defensive behavior from one generation to the next. Differences between the two groups of workers may be due to the feeding types, in accordance with Bienefeld (1991) who mentioned that the feeding type and environments in many cases are the correct reasons for the fall-off in quality and reduced morphological characters of the offspring. On the contrary, characters which were affected by conditions other than genetic showed a different value like tongue length.

It could be concluded that rearing bees with unnatural feeding type resulted in the differentiation of morphological characters. The obtained results are in agreement with those obtained from the study done by El-Aw *et al.* (2012) in the El-Beheira governorate of Egypt during two years (2006 and 2007) where characters were generally decreased

from the first year to the second year with respect to feeding types and environmental conditions.

By using fore wing characters, standard morphometric analysis has shown that the length and width of the inner wing were the most successful characters in discerning between the two races without overlapping. In all measured fore wing characters, significant differences were discovered between the different colonies. Concerning the discrimination between the two feeding types hind and fore wing characters were used successfully for the discrimination between the two groups. Between means of hind and fore wings length, a significant difference was observed while there were significant differences between means of wings.

Importance of morphological measurements:

The measurements of morphological characteristics are carried out for various reasons. Some of the major uses are to characterize honeybee races and individuals (Ruttner, 1988; Meixner *et al.*, 2007), to determine the degree of hybridization with foreign races (Bienefeld *et al.*, 1996; Radloff *et al.*, 2003), and also for the discrimination between honeybee subspecies (Tofilski, 2004; Abou-Shaara and Al-Ghamdi, 2013). In order to investigate the impacts imported queens have on honeybee populations (Guler, 2010) or to check populations purity (Miladenovic *et al.*, 2011), morphological characteristics were measured. Also, to differentiate between honeybee subspecies, multiple body characteristics such as tongue length, wing length, and wing width were used (Buco *et al.*, 1987; Rinderer *et al.*, 1993; Crewe *et al.*, 1994; Ftayeh *et al.*, 1994; Diniz-Filho *et al.*, 1995; Szymula *et al.*, 2010).

Indirect prediction of colony productivity or selection of productivity can be made through body characteristics. Honeybees with bigger legs and wings have been known to have higher flight power and thus could be more useful in the collection of pollen and nectar, brood rearing and colony population. (Mostajeran *et al.*, 2006). Honey production and corbicular area have a positive correlation (Milne and Pries, 1984). It has been found that honey production has significant and positive correlations with fore and hind wing areas (Szabo and Lefkovich, 1988). The relationship between honey production and fore wing length, tongue length, hind wing length, length and width, leg length, tibia length, femur length, and metatarsus width was found by Mostajeran *et al.* (2002). The correlation between body size and colony productivity was found by Waddington (Waddington, 1989). Improved honey production through the selection of the forewing width was proposed by Edriss *et al.* (2002). Therefore body morphological characteristics correlate with colony productive characteristics and are therefore very important. However, it must be noted that these relations should not be regarded as general rules but attributed only to specific conditions.

Some studies (Morimoto, 1968; Souza *et al.*, 2002; Marghitas *et al.*, 2008) have shown that tongue length is a good indicator of geographical variation. Between *A.m.mellifera*, *A.m.carnica*, and *A.m.caucasica* (Souza *et al.*, 2002), proboscis length was also found to be the most differentiated characteristics among others. Furthermore, body measurements and honey yield may show some correlations. Kolmes and Sam (1991) found that honey production was highly correlated to overall

size, corbicular area and wing measurements in Carniolan honeybees.

Body characteristics may thus be used for indirect prediction of colony productivity or for selection of productivity where honeybees with bigger legs and wings have higher power flight and could gather more pollen and nectar for brood rearing and consequently colony population (Mostajeran *et al.*, 2006). There is a positive correlation between honey production and corbicular area (Mile and Pries, 1984). Szabo and Lefovich (1988) found that honey production had significant and positive correlations with both fore and hind wing area. Mostajeran *et al.* (2002) found that honey production was related to tongue length, fore wing length, and width, hind wing length, leg length, femur length, tibia length, and metatarsus width. Waddington *et al.* (1989) found a correlation between body size and colony productivity. Edriss *et al.* (2002) indicated that honey production can be improved through the selection of the fore wing width. Therefore, there is evidence that body morphological characteristics are very important and correlated with colony productive characteristics. However, it must be noted that these relations may be attributed to specific conditions rather than indicating general rules.

Ongoing evaluation of morphological characteristics could help in understanding racial fluctuations due to beekeeping, hybridization and environmental factors. In addition, morphological characteristics are also correlated with colony productive characteristics. Body morphological characteristics can be used as a simple indicator for estimating variations in genetic and productive characteristics of honey bee colonies. It is apparent that still more work is

required to provide insights into the feeding types impacts on body morphological characteristics.

Several morphometric studies of the mouthparts of bees, mainly European bees, have been conducted, especially with respect to the qualities of the tongue as a factor of selection and discrimination among the various subspecies of *Apis mellifera* (Goetze, 1940; Hachinohe and Onisshi, 1954; Weiss, 1955; Abdellatif *et al.*, 1977; Mattu and Verma, 1983; Michener and Brooks, 1984).

Rinaldi *et al.* (1970), established indices for different traits, including the glossae of Africanized, Italian and Caucasian bees, in order to differentiate these bee types. Funari (1983) also showed that the qualities of the glossa are important for the separation of bee groups. Woke (1977), in a study of Africanized and Italian bees, showed that diploid drones are closer to workers and haploid drones are closer to queens in measurements of lips and labial palpi. Proboscis characteristics is also an important trait affecting plant-pollinator interactions.

The chemical senses may be divided into taste, for detection of aqueous chemicals, and smell, for air-borne ones — but the distinction is relative. Alternative terms are contact (taste, gustatory) and distant (smell, olfactory) chemoreception.

Chemosensors trap chemical molecules, which are transferred to a site for recognition, where they specifically depolarize a membrane and stimulate a nerve impulse. Effective trapping involves localization of the chemoreceptors. Thus, many contact (taste) receptors occur on the mouthparts, such as the labella of higher Diptera where salt and sugar receptors occur, and on the ovipositor,

to assist with the identification of suitable oviposition sites. The antennae, which often are forward-directed and prominent, are first to encounter sensory stimuli and are endowed with many distant chemoreceptors, some contact chemoreceptors, and many mechanoreceptors. The legs, particularly the tarsi which are in contact with the substrate, also have many chemoreceptors. In butterflies, stimulation of the tarsi by sugar solutions evokes an automatic extension of the proboscis. In blow flies, a complex sequence of stereotyped feeding behaviors is induced when a tarsal chemoreceptor is stimulated with sucrose. The proboscis starts to extend and, following sucrose stimulation of the chemoreceptors on the labellum, further proboscis extension occurs and the labellar lobes open. With more sugar stimulus, the source is sucked until stimulation of the mouthparts ceases. When this happens, a predictable pattern of search for further food follows.

Insect chemoreceptors are sensilla with one or more pores (holes). Two classes of sensilla can be defined based on their ultrastructure: uniporous, with one pore, and multiporous, with several to many pores. Uniporous sensilla range in appearance from hairs to pegs, plates, or simply pores in a cuticular depression, but all have relatively thick walls and a simple permeable pore, which may be apical or central. The hair or peg contains a chamber, which is in basal contact with a dendritic chamber that lies beneath the cuticle. The outer chamber may extrude a viscous liquid, presumed to assist in the entrapment and transfer of chemicals to the dendrites. It is assumed that these uniporous chemoreceptors predominantly detect chemicals by contact, although there

is evidence for some olfactory function. Gustatory (contact) neurons are classified best according to their function and thus, in relation to feeding, there are cells whose activity in response to chemical stimulation either is to enhance or reduce feeding. These receptors are called phagostimulatory or deterrent.

In comparison with vertebrates, insects show a more profound use of chemicals in communication, particularly with other individuals of their own species. Insects produce chemicals for many purposes. Their perception in the external environment is through specific chemoreceptors.

Honeybees (*Apis mellifera*) are highly social insects who live in dense colonies. For this reason, they require a sophisticated set of senses for communication. They also use their senses for food and threat detection. The majority of bees' sensory organs are located in the antennae. This is the first part of the bee to come into contact with scent, flavor and the physical world. Aside from the antennae, their hairs are highly useful for making sense of the world around them.

Honeybees use their antennae to detect odor. According to research by the National Institutes of Health, published in the "Genome Research" journal, honeybees have 170 odor receptors or chemoreceptors in their antennae. The honeybee's sense of smell is so sensitive that it can detect the trace of a scent in flight. This ability equips the bee to effectively and efficiently locate pollen-rich flowers. Once the scent is detected on the antennae, the bee's hyper-sensitive olfactory path processes the information, enabling the bee to determine the relevance of the scent to her search for pollen. As well as for finding

food, honeybees use their sense of smell to locate other bees.

Bees' sense of taste is somewhat simple in contrast to their highly developed sense of smell. The tongues of bees contain taste buds which can detect sweet, sour, bitter and salty tastes with the same range of taste detection like humans. Before the pollen touches the tongue, it has been tasted by the bee because it gathers lots of information from smell. As a result, they have effectively carried out all the research necessary before the sustenance reaches their mouths. Plants have not evolved any form of defense mechanisms against bees since they provide benefit to them through pollination. This means that distasteful or harmful pollen are not produced by plants. Bees are more sensitive to salts compared to humans but less sensitive to bitter flavors.

The honeybee's tongue is called a glossa, and it resembles a mop because it is covered in long hairs. The mouthparts surrounding the glossa form a sucking tube when the bee is ready to drink nectar from a flower. The long glossa then stretches down through the tube to reach into the liquid.

According to researchers at the University of Illinois, honeybees have a much better sense of smell than fruit flies or mosquitoes but with a much worse sense of taste. A remarkable expansion of the insect odorant receptor family in honeybee genome was revealed by Robertson (2003) compared with those found in fruit flies or mosquitoes. The bee genome also reveals gustatory or taste receptors that are far fewer. The family of honeybee chemoreceptors that deal with smell and taste was identified by Robertson *et al.* (1999).

The researchers found 170 odorant receptors in honeybees (*Apis mellifera*), compared with 62 in fruit flies (*Drosophila melanogaster*) and 79 in mosquitoes (*Anopheles gambiae*).

The honeybee's remarkable olfactory abilities that include kin recognition signals, the perception of pheromones, and social communication within the hive reflects the enhanced number of odorant receptors they have.

Honeybees can also find food using odor recognition. By using subtle olfactory cues, foraging worker bees can discriminate between a confusing number of flowers they encounter to choose from (Robertson *et al.*, 2003). Bees can find food and communicate its location to other bees by using a large number of odorant receptors. The researchers found only 10 gustatory receptors in *A. mellifera*, compared with 68 in *D. melanogaster* and 76 in *A. gambiae* in a striking contrast. The low number of gustatory receptors for the sense of taste was unexpected (Robertson *et al.*, 2003). The relationship between plants and honeybees is beneficial and non-antagonistic, so plants don't have to defend themselves with toxins (Robertson, 2000). Bee larvae are provisioned by adults with food that is free of toxins in the nurturing environment. Bees don't need many gustatory receptors since they don't have to detect toxins. Honeybees require a very good sense of smell in order to detect chemical signals, such as pheromones, that control their behavior within and outside the hive but they don't need many taste buds. The sole task of male drone bees, for example, is to mate with virgin queen bees, and so in order to detect the queen pheromone, the male's antennae are specifically designed for this purpose.

Several honeybee odorant receptors that are abundantly expressed in male antennae have been identified (Robertson *et al.*, 1999).

Solutions of sucrose of strength in two different concentrations were freshly prepared using distilled water (Marshall *et al.*, 1934). A group consisting of twelve bees was taken at one time and solutions of sucrose were applied at intervals of twenty minutes. A response was recorded if there was an extension of the proboscis within ten seconds after the application of sucrose solutions. Based on the threshold values of sucrose solutions which elicit a response, the results of the experiments demonstrate that the antenna normally responds to dilutions. In respect to gustatory reaction, amputation of the antenna does not impair the normality of the bee. Threshold experiments on contacted chemoreceptors show that the bee responds when a solution of sucrose of an average strength of 12 times concentrations comes into contact with the antenna when the extension of the proboscis is taken as the response. The completion of the test indicates that choosing and sucking is the responsibility of the proboscis with support of the honeybee's antenna to locate the source of nectar.

The glossa or tongue of the honeybee is covered with bushy hairs looks like a mop or a brush. The dimensions of glossal hairs of the Italian honeybee (*Apis mellifera ligustica*) were examined by (Amirouche *et al.*, 2009) and they found that the average length of hairs increased from the proximal segment to the distal end. Wu *et al.* (2015) discovered the function of the glossal hair and reported that when drinking nectar, the glossal hairs erected rhythmically; specifically, hairs on the proximal segment erected before those on the

distal end of a honeybee's tongue, and this was described as asynchronous hair erection. Moreover, they found that the flabellum was the most hydrophilic and the root of the tongue was hardest to be wetted by measuring the wettability of the tongue. According to their observations, they suggest that the honeybee has an optimal hair-erection pattern that could balance nectar intake and viscous drag. The liquid-feeding mechanism of honeybees, and most importantly the function of erectable glossal hairs can be understood with help from these results (Yan and Yang, 2015).

Using 25%, 35%, and 45% (wt/wt) sucrose solution, Wu *et al.* (2015) measured the wettability of the different regions on the glossa and proposed that a smaller contact angle indicates higher wettability. There is a sharp rise in viscosity when sucrose concentration increases (Yang *et al.*, 2014). Wu *et al.* (2015) had a similar result when they used high concentrations of sucrose solution. Amirouche *et al.* (2009) discovered that the contact angles were getting smaller when a higher concentration of sugar solution was used, and this suggests that the glossa surface exhibits stronger hydrophilicity to the thicker nectar. It can be concluded that the dorsal part was much easier to wet than the ventral part. Moreover, the tongue tip was more hydrophilic than the middle region of the tongue and the proximal segment was hardest to wet with the artificial nectar (Wu *et al.*, 2015).

The asynchronous erection of glossal hairs might be caused by the variations in the internal pressure of the humor cavity of metameric glossa, which is compressed by the muscular rod (Snodgrass, 1956). Amirouche *et al.* (2009) focused on the function of the

asynchronous erection of glossal hairs and tried to explore its role in improving the feeding efficiency of honeybees. According to the erection pattern and the section-wise wettability of the glossa, some implications can be drawn. First, the glossal surface, covered by dense hairs, performed wettability of hydrophilicity in that the contact angles were all less than 90°. The volume of nectar collected during lapping is thus directly related to the dimensions of the tongue, as well as the thickness of the adhered-nectar layer (Kim *et al.*, 2011). The nectar intake mass Q can be expressed as: with the assumption that the tongue has the ability to entirely fill gaps between the glossal hairs with nectar. The glossal hairs transform from their resting to erect postures during lapping, increasing the erection angles directly, thereby promoting the ability of nectar intake.

The viscous drag equation suggests that if the erection angles are bigger, the drag together with the energy needed for overcoming drag will be larger. So honeybees may achieve an efficient energy-saving mechanism by adjusting the erection angles to balance the volume of trapped nectar and the energy used for overcoming viscous drag. The basal parts were immersed in nectar for white 100 W light source and a high-speed camera (Olympus, iSpeed TR), the shortest time compared with the anterior and middle parts, of section I need to perform rapid erection to take more nectar. Intriguingly, both the erection angles and hair length on the proximal segment were greater than those on sections II and III, which increased the amount of lifted nectar and overcame the shortcomings of the lower wettability of basal hairs. In addition, the anterior tongue was the most hydrophilic, so prolonging the time

immersed in the nectar might improve the amount of trapping sugar water. It is likely that the dynamic surface of the honeybee's glossa helps *Apis mellifera ligustica* make use of a limited nectar resource to accommodate the energy-intensive lifestyle (Harper *et al.*, 2013).

The honeybee's drinking process has generally been simplified because of its high speed and small scale. These studies clearly observed the morphological changes in proboscis. They described the pattern of hair and the dimension of the proboscis by measurements. Results showed that the honeybee used two special protraction strategies to save energy. The proboscis structure and hair pattern differences lead to suppose the effect in the quality of sucking process according to (Wu *et al.*, 2015). First, the glossal hairs remain addressed until the end of the protraction, which indicates that the hydraulic resistance is reduced to less than 1/3 of that in the case if the hairs remain erect. Second, the glossa protracts with a specific velocity profile this moving strategy helps reduce the total energy. We need to study the effect of feeding on soft drinks on the formation and effectiveness of sensory receptors in sensory hairs.

Some bee families presumably have provided the diversity of odorant receptors that allow bees to recognize diverse floral odors as well as employ complex pheromone blends to coordinate caste-specific tasks within the social colony. The evolution and divergence of bees and angiosperm plants have been closely linked, with the angiosperms evolving new flower characteristics to attract bees (such as odor, color, and food rewards) and with the bees

evolving mechanisms to collect and transfer pollen. The expansion of the bee odorant receptor (Or) family may reflect this coevolution between bees and angiosperms. Determining the expression patterns of these Ors in the various bee castes and life stages should provide indications of their involvement in these various roles and eventually lead to the determination of ligand specificities (Hallem *et al.*, 2004). For example, the sole task of male drone bees is to mate with the virgin queen.

Robertson and Wanner (2006) stated that the lack of expansion of the bee gustatory receptors (Grs), Gr lineages stand in stark contrast to the multiple expansions of different Gr subfamilies in both the *Drosophila* fly and *Anopheles* mosquito genomes. It also contrasts with the massive expansion of the bee Ors. Robertson and Wanner (2006) speculate that the coevolution of bees and angiosperm plants, as well as the social nature of bees, may account for this. First, unlike many insects, bees have mutualistic relationships with plants, which provide nectar in return for pollination services. Nectar and pollen alone provide for all of the nutritional requirements of a hive, and 98% of the nectar is composed of sugars and water. Since plants have evolved mechanisms to attract and reward bees, bees have not required the ability to detect and discriminate between the numerous plant secondary chemicals and toxins usually deployed in the chemical ecological arms races between most plants and many insect herbivores (Robertson and Wanner, 2006). Second, bee larvae are sequestered in cells in the hive and are provisioned by adult nurse bees; hence, they have little need for gustatory receptors to locate and recognize food. Third, bees

commonly use their antennae to touch objects, including kin, in a form of contact chemoreception. Therefore, some Ors on the antennae may function in a manner similar to that of contact gustatory receptors and effectively replaced the need for actual Grs. (Szabo and Lefkovitch, 1988; Robertson and Wanner, 2006).

Robertson and Wanner (2006) definition of the Or and Gr repertoires of the honeybee has enabled recognition of several lineages of Grs that are unusually conserved and might serve particular functions in diverse insects, as well as a massive expansion of the Or family. Determining the expression patterns of these bee Ors and Grs should provide indications of their possible roles and will be the first step toward determining their ligand specificity and defining their roles in bee ecology and social organization.

Our most remarkable finding is the proboscis in the mouthpart of the honey bees feeding on the Pepsi is wider, thicker, and very hairy compared to the natural feeding honey bees. From the above, the importance of the sensory hair as chemoreceptors necessary for all physiological, behavioral and environmental needs of bees becomes clear. The changes seen in this study may be due to the nutrition deficiency/malnutrition resulting from the metabolism of soft drinks, which led to one of these observed abnormalities that may have a logical explanation if studied at the level of the genome to evaluate the potential physiological and genome effects on the next-generation in microfluidic sucking processes.

The relationship between bees and the process of pollination and continuity is a strong relationship that makes attention to the

tripartite relationship between the bees, plants and environment which lead to concentrate on studies on the safety of honeybee in general and its morphological structures in a particular way to avoid malformation reasons to keep it suitable for carrying out various tasks, including nectar collecting to complete the transfer of pollen, in conjunction with the safety of plants and its diversity to ensure the integrity of the balance of the environment worthy of study. Research on this triangular relationship (insects, plants, environment) in various biological studies were conducted.

Regarding the evolution of floral spur length, two non-mutually exclusive hypotheses have been proposed: Darwin's 'coevolutionary race' and the 'pollinator shift'. The coevolutionary race is based on Darwin's observations of the orchid *Angraecum sesquipedale* and his prediction that it is probably pollinated by a moth with a proboscis thick enough at the base to pick up pollinia and long enough to reach the nectar in the long spur, Darwin 1862 (Robertson and Wanner, 2006). Darwin's coevolutionary race hypothesis assumes a gradual increase in the length of a plant's spur together with the prolongation of the pollinator's tongue within a species lineage. The driving forces of the coevolutionary race are the advantage for long-proboscid visitors in reaching the nectar and the advantage for long-spurred plants in more effective contact between their reproductive organs and the visitor's body (Nilsson, 1988; Pauw *et al.*, 2009). The pollinator shift hypothesis considers spur length to be the result of an adaptation to a series of unrelated pollinators, each with a longer proboscis than the species before it (Wasserthal, 1997; Whittall and Hodges,

2007). The pollinator shift hypothesis is characterized by rapid changes during a switch to new pollinators by creating reproductive barriers and subsequent speciation. This hypothesis predicts that short-proboscid visitors will not be able to reach the nectar and, consequently, will not visit long-spurred flowers and/or that they will not be effective pollinators because they no longer at the flower's reproductive organs. Therefore, the plant will become more phenotypically specialized and also more ecologically and functionally specialized (Ollerton *et al.*, 2007). Thus, visitors with shorter proboscises may easily reach at least some of the nectar. Furthermore, several previous studies showed that the sugar concentration in the nectar of some spurred (Martins and Johnson, 2007) or tubular (Petit *et al.*, 2011) flowers is not homogenous and suggest that different types of nectar are consumed by different visitors, a fact that probably favors the generalization of pollination systems (Petit *et al.*, 2011). Moreover, other studies revealed diurnal changes of both nectar availability and visitor spectra, indicating possible temporal niche separations (Herrera, 1990).

In the present study, we supported our working hypotheses that (1) nectar type is very important to be consumed by honeybee with different proboscis qualities, and (2) nectar types affect proboscis formation. Moreover, these results may contribute to the discussions of the diversification of feeding types that affect the formation of proboscis due to the different ecological and biological requirements. Suggesting that the proboscis in honeybee fed on natural nectar from flowers is closer to the characteristics of the capillary tube, ideal for the completion of absorption by

osmosis processes more efficiently compared to honeybee proboscis that resulted from feeding on Pepsi by placing it in shallow open containers.

Both the coevolutionary race hypothesis of Darwin, (Darwin, 1862) and the pollinator shift hypothesis (Wasserthal, 1997; Whittall and Hodges, 2007) predict that during the evolution of long-spurred flowers, the short-proboscid pollinators are excluded from the pollination system, and the long-proboscid pollinators are expected to be the only ones producing selection pressure on flower traits. By contrast, our results show that the shape of proboscis can be affected by nectar types and feeding style and that the possible selection pressures on proboscis traits can affect the configuration.

We believe that unless there is a better understanding of the roles of feeding types on proboscis traits and related selection, different mechanisms of nectar consumption can enable visitors to use different feeding niches and lead to higher generalization of the *I. burtonii* pollination system. Syrphid mouthparts form a tubular sucking organ (proboscis and labella) with pumps operated by muscular contraction (Gilbert, 1981). By contrast, the mouthparts of the honeybee, a tongue (glossa) with erectable hairs (Snodgrass, 1956; Zhu *et al.*, 2016), capture nectar on the outer surface (Kim and Bush, 2012), and with the shortest proboscis, sucks nectar remnants from the spur walls.

Both Darwin's coevolutionary race hypothesis of Darwin (1862) and the pollinator shift hypothesis (Wasserthal, 1997; Whittall and Hodges, 2007) predict that during the evolution of long-spurred flowers, the short-proboscid pollinators are excluded

from the pollination system, and the long-proboscid pollinators are flower traits. By contrast, Anna *et al.* (2017) results show that feeding type and styles can affect the formation and the characteristics of proboscises which we assume can be ineffective pollinators and that the possible selection pressures on flower traits can, therefore, be much less diverse. We believe that unless there is a better understanding of the roles of feeding type and styles on proboscises formation and efficiency, it will be impossible to understand the functioning and evolution of mouthparts growth and development process. For example, spur and proboscis lengths may be influential in some pollination systems on the quantity component of pollinator effectiveness (i.e. on visitation frequency), whereas other traits may be relevant for the quality component of pollinator effectiveness (i.e. pollen deposition per visit). In the case of Darwin's system with *A.sesquipedale* and *X. morgani*, these quality-related traits are the arrangement of pollinia and the thickness of the proboscis at the base, respectively. Only a thick (not necessarily long) proboscis can effectively touch and remove pollinia. Mayfeld *et al.* (2001) suggested that the hairy bodies of bumblebees may ensure excellent contact with sexual organs of *Ipomopsis aggregata* (Pursh) V.E. Grant (Polemoniaceae). Wu *et al.* (2015) stated that the insect must be large enough to touch the anthers and stigma when sitting on the lower petals during feeding. It is also possible that the hairs on the honeybee thorax can increase its effectiveness, similar to the suggestions mentioned by Mayfeld *et al.* (2001). Such adaptations or malformation in proboscis may include, for example, a wide proboscis and less hair. Our observations and

results support a quite different scenario for the pollination system of spurred flowers if the long-proboscid pollinators disappear, in contrast to the coevolutionary race and pollinator shift hypotheses. The possibility of an evolutionary shift to the wide and hairy – proboscis in honeybees that feed on *Pepsi* should be corroborated in further studies. These studies should include the (1) genome studies should include comparative studies on different feeding styles and types, (2) selection pressures in individual populations would be much more diverse; and (3) this geographical co-evolutionary mosaic (in the sense of Thompson's theories; Thompson, 2005) would lead to rapid diversification. Based on recent global changes, this scenario presents both bad and good news. Although it supports the robustness of plant-pollinator interactions and reduces the probability of the extinction of individual species, it also predicts much greater ease of invasion of alien species into new areas.

The microstructures found in various organisms, especially insects, have shown interesting functions that can provide promising applications. Hairy structures in biological systems are found to significantly influence friction and adhesion control, interlocking mechanisms, water transport, self-cleaning (Bienefeld, 1991) systems and water absorption of plants. In the current work, we supposed another feature of the insects' hairs may cause the moving pattern of glossal hairs to play an essential role in the bee's nectar feeding. Some studies used a video capture system to elucidate the drinking process of the Italian honeybee. They examined the erection pattern of the glossal hairs and the protraction kinematics of the dipping glossa. Also, they demonstrated that

the honeybee achieves an efficient energy-saving mechanism by adopting the two drinking strategies. Harper *et al.* (2013) found that the bat tongue is like a specialized mop, that is, the erection pattern of the hair-like papillae in the tongue of the bat helps nectar trapping. The tongue of a honeybee is similar to a specialized brush controlling the erection pattern of glossal hairs. During feeding, bats and honeybees stick out their tongues deep into their liquid food sources. With respect to the living environment of these species and their feeding habit, a trapping strategy using a hairy system is deployed.

Conclusion

In taking morphological measurements, different methods were employed. However, time is saved and precise measurements are obtained when computer programs like Photoshop, AutoCad, etc., are used. It is important to note that the size of the sample, sampling season, sampling method/technique and measuring method differ from one author to another and from one country to another and these should be coordinated. In order to facilitate the comparison of results of different subspecies and countries, it is advised to use standard methods for measuring these characteristics. The steps recommended for morphometric analysis using body characteristics are shown in Fig. 1. Fifteen workers per colony and six colonies per district should be sufficient for sample size. It is easier to take samples from colony combs than from forager bees. Racial fluctuations that may result from beekeeping, feeding types, hybridization, as well as environmental factors can be understood by the help of ongoing evaluation of morphological characteristics. In addition, morphological characteristics are also correlated with colony

productive characteristics. Fluctuations in genetic and productive characteristics of honeybee colonies can be estimated using morphological characteristics as a simple indicator. More research is required to provide insights into the feeding type impacts on body morphological characteristics (Snchez-Gracia *et al.*, 2009).

Examining The anatomy of the glossa of a honeybee is an interesting topic for future research. In addition, the process by which honeybees coordinate different structures within the tongue to control hair erection should be explored. The drinking strategy of a honeybee may inspire some new concepts to facilitate the design of micropumps (Wu *et al.*, 2015).

Snchez-Gracia *et al.* (2009) in the recent genomic data support the BD model for chemosensory family evolution, with progressive divergence and functional diversification among their members. In spite of high BD rates, the number of genes in each chemosensory family has remained fairly conserved across *Drosophila*, and a large fraction of members evolve under purifying selection. The large variation in gene repertoire size observed among distantly related insects might thus be explained by genomic drift accumulated over long time scales. Indeed, gene gains and losses might provide the raw source of variation for evolutionary change. Given the crucial role of the chemosensory system in the survival and reproduction of individuals, adaptive changes likely arise in response to the demands of new environmental conditions. Molecular adaptation may entail, for instance, changes in the detection of pheromones (changes in chemical sensibility or specificity; for example, Willett (2000)), and it might be fostered by

shifts in ecological interactions (Matsuo *et al.*, 2007; McBride, 2007; McBride and Arguello, 2007; Vieira *et al.*, 2007; Gardiner *et al.*, 2008) or even by changes in some aspects of social behavior. (Snchez-Gracia *et al.*, 2009) genomic studies analyzing closely related species with well-resolved phylogenies have provided valuable insight into evolutionary patterns and processes and illuminated features that usually are hidden in analyses using distantly related species or a small number of genes. Conduction of future studies may be useful for clarifying the relative contribution of neutral mutation and natural selection to the BD evolution of chemosensory multigene families and in the molecular evolution of insects in general.

This study leads to more questions that need to be answered by conducting studies based on molecular biology, sophisticated computers, and advanced imaging techniques is an important step closer to understanding the molecular details of how bees, and insects in general, smell, and their morphological malformations effects.

The interrelationships between insect structures and the success of tree fertilization processes create attention to the detailed structures of body appendages in general and mouthparts in particular, hair, sensory and chemoreceptors. We recommend extensive studies to preserve the environment and insect integrity from harmful mutations that may result from food contamination or pesticides that may impair their innate potential. Feeding on soft drink for example, may affect embryological differentiation and ecological balance which may cause genetic changes in the long-term. This can lead to the extinction of some species important for the continuation of ecological balance.

The close relationship between smell and taste in bees to achieve the successful nutrition process requires work on future comparative studies on bees fed with the two methods we applied in our study at the level of insects in general, smell, test processes, and morphological malformations effects as an assumed result, according to our observations in the mouthparts feeding on soft drinks known internationally for their bad health effects, and their toxic chemical components.

Reasons for requesting the inclusion of videotaped work in future studies:

Glossal hair behavior per second was revealed through a series of high-speed photographic images. By adjusting the height of the positioner, we were able to capture images in different regions of the glossa. Using Matlab (R2013b, MathWorks, Natick, MA, USA), a series of photographs with high-resolution captured by the high-speed camera in every microsecond were processed. This is to ensure that there is repeatability and correctness of the hair erection results during nectar feeding.

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