

Artificial Feeding as Stress Factor Affecting Wing Venation Symmetry of Worker Honeybees

Fathy, H. M.¹; A. M. Mazed²; E. A. Nafae³ and M. R. Abd El Dayem³

¹Department of Economic Entomology, Faculty of Agriculture, Mansoura University.

²Department of Economic Entomology and Pesticides, Faculty of Agriculture, Cairo University.

³Department of Apiculture, Plant Protection Research Institute, Agriculture Research Center, Dokki, Giza.



ABSTRACT

Directional and fluctuating asymmetry were estimated in worker bees fed on sugar syrups in comparison with feeding with honey (control). By using 25 traits of wing venation pattern on left and right forewing of worker honey bees, it could be stated that directional asymmetry (DA) was not related to the type of feeding. Fluctuating asymmetry (FA), however, differed between characters and between feeding treatments, but no interaction was found between them. The individual characters were combined and analyzed as composite fluctuating asymmetry to maximize the probability of detecting (FA)-stress relationship when it exists. The result showed that (FA) of distances between vein junctions were significantly higher in sugar-feeding than in honey-feeding colonies.

Keywords: Honeybees, workers, forewing venation, vein distance, vein angels, polar coordination, fluctuating asymmetry, directional asymmetry, composite fluctuating asymmetry.

INTRODUCTION

Nutrition is considered the first line of defense of honeybee colonies and is the key in dealing with major honeybee diseases. Honeybee Larvae fed on a nutritionally poor diet were found to be significantly more susceptible to various diseases (Foley, *et al.* 2012; Li, *et al.*, 2007). In addition, Longevity of worker bees (Chengcheng, Xu, *et al.* 2014). and body size (Daly, *et al.* 1995) decrease when their larvae experience poor maturational conditions.

During development, individuals of an organism species may be exposed to unfavorable conditions which may impair optimal growth or some physiological functions (Møller and Thornhill 1998; Field and Yuval 1999; Schmid-Hempel 2003). So, they may be unable accurately to develop their expected phenotype given their genotype and the environment (Palmer and Strobeck 1986). Accordingly, departures from the ideal expression of particular traits are expected which could be used as evidence that organisms have been exposed to sub-optimal resources as mediated through environmental or genetic constraints (Schmid-Hempel, 2003).

Bee colonies suffer greatly from shortage of natural sources of nectars as well as hard conditions for foraging during winter. Beekeepers usually supply their colonies with artificial feeding during dearth periods of the year, especially cold times of winter. This is accomplished by feeding sucrose solution, invert sugars, high fructose solution or various fruit syrups (Neupane & Thapa, 2005) inside the hive.

One of the most important morphological characteristics being used as stress-bioindicator for measuring developmental instability in many insects is wing fluctuating asymmetry (FA) (Parsons and Hoffmann 1991; Clarke and McKenzie 1992; Bjorksten *et al.* 2000; Rantala, *et al.* 2004). So, the aim of our study was to show the possible effect of artificial feeding on the fore wing asymmetry of honeybee workers.

MATERIALS AND METHODS

This experiment was carried out in the Apiary of Beekeeping Research Center at the Ministry of Agriculture in 2016. The treatments were applied on 12 colonies belonging to Carniolan race (*A.m carnica*) each supplied with one kilogram of bee worker on 3 empty combs. The

queens were all sisters and mated naturally in Manzala (where Carniolan bee are preserved purely) at the apiary from which the experimental colonies were prepared. The colonies were divided randomly into 4 groups each of three colonies, and were fed with different types of sugar nutrition:

I Sugar Cane syrup: Sucrose-water solution 1:1.

II Sugar beet syrup: Sucrose-water solution 1:1.

III Sugar beet & Citrus honey syrup: Honey-Sucrose-water solution 0.5:0.5:1.

IV Citrus honey syrup (Control): Honey-water solution 1:1.

Each colony was fed twice a week by one-liter solution. Each group was fed exclusively with its treatment for 6 weeks before taking of bee samples. From each colony in each group, a worker sealed brood comb was taken and put in a cage into their colony until emerging of worker bees, then about 15 workers were collected from each group and preserved in ethanol 70% until dissection.

From each honeybee worker, the pair of forewings was dissected, and then they were air-dried to the glass slides while ethanol evaporated, and the dry mounts were digitally photographed by a slide-scanner.

1. Asymmetry of wing venation:

The forewing measuring characters were chosen according to (Kauhausen 2002; Szymula, *et al.* 2010), who used polar coordinate system for description the location of the different intervention points in terms of lengths and angles between these points. The number of points chosen was 17 (Fig. 1).

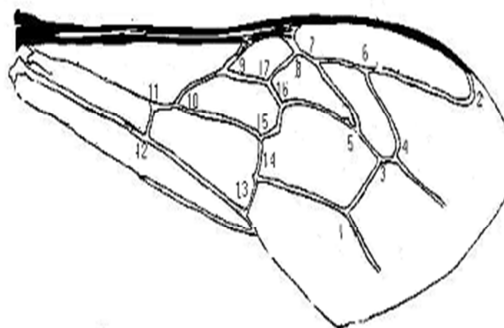


Fig. 1. Forewing of worker bees showing 17 points used to establish wing coordinates

The first point has no values on the coordinate system ($X = 0$ and $Y = 0$). The second point, however, was represented by one value on the first reference point. All the remaining points are represented by two values, so the number of the coordinates is $(2 \times 17) - 3 = 31$ points, which represent the measured characters (Table, 1). The coordinates characters measured represent 16 intervention distances and 15 angles coordinates. For technical reasons and to ensure precision of the measurements values, 6 characters were excluded from the results.

Table 1. Characters measured on the forewing

No	Distance (L) and Angles (A) between coordinates	Morphometrical characters in relation to the first coordinates (L,2) (Fig 1)	No	Distance (L) and Angles (A) between coordinates	Morphometrical characters in relation to the first coordinates (L,2) (Fig 1)
1	L1	1-2	17	A9	*2-1-10
2	L2	1-3	18	L10	*1-11
3	A2	2-1-3	19	A10	*2-1-11
4	L3	1-4	20	L11	*1-12
5	A3	2-1-4	21	A11	*2-1-12
6	L4	1-5	22	L12	1-13
7	A4	2-1-5	23	A12	2-1-13
8	L5	1-6	24	L13	1-14
9	A5	2-1-6	25	A13	2-1-14
10	L6	1-7	26	L14	1-15
11	A6	2-1-7	27	A14	2-1-15
12	L7	1-8	28	L15	1-16
13	A7	2-1-8	29	A15	2-1-16
14	L8	1-9	30	L16	1-17
15	A8	2-1-9	31	A16	2-1-17
16	*L9	1-10			

* characters being excluded from the analysis

1. Equipment and measurement procedure

The bees were prepared and the forewing characters were measured at Bee Section of Faculty of Agriculture, Cairo University.

The morphometrical system used consisted of the following parts:

1. Computer unit with a suitable analyzing program developed by Kauhausen (2002).
2. A Slide-Scanner (Minolta Dimage Scan Dual II).

The wing measurements were made by using a 35 mm slide projector to project the images of mounted wings onto a monitor screen. A particular type of slide known as a Gepe consists of two plastic half frames containing thin metal masks and each half frame is separately glazed.

From each honeybee worker, the left and right forewings were cut off at the base with a fine forceps and dry-mounted onto slides. Each slide bear 15 wings and each 4 slides were put in a slide mount holder, which was then put inside the Scanner, in order to scan the mounted wings. After setting a suitable display-program, the wings were displayed on the screen monitor of a computer, and by the computer-mouse, the different intervention points were marked. The measuring-program converted these coordinate points into actual lengths and angles of the different intervention points relative to the first two points. The converted measurements are then stored in a new file.

For every bee sample, the converted data, the information about its origin, the type of bees, to which it belongs, and the date of collection were registered in a data-bank.

2. Statistical analysis:

To obtain the measure of directional asymmetry (DA), paired t-test between left and right wing for each trait was carried out according to (Palmer 1994). Because we have several comparisons (31), and because the individual tests may not be independent, the decisions based on conventional levels of significance might be in doubt, so we employ a conservative approach in which one lower the type 1 error of the statistic of significance for each comparison so that the probability of making any type 1 error at all in the entire series of tests does not exceed α . For all characters, a significant p value was set at 0.002 based on Bonferroni's correction for 25 comparisons (Sokal & Rohlf, 1995).

It was advised that characters that show directional asymmetry should not be used for analysis of fluctuating asymmetry, because its presence make the interpretation of fluctuating asymmetry more difficult, (Palmer and Strobeck 2003), so the characters showing (DA) in workers were excluded from our analysis when calculating (FA).

(FA) was calculated as (FA)1 of Palmer (1994), which is the (FA) measure reported in most of studies as the mean of absolute value of the difference in trait size between the right and the left sides of the body $|R - L|$.

To test the significance of AF between the four groups, we carry out a significance test for absolute asymmetry difference by using two-way ANOVA, to test the effect of both traits and feeding treatments on the value of (FA).

After doing individual tests, individual data of absolute asymmetry were pooled to make composite variable of all 25 variables (Clarke and McKenzie 1992; Whitlock 1993; Leung, et al. 2000; Mazed, et al 2015). The composite variable is the sum of absolute (FA) values for all traits for each individual. Absolute (FA) values are used because (FA) of individuals should be directionally random, and it is the magnitude of asymmetry in either direction that may indicate stress. The new composite variable was analyzed by using t-test. All the analyses were carried out by using Almo-Statistik-System, Version 15 (Holm, 2015).

RESULTS AND DISCUSSION

1. Testing of directional asymmetry (DA):

The results of T-Paired test of 25 characters in the four groups are shown in table (2). In worker bees, and from 25 characters measured, 3 characters showed significant differences between left and right wing. Two of them were in favor of the right wing (L1 and A6) and one in favor of left wing (A7). In sugar cane syrup, and one character in sugar beet syrup shoed (DA) in favor of left wing (A6), suggesting the presence of directional asymmetry (DA) in data.

Table 2. Effect of different feeding on directional asymmetry of the forewing venation characters of worker honey bees (T Paired Test)

Venation characters	Sugar Cane feeding		Sugar Beet feeding		Sugar Beet & Citrus honey feeding		Citrus honey feeding	
	t-value	Sig. (2-tailed)	t-value	Sig. (2-tailed)	t-value	Sig. (2-tailed)	t-value	Sig. (2-tailed)
L1	3.937*	.001	1.317	0.204	2.351	0.029	0.515	0.616
L2	0.736	0.468	-0.325	0.749	-0.719	0.480	0.000	1.000
A2	1.259	0.219	0.619	0.544	-0.987	0.335	0.214	0.834
L3	0.902	0.375	0.314	0.757	-0.118	0.908	1.100	0.293
A3	1.495	0.147	1.380	0.185	0.271	0.789	1.652	0.124
L4	2.791	0.010	1.661	0.114	-1.872	0.076	-0.493	0.631
A4	0.911	0.371	-0.678	0.506	-1.374	0.185	-1.107	0.290
L5	-0.328	0.746	-0.615	0.546	0.116	0.909	-0.877	0.398
A5	-2.449	0.021	-1.661	0.114	0.740	0.468	-0.574	0.577
L6	0.440	0.663	-0.775	0.448	-3.619	0.002	-0.615	0.550
A6	5.443*	0.000	-3.741*	0.001	-2.250	0.036	-0.581	0.572
L7	-0.542	0.592	-0.884	0.388	-1.376	0.184	-1.514	0.156
A7	-3.797*	0.001	-2.257	0.037	0.856	0.402	-0.004	0.997
L8	1.213	0.236	0.134	0.895	-1.210	0.240	-0.656	0.524
A8	-1.589	0.124	-1.070	0.299	1.421	0.171	-0.108	0.916
L12	0.049	0.961	-0.417	0.682	0.920	0.368	0.000	1.000
A12	-2.712	0.012	-1.479	0.156	2.994	0.007	-0.523	0.610
L13	1.433	0.164	-0.297	0.770	-0.603	0.553	-0.610	0.553
A13	-2.048	0.051	-1.281	0.217	2.119	0.047	-0.477	0.642
L14	0.166	0.869	-0.071	0.944	0.072	0.943	-0.399	0.697
A14	-1.511	0.143	-1.137	0.271	0.745	0.465	-0.442	0.666
L15	3.218	0.003	0.218	0.830	1.770	0.092	0.693	0.502
A15	-2.334	0.028	-0.733	0.473	0.808	0.428	0.252	0.805
L16	2.935	0.007	1.157	0.263	0.277	0.785	0.000	1.000
A16	-2.324	0.028	-0.904	0.378	1.962	0.064	-0.121	0.906

* p < 0.002

2. Fluctuating asymmetry (FA).

Results from two-way ANOVA test that combine information from different characters to test the difference in (FA) among feeding groups are shown in table (3).

As shown in table (3) and concerning (FA) in workers the different characters has a significant effect on the value of (FA). Also, the feeding-type has a significant effect on the value of (FA). No Interaction was found between traits and feeding groups.

Table 3. ANOVA table for analyzing the effect of trait and feeding treatment on FA of forewing venation in workers

S. V	df	MS	F
Traits	24	19.6	63.02**
Feeding treatments	3	0.942	3.03*
Interaction	72	0.194	0.624 ^{ns}
Error	1900	0.311	

* p < 0.05

For summarizing the results of (FA), we use an index of (FA) across traits and the results of t-test were shown in figure (2 and 3). This index is composite fluctuating asymmetry.

The results of composite (FA) indicated that feeding treatments did not affect wing angels in worker bees, but it has an effect on veins distance, as feeding with sugar cane caused significant higher (FA) than with feeding with honey.

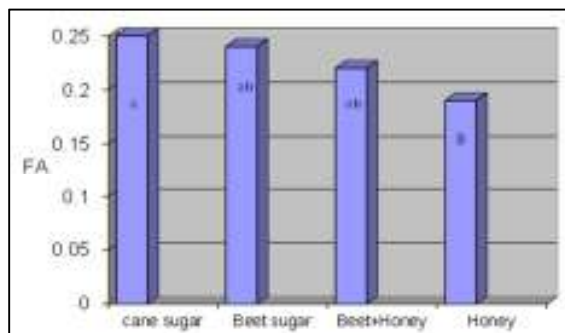


Fig. 2. Workers average values of composite fluctuating asymmetry in veins distances of forewing in different treatments (similar letters indicate non-significant)

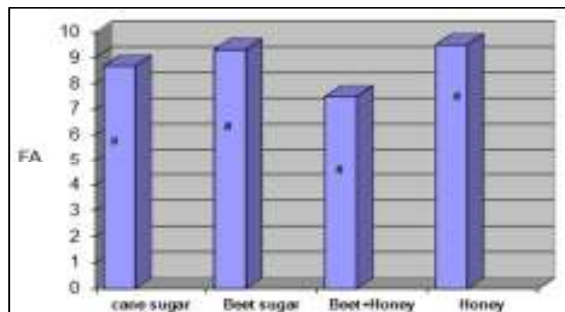


Fig. 3. Workers average values of composite fluctuating asymmetry in veins angels of forewing in different treatments

In this study we investigate the influence of artificial feeding in comparison to honey-fed colonies on the degree of symmetry between right and left wing of honeybees, as many researches established that the use of wings seems to be effective in measuring developmental instability in many insects (Parsons and Hoffmann 1991; Imasheva *et al.* 1997; De Block and Stoks 2007; Berggren and Low 2009; Mazeed 2012; Mazeed, *et al.* 2015).

The presence of directional asymmetry of honeybee wings was demonstrated in some studies (Smith, *et al.* 1997; Schneider *et al.* 2003), but in most of them it was not significant (Clarke and Oldroyd 1996; Clarke 1997; Clarke, 1998; Jones *et al.* 2005; Ondo Zue Abaga *et al.* 2011).

In our results, only 3 forewing characters in sugar cane fed-colonies and one character in sugar beet fed-colonies showed significant directional asymmetry after bonferonii correction of significance level, so, it could be concluded that (DA) is less associated with the type of feeding under study. This result is in agree with that of (Mazeed, *et al.* 2015), who found that (DA) was not related to the type of food. It was earlier suggested that directional asymmetry is genetically determined and adaptive (VanValen, 1962; Windig & Nylin, 1999), therefore, it should not be used as a measure of developmental stability (Palmer and Strobeck 1992).

As the results of (FA) analysis indicated, the (FA) differed significantly between all wing characters across feeding treatments, so, some traits are repeatably less stable developmentally than others. Also the four treatments had different effect on the fluctuating asymmetry of the pooled characters, suggesting that the level of developmental stability varies among feeding groups. The non-significant of the interaction indicated that, the variation of (FA) of the traits do not depend on feeding type, and all traits are the same at revealing presumed differences in developmental stability among samples.

It was suggested that some of these differences between characters may result from the fact that the impact of stress on fluctuating asymmetry seems to be trait- or stressor-specific (Beasley, *et al.* 2013) or simply be a consequence of their under sampling (Babbitt, *et al.* 2006).

According to the results of composite asymmetry, the vein distances of workers are more sensitive to the treatments than vein angles and caused higher (FA) values when feeding on cane sugar than feeding with honey.

Fluctuating asymmetry has already been used as a measure of developmental stability in honeybees. It was found that drones are more asymmetrical than workers (Brueckner 1976, but see Clarke 1997). Fluctuating asymmetry was also used in bee studies of rearing temperature (Jones *et al.* 2005), inbreeding (Brueckner 1976; Clarke, *et al.* 1986) and hybridization (Smith, *et al.* 1997) or even toxicity (Ondo Zue Abaga *et al.*, 2011). However, the results of these studies are inconsistent, and some failed to demonstrate detectable changes in the level of fluctuating asymmetry (Jones *et al.* 2005; Smith, *et al.* 1997; Clarke, *et al.* 1986) while others did (Brueckner, 1976; Ondo Zue Abaga *et al.*, 2011).

Few Studies had dealt with the impact of feeding on the (FA) of members of honeybee colonies. (Mazeed, *et al.* 2015) stated that sugar feeding caused significantly higher

FA in comparison to feeding with honey, and this effect was not influenced by neither the time of year nor the genotype of the colonies. Szentgyörgyi, *et al.* (2016), stated that, limited access to pollen caused some differences in the fluctuating asymmetry of size and shape in pollen-deprived workers and drones compared to the control bees, and there are more pronounced differences in (FA) due to replication than to pollen deprivation itself. They also reported that, malnutrition is a strong stressor affecting development and can cause changes in various morphological traits. One of these traits is the body symmetry of an organism.

In all mentioned (FA)'s Studies, the researchers aimed to study the effect of specific factor on the value of (FA) to investigate if a factor could be considered as a parameter for the existence of FA. Only few studies are dealing with the biological meaning of the presence of FA in terms of its effect on specific behavior, such as (Jaffé and Moritz, 2010) who stated that wing (FA) was found to be significantly lower in the drones collected at the drone congregation areas than in those collected from the hives. They concluded that a strong selective pressure may act on drones to ensure that queens in the congregation areas would mate with high quality drones. So, as reported by (Harvey And Walsh 1993; De Block and Stoks 2007), individuals with more symmetric wings may have a higher mating success due to flight mechanistic reasons as suggested in damselfly

Comparing the chemical composition of sugar cane with honey, it could be detected that the sugar cane has only one type of sugar, sucrose, in comparison with honey which has many types of sugars, amino acids, minerals, enzymes, hormones, vitamins, organic acids, and natural antibiotics in its components (Herold, 1982). So, sucrose syrup as artificial feeding might be one of nutritional stresses which are responsible of development instability (Polak, 1993). However, most of beekeepers cannot dispense with using artificial sugar feeding to feed their colonies, since it is used to supplement a shortage of stored honey to prevent starvation of the colony, or to stimulate a colony to artificially promote breeding. Accordingly, more studies are needed in order to find any deleterious effect of the presence of (FA) and other abnormal behavior on workers when feeding continuously with sugar cane in order to determine if (FA) had a biological meaning for bees or it would be only used as a marker to distinguish colonies being fed with artificial sugar syrup.

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التغذية الصناعية كعامل إجهاد على تماثل تعريق الجناح في شغالات نحل العسل

حسن محمد فتحي¹، عادل محمود مزيد²، عماد أحمد عبد الحميد نافع³ و محمد رمضان محمد عبد الدايم³

¹قسم الحشرات الاقتصادية - كلية الزراعة - جامعة المنصورة

²قسم الحشرات الاقتصادية والمبيدات - كلية الزراعة - جامعة القاهرة

³معهد بحوث وقاية النباتات - مركز البحوث الزراعية

تم تقدير صفة عدم التماثل ذو الاتجاه الواحد وذو الاتجاهين في الجناح الامامي لشغالات نحل العسل التي تم تغذيتها بمحاليل سكريه مختلفه و مقارنتها بمحلول العسل ككنترول. وقد تم التركيز على خمسة وعشرون صفة من صفات التعريق في الجناح الأمامي الأيمن والأيسر لشغالات نحل العسل. هذا وقد اوضحت النتائج أن عدم التماثل ذو الاتجاه الواحد لم يتأثر تقريباً باختلاف نوع التغذية السكريه. وعلى الجانب الآخر وجد ان عدم التماثل ذو الاتجاهين تأثر باختلاف نوعيه محلول التغذية، وباختلاف الصفة وذلك بدون وجود أى تفاعل فيما بينها. بالإضافة الى ذلك فعند تجميع قيم عدم التماثل ذو الاتجاهين لكل الصفات تحت الدراسة وتحليلها احصائياً فقد أظهرت النتائج أن عدم التماثل بين الجناحين الأيمن والأيسر بالنسبة للمسافات بين نقاط التقاء العروق كانت عالية بشكل معنوى في التغذية السكريه بقصب السكر عن التغذية بالعسل.