Volume 45 | Number 3 | 2006

Journal of Apicultural Research

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ORIGINAL RESEARCH ARTICLE

Detection of fluvalinate resistance in *Varroa destructor* in Spanish apiaries

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Received 4 October 2005, accepted subject to revision 5 July 2006, accepted for publication 26 July 2006.

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Summary

A laboratory assay developed by Milani (1995) was used to assess the susceptibility to the acaricide fluvalinate in 10 populations of *Varroa destructor* Anderson and Trueman from apiaries in Aragón (northeastern Spain). We assayed mites at the diagnostic concentration (200mg/kg) predicted to kill all susceptible mites. In some populations, LC_{50} values were obtained. Some mites were resistant to fluvalinate, which is the first reported case of fluvalinate resistance in *Varroa destructor* in Spain. Thus, it is inadvisable to base mite control strategies on chemical treatment alone. There is urgent need to expand and implement more biorational strategies in the fight against *V. destructor*.

Keywords: *Apis mellifera, mite resistance, Varroa destructor,* fluvalinate, control, Spain

Introduction

Varroa destructor Anderson and Trueman (2000) is a deadly non-natural parasite of *Apis mellifera.* Until recently, the mite was effectively controlled by the pyrethroid fluvalinate (Apistan®); however, resistance to this acaricide seems to be increasing in mite populations in several countries (Londesani *et al,* 1995; Colin *et al.,* 1997; Trouiller, 1998; Baxter *et al.,* 1998; Thompson *et al.,* 2002;), and regular surveys to verify susceptibility are needed.

One method to verify susceptibility to acaricides involves bioassays of mites in the laboratory. There are several methods to test *V. destructor* resistance to pyrethroids (Milani, 1995; Vandame *et al.,* 1995; Faucon *et al.,* 1996). We used Milani's (1995) method because it is accurate and easy to perform and can be compared with data from studies in other countries. The prompt detection of yrethroid-resistant populations of *V. destructor* can reduce the loss of bees and help prevent the spread of resistant strains by indicating the need for alternative control techniques.

In Spain, previous studies did not detect resistance (Trouiller, 1998), but there is no information about the resistance of *V. destructor* to fluvalinate. In 2001, we assessed the susceptibility to fluvalinate by *V. destructor* in Aragón. We tested mites using a laboratory assay at a fluvalinate concentration (200mg/kg) that discriminates resistant and susceptible populations. In some apiaries, we determined the median lethal concentration (LC_{50}) and level of resistance.

Materials and Methods

Mites

In 2001 and 2002, we sampled mites from naturally infested colonies in different sites in Aragón (northeastern Spain) and used 10 apiaries that had received various fluvalinate treatments. The long-term treatment based on the use of fluvalinate strips year-round, overdosing, and the use of agricultural formulations makes the development of resistance more probable than does the emigration of resistant mites (Watkins, 1997). Therefore, we chose apiaries (Fig. 1) never treated with fluvalinate (A and B), apiaries routinely treated with fluvalinate (C, D and E), and others with a history of fluvalinate treatment until 1999, when the treatment agent changed to amitraz (F, G, H, I and J).

We conducted all assays in apiaries that had 25–50 colonies and on a minimum of five colonies in each apiary. Infested combs (or parts of combs) were brought to the laboratory and adult female mite removed from capped brood by opening and inspecting individual cells. Mites were put into Petri dishes containing damp filter paper and honeybee larvae from just-capped cells within 24 h before the assay. Combs were sampled as soon as possible after they arrived in the laboratory and no more than 24 h after being collected. Combs were stored at 34°C and 60–80% relative humidity in the dark.

We discarded diseased bee larvae and mites found dead or moribund. The mites from colonies belonging to the same apiary were pooled.

Laboratory tests

Bioassays were performed using fluvalinate (Ehrenstorfer, 76% purity) following the technique described by Milani (1995). The active ingredient was dissolved in hexane, incorporated into paraffin wax (Merck 7151, melting point 46-48° C), and the solvent evaporated. Control paraffin was prepared using the same volume of solvent as used with the treated paraffin. The glass disc and steel ring were coated with the treated paraffin. The remaining solvent was evaporated overnight and the discs placed together to form a capsule (before being used, the samples were stored in the dark at 30–32° C and 60–80% relative humidity).

Ten or 15 mites were introduced into each capsule. After 6 h, the mites were transferred to a clean Petri glass containing two or three worker larvae taken from cells 0-24 h after capping. Typically, we used two or three replicates (sometimes five) per concentration. The mites inside the capsules and in the Petri dishes were kept in an incubator at 32.5° C and 70% RH. We observed mites under a dissecting microscope at 6 h (when transferred to the Petri dish), 24 h and 48 h after the beginning of the treatment, and each was classified as being either mobile, paralyzed, or dead (Milani, 1995). Mites that were lost or accidentally killed were not included in the analysis.

In 2001, we collected mites from 10 apiaries, which we assayed in capsules coated with paraffin containing 200 mg/kg fluvalinate which is the diagnostic dose (susceptible mites die and resistant mites survive: Milani, 1995; Trouiller, 1998). In 2002, to obtain the median lethal concentration (LC_{50}), assays were performed on mites from six apiaries using various concentrations of fluvalinate: 0, 2, 5,10, 20, 50, 100 and 200 mg/kg. The apiaries were chosen from among the three types that differed in the use of fluvalinate.

To determine the resistance level, we compared the LC_{50} data with the susceptible *V. destructor* strains in the same assay and the data from other susceptible populations (Milani, 1995 and Trouiller, 1998).

Statistical analysis

The data were analyzed using the probit transformation (Finney, 1971) as in Milani (1995), with an Excel 4.0 spreadsheet.

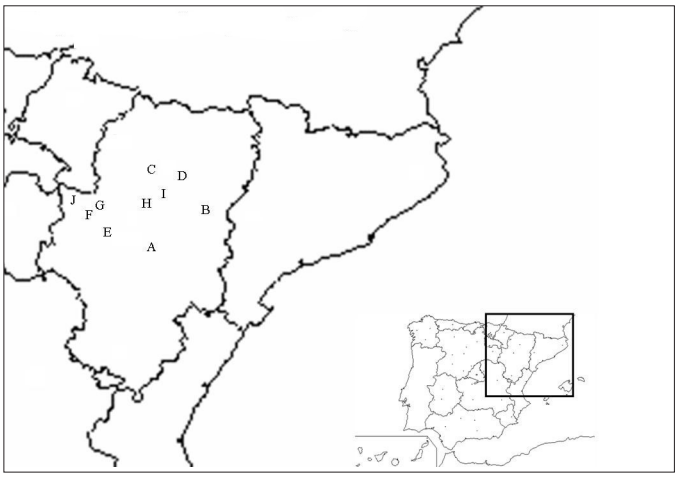

Fig. 1. Location of the apiaries sampled

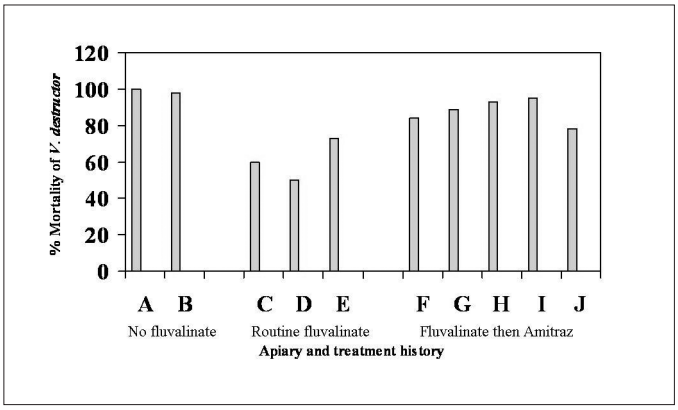

Fig. 2. Percent mortality of *Varroa destructor* mites at the diagnostic concentration (200 mg/KG)

Results

The results of the application of the diagnostic dose (200mg/kg) of fluvalinate carried out on the 10 apiaries are indicated in figure 2. The 10 apiaries varied greatly in their sensitivity to the diagnostic dose. The mortalities varied from an average of 50 to 100%.

The median lethal concentrations (LC_{50}) in six mite populations are presented in Table 1. Apiary A stands out for its low LC_{50} (0.13 mg/kg), followed by apiary H with a LC_{50} of 23.27 mg/kg, apiary G with a LC_{50} of 73.52 mg/kg, apiaries C and J with similar LC_{50} (142.08 and 150.57 mg/kg respectively) and finally the extraordinarily high LC_{50} of the apiary D (1882.38 mg/kg).The ratios of resistance are presented in Table 2. Due to the high sensitivity to fluvalinate observed in apiary A, which differed significantly from the other values, we calculated the ratio using apiary H. When we compare the LC_{50} data of apiaries with the LC_{50} of the sensitive strain observed in apiary H and with the reference sensitive strains reported by Milani (1995) and Trouiller (1998), we observe resistances that are 1.4 to 110.7 times higher.

Table 1. Median lethal concentration (LC_{50}) and its fiducial limits (and slope) in six *V. destructor* populations.

Origin of mites	LC_{50}	Fiducial limits	Slope
A	0.13	-1.82	0.2
H	23.27	9.69-42.01	1.1
G	73.52	34.66-149.46	2.1
C	142.08	77.86-478.82	1.1
J	150.57	72-16-1243.22	1.2
D	1882.38	182.98-	0.5

Table 2. Ratios of resistance calculated as LC_{50} in the considered population / LC_{50} in the most susceptible population.

	LC_{50} MOST SUSCEPTIBLE POPULATION		
Apiary (origin of mites) H*	**Present study, (LC_{50} 23.27)**	**Milani (LC_{50} 17)**	**Trouiller (LC_{50} 25)**
A (LC_{50} 0.13)			
H (LC_{50} 23.27)		1.4	
G (LC_{50} 73.52)	3.2	4.3	2.9
J (LC_{50} 150.57)	6.5	8.8	6
C (LC_{50} 142.08)	6.1	8.3	5.7
D (LC_{50} 1882.38)	80.9	110.7	75.3

* see explanation in text

Discussion

In the treated mites, the evidence of fluvalinate intoxication (death and paralysis) changed with time. The percentage of paralyzed mites decreased from 24 h.

(8.5%) to 48 h (3.2%). In the most sensitive populations, the effect at 24 h was more rapid and evident and less variable than at 48 h. In the most resistant populations, the effect was delayed. In apiary E, but not in the others, there were differences in mortality after 24 h and after 48 h. It was difficult to differentiate immobile from dead individuals, and the response was more apparent and uniform with few immobile individuals; therefore, we believe that the observations after 48 h are more reliable. The method described by Milani (1995) was easy to use and we support the view that death should be evaluated after 48 h.

The 10 apiaries varied (50-100% mite mortality) in their sensitivity to the diagnostic dose of fluvalinate. Other studies reported a positive correlation between laboratory and field data (Trouiller, 1998; Thompson *et al.,* 2002) or concluded that the laboratory test is more sensitive than a field efficacy test (Trouiller, 1998). Even if normal efficacy occurs in the field, some mites may be resistant. Thompson et al. (2002) used the diagnostic dose (200 mg/kg), and resistance was confirmed if efficacy was #70%. In our study, efficacy was <70% in two apiaries (C and D).

Nevertheless, in our study, 90% efficacy after applying the diagnostic dose suggests a borderline between populations that are strictly sensitive to fluvalinate and those that have a decreased sensitivity of *V. destructor* to this pyrethroid. Consequently, we believe that mortality >90% (after application of the diagnostic dose) might indicate sensitivity of *V. destructor* to fluvalinate. From those apiaries with a mortality above 90% after the application of the diagnostic dose and with LC_{50} data, the results from apiary A are surprising due to the low LC_{50} (LC_{50} 0.13 mg/kg) compared to those reported by other authors (Milani, 1995 and Trouiller, 1998). In apiary A, the absence of previous fluvalinate treatments may explain the apiary's extreme sensitivity. The LC_{50} obtained in apiary H (LC_{50} 23.27) is the only one that was similar to other studies for reference sensitive populations. Indeed, the toxicity of fluvalinate to the susceptible mites from apiary H was similar to that reported by Trouiller (1998), (LC_{50} 25 mg/kg) and slightly higher than that reported by Milani (1995), (LC_{50} 13-19 mg/kg).

In the other apiaries, all of which exhibited mite mortality < 90% after the application of the diagnostic dose, there was a decrease in the sensitivity of *V. destructor* to fluvalinate. The LC_{50} values obtained in apiaries G, C, and J were higher than those in A and H, and the sensitive reference populations studied by Milani (1995), (13–19 mg/kg), Trouiller (1998), (25 mg/kg), Mozes-Koch et al. (2000), (41.5 mg/kg), and Thompson et al (2002), (42.7 mg/kg). Nevertheless, the values from A and H were lower than the values of those strains considered resistant in the studies of Milani (1995), Trouiller (1998), Mozes-Koch et al. (2000), and Thompson et al (2002).

Since one population is formed with resistant and susceptible phenotypes, the intermediate stage probably consists of a low frequency of resistant individuals. However, the presence of a few resistant mites represents the beginning of the resistance but not the immediate ineffectiveness of the treatment. That is, although

the treatment is efficient, it suggests that alternative treatments should be considered.

In apiary D, we obtained the highest LC_{50} (1882.38 mg/kg). Although the LC_{50} in that apiary is lower than that observed by Trouiller (1998), (9234 mg/kg) in his reference resistant strain, it is higher than the resistant strains reported by Milani (1995) in Italy (857 mg/kg) and Thompson et al (2002) in the UK (477 mg/kg). We observed ratios of resistance that were 1.4 to 110.7 times higher (Table 2). Specifically, apiaries G, C, and J, when compared to apiary H and two reference sensitive strains (Milani, 1995 and Trouiller, 1998) were three to almost nine times more resistant. The ratio between apiary D and the reference sensitive strains from H, Milani (1995) and Trouiller (1998) had populations of *V. destructor* that were 80, 110 and 75 times more resistant than the reference populations, respectively.

Milani (1995) found that the LC_{50} of mites from areas where treatment with fluvalinate was no longer effective was about 25–50 times higher than areas where the mites were susceptible. In our study, the LC_{50} was up to 110 times higher, so that although we do not have efficacy data from the field, we would expect it to be low.

Our study is the first to report *V. destructor* resistance to pyrethroids in Spain. Trouiller (1998) monitored the spread of resistance to pyrethroids throughout Europe and his data support the theory that the resistant strain originated in Italy in the early 1990s and spread to Slovenia, Switzerland, France, Belgium, and Austria. The samples from Spain did not show resistance. Trouiller (1998) remarked that if resistance was confirmed in Spain, the country could be the site of another primary resistance centre, independent of the Italian strain.

The long-term, continuous use of fluvalinate strips year-round, overdosing, and the use of agricultural formulations of pyrethroids (Watkins, 1997) has increased the selection pressure on mites and makes the development of resistance within the area more probable than does the importation of resistant mites. In general, we observed a very close relationship between mite sensitivity to fluvalinate and the use of this acaricide. There was a trend for differences between apiaries in their resistance and history of use of fluvalinate. The apiaries that still use fluvalinate (C and D) have the highest LC_{50}, while those that never used it (A) or stopped using it in 1999 (H) have the lowest LC_{50} values. That said, the results from apiary J are surprising because apiary H has not used fluvalinate since 1999, yet it had a high LC_{50}. It is possible that resistant mites did not undergo the same degree of selection when the fluvalinate treatment ended and at that time selection for resistant mites in apiary J was high.

We do not know the previous selection pressures on the apiaries, so we cannot measure the extent of the recovery. The frequency of resistant mites declined after the acaricide treatment ceased (reversion). Milani and Della Vedova (2002) pointed out that the percentage of resistant mites decreased by approximately ten times in three years in populations of *V. destructor* not treated with this pyrethroid.

In areas that have resistant strains of *V. destructor* (and assuming a ten-fold decline in resistance over three years), treatments with these acaricides might be effective if they are administered every 4–6 yr and promptly followed by another treatment to eliminate as many resistant mites as possible before mites are exchanged between colonies (Milani and Della Vedova, 2002).

If we assume this in our case, a minimum of an additional three years would be necessary before fluvalinate use could resume. In this interval, the active ingredient should not be used. The persistent use of a single acaricide (fluvalinate) will exert a strong selection pressure for fluvalinate resistance in mite populations. Our results indicate a need for an alternative class of miticide for mite control. In Spain, the only registered products for *V. destructor* in the presence of brood are the pyrethroids fluvalinate (Apistan®) and flumethrin (Bayvarol®), and one formamidine amitraz (Apivar®).

The detection of resistance to fluvalinate justifies an assessment of cross-resistance with the other pyrethroid, flumethrin. The presence of cross-resistance is not unexpected because of the similarity of the molecular structures of the compounds. Milani (1995) and Thompson *et al* (2002) clearly demonstrated cross-resistance between fluvalinate and flumethrin. Furthermore, resistance is not limited to the pyrethroid varroacides because it has been reported for coumaphos and amitraz (Milani, 1999).

In the use of new products, it is important to avoid the problems observed with fluvalinate. The improper use of varroacides will probably accelerate the selection of resistance in mites. Pettis (2004) detected resistance in mites after only three years of using coumaphos. That result emphasizes the risk of basing strategies for control on chemical treatments only and the urgent need to implement more rational approaches to the fight against *V. destructor.* There is a renewed interest in substances of natural origin such as essential oils and their components, or organic acids, especially formic acid and oxalic acid (Colin et al, 1999). Those measures could be coupled with the selection of resistant bee strains.

In short, an integrated pest management approach needs to be developed to manage the growing problem of acaricide resistance in V. destructor populations worldwide.

Acknowledgements

We thank N. Milani of Udine University for his scientific assistance in the operative stages and especially for his help with the computing. We are grateful to the beekeeping associations in Aragón that made the assays possible. This study was carried out with the financial contribution of the Spanish INIA (project API 98-008).

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ORIGINAL RESEARCH ARTICLE

Production Costs Of Conventional And Organic Honey In The Yucatán Peninsula Of Mexico

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Received 15 June 2005, accepted subject to revision 29 July 2005, accepted for publication 11 July 2006

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Summary

In the Yucatán peninsula of México, ca 16,000 beekeepers are indigenous Mayan farmers living in relatively natural, undisturbed regions and practicing traditional forms of *Apis mellifera* beekeeping. The economics of family survival, where large profits from honey sales are not possible, are distinct from the economics of the large market economy surrounding them. According to market economic theory, in the years 2002 and 2003 those beekeepers who did not produced more than 1,200 kg honey per year did not recover their investments, at least as regards variable costs; in other words, they paid more than they gained through sales. In addition, it is shown that organic (ecologic) honey production increases costs about 70% above that of conventional honey.

Keywords: honey, honey markets, economy, ecological honey, cost of production, Yucatán Peninsula

Introduction

The exploitation of bees is a long tradition in southeastern México where it was practiced with the native stingless bee *Melipona beecheii* before the arrival of the Spaniards in the 16th century. Beekeeping experienced a huge transformation as a result of the introduction of the European honey bee, *Apis mellífera.* Today *A mellifera* beekeeping predominates in the Yucatán, but there remain large demographic and social distinctions among beekeepers. The commercial sector, comprised of around 1,700 beekeepers of largely non-Mayan descent, is responsible for making the Yucatán peninsula a world leader in honey production, with 95% of its production going to international markets (Cajero, 1999). Another sector, comprised of ca 16,000 loosely-organized beekeepers of indigenous Mayan descent (SAGARPA 2003), lives in relatively undisturbed natural areas in the states of Yucatán, Campeche and Quintana Roo and practices traditional forms of *A mellifera* beekeeping.

These traditional beekeepers have, on average, <25 hives with an average yield of 30 kg honey per year (Secretaría de Desarrollo Rural y Pesca 2002, SEDARI 2001, Tron 2001). For many of them, beekeeping represents their only source of cash income aside from wages from occasional jobs (Villanueva & Collí Ucán 1996). Other activities such as hunting, agriculture and livestock management are directed toward home consumption. Sands (1984) explains how traditional beekeeping in the Yucatán is distinguished from that of the market economy in that the activity is not pursued for optimum economic gains. Echazarreta (1999) and Villanueva & Collí Ucán (1996) make the point that beekeeping complements other subsistence pursuits such as plant and animal agriculture and forestry.

According to the European norm (Art 11 CEE/ No 2092/91 of June 24, 1991), "ecologic apiculture" (organic honey production) is that practiced in non-disturbed natural reserves or in regions unaffected by modern agriculture. The requirement is a

production area characterized by minimal human-induced impacts on the environment, especially to the wild flora that impacts foraging bees. Additionally, the use of chemicals is not permitted in the feeding or health management of bees or in the maintenance of hive equipment (SAGARPA 2003). There are several natural reserve zones in the Yucatán peninsula that ensure large tracts of natural vegetation and make possible the production of certifiably organic honey. Two organizations, "SSS Chilaan Kabo'ob" and "Hai Ich Cab" in the state of Quintana Roo produce honey certified organic by Naturland-Verband, Germany.

The problems that vex Yucatán beekeepers in general have hit the traditional sector particularly hard. These include the arrival of Africanized honey bees (*Apis mellifera scutellata*) and tightening requirements for product quality and inventory tracking for the international market (Villanueva G & Collí Ucán 1996, Jiménez 1998, Echazarreta-González 1999, Güemes-Ricalde & Pat 2001, Güemes-Ricalde & Villanueva G 2002).

The objective of this work is to describe the costs incurred by traditional Yucatán beekeepers in the production of conventional and organic honey. This was done taking into account the present production conditions and the social and economic constraints of beekeepers.

Materials and Methods

The information reported herein is the result of a survey carried out in 2002 on a sample of 538 of the ca 16,000 traditional beekeepers living in the Yucatán peninsula (CIAP-SAGARPA, 2003). Respondents were distributed over 47 communities representing the three states of Yucatán, Campeche and Quintana Roo. The survey consisted of two parts: the first related to general conditions for bee production and the second to details about fixed and variable costs. Survey questions addressed a number of activities related to Mayan beekeeping such as the degree of family labor employment, number of colonies owned, distance (km) between apiaries, distance between apiaries and the beekeeper's houses, yearly honey yields, capital invested throughout the production period, and the type of equipment and materials used for honey production. Data on prices received and summaries of costs incurred were obtained directly at production sites, and cost / benefit relations subsequently determined.

The protocol employed to obtain information about costs was derived from the Organization of United Small-Scale Beekeepers of Latin America (PAUL) and the Fair Trade Labeling International Organizations (FLO) as set out by Munguía (2000). The handbook for calculating the cost of honey in Yucatán by Echazarreta (2002) was also used. Costs included manpower, materials, depreciation and transportation costs for marketing or selling honey in retail centers, with the exclusion of retail centers' operational costs as these are not borne by beekeepers. The so-called implicit costs included the rent value of resources employed in production, costs for capital use, and opportunity costs when the use of production resources necessarily implied the partial or total neglect of other activities (Martinez, 1999).

Concerning organic honey production, the implicit costs were calculated, unlike in the case of conventional honey in which the subsidies granted through programs such as "Alianza para el Campo" (Alliance for Agriculture) and programs for temporary employment, among others, correspond to the implicit costs. For this reason, in reckoning the costs of organic honey we estimated a 70% updating factor on top of the overall costs related to conventional honey production. This factor was calculated on the basis of market prices of the necessary new equipment, certification costs, and according to what beekeepers waiting for certification for the organic market declared. This 70% also includes the greater explicit production costs required to produce organic honey.

We used Ordinary Least Squares (OLS) regression analysis as a method appropriate for studying the relationship between two or more variables (Gujarati 1995, Wooldridge 2002). We were interested in explaining how *y* varies with changes in *x*. OLS lets us estimate a linear function of *x*, linear meaning that a one unit increase in *x* changes the expected value of y by an amount *B*. The task is to estimate the unknown parameters $B_1, B_2, B_3, \ldots B_n$.

After carrying out the following statistical significant tests: t tests for parameter values, *F* tests for the pattern as a whole, and homoscedasticity, colinearity and Durbin Watson trial statistic tests to detect self-correlation (Table 1), the results of the OLS regression for the function of Total Variable Cost (TVC) enable us to demonstrate that the research results are valid and generally applicable for the Yucatán peninsula.

Table 1. Results of the regression analysis of the total variable cost (TVC) curve through the ordinary least squares method.

CURVILINEAR ESTIMATE

Dependent variable: TVC, Method: cubic	
Independent variable: 2002 production ($Prod_{2002}$)	
Listwise deletion of missing data	
Multiple *R*	0.76
R^2	0.57
Adjusted R^2	0.57
Standard error	3684.9
Durbin-Watson	1.7
Usual estimate error	3684.9

ANALYSIS OF VARIANCE

	df	Sum of Squares	Mean Squares
Regression	3	9209829045.2	3069943015.1
Residuals	510	6925011374.1	13578453.7
F	226.1		
P > *F*			0.0000

VARIABLES IN THE EQUATION

Variable	B	SE B	BETA	t	∀
$Prod_{2002}$	4.624763	0.297526	2.185632	15.5	0.0000
$Prod_{2002}$**2	-0.000423	4.4374E-05	-3.632739	-9.5	0.0000
$Prod_{2002}$**3	1.18126773E-08	1.4358 E-09	2.230841		0.0000
(Constant)	3461.875558	300.023351	11.539	11.5	0.0000

The cost function is the most useful tool for studying the economic behavior of a firm. The cost function summarizes all economically relevant information about the firm's inputs. The total cost curve relates output and costs. In the short term the cost function has two components: fixed costs and variable costs. The average cost curve, however, can increase or decrease with output. The reason for this is the variable costs. Average variable costs reflect first increasing and then decreasing average product of labor. The total variable cost (TVC curve) is the total variable costs divided by the quantity of output produced (Hall and Lieberman 1998).

It is demonstrated that the TVC function of the Sample Regression Function (SRF) or that obtained by the sample (Table 1) is statistically valid for assessing the Population Regression Function (PRF). This means that on the basis of the SRF data or those obtained by the sample, it is possible to predict 57% of the time the average TVC of the overall studied population with a 95% confidence level at ∀0.05 precision. Although R^2=0.5780 does not necessarily implicate a robust model for prediction, in the case of such a large sample which includes cases of high production it was decided not to eliminate them from the sample, even though adjustment in the pattern could be lost since at a given moment they would help show the real production costs with different production and efficiency levels, which does not limit the results obtained in our SRF with the purpose of predicting PRF (Fig. 1).

When comparing the theoretical cost curves with the results obtained from the SRF for the Yucatán peninsula, we see that they coincide in their development and in what they show in the form of mathematical and graphic functions. This gives us confidence to use this model in order to explain gain and loss situations of our subject beekeepers.

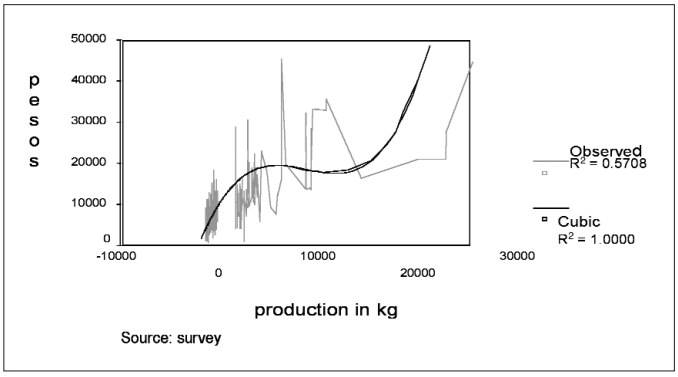

Fig. 1. Curve of Total Variable Cost in honey production in the Yucatán peninsula

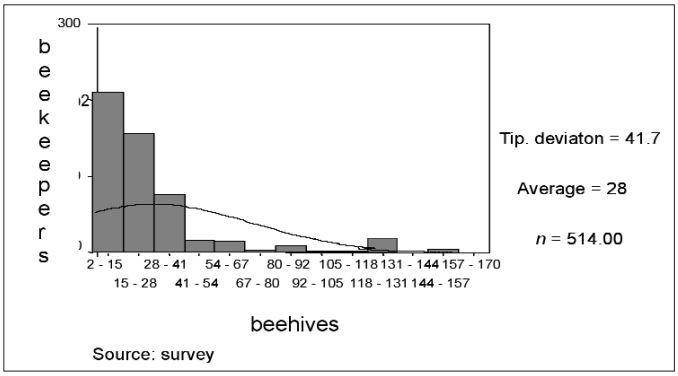

Fig. 2. Number of beehives for each beekeeper in the Yucatán peninsula

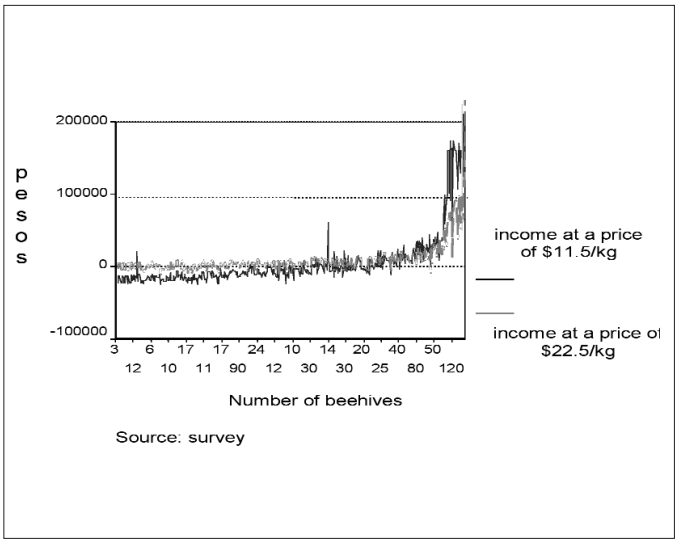

Fig. 3. Income difference for beekeepers in the Yucatán peninsula at 2002 and 2003 prices

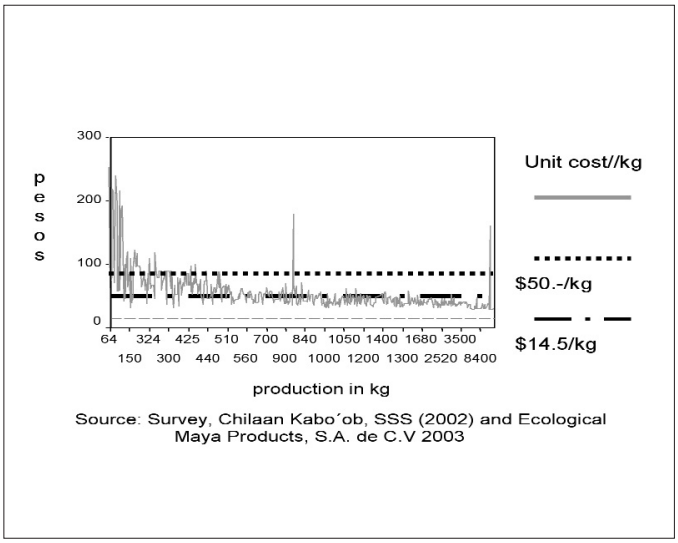

Fig. 4. Gain or loss situations in ecologic beekeeping in the Yucatán peninsula

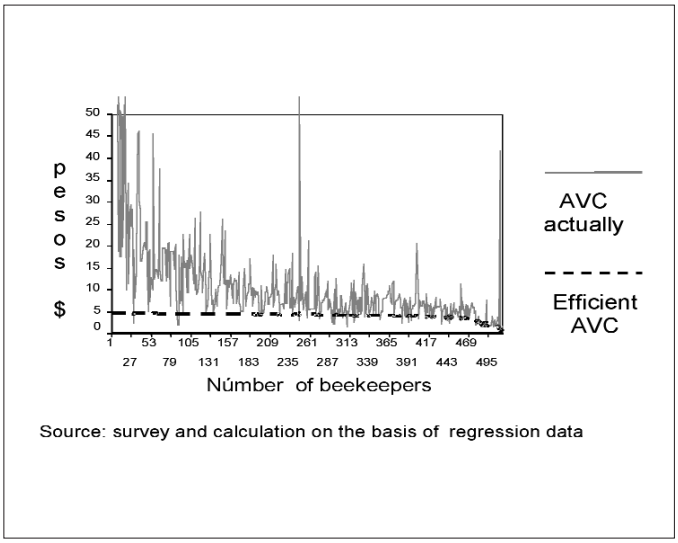

Fig. 5. Cost efficiency levels in beekeeping production in the Yucatán peninsula in 2002

Results

The average number of beehives for each beekeeper is 28. However, 40% of beekeepers have between 2 and 15 beehives; 31% have 15 to 28 beehives, and 16.5% have 28 to 40 beehives. Only the remaining 12.5% have more than 40 beehives (Fig. 2).

Cost and income analysis

As regards overall income, more than 50% of beekeepers do not get more than 10.53 pesos[1] per year. Conversely, in some cases some beekeepers earn more than 40,000 pesos per year.

The average overall investment per beekeeper in the Yucatán peninsula is 14,453.2 pesos. The state of Yucatán shows the highest average figures, amounting to 19,597 pesos. However, also in this state is found the widest deviation (14,035.6) from the average. As regards overall costs, it is followed by the state of Quintana Roo with 14,566.7 ∀ 6,464 pesos and the state of Campeche at 8,681 ∀ 6,520 pesos.

In 2002, total production costs were just covered by those who worked efficiently with 35 beehives, whereas others reaped some benefits with 50 beehives.

Figure 3 shows the different price levels received by beekeepers of the Yucatán peninsula and the benefits obtained in the following two situations. In 2002, the price of 11.5 pesos per kg honey began to generate income after covering total investment only when the beekeeper had at least 35 beehives, whereas in 2003 with a price of 22.5 pesos per kg the beekeeper would have obtained benefits at 11–15 beehives at 2002 production costs.

At the 2002 price of 11.5 pesos per kg, most beekeepers with an approximate production level lower or slightly higher than 2.5 kg honey suffered losses. Namely, what they got for 1 kg honey was not enough to compensate fixed and variable production costs. Roughly 87.5% of beekeepers registered losses, since in that year they produced less than 1,200 kg honey, owing to the limited number of beehives owned.

In 2003, the average price was 22.5 pesos per kg honey. Those who did not suffer damage from hurricanes reaped benefits in spite of reduced production levels over 2002 since at production levels slightly higher than 1,000 kg they could still obtain enough income to cover unit costs and obtain a profit (Fig. 3).

Figure 4 shows gains and losses of ecologic honey production in the Yucatán peninsula. In 2002 losses were registered below a production level of 84,000 kg honey, since in that year the price was low at 14.5 pesos per kg, and production costs were 70% higher than those for conventional honey.

[1] On 5 July 2003, the exchange rate of the Mexican peso against the US dollar was 11.42 PESOS=1 USD.

Total variable cost curve by OLS method

Through the regression results the following Total Variable Cost (TVC) function was obtained:

$$TVC = 4.624763Q - 0.000423Q^2 + 0.00000011Q^3$$

Starting from this equation, the marginal cost curve (MGC) was obtained, and with respect to Q it derives that:

$$MGC = 4.624763 - 0.0000000846Q + 0.000000033Q^2$$

Equalizing the previous MGC equation against marginal income (MGI) = average beekeeper price in 2002.

$$MGC = 4.624763 - 0.0000000846Q + 0.000000033Q^2 = 11.5$$

We then clear and equalize to zero.

$$6.875237 - 0.000846Q + 0.000000033Q^2 = 0$$

In order to obtain the extreme value of "Q" optimum we use

$$Q = \frac{-b \pm \sqrt{b^2 - 4ac}}{2c}$$

where Q=25,641 kg.

This extreme value of (Q) corresponds to the point at which the marginal cost and the average variable cost curves intersect. This point represents the balance point or the "optimum production" level for the average beekeeper in the Yucatán peninsula. It represents the lowest production unit cost. Beyond this point, an increase in production will bring about a higher increase in costs than in profits.

Producing below this level would mean not seizing the chance of obtaining a greater total income with relation to total production cost. The existence of losses or gains will depend on the relation between the unit price paid to the producer and the production unit cost. In order to generate this production level it is estimated that, given the technical condition of production, and with an average productivity of 33 kg per beehive, an average of 777 beehives will be needed.

In order to obtain the minimum of production, it is necessary to obtain the Average Variable Cost function (AVC), which can be obtained by dividing the AVC function by the level of production as follows:

$$AVC = \frac{4.624763Q - 0.000423Q^2 + 0.00000011Q^3}{Q}$$

$$\rightarrow AVC = 4.624763 - 0.000423Q + 0.00000011Q^2$$

$$\frac{d\,AVC}{d\,Q} = -0.000423 + 0.00000022Q = 0$$

Where Q= 19,227.

This represents the minimum point of the average variable cost curve, namely, 583 beehives producing an average of 33 kg per year. This is the production level necessary to obtain at least the variable costs invested in production in terms of maximum efficiency.

In order to obtain the price at this point which guarantees retrieving at least the AVC per production unit, one substitutes the value obtained from Q minimum = 19,227 as follows:

$$AVC = 4.624763 - 0.00423(19{,}227) + 0.00000011(19{,}227)^2$$

AVC = minimum price to produce = 0.56

This price would be sufficient to make up for variable costs for one unit of production, provided that this level of production is reached with maximum efficiency. Likewise, by applying the same formula we calculate the maximum efficiency price according to the 2002 production of 1.2 kg which represents the average production level of more than 87.5% of beekeepers in the Yucatán peninsula. We found out that, to reach maximum efficiency in production, a price of 4.13 pesos/kg would be enough to cover variable costs to produce 1 kg of honey, provided that 1.2 kg of honey are produced with approximately 36 beehives.

Likewise, beekeepers who own about 15 beehives, with a production level of 495 kg honey, should reduce their variable costs to 4.62 pesos per kg honey. In this way it is possible to calculate the minimum price level needed to compensate for the variable costs per unit, which would allow beekeepers to remain in the market (Fig. 5).

After substituting the data for each product obtained in the sample in the regression functions, thereby obtaining the minimum AVC used to measure production economic efficiency, it becomes evident that in few cases typified in the sample the AVC can be found at minimum production levels. That is to say that beekeepers in the Yucatán peninsula operate under inefficient economic conditions, which entail increase in losses and reduction in net incomes due to the fact that production levels and the average productivity of variable costs are low, mainly with reference to labor.

Discussion

Our results can be considered a tool for analyzing the present situation and to appraise the likely future of beekeeping in this region which has been influenced by expanding markets and increasing market controls.

The use of the statistical method, even though limited to a sample of the overall population, allows us to affirm that the results derived in this analysis are representative of the Yucatán peninsula, unlike other works which have focused on micro-regions, organizations and case studies (Parra, 2002; Mayo, 2002).

As Cajero-Avelar (1999) points out, beekeeping in the Yucatán has been influenced by expanding market opportunities. This obliges producers to compete in an international market where prices are dependent on worldwide supply, and consumer countries apply higher non-tariff barriers to their markets with the aim of protecting domestic producers.

In contrast to the increasingly globalized free market, there remains a sustenance economy at the local scale as described by Labougle and Zozaya (1986). Beekeeping is a small-scale activity for most of the 16,000 beekeepers of Maya descent in the region. For these individuals, beekeeping is considered an investment since they know that sometime over the year an income will result. Generally, production is not sufficient to grant a permanent income. The bulk of income is devoted to debt payment and household expenses, mainly clothing, food, education and health. Two-fifths of it are used to pay for beekeeping equipment depreciation.

As pointed out by Villanueva G & Collí Ucán (1996), Echazarreta-González & Quezada-Euán (1997), Echazarreta-González (1999) and Güemes-Ricalde & Villanueva G (2002), beekeeping in the Yucatán peninsula has had difficulty moving beyond a sustenance level. The loss of assets and the decapitalization of beekeepers to make up for losses are repeated patterns emerging from the survey. Beekeepers claim that state and federal support to beekeeping is constantly decreasing, or comes never or too late. Moreover, damage from sporadic hurricanes is an unpredictable and dire difficulty. These problems are forcing beekeepers to reconsider their business and management habits.

It has been observed that more than 80% of beekeepers obtain an annual income of less than 20,000 pesos, which is linked with high costs and low production levels. About 87.5% of beekeepers own <40 bee hives, and 40% own 2 to 15 bee hives. These figures, compared with income levels, costs and overall production show that, according to the current average market price of 11.5 pesos in 2002, the number of beehives reported in the survey was not sufficient to generate an economic benefit for beekeepers.

According to market economic theory for the production of 2002 conventional honey, those beekeepers who had not produced more than 1.2 kg of honey per year did not recover their investment, at least as regards their variable costs; that is to say, they paid more than they gained through sales. In case beekeepers had to pay for labor, they would have had to produce at least 90 kg more honey over present amounts.

Although prices were high in 2003, the effect of hurricane Isidore in late 2002 was devastating for beekeeping in Yucatán due to hurricane-related losses of equipment and interruption of flowering. More than one year after the hurricane, beekeepers were still trying to recover assets.

One of the most sustainable attributes of beekeeping in the Yucatán peninsula is the availability of household manpower which is seldom reckoned in production costs. Employing household manpower does not represent a cost, and failing this, one can look for support from other beekeepers. This situation underlies the estimate that 80,000 people or more are linked with beekeeping throughout the year.

It is important to mention that low prices paid for ecologic honey in 2002 led many beekeepers to return to conventional honey production. However, in 2003 those who continued producing ecologic honey reaped 50 pesos per kg, which translated to a net benefit for those beekeepers who reached an approximate minimum production of 560 kg.

The fact that beekeeping is seen as a savings investment rather than a source of income may be the reason why beekeepers remain active despite losses, contrary to market theory. Through the Price or Market Theory, this logic seeks the best possible efficiency in manpower use by increasing average productivity with the aim of reducing costs and increasing gains (Samuelson & Nordhaus, 2002).

By this we do not mean that beekeepers should not improve efficiency of production or consider the market, but rather that the market consider the sociology of beekeepers as a tool toward sustainability rather than fixate on controlling production.

Without abandoning his production habits, the beekeeper must understand current markets in order to make his/her management more efficient, to avoid non-compliance of standards, and to meet consumer expectations in various markets. A goal should be to increase the product's added value and his/her profits. This can be done by packaging honey in consumer-ready increments and labeling honey with information about floral and geographic origins.

We agree with the calculations made by MABEVI (2002) about the benefit beekeepers could reap by efficiently working with 30 bee hives, but we disagree as regards the cost structure and accounting, particularly as to depreciation costs (fixed costs) and manpower costs (variable costs). We also disagree with Parra (2002) when he considers manpower a fixed cost, since this concept is variable and depends on the level of production.

It is important to carry out recapitalization of beekeepers to make up for losses due to climatic phenomena, aiming at transforming the equipment in line with market needs. Production levels must guarantee profit, but they must not be too high, as an excess of supply would have a negative effect on prices. This strategy, however, must be accompanied by a restructuring of the activity aiming at its diversification in order to cater for new market sectors.

The difference in prices of conventional honey and organic (ecologic) honey stems from market criteria as regards quality and production conditions of the two products. Organic honey quality must be higher in order to obtain certification on the world market. However, costs are substantially higher, and so it is necessary to increase productivity and the number of beehives for each beekeeper in order to make beekeeping an economically viable activity.

Acknowledgments

We thank Dr. Stephen Buchmann for his comments and help in translating the manuscript.

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ORIGINAL RESEARCH ARTICLE

Toxic effects of methanolic and dichloromethane extracts of flowers and peduncles of *Stryphnodendron adstringens* (Leguminosae: Mimosoideae) on *Apis mellifera* and *Scaptotrigona postica* workers

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Received 5 September 2005, accepted provisionally 10 March 2006, accepted for publication 14 June 2006.

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Summary

Laboratory bioassays were conducted to evaluate toxicity of methanolic and dichloromethane extracts obtained from *Stryphnodendron adstringens* to *Apis mellifera* and *Scaptotrigona postica* workers. The extracts were incorporated into the diet of the bees for evaluation of mortality rates. The ingestion bioassays were made with three concentrations (0.002mg/g, 0.005mg/g and 0.01mg/g) for each bee species. The workers were kept in cages, with twenty workers per cage for each concentration tested. All bioassays had sixty workers in three cages that where maintained in a biological oxygen demand incubator with controlled temperature and humidity. The data obtained in the toxicity bioassays were analyzed statistically by Log Rank test and all methanolic and dichloromethane extracts showed significant (P<0.0001) toxic effects in all tested concentrations.

Keywords: botanical extracts, honey bees, "barbatimão," toxicology

Introduction

Fossil evidence for relations between plants and insects date from the end of the Jurassic and the beginning of the Cretaceous periods (Zwölfer, 1982). Plants probably are the richest source of bioactive organic chemicals (Harborne, 1988). Secondary compounds in nectar and toxic nectar are geographically and phylogenetically widespread, but their ecological significance is poorly understood. Several hypotheses have been proposed for the possible functions of toxic nectar, including encouraging specialist pollinators, deterring nectar robbers, preventing microbial degradation of nectar, and altering pollinator behavior (Adler, 2000).

The western honey bee, *Apis mellifera* was introduced into Brazil several times in the 19th and 20th centuries, including the east African subspecies *Apis mellifera scutellata* (Ruttner, 1988). The native stingless bee, *Scaptotrigona postica* is also present. Known locally as mandaguari, this bee has a tropical and subtropical distribution and has been observed in most parts of Latin America (Nogueira-Neto, 1997).

Stryphnodendron adstringens (Fabaceae) is a common tree that occurs from south to west in Brazil. There are no references about the occurrence of this species in other parts of the world. Two plant species have been popularly known as "barbatimão:" *Stryphnodendron adstringens* (true "barbatimão") and *Dimorphandra mollis* (false "barbatimão") (Lorenzi, 1992). In spite of belonging to different genera, both species are sympatric (similar blooming period) from September to January, and the pollen of *Stryphnodendron polyphyllum* has been reported to be toxic to bees (Carvalho and Message, 2004).

In Brazil, studies done with "barbatimão" showed toxic activity of dehydrated and milled flowers from these plants when

incorporated into bee diets. The authors studied two species, *D. mollis* and *S. adstringens,* and even though both were toxic to bees there was no statistical difference between the two (Cintra *et al.,* 2003). These authors also worked with methanolic extracts from flowers and peduncles of *D. mollis* and isolated the flavonol astilbin which showed toxicity to honey bees under laboratory conditions (Cintra et al., 2002, 2005).

Studies conducted on the congeneric plant species *Stryphnodendron polyphyllum* have shown toxic effects on *A. mellifera larvae*. Pollen from this plant cause sacbrood virus-like disease symptoms in last stage honey bee larvae (Carvalho and Message, 2004).

In order to supplement this knowledge base on botanical toxicity toward bees, we examined the toxicity of methanolic and dichlorometane extracts of *S. adstringens* flowers and peduncles toward adult *A. mellifera* and *S. postica* workers.

Materials and Methods

Plant Material and Extraction

The flowers and peduncles of *S. adstringens* were collected in Corumbataí, São Paulo State, Brazil (22°15' S, 47°00' WE), from October through December 2004. This species has inflorescences separated into flowers and peduncles. The plant parts were dried separately in an oven (40° C) with air circulation and then milled. Extraction of dry plant materials was performed with dichloromethane (3 times) followed by methanol (2 times). The amount of solvent used each time was 0.5 L per 88.5 g dry mass for peduncles and 1.0 L per 183 g dry mass for flowers.

Biological assay

Apis mellifera worker bees were collected at the UNESP Rio Claro apiary. Newly-emerged bees are visually distinguishable from adults up to ca 24 h age. Therefore, test cohorts of honey bees were nearly the same age (# 24 h) and collected from the same colony. The *S. postica* combs were collected from the same colony at the UNESP Rio Claro meliponary and maintained in an incubator (28 ± 1°C) until emergence of the workers.

The methanolic extracts were incorporated in small percentages (0.002 mg/g, 0.005 mg/g or 0.01 mg/g) into artificial diet for the bees. For *A. mellifera,* the artificial diet consisted of 500 g dry sucrose and 100 g honey (5:1) and given as such to the control group. For *S. postica* the diet was the same, but supplemented with pollen collected from their hives. At each five days of bioassay the supply of pollen was replaced at a rate of about 1.5 g. Experience at our laboratory indicates that supplemental pollen is necessary for laboratory maintenance of *S. postica,* but not *A. mellifera.* In any case, we were not interested in comparative results between bee species.

The diet was offered to 60 bees, divided into three groups of 20 bees confined in wooden cages (11 cm x 11 cm x 7 cm). Twenty bees has been shown to provide adequate survivorship under these conditions (Betioli, 1989). The control group received only the diet and water daily. The diet was placed in plastic containers, each 2.8 cm in diameter and 5 mm tall and covered with a fine metalic mesh (3.5 cm diameter) to avoid contamination by contact and permit access to bees feeding. The diet was replaced at each 5 days. The experiments were performed over a period of 25 days, at 32 ± 1° C for *A. mellifera* and 28 ± 1° C for *S. postica* with controlled humidity of about 70% in a BOD apparatus (Biological Oxygen Demand incubator). The number of dead bees was recorded daily.

Statistical analysis

The daily survival rate for each treatment was calculated, and the non–parametric Log Rank test (Motulsky, 1995) applied a posteriori to compare each treatment with the control group. The Log Rank test considers each bee one replication, so sample size (*n*) in tables 1-8 is 60, the sum of three sets of 20 bees.

Results

The methanolic extracts of flowers and peduncles showed toxic effects to both bee species (Tables 1, 3, 5, 7). Signs of poisoning, including paralysis of legs and difficulty walking, were observed for all extracts tested. The dichloromethane extracts were also toxic, and showed toxic effects in all concentrations tested for both bee species (Tables 2, 4, 6, 8).

Discussion

In experiments with methanolic extracts of *S. adstringens,* we observed reduced survivorship for treated bees (see tables), confirming results obtained by Cintra et al (2003) with dehydrated flowers. Cintra et al (2003) dehydrated flowers from both species and offered them to bees incorporated into artificial diet at low concentrations (2.5% and 5%). The obtained results indicated that flowers of "barbatimão" reduced the median survivorship of bees. In considering the choice experiment, bees of the treated group had low survivorship.

Cintra et al (2002) also evaluated the toxic properties of methanol extracts of flowers, peduncles, leaves, petioles and stem bark of *D. mollis* against *A. mellifera* workers. Astilbin was isolated from the peduncles and flowers of this plant in large amounts. In this case, the concentrations utilized were the same as the present study: 0.2, 0.5 and 1%. The results found for *S. adstringens* indicated that this species is as toxic as D. mollis. Corroborating these results, the methanolic and dichloromethane extracts from flowers and peduncles of *S. adstringens* present toxic properties against bees.

However, dichloromethane extracts of *D. mollis* were not toxic to *A. mellifera* bees (Cintra, unpublished). *S. adstringens* extracts tested in this work presented significant results against both bee species in all concentrations tested, suggesting the possibility that more than one substance that can cause mortality to bees.

Some experimenters have removed nectar from flowers, performed laboratory bioassays, and demonstrated that nectar was the cause of toxicity (Palmer-Jones and Line, 1962; Clinch et al, 1972; Sharma et al, 1986). An emphasis on toxicity toward honey bees, which are not the native pollinator for many of these plants, raises questions whether native pollinators are as affected as introduced species. In the case of *S. adstringens,* the toxic

effects of methanolic and dichloromethane extracts to bees of a native species, *S. postica* as well as *A. mellifera* are shown in the present study.

Numerous studies have demonstrated secondary compounds in nectar without testing the effects of these compounds on floral visitors. Therefore it is not known whether these compounds occur in sufficient concentrations to have ecological consequences (Adler, 2000). S. adstringens has extrafloral nectaries, very small flowers, and small pollen grains ca 33μm/30μm (Cintra et al, 2000). The small quantities of nectar and pollen do not facilitate ingestion experiments.

Only one study offered both nectar and a sugar solution in field tests; sugar solution was preferred over nectar by ants in two of four plant species (Feinsinger and Swarm, 1978). Besides these studies, honey bees have contacts with vegetative parts of plants to collect exudates to produce propolis (Park and Inegaki, 1998). Since is not possible to test nectar or pollen directly, it is important to use other methods to infer plant toxicity.

The next step of research would be the isolation and identification of components present in *S. adstringens* methanolic and dichloromethane extracts to evaluate their toxic effects on bees.

Table 1. Mortality percentage of *A. mellifera* (*n*=60) in feeding experiments with methanolic extract of flowers of *S. adstringens*

Concentration	DAYS										
(mg/g)	1	2	3	6	8	10	14	17	21	25	S_{50*}
Control	0	0	0	0	1.6	3.2	26.6	60	80	95	17*b*
0.002	0	0	0	1.6	3.2	16.6	65	93			13*c*
0.005	0	0	0	1.6	5	11.6	76				12*c*
0.01	0	1.6	1.6	5	45	86.6					9*c*

* S_{50} = survival median. Different letters after the S_{50} values show a significant difference according to the log-rank test.

Table 2. Mortality percentage of *A. mellifera* (*n*=60) in feeding experiments with dichloromethane extract of flowers of *S. adstringens*

Concentration	DAYS										
(mg/g)	1	2	3	6	8	10	14	17	21	25	S_{50*}
Control	0	0	0	1.6	5	13.3	23.3	45	70	86.6	18*b*
0.002	0	0	0	1.6	5	6.6	35	60	80	95	16*c*
0.005	0	0	0	0	5	6.6	30	53	81.6		15*c*
0.01	0	0	0	8.3	13.3	26.6	48.3	83.3			14*c*

* S_{50} = survival median. Different letters after the S_{50} values show a significant difference according to the log-rank test.

Table 3. Mortality percentage of *A. mellifera* (*n*=60) in feeding experiments with methanolic extract of peduncles of *S. adstringens*

Concentration	DAYS										
(mg/g)	1	2	3	6	8	10	14	17	21	25	S_{50*}
Control	0	0	0	0	6.6	10	45	60	86.6	95	16*b*
0.002	0	0	0	0	6.6	13.3	58.3	86.6			13*c*
0.005	0	0	1.6	5	10	26.6	80	95			13*c*
0.01	0	0	1.6	8.3	26.6	45	95				11*c*

* S_{50} = survival median. Different letters after the S_{50} values show a significant difference according to the log-rank test.

Table 4. Mortality percentage of *A. mellifera* (*n*=60) in feeding experiments with dichloromethane extract of peduncles of *S. adstringens*

Concentration	DAYS										
(mg/g)	1	2	3	6	8	10	14	17	21	25	S_{50*}
Control	0	0	0	0	5	5	18.3	45	63.3	80	18*b*
0.002	0	0	1.6	5	10	15	33.3	60	76	90	16*c*
0.005	0	0	3.2	6.6	8.3	15	35	61.6	86.6		16*c*
0.01	0	0	5	8.3	10	20	40	65	95		16*c*

* S_{50} = survival median. Different letters after the S_{50} values show a significant difference according to the log-rank test.

Table 5. Mortality percentage of *S. postica* (*n*=60) in feeding experiments with methanolic extract of flowers of *S. adstringens*

Concentration	DAYS										
(mg/g)	1	2	3	6	8	10	14	17	21	25	S_{50*}
Control	0	0	0	0	5	5	36.6	55	76.6		17*b*
0.002	0	0	0	0	1.6	13.3	58.3	80			14*c*
0.005	0	0	0	0	0	15	51	96.6			14*c*
0.01	0	0	0	1.6	3.2	20	70	98.3			13*c*

* S_{50} = survival median. Different letters after the S_{50} values show a significant difference according to the log-rank test.

Table 6. Mortality percentage of *S. postica* (*n*=60) in feeding experiments with dichloromethane extract of flowers of *S. adstringens*

Concentration	DAYS										
(mg/g)	1	2	3	6	8	10	14	17	21	25	S_{50*}
Control	0	0	0	0	5	5	36.6	55	76.6	95	17*b*
0.002	0	0	0	1.6	3.2	10	35	85			15*c*
0.005	0	0	0	5	10	15	60	91.6			14*c*
0.01	0	0	0	10	16.6	21.6	61.6	90			14*c*

* S_{50} = survival median. Different letters after the S_{50} values show a significant difference according to the log-rank test.

Table 7. Mortality percentage of *S. postica* (*n*=60) in feeding experiments with methanolic extract of peduncles of *S. adstringens*

Concentration	DAYS										
(mg/g)	1	2	3	6	8	10	14	17	21	25	S_{50*}
Control	0	0	6.6	13.3	15	16.6	43.3	56.6	75	95	15*b*
0.002	0	0	6.6	28.3	30	41.6	53.3	91			13*c*
0.005	0	0	5	25	30	51.6	85	93.3			10*c*
0.01	0	0	10	23.3	28.3	58.3	90				10*c*

* S_{50} = survival median. Different letters after the S_{50} values show a significant difference according to the log-rank test.

Table 8. Mortality percentage of *S. postica* (*n*=60) in feeding experiments with dichloromethane extract of peduncles of *S. adstringens*

Concentration	DAYS										
(mg/g)	1	2	3	6	8	10	14	17	21	25	S_{50*}
Control	0	0	6.6	13.3	15	16.6	43.3	56.6	75	95	15*b*
0.002	0	5	8.3	21.3	21.6	26.6	53.3	71.6	96.6		14*c*
0.005	0	3.3	3.3	8.3	13.3	20	86.6	96.6			11*c*
0.01	0	6.6	10	28.3	30	45	66.6	81.6			12*c*

* S_{50} = survival median. Different letters after the S_{50} values show a significant difference according to the log-rank test.

Acknowledgments

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (Fapesp), CNPq-MCT-Pronex, CNPq-PIBIC.

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ORIGINAL RESEARCH ARTICLE

A model for predicting geographic origin of honey from the same floral source

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Received 15 February 2006, accepted subject to revision 24 April 2006, accepted for publication 27 June 2006.

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Summary

A method for predicting geographic origin of honey of the same floral source coming from different areas is presented. For this purpose, Greek thyme honey was selected, and melissopalynological analysis of 180 samples produced in different areas was carried out. Discriminant analysis was applied to the results and a discriminant model was made by using absolute pollen grains frequency. The analysis showed a high degree of discrimination for honeys examined and a significant discriminative power for the model. The simulation performance of the model was estimated from an external testing set with verification from 90% to 100%, according to geographic area. Emphasis was given to the sampling method which limits the influence of beekeeping handling and accidental contamination and increases validity of the predictive model.

Keywords: honey, melissopalynology, geographical origin, discriminant analysis, predictive model

Introduction

Melissopalynology is considered as a reference method for the determination of botanical and geographical origin of honey. Botanical origin can be detected by recording the predominant, or in some cases, under-represented pollen types, while the determination of the geographical origin is more complicated. It is achieved since the pollen spectrum of the honey sediment reflects the flora of the area where the honey was produced. The honey production of an area mainly depends on the flora and climatic conditions, as the flowering and nectar production season can vary for the same species in different areas (Zimmerman, 1988). Alternatives to defining the geographical origin, such as metal content (Latorre *et al,* 2000; Paramas *et al* 2000), organic acids (Mato *et al,* 2004) and chemical composition (Krauze & Zelewski, 1991; Sanz *et al,* 1995; Gomez-Barez *et al,* 2000; Paramas *et al*, 2000; Devillers *et al,* 2004), have also been reported.

Differences in pollen spectrum among honeys from different territories and climatic zones are easy to detect. However, if the geographical zones are close or the honeys produced from the same floral source, variations are more difficult to distinguish (Bogdanov & Martin, 2002). Statistical analysis is useful for the geographic characterization of honeys originating from proximal geographical zones by using discriminant and cluster analyses (Sancho *et al,* 1991; Aira *et al,* 1998; Seijo & Jato, 1998; Herrero *et al,* 2002). Geographical distinction of honeys of the same botanical origin is accomplished through the characteristic combination of dominant and secondary pollen grains (Ricciardelli D'Albore, 1998). Honey characterization is essential when its commercial quality must be assessed. The establishment of geographical identification of honey production, with the aim to protect the production zones, leads to marketable honeys from particular areas. The objective of this study was to determine a general method for predicting the geographic origin of honeys of the same botanical origin produced in different areas. For this purpose, greek thyme honey was selected as it is considered of high commercial value and has been famous since the ancient times for its special aroma and flavor (Crane, 1979). Thyme honey is produced mainly from the plant *Thymus capitatus* (L) Hoffmanns & Link, and melissopalynological and statistical analyses have been used to create a model in order to obtain the best classification of honeys according to their area of origin. For this purpose, three areas of Greece were chosen wherein the vast majority of plants were *T capitatus.* Honey samples were collected from bee colonies located at the specific areas during three successive years. Pollen analysis was carried out and discriminant analysis applied to the results.

Materials and Methods

Sampling areas of plant material

The study was performed over three successive years: 2003, 2004 and 2005. As sampling stations, three areas of Greece were selected (Fig. 1). Area 1 is Grambousa at the west side of the island of Crete (35° 31'N and 23° 36'E); area 2 Dombrena (38° 13'N and 22° 59'E), and area 3 Fanos (38° 58'N and 22° 59'E) on the mainland. The sampling areas are considered traditional beekeeping zones, producing honey of special organoleptic characteristics. The areas are close to the sea, and flora consists mainly of herbs and low vegetation (phryganic) and in all areas the same thyme species (*T capitatus*) dominates.

Samples from plants that were at anthesis were collected in two periods per year: the first just before blooming of thyme and the second when 50% of the thyme flowers were at anthesis. The sampling area extended up to 2 km around the bee colonies, which is considered the maximum distance a bee can fly in the period of blooming (Steffen-Dewenter & Kuhn, 2003). Sampling consisted of collecting whole plants for accurate species determination. The identification of plant species and the nomenclature of plant families and species was carried out according to Flora Europaea (Tuttin *et al,* 1968–1993).

Bee colonies and honey collection

In order to compare pollen grains in honey with those collected from blooming plants, five bee colonies where transferred to each area before the blooming of *T. capitatus*. The hives were placed in one group fairly close to each other on a surface of about $20m^3$. The colonies had no stored honey and new built combs were added.

Four samplings were made in each experimental apiary: the first at the beginning of blooming of thyme, the second one week after the beginning of the blooming, the third when 50% of the thyme flowers were in anthesis, and finally at the end of the blooming period. Sampling consisted of collecting whole honey combs with at least half their cells sealed. Honey was extracted by straining and analyzed immediately after collection. Frames were checked against light for cells with stored pollen, which was removed.

Preparation of reference slides

Collected plant samples were carried to the laboratory while their flowers were still closed. Anthers were dissected under protected conditions in order to avoid cross contamination. Reference slides were prepared using the method of Louveaux *et al* (1978). The anthers of the flowers were washed in a watch-glass filled with ether. Ether was then decanted and pollen grains rinsed with fresh ether. After drying, a drop of 20% fructose solution was added. Fructose solution helps the pollen grains to be transferred easily and accelerate their swelling. The preparation was dried on a warm plate at a temperature up to 40°C and finally mounted with Entellan Rapid mounting media for microscopy (Merck 1.07961.0500). All samples were photographed with an OLYMPUS BX 40 light microscope equipped with a Sony digital video camera (CCD color camera a SS-DC 38P/45, Sony Corporation, Tokyo, Japan) and a reference collection was produced.

Honey sample analysis

In total 180 honey samples were gathered and analyzed during 2003, 2004 and 2005. Melissopalynological analysis was carried out according to the method of Louveaux *et al* (1978) using a nonacetalytic technique. For each sample, 10 g of honey was centrifuged and two independent slides prepared. The sediment was dried in 40° C and mounted with Entellan as above. 1200 pollen grains were counted and identified. Results were expressed in percentages. Microscopical observations were carried out in an OLYMPUS BX 40 light microscope at 200x. Pollen grain types were included only if they appeared at frequencies >1%. Also, pollen grains of nectarless plants were recorded but were not expressed in percentages (Louveaux *et al*, 1978).

Statistical analysis

For the evaluation of the results we performed discriminant analysis using SPSS software (Standard Version, SPSS Inc. 1999). Discriminant analysis is useful for building a predictive model of group membership based on observed characteristics of each case. The procedure generates a discriminant function for more than two groups, based on linear combinations of the predictor variables that provide the best discrimination between groups. The functions are generated from a sample of cases for which group membership is known; the functions can then be applied to new cases with measurements for the predictor variables but unknown group membership. The statistical study was conducted using a data matrix of absolute frequencies of each pollen type that appeared in each honey sample. Statistical analysis was carried out with the use of Wilks'λ method, a multivariate test of significance, which tests the equality of comparing groups. The values of this criterion show the differences between the groups and varies from 0 to 1. Values close to 0 indicate that group means are different, and values close to 1 indicate that group means are not different (equal to 1 indicates that all means are the same).

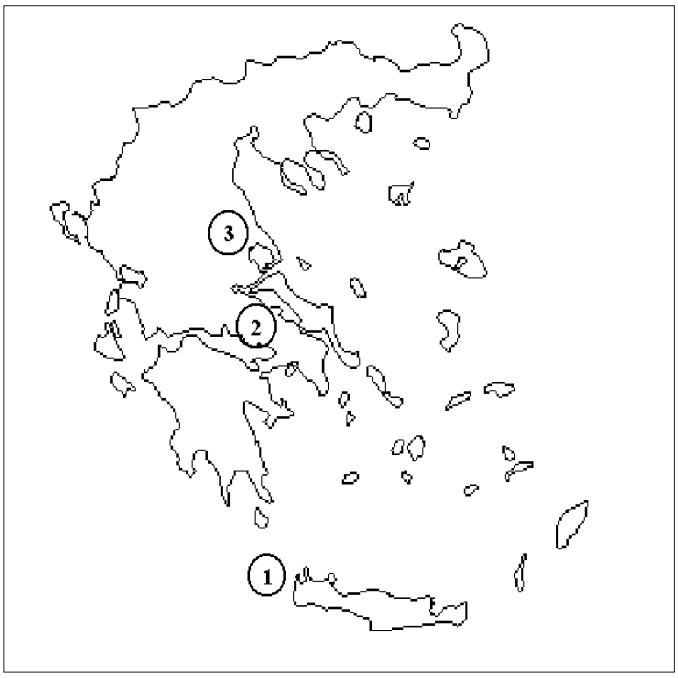

Fig. 1. Geographical areas of Greek honey production used in this study. Area 1: Grambousa, Area 2: Dombrena, Area 3: Fanos

Results

Melissopalynological analysis

In total, 122 taxa were collected in the three areas representing 36 plant families. Melissopalynological analysis of the honey samples show a wide variability. 40 taxa were found in honey sample coming from plants with beekeeping interest (Crane *et al,* 1984; Harizanis, 1996). From those, 21 were found in honey samples from area 1, 14 in honey samples from area 2, and 25 in honey samples from area 3. These taxa were classified in 14 families with highest representation from plants of the families Compositae, Labiatae, Leguminosae and Boraginaceae. Pollen grains of plants *T. capitatus, Ammi majus* L, *Echium italicum* L, *Hypericum empertifolium* Willid. and *Hirschfeldia incana* L were common in honey samples from the three areas (Table 1). The other 82 collected plants had no representation in honey.

From the 120 honey samples collected during 2003 and 2004, 90 were considered monofloral thyme type honeys and the other 30 as multifloral. According to Greek legislation (Greek Ministry of Agriculture, 2005), thyme honey must have a minimum representation of thyme pollen grains of 18%. In collected samples, the percentages of *T. capitatus* pollen grains varied from 27.3 to 95.7%. Table 1 describes the pollen grains which were identified in honey samples from the sampling areas. Mean of percentages and standard deviation are included.

Evaluation of predictive models

In order to achieve the best results from the statistical analysis, two predictive models have been created and tested. For model 1, the samples collected during 2003 and 2004 were used for creating the model and those of 2005 for its validation. For model 2, 60% of the collected samples consisting of equal numbers from each harvest year were used for developing the discriminant model and the remaining 40% for its validation. Models 1 and 2 correctly classified 99.2% and 100% respectively of the training sets and 92 % and 95% of the test sets. Model 2 has been chosen and presented here, as it provided a much more balanced evaluation, limited the accidental effect of environmental conditions to the model, and gave better results during validation.

Statistical approach

The Wilks'λ method evaluates the discriminatory power of functions. In the present study the Wilks'λ has a value of 0.014 for function 1 and 0.163 for function 2, declaring that both discriminant functions were significant and sampling areas were considered as different groups (Terrab *et al*, 2004).

For the evaluation of the geographical origin, the Canonical Discriminant Function Coefficients were used. The discriminant program calculates the canonical correlations between the variables entered. A canonical variable is a linear function of the variables selected by the program. The first canonical value is one, which best discriminates between honey samples. The second is the next best linear function orthogonical to the first. The method extracts n-1 canonical variables, where n is the number of groups to discriminate (Terrab *et al,* 2004).

In order to carry out the dicriminant analysis, data of the pollen analysis results were created. Each honey sample was considered as an assembly of variables represented by its pollen grain frequency. The coefficients create a quantitative model for the classification of unknown honey samples according to their area of origin.

The coefficients and constants for these functions are shown in Table 2 and plotted graphically in Fig 3.

The given value for the first function captures most of the differences among the areas (67.1%), followed by the second function (32.9%) with coefficients of canonical correlation close to unity, denoting that the variation of the data is due to differences among groups. The loading matrix of correlations between pollen grains and discriminant functions suggests that function 1 discriminates samples of area 3 from 1 and 2, while function 2 separates samples from areas 1 and 2.

The pollen grain type with the highest discrimination power for each function is described by the largest absolute correlation between each pollen grain type variable and the discriminant functions (Table 3).

The canonical scores of group means are presented in Table 4. The first canonical discriminant function in area 2 (2.441) is followed by that of area 1 (2.069), and both are significantly greater than area 3 (-4.509). The second discriminant function in area 1 (2.810) is followed by area 3 (-0.150) and 2 (-2.660). The canonical scores suggest that areas are well separated without overlap (Fig 2).

Furthermore, the area of origin was declared and we investigated the indentification between the actual and predicted area of origin. The results were classified into groups with the highest classification score, each representing the area of origin. All honey samples were classified correctly. In Fig. 2 the samples in each area are represented with their centroid as a function of the values of the two canonic functions showing the separation between the zones.

Validation of coefficients

A validation was performed in order to detect the correct classification of samples with unknown area of origin. For this purpose, 72 samples representing 40% of the collected samples during 2003, 2004 and 2005 with equal representation of each year were considered as a testing set for estimating the simulations performance of the discriminant analysis model. The results of melissopalynological analysis were applied to the function coefficients of Table 2. Correct classification was obtained to the majority of samples. The results are presented in Table 5. The correct classification for samples from area 1 is up to 100%; from area 2 it is 95% and from area 3 it is 90%, indicating a fairly good separation.

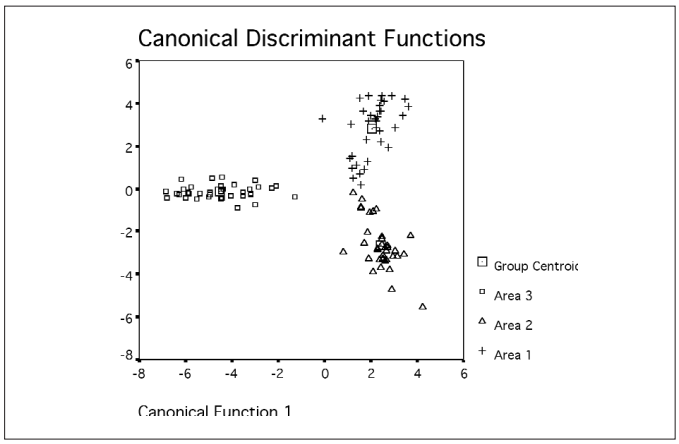

Fig. 2. Plot of canonical functions 1 and 2 for 108 honey samples used for creating the predictive model

Discussion

In the literature, geographic discrimination of honey is the aim of many studies as honeys from specific areas have higher value in the market. The vast majority of these works try to identify the area of origin using chemical methods (Krauze & Zelewski, 1991; Sanz *et al*, 1995; Gomez-Barez *et al*, 2000; Latorre *et al,* 2000; Paramas *et al* 2000; Devillers *et al*, 2004) and only a few include melissopalynological analysis or results from both (Sanz *et al*, 2004; Soria *et al*, 2004). In all cases the statistical analysis is essential for the evaluation of the results.

Physicochemical parameters like enzymes, hydroxymethylfurfural (HMF), moisture, acidity and conductivity have discrimination power varying from 51.67% (Paramas *et al*, 2000) to 83% (Sanz *et al*, 1995) for spanish honeys and 75% for french honeys (Devillers *et al*, 2004). Carbohydrate composition appears better, with results reaching absolute discrimination, but it is based on the fact that in different areas grow different plant species producing blossom or honeydew honeys (Sanz *et al*, 2004; Gomez-Barez *et al*,2000; Krauze & Zelewski, 1991). The sugar patterns of blossom and honeydew honeys can be different, especially in disaccharides and trisaccharides (Bogdanov *et al*, 2004). Mineral composition as an indicator of geographical discrimination is reported for spanish honey samples (Paramas *et al*, 2000; Latorre *et al*, 2000). Minerals in honey are related to the soil from the area where it is produced. Although the discrimination is up to 86.21%, authors indicate the high variability of results as well as possible contamination from processing equipment and containers. In most cases, a combination of the above criteria are suggested. This leads up to 30 different parameters which must be evaluated in different methods of analysis.

Organic acids seem to be promising for the determination of geographical origin, but may also be useful indicators of fermentation or the treatment of *Varroa destructor* infestation (Mato *et al*, 2004).

Melissopalynological analysis has some limitation related to the pollen production according to plant species (Molan, 1998) and also requires previous knowledge of pollen morphology in order to achieve reliable results (Downey *et al*, 2005). But still it seems to be the most important for the determination of the geographical origin of honey, especially when complemented by sensory analysis (Anklam, 1998; Paramas *et al*, 2000).

The pollen analysis is followed by a statistical analysis using discriminant or cluster analysis. Cluster analysis classifies samples into groups and seems more appropriate for distinguishing honeys of different botanical origins from different areas rather than distinguishing honeys of the same botanical origin from different areas (Herrero *et al*, 2002; Seijo*et al*, 2003). Discriminate analysis has the advantage of creating predictive models by using analysis of the absolute percentages of pollen and includes all pollen types present in samples connected to a certain honey of a certain area.

Results obtained in this study indicate that a high degree of discrimination has been obtained. The correct classification of unknown samples using the created predictive model varied from 90 to 100% according to the area of origin. The high verification confirms the discrimination power of the model and allows us to reach the goal of predicting the honey's area of origin.
The higher discrimination obtained here compared to those reported in previous studies (Sancho *et al*, 1991) is due to

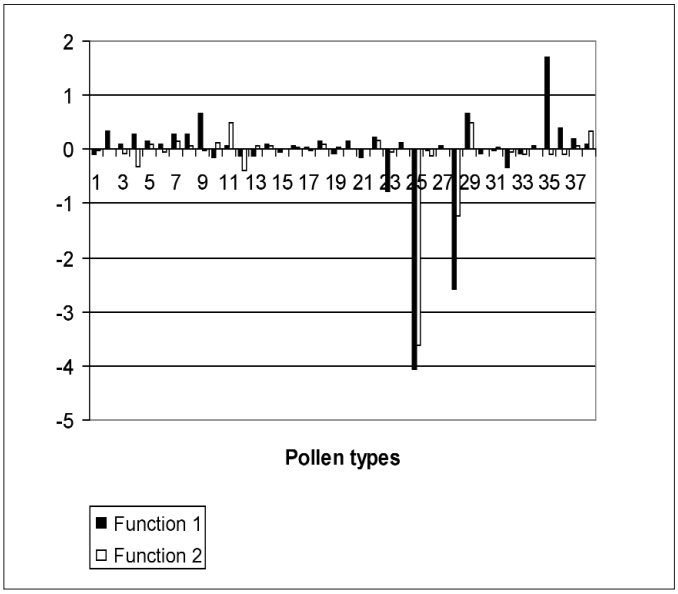

Fig. 3. Plot of the functions 1 and 2 of various pollen types. The numbers at the horizontal axis represent the increasing number of the plant species presented in Table 2.

sampling method. Honey samples collected from experimental bee hives and not directly from beekeepers, reduce the effect of beekeeping handling or techniques. Beekeepers frequently extract honeys from different areas together or store them in the same tanks. This is a common practice that complicates efforts to analytically assign geographic origin to honey. Precautions have been taken for the extraction of honey from combs using straining instead of mechanical extraction. The use of freshly-built combs and the removal of cells of stored pollen limited the possibility of contamination. Pollen can be stored for long periods in honey combs, representing pollen collected weeks or even months prior to a particular nectar flow.

The successive samplings during a nectar flow and the collection of all blooming plants in each area have the advantage of revealing the presence of pollen grains of low percentages. These scarcely-represented pollen types can relate a certain honey type to a specific geomorphological area. For the creation and validation of a predictive model, sampling should proceed for at least three years. Fewer years are insufficient as climatological conditions influence the nectar and pollen production of the plants.

In conclusion, the method described here is promising for the determination of geographical origin of honeys of the same botanical origin produced in different areas. The possibility of assigning geographic origin to honeys of the same botanical origin has numerous applications in marketing and conservation.

Table 1. Taxa found in honey samples, mean and standard deviation – *n is the number of samples in which the pollen grains of the plant were found

Taxa	Area 1			Area 2			Area 3		
	n	Mean	SD	*n*	Mean	SD	*n*	Mean	SD
Ammi majus L	16	4.8	4.36	1	4.3		7	2.8	0.57
Anchusa azurea Miller	4	7.5	2.43						
Anthylis vulneraria L							17	6.8	6.99
Ballota acetabulosa (L) Bentham				39	6.4	4.17			
Ballota phseudodictamnus (L) Bentham	39	21.9	14.71						
Bubleurum glumaceum Sibth & Sm				27	17.4	18.12	18	10.5	6.65
Carthamus lanatus L				21	2.2	1.09	8	1.9	0.13
Chamomilla recutita (L) Rauschert							6	6.8	6.69
Cichorium intybus L				1	2.1		2	2.3	0.32
Cirsium arvense (L) Scop							18	5.0	3.91
Crepis type *(C.dioscoridis* L,C. *foetida* L)	7	2.2	0.61	8	3.4	0.76			
Chrysanthemum coronarium L	4	1.7	0.70						
Dorycnium hirsutum (L) Ser. In DC							11	24.1	11.75
Echium italicum L	19	5.4	4.95	3	3.7	4.31	5	4.8	2.84
Echium plantagineum L							18	36.1	20.28
Eucalyptus camaldulensis Dehnh	20	9.1	8.68						
Galactites tomentosa Moench	20	7.7	8.21						
Helichrysum stoechas (L) Moench	1	7.5							
Hirschfeldia incana (L) Lagreze-Fossat	13	8.0	5.05	22	8.8	7.72	11	17.4	6.97
Hypericum empertifolium Willd	11	9.0	6.51	11	17.8	14.20	27	14.4	10.76
Medicago sativa L							10	7.2	7.73
Micromeria juliana (L) Bentham	1	5.0					20	7.0	8.05
Myrtus communis L	29	5.4	4.26						
Origanum vulgare L							23	1.9	0.92
Paliurus spina-christi Miller							11	1.9	4.73
Pallenis spinosa (L) Cass	1	1.1							
Petroragia prolifera (L) PW Ball & Heywood				17	5.5	4.33	8	1.2	0.04
Phlomis fruticosa	24	17.1	8.89	25	8.1	5.88			
Polygonum aviculare L							2	1.9	1.41
Rubus sanctus	4	3.2	2.86						
Satureja thymbra L	28	4.4	3.60				39	3.2	2.39
Scolymus hisasnicus L	2	1.3	0.15				9	4.6	0.02
Silybum marianum (L) Gaertner							17	3.4	2.35
Teucrium polium L				38	8.1	7.36	27	3.4	3.07
Thymus capitatus (L) Hoffmanns & Link	30	62.3	22.97	30	60.0	24.62	30	57.8	14.54
Trifolium repens L							5	1.5	0.03
Verbascum phlomoides L				38	7.6	5.48			
Verbascum undulatum Lam	8	1.7	0.35				8	1.8	1.13
Vitex angus-castus L	18	5.6	3.30						

Table 2. Canonical discriminant function coefficients

Number	Name	Function 1	Function 2
1	*Ammi majus* L	-0.095	-0.021
2	*Anchusa azurea* Miller	0.330	-0.004
3	*Anthylis vulveraria* L	0.087	-0.073
4	*Ballota acetabulosa* (L) Bentham	0.271	-0.323
5	*Ballota phseudodictamnus* (L) Bentham	0.135	0.077
6	*Bubleurum glumaceum* Sibth & Sm	0.078	-0.058
7	*Carthamus lanatus* L	0.283	0.141
8	*Chamomilla recutita* (L) Rauschert	0.282	0.056
9	*Cichorium intybus* L	0.663	-0.014
10	*Cirsium arvense* (L) Scop	-0.155	0.126
11	*Crepis* type	0.049	0.482
12	*Chrysanthemum coronarium* L	-0.116	-0.408
13	*Dorycnium hirsutum* (L) Ser	-0.119	0.061
14	*Echium italicum* L	0.076	0.067
15	*Echium plantagineum* L	-0.050	0.002
16	*Eucalyptus camaldulensis* Dehnh	0.049	0.030
17	*Galactites tomentosa* Moench	0.023	-0.007
18	*Hirschfeldia incana* (L) Lagreze-Fossat	0.147	0.091
19	*Hypericum empertifolium* Willd	-0.074	0.033
20	*Medicago sativa* L	0.133	-0.005
21	*Micromeria juliana* (L) Bentham	-0.149	0.021
22	*Myrtus communis* L	0.218	0.174
23	*Origanum vulgare* L	-0.777	-0.045
24	*Paliurus spina-christi* Miller	0.110	0.014
25	*Pallenis spinosa* (L) Cass	-4.046	-3.626
26	*Petroragia prolifera* L	-0.010	-0.129
27	*Phlomis fruticosa* L	0.059	0.021
28	*Polygonum aviculare* L	-2.584	-1.234
29	*Rubus sanctus*	0.643	0.490
30	*Satureja thymbra* L	-0.083	-0.003
31	*Scolymus hispanicus* L	-0.027	0.039
32	*Silybum marianum* (L) Gaertner	-0.349	-0.040
33	*Teucrium polium* L	-0.069	-0.092
34	*Thymus capitatus* (L) Hoffmanns & Link	0.072	0.013
35	*Trifolium repens* L	1.703	-0.092
36	*Verbascum phlomoides* L	0.398	-0.101
37	*Verbascum undulatum* Lam	0.186	0.054
38	*Vitex angus-castus* L	0.088	0.320
	Constant	-5.671	-1.300

Table 3. Correlations between pollen grains type and standardized canonical discriminant functions

Name	Function 1	Function 2
Origanum vulgare L	-0.251*	-0.017
Hypericum empertifolium Willd	-0.155*	-0.037
Silybum marianum (L) Gaertner	-0.151*	-0.010
Cirsium arvense (L) Scop	-0.149*	-0.010
Micromeria juliana (L) Bentham	-0.141*	-0.010
Anthylis vulveraria L	-0.138*	-0.009
Dorycnium hirsutum (L) Ser	-0.124*	-0.008
Phlomis fruticosa L	0.114*	-0.012
Echium plantagineum L	-0.111*	-0.008
Verbascum phlomoides L	-0.103*	-0.007
Medicago sativa L	-0.098*	-0.007
Paliurus spina-christi Miller	-0.090*	-0.006
Trifolium repens L	-0.087*	-0.006
Chamomilla recutita (L) Rauschert	-0.071*	-0.005
Crepis type	0.069*	0.029
Scolymus hispanicus L	-0.069*	0.001
Polygonum aviculare L	-0.043*	-0.003
Cichorium intybus L	-0.038*	-0.032
Ballota acetabulosa (L) Bentham	0.188	-0.417*
Ballota phseudodictamnus (L) Bentham	0.133	0.369*
Verbascum undulatum Lam	0.146	-0.291*
Myrtus communis L	0.104	0.288*
Teucrium polium L	0.027	-0.261*
Carthamus lanatus L	0.028	-0.221*
Bubleurum glumaceum Sibth & Sm	0.063	-0.205*
Satureja thymbra L	-0.112	0.201*
Eucalyptus camaldulensis Dehnh	0.071	0.196*
Vitex angus-castus L	0.070	0.195*
Galactites tomentosa Moench	0.065	0.180*
Petroragia prolifera L	0.059	-0.174*
Ammi majus L	0.032	0.158*
Echium italicum L	0.046	0.147*
Anchusa azurea Miller	0.039	0.108*
Chrysanthemum coronarium L	0.039	0.108*
Rubus sanctus	0.032	0.088*
Hirschfeldia incana (L) Lagreze-Fossat	0.030	-0.056*
Pallenis spinosa (L) Cass	0.020	0.054*
Thymus capitatus (L) Hoffmanns & Link	0.020	-0.022*

* Largest absolute correlation between each variable and any discriminant function

Table 4. Functions at group centroids

Area of sampling	Function 1	Function 2
Area 1	2.069	2.810
Area 2	2.441	-2.660
Area 3	-4.509	-0.150

Table 5. Classification matrix of unknown honeys on the area of origin

Area of origin		PREDICTING AREA		
	Percent correct	Area 1	Area 2	Area 3
Area 1	100	24		
Area 2	95	1	23	
Area 3	90	1	1	22

Acknowledgements

The help of lecturer John Tsiripidis for the identification of plant species and of professor Stefanos Manias and the graduate student Haris Doucas for the statistical approach is gratefully acknowledged.

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ORIGINAL RESEARCH ARTICLE

Pollination of habanero pepper *(Capsicum chinense)* and production in enclosures using the stingless bee *Nannotrigona perilampoides*

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Received 3 March 2006, adccepted subject to revision 23 May 2006, accepted for publication 12 July 2006.

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Summary

A flower isolation experiment was conducted to determine the effect of self and cross pollination in habanero pepper (*Capsicum chinense*) in Yucatán, Mexico. The number of fruits increased, differing significantly ($p<0.01$) between bagged flowers (BF=25%), flowers that received one visit (V= 79%) and those with unrestricted visits (UV=86%). Likewise the numbers of seeds and the fertilization index differed significantly between treatments ($p<0.01$). In a second experiment, the efficiency of the stingless bee *Nannotrigona perilampoides* (SB) for pollinating this crop was evaluated. The quality of the fruit and the production in kg/m^2 were not different between SB and mechanical vibration (MV) but the latter showed significantly lower values in the polar diameter, weight and number of seed per fruit ($p<0.01$). Both SB and MV showed significantly higher values compared with a test treatment where no pollinator was used in all the evaluated variables ($p< 0.01$). We conclude that C. *chinense* can be self pollinated but cross pollination is needed to increase the number and quality of fruits and especially for high seed production probably due to the increased transference of pollen grains. The use of *N. perilampoides* seems a good alternative to the use of mechanical vibration and non native bees for pollination of peppers in enclosures under tropical conditions.

Keywords: pollination, Solanaceae, pepper, *Capsicum chinense,* stingless bees.

Introduction

The genus *Capsicum* is native to the Neotropics and includes five domesticated species, including the habanero pepper, *C. chinense,* which is rated as one of the most pungent worldwide (Cázares - Sanchez, *et al.* 2005). This species, native to the Amazon basin (D'Arcy and Esbaugh, 1974), was introduced into the Yucatán Peninsula in Mexico from the Antilles (Zarco-Padrón de Coronado, 2004). The Yucatán peninsula is the largest producer of habanero pepper worldwide with ca. 732 ha cultivated and 3,700 tons harvested annually (Herrera, 2001; Montes-Hernández, 2002). In the last five years, habanero pepper has experienced a substantial increase in domestic and international demand. There has been a corresponding increase in the cultivated areas all over the Yucatán Peninsula (Trujillo-Aguirre and Jorge , 2002; Tun-Dzul, 2002) .

There are no studies on *C. chinense* pollination. Extensive study has shown that the common pepper, *C. annum,* is largely self pollinated but cross pollination is common and that yields are increased by the presence of external pollinating agents (Rasmussen 1985; Jarlan, *et al.* 1997; Meisels and Chiasson, 1997). Similar to commercial production of *C. annuum,* there has been increasing interest in growing *C. chinense* in enclosures in order to reduce the use of pesticides and fertilizers, and allow year round production (Espinosa-Zapata *et al.* 2002; Zuñiga-Estrada et al. 2002; Jovicich *et al.* 2005). Pure seed production is also needed as part of a germoplasm rescue program of local habanero pepper (Trujillo-Aguirre, 2002). At other latitudes, honey bees and bumble bees are efficient pollinators of peppers cultivated in greenhouses. In the Yucatán, however, the highly defensive behaviour of Africanized honey bees and the high cost of bumble bee colony importation prevent extensive use of these bees for pollination (Palma, *et al.* 2004).

Recently, the use of native stingless bees has gained attention due to promising results obtained in evaluations of their

pollinating efficiency on various local crops (Cauich, *et al.* 2004; Melendez, et al. 2004; Can, *et al.* 2005; Palma, 2005). The species *Nannotrigona perilampoides* has been evaluated in Yucatán for tomato pollination with promising results in its adaptation to the high temperatures and humidity in enclosures and with good amounts and quality of fruits produced (Cauich, *et al* 2003; Palma, 2005). The genus *Nannotrigona* is widespread throughout the Neotropics and has also been successfully tested for pollination at other latitudes (Maeta , *et al.* 1992; Slaa , *et al.* 2005) .

In this paper, we evaluated for the first time the pollination needs of *C. chinense* and also determined the effect of using native *N. perilampoides* on fruit yield and seed production in enclosures. The aim is to provide local growers with accessible pollinators for this crop in the light of an expected increase in the demand and production of seeds and fruits in greenhouses.

Materials and Methods

The study was conducted at the Universidad Autónoma de Yucatán in Mérida located at 21° 10'N and 89° 27' W. The climate in this region is classified as sub humid type Awo. The annual average for temperature is 26.5 °C (range 17 – 42°C) and for rainfall is 940 mm (García, 1988).

A pollination cage (4 x 12 x 3 m tall) of iron frames covered with anti-aphid mesh (40 x 40 threads per square inch) was used in the experiments. Neither plastic nor glass was used on the walls or roof. Habanero pepper plants of a local variety (criollo) were grown using seed from a program of habanero rescue conducted by the Technological Institute-2 at Conkal (ITA-2 Conkal) (Trujillo-Aguirre 2002). The plants were sown at a distance of 1 m between rows and 0.4 m between plants within a row using the management recommended for this crop (Soria-Fregoso, *et al.* 2002).

Cross pollination

In this experiment 100 habanero plants were grown in the pollination cage. We evaluated the need for cross pollination in C. *chinense* using two approaches. First, we recorded the number of fruits and seeds produced from 100 flowers left open to bee visits, 70 flowers that received one bee visit and then were isolated from bees using cotton bags, and 70 flowers which were bagged throughout to eliminate bee visits. The flowers were marked in different plants and were all of the same age. After one week, when flowers had senesced, all bags were opened and fruit allowed developing. The number of seeds produced in the three treatments were counted in the lab and used to calculate Spear's index (1983) as follows: pollinator efficiency (PE) = $(P_i - Z) / (U - Z)$, where: P_i = average number of seeds in the fruit that received one visit by pollinator *i*; Z= average number of seeds in the fruit that received no visits; and U= average number of seeds in the fruits that received unrestricted visits. This index is a direct method for evaluating the relative importance of different visitors to a plant's pollination by allowing virgin flowers to be visited by one visitor and monitoring the subsequent fruit, seed or nut set (Spears, 1983). The values of the index range from 0 when no contribution by a given pollinator is observed, to 1 when the production of seed or fruit by a given pollinator is equal to that in flowers that received unrestricted visits by pollinators.

The value of cross pollination was also measured by the number of ovules that developed into seeds, called the fertilization index (Stoddard, 1985). Thirty flower buds were randomly removed from different plants and dissected to obtain the average number of ovules per flower. The average number of ovules was used as an indicator of the potential seeds that could develop after efficient pollination. When the fruits developed, six fruits per treatment were removed and opened to count the number of seeds (i.e., fertilized ovules). This was done weekly during six weeks. The fertilization index per treatment was determined using those values according to the following formula: fertilization index = (ASi) x 100 (AO), where: ASi = average number of seed for the xi treatment; and AO = average number of ovules in flowers.

Seed number was compared by an analysis of variance (ANOVA) and a Tukey test. The percentage of set fruit was arcsine transformed and compared using an ANOVA test too. SAS software (SAS 1990) was used for all statistical analyses.

Bee behaviour

The next experiment was to evaluate the behavior of the bees on the crop and the amount and quality of the fruits produced using different pollination methods. For this, the pollination cage (4 x 12 x 3 m tall) was divided into three sections of 4 x 4 m. In each section, one of the following treatments was used: stingless bees (SB), mechanical vibration (MV) and no pollination (NP). An average of 40 plants was grown in each of the three sections of the pollination cage. Observations were replicated three times using different plants and colonies. Data were collected only for six weeks each time, at the peak of production. The experiments started seven weeks after planting the seedlings, when approximately 60% of the plants had open flowers (ca. 50 flowers per section). In the SB section of one cage, a colony of *N. perilampoides* was introduced following the procedures described in Cauich *et al.* (2004). Three different colonies were used, one in each repetition. Each colony was in average 2.5 l of volume and ca. 700 workers, each worker is approximately of 0.5 cm long. In the MV treatment each plant was vibrated for 5 sec each time between 9:00 and 16:00 h using the electronic device called pollinator II from HGI worldwide Inc. The plants in the NP group received no pollination treatment.

The number of bees that left the colony during five minutes was registered eight times per day (every hour between 9:00 and 16:00 h) on one day of each week during each of the three 6-week replications. The numbers of flowers the bees visited per trip was registered by visually following one bee across its trips between plants with the help of a video camera. Twenty bees were followed in each repetition (n=60). Additionally, we marked 20 flowers in each repetition and registered the number of visits that they received in 5 min (n=60).

The number of open flowers was counted each week for each treatment. Pearson's correlation analysis (Zar, 1984) was used to determine if these data were related to the number of bees on the flowers. Temperature, humidity, wind speed and light intensity were recorded in the section of the cage with SB using a thermo hygrometer (Sato Keiryoki MFG Co.), an anemometer (Turbo meter-Davis instrumental) and a luxometer (Luton Lx) respectively. These environmental variables were measured at the same time and evaluated for correlation with bee activity.

The amounts and quality of fruits produced in each treatment were evaluated for each 6-week replication. The fruits were harvested when they turned orange as is required for market. The average weight of fruits was recorded by individually weighing all the fruits produced on eight plants selected randomly in each treatment. The polar and equatorial diameters of each fruit were measured. Fruits were opened and the number of lobules and seed were counted. The production of all plants per section was registered and thus, we obtained a total production in kg per treatment. The percentage data were arcsine transformed. Comparisons between treatments were made using a two–way ANOVA and Tukey tests.

Results

Cross pollination

Inside the pollination cage, the only insect observed visiting the flowers was *N. perilampoides*. Greater numbers of visits per flower increased the number fruits set, ovules fertilized and seeds produced (Table 1). The numbers of fruits produced were 18 (25.7%) from the bagged flowers, 55 (79.4%) from the flowers that received one visit and 86 (86%) from the open ones. Fruit set differed significantly among the treatments ($p<0.01$). Highly significant differences were also found between treatments for the number of unfertilized ovules (F df= 2,239= 26.9), the number of seeds per fruit ($F_{df= 2,239}$= 133.8) and the number of seeds per lobule ($F_{df= 2,239}$= 133.76) ($p< 0.01$; Table 1). The mean number of ovules in the flowers was 84.8 ± 22.6 (range 58 to 143). The percentage of fertilization obtained was significantly more in flowers with one bee visit (68%) and with unrestricted visits (83.5%) compared with flowers that bees did not visit. Although fertilization occurred in bagged flowers, the increase was significant with one visit (71%) and even more with unrestricted visits (112%).

The Spear's index for *Nannotrigona perilampoides* was 0.89. This high value, close to 1.0, indicates that this bee is a good pollinator of *C. chinense*.

Bee behaviour

In the second experiment, the number of bees exiting the hives was correlated with the number of open flowers on the plants ($r = 0.675$ $p<0.01$) (Fig. 1). Only one of the environmental parameters registered had a significant influence on the activity of the bees (Table 2). Light intensity had a positive influence on both the numbers of bees exiting the hive ($r = 0.86$, $p< 0.001$; Fig 2) and the numbers of bees visiting the flowers ($r = 0.97$, $p< 0.01$). Light intensity was similar in the three sections of the cage. The average number of flowers visited by a bee per trip was 7 + 0.6 and the average number of visits a flower received in 5 min was 2 + 0.4.

The total numbers of fruits evaluated for each treatment in the three repetitions were: 954 for SB, 938 for MV and 985 for NP. The treatment with stingless bees gave significantly better values than the other two. There were statistical differences for the weight of fruit ($F_{df=2,8}$ 141.1); polar diameter ($F_{df= 2,8}$ 117.1), equatorial diameter ($F_{df= 2,8}$ 139.1), number of seed (F df= 2,8 169.2) and number of seed per locule ($F_{df= 2,8}$ 169.1) (Table 3).

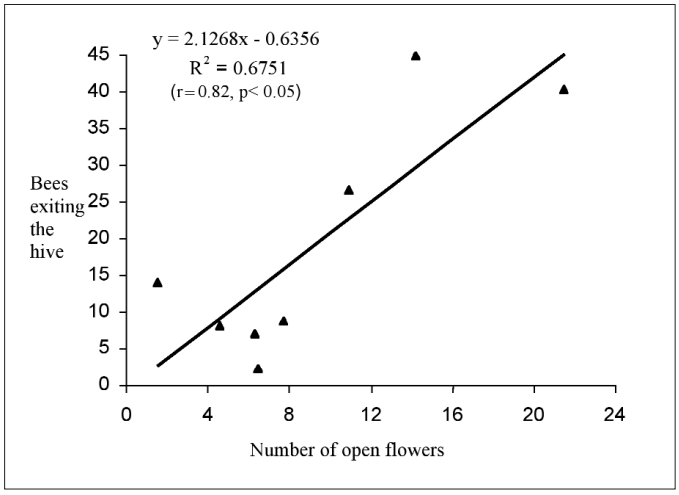

Fig. 1. Correlation of the number of open flowers per plant with the number of bees exiting the hives.

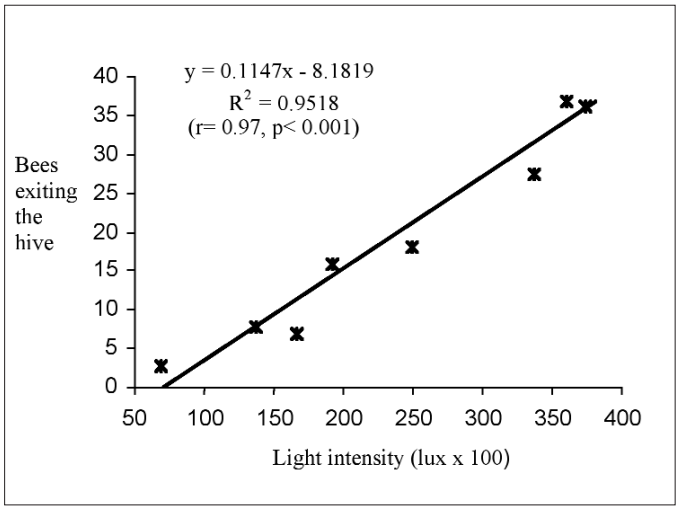

Fig. 2. Correlation of light intensity with the number of bees exiting the hives.

Discussion

This study is the first to provide information of the effect of self and cross pollination and the pollination efficiency of stingless bees on *Capsicum chinense* in enclosures. In our study we found that bagged flowers of C. *chinense* bear fruits in the absence of external pollinating agents but the visits of bees substantially increased the percentage of fruit set, as has been reported for C. *annuum* (Meisels and Chiasson, 1997; Delaplane and Mayer, 2000). There was also a significant increase in the number of fertilized ovules and the amount of seed produced after one bee visit, thus the increase in seed numbers suggests that in C. *chinense* the release of pollen grains was facilitated by the bees that visited the flowers. More interesting was the fact that the number of fertilized ovules increased with greater numbers of bee visits, suggesting that bees can increase seed production by increasing the number of pollen grains transferred to the stigmas of other plants. Alternatively, this could have been a result of an increased efficiency of the transferred pollen grains in fertilizing the ovules of genetically unrelated plants (Monteiro *et al.* 2004).

Similarly, in C. *annum*, the use of hand or mechanical vibration also resulted in an increase in fruit-yield, but crossed flowers develop more fruits than selfed ones (Rasmussen, 1985). In experiments where C. *annum* plants were grown in enclosures, the production of fruits was significantly less than in those allowed open pollination by wind and insects (Raw, 2000). This could be related with the fact that in some pepper varieties the anthers do not fully release the pollen grains and thus, the presence of an external pollinator agent can positively affect fruit set and seed production (McGregor, 1976). Our results clearly indicate that in enclosures, the flowers of *C. chinense* can self pollinate, but the presence of bees can increase both self pollination and cross pollination which results in a greater fruit set and more seeds per fruit.

In the second experiment, *N. perilampoides* showed a good effect on the amount and quality of *C. chinense* fruit in enclosures. In open fields, in Yucatán, México, Cauich (2004) obtained the frequency of bee species visiting flowers of *C. chinense* on a commercial crop. The visitors included six species of stingless bees of which *N. perilampoides* was the most frequent species (> 15 %) followed by *Melipona beecheii* and *Trigona nigra* (5%) while *Scaptotrigona pectoralis, Trigona fulviventris, Partamona bilineata* were the least represented in the samples (1%). These results suggest that *N. perilampoides* is naturally attracted in significant numbers to *C. chinense.* In the present and other studies in the same region, *Nannotrigona* bees started foraging evenly after one week of introduction to the cages and responded in number of foragers to the increase of flowers per plant and provided good numbers of visits (Macias, 2003; Cauich, *et al* 2004), probably as a result of their natural attraction to the crop.

However, Raw (2000) reported eight genera of bees visiting flowers of C. *annuum* in Brazil, none of which were stingless bees despite *Nannotrigona* being common in this country (Maeta , *et al.* 1992). These differences have been attributed to the fact that colonies of stingless bees were absent nearby (Raw 2000) but in the case of the Yucatán could derive from a heavy use of pesticides during crop production that can alter the numbers of stingless bees in the area (Ancona-Xiu, *et al.* unpubl.data).

As with tomato (Cauich, *et al.* 2004), high temperatures and humidity did not have a significant effect on the foraging activity of the bees providing additional evidence of their rapid acclimation to the cages. This suggests that *N. perilampoides* may be a suitable species to use in tropical greenhouses where high humidity and temperature prevail.

The fruits obtained using *N. perilampoides* were heavier, longer and more homogeneous (fruit of first quality) compared to the other two treatments although the production per area did not differ between mechanical vibration and the presence of stingless bees. Moreover, there were few misshapen fruits using stingless bees indicating that *N. perilampoides* had a positive effect in the production of fruits of *C. chinense* in enclosures.

On sweet pepper and tomato the use of external pollinators may increase fruit set and the number of seeds by their mechanical, albeit non-specialized action (Banda and Paxton, 1991; Roubik 1995; Meisels and Chiasson, 1997; Dogterom, *et al.* 1998; Morandin, *et al.* 2001). In this context, stingless bees can act as other Capsicum pollinators such as ants (McGregor , 1976) hover-flies (Jarlan, *et al.* 1997) and different species of bees (Kristjansso and Resmussen, 1991; Shipp, *et al.* 1994; Dag and Kammer, 2001; Cruz, et al. 2005). Something similar can be occurring in *C. chinense.*

The production of uncontaminated seeds under genetic programs for the preservation of certain varieties of *C. chinense* from the Yucatán could also benefit from pollination of plants under isolated conditions (Free, 1993; Trujillo, 2002) and especially by the small flight distance of Nannotrigona compared with *A. mellifera* and *Bombus sp* (Slaa, *et al.* 2000) and possibly the reduced gene flow between plants due to the small numbers of flowers small bees visit (Raw 2000). In this context, we suggest that *N. perilampoides* could be an alternative for both seed and fruit production in Solanaceae cultivated in greenhouses under tropical conditions, where the use of Africanized honey bees or temperate originated bumblebees could be restricted.

Table 1.

Treatment	Fruit set (%)	Seed/fruit	Seed/lobule	Fertilization index	% seed increase/relative to no visits
No visits (n=70)	25.7 **c**	31.1 ± 19.8 **c**	10.6 ± 6.6 **b**	39.3	
One visit (n=70)	79.4 **a**	51.6 ± 24.7 **a**	17.5 ± 8.2 **a**	68.3	71.4
Unrestricted visits (n=100)	86.0 **b**	65.4 ± 18.8 **b**	21.8 ± 6.3 **a**	83.5	112.6

Different letters in the same column indicate significant differences at $p<0.05$.

Table 2. Fruit set, weight, diameters, number of seed per fruit and production per m^2 obtained using three pollination methods.

Treatments	Fruit set (%)	Weight (g)	Polar diameter (cm)	Equatorial diameter (cm)	Number seed/fruit	Production kg/m^2
Stingless bees N= 954	89 **a**	10.3 ± 2.3 **a**	4.8 ± 0.6 **a**	3.1 ± 0.4 **a**	59.8 ± 20.5 **a**	3.5 ± 0.3 **a**
Mechanical vibration N= 938	90 **a**	9.4 ± 2.7 **b**	4.4 ± 0.6 **b**	3.1 ± 0.4 **a**	54.4 ± 24.7 **b**	3.3 ± 0.3 **a**
No pollination N= 985	29 **b**	7.3 ± 2.7 **c**	4.0 ± 0.8 **c**	2.7 ± 0.4 **b**	32.1 ± 19.9 **c**	1.8 ± 0.2 **b**

Different letters in the same column indicate significant differences at $p<0.05$.

Table 3. Correlation values (r) of environmental factors with flight activity of *N. perilampoides* in cages.

	Temperature (°C)	Wind speed (m/s)	Humidity (%)	Light Intensity (lux x 100)
Bees exiting the hive	0.46	-0.28	-0.17	0.86**
Number of seeds/umbel	0.44	-0.18	-0.07	0.97*

*p< 0.001, **p< 0.01

Acknowledgements

We are thankful to CONACYT-SISIERRA (project 2002-0106) and Fundación Yucatán Produce for funding this research.

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ORIGINAL RESEARCH ARTICLE

Effect of venom from the Asian honeybee (*Apis cerana* Fab.) on LPS-induced nitric oxide and tumor necrosis factor-α production in RAW 264.7 Cell Line

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Received 25 July 2005, accepted subject to revision 23 March 2006, accepted for publication 21 June 2006.

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Summary

To explain the composition of Asian honeybee (*Apis cerana*) venom (ABV) and the anti-inflammatory effect of ABV. The major components of ABV by LC chromatography and SDS-PAGE were identified. We demonstrated that the component of ABV and Western honeybee (*A. melifera*) venom is similar in its composition but the melittin and apamin content of ABV is lower content than that of *A. melifera.* The production of pro-inflammatory cytokines, nitric oxide (NO) and tumor necrosis factor-alpha (TNF-α) was examined by lipopolysaccharide (LPS) in a macrophage cell line, RAW 264.7 cells, with ABV. The effects of ABV on the expression of inducible nitric oxide synthase (iNOS) and TNF-α were investigated by western blot and RT-PCR in LPS-stimulated RAW 264.7. ABV suppressed the NO, iNOS and TNF-α production, and decreased the levels of iNOS and TNF-α mRNA . These results suggest that ABV has an anti-inflammatory effect by inhibiting iNOS and TNF-α expression. Therefore, ABV may be useful for inhibiting the production of inflammatory cytokine and NO production in immune cell-mediated inflammatory diseases. Further studies on the pharmacological aspects of the individual components of ABV are recommended for future trials.

Keywords: bee venom, *Apis cerana,* inflammation, nitric oxide, *i*NOS, TNF-α

Introduction

As a traditional alternative medicine approach, honeybee (*Apis mellifera.* L) venom has been utilized to relieve pain and to treat inflammatory diseases since ancient times (Billingham *et al.,* 1973). It became known as apitherapy and involves the medicinal use of honeybee products including honeybee venom. Similar references are to be found in Korean beekeeping history (Kim *et al.,* 2002; Nam *et al.,* 2005). Honeybee venom, as a therapeutic modality, has been extensively researched and practiced in Korea, focusing on clinical applications as a meridian therapy (Kwon *et al.,* 2001; Kim *et al.,* 2003; Lee et al., 2003; Yin *et al.,* 2005). Apitherapy using live honeybee stings proved to have therapeutic value in Korea for pigs with respiratory diseases such as atrophic rhinitis, pleuropneumonia and Glasser's disease (Choi *et al.,* 2003). The bee venom treatment is known to be as effective, if not more so, than antibacterial drugs an to have no side effects, and is not expensive (Eiseman *et al.,* 1982; Akdis *et al.,* 1996; Kwon *et al.,* 2001).

Two honeybee species in Korea, *A. mellifera* L. and *A. cerana* Fab., are kept by the commercial beekeeping industry. Recently, Western honeybee (*A. mellifera*) venom has been tested for its components and their possible anti-inflammatory functions (Dotimas & Hider, 1987; Choi *et al.*, 2003; Lee *et al.,* 2004). In Korea, however, there is not sufficient knowledge on the components and pharmacological effects of Asian honeybee (*A. cerana*) venom (ABV) compared to that of Western honeybee venom.

Serious inflammation can cause septic shock, rheumatoid arthritis or other diseases in which the excessive production of Nitric Oxide (NO) and Tumour necrosis factor (TNF)-α plays an essential role (Jang *et al.,* 2005). NO is a multi-functional mediator,

which is implicated in a wide variety of biological functions such as neurotransmission, non-specific immune defence and vasodilatation (Moncada *et al.,* 1991). NO produced in large amount by inducible NO synthase (iNOS) and its derivatives, such as nitrogen dioxide, also play a role on inflammation (Park *et al.,* 1993). TNF-α is a multi-functional cytokine mostly secreted by inflammatory cells and is involved in numerous pathological states (Aggarwal & Natarajan, 1996; Crane-Godreau & Wira, 2005). TNF-α has a profound effect in tissue remodelling, repair and inflammation by activated macrophages (Novgrodiski *et al.,* 1994). Because of their important role in inflammatory diseases, a significant effort has been focused on developing therapeutic agents that could interfere with NO and TNF-α production (Manogue *et a*l., 1992; Faris-Eisner *et al.,* 1994; Chen *et al.,* 2001). Hence, there is a considerable interest in inhibiting agents of NO and TNF-α production such as a therapeutic intervention in inflammatory diseases (Kondo *et al.,* 1993; Park *et al.,* 1993; Lang *et al.,* 1995). As a potential therapeutic agent, ABV needs to be evaluated in terms of its anti-inflammatory effect in inflammatory environments.

This study was carried out to determine the composition of ABV in Korea and to evaluate the inhibitory activity of NO and TNF-α production in LPS-stimulated RAW 264.7 cells, a macrophage cell line.

Materials and Methods

ABV collection

Asian honeybee workers were collected from two experimental colonies maintained in National Institute of Agricultural Science and Technology (NIAST), Suwon, Korea. Pure venom was obtained by using the Schmidt method (Schmidt, 1986). In brief, worker bees were killed by freezing and then their venom was obtained by squeezing venom sacs. The extracted venom was diluted twice in cold water and then centrifuged at 10,000 g for 5 min at 4°C to discard cellular residues from the supernatant. The venom was lyophilised by freeze dryer and stored at refrigerator for later use.

ABV analysis

The 100 mg frozen and dried whole ABV was dissolved in 0.1 M ammonium formate (pH 4.5). Particles were removed by centrifugation and filtration prior to sample application to a Sephadex TM200 column (AKTA explorer, Pharmacia, USA) equilibrated in 0.1 M ammonium formate. Honeybee (*A. mellifera*) venom standard proteins were purchased from Sigma. All fractions collected were examined for total protein, hyaluronidase, phospholipase A2, mellitin and apamin. Protein concentration was determined by the Bradford method (BioRad, CA, USA). The purity of proteins and peptides was assessed by SDS-PAGE on 4-20 % gradient tricine gels (Novex Tricine Gels, Invitrogen, USA). Proteins and peptides were stained with Coomassie blue R-250.

Cell culture

The murine macrophage cell line, RAW 264.7 cells were obtained from the Korean Cell Line Bank (KCLB). The cells were cultured in Dulbecco's modified Eagle's medium containing 10 % foetal bovine serum (FBS) and 1% penicillin (10,000 U/ml)/streptomycin (10,000 U/ml). The cells were maintained in a humidified atmosphere containing 5 % CO_2 at 37°C. For experiments, the cells were added to 24-well plates at a density of 5×10^5 cells/well and adhered overnight for examination. Various amounts of ABV and LPS (100 ng/ml) were added and incubated. The supernatants were then collected for NO and TNF-α assays.

Cytotoxicity

Cytotoxicity was assessed by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assays. RAW 264.7 cells were seeded in 96-well plates and treated with various reagents. They were cultured in a CO_2 incubator for the indicated time periods. The medium was removed and MTT was added followed by incubation at 37°C for 4 h in an incubator. Supernatants were carefully removed and DMSO was added. They were incubated at 25°C for 30 min and measured by a microplate reader (UV max, Molecular Devices, USA) at 540 nm. Percent viability was calculated as follows (Orsolic *et al.,* 2003):

Percent viability

= (optical density experimental wells – background),∞100 /optical density control wells – background

NO generation assay

Nitrite was carried out as another method reported by Stuehr *et al.* (Stuehr *et al.,* 1991). 100 Ìl of supernatant from a 24-well plate culture at 22 h was transferred into new 96-well plate and mixed with an equal volume of Griess reagent (Sigma, USA). The new plate was then incubated for 10 min at room temperature and measured by a microplate reader (Green *et al.,* 1982). The standard calibration curve was prepared using sodium nitrite as a standard.

TNF-α assay

TNF-α secretion was measured using the ELISA kit (Endogen, USA). 50 μl of either various samples was added in 96-well ELISA plate. Biotinylated antibody reagent to each well was incubated in the plate at room temperature for 2 h. After washing the plate with PBS-tween 20, diluted streptavidin-HRP was added, and the plate was incubated at room temperature for 30 min. After washing the plate, premixed TMB substrate solution was added, and then the plate was developed in the dark room for 30 min. The plate was read at 490 nm using a microplate reader. The concentration of TNF-α was calculated using murine rTN-α as a standard.

Western blot analysis

RAW 264.7 cells were cultured in a 100 mm plate in the presence of LPS and ABV for 22 h. The cells were washed with phosphate buffered saline (PBS) and lysed by boiling with a lysis buffer for 30 min on ice. 50 μg protein from the cell lysates was applied to 15% SDS-PAGE gels and transferred to a nitrocellulose membrane. The membranes were blocked with a 5% skimmed milk solution for 1 h. They were then incubated with anti-*i*NOS (abcam, UK), anti-TNF-α (Koma Biotech, Korea) and anti-β-actin antibody (Santa Cruz Biotechnology, USA) as the primary antibodies for 2 h, respectively. Expression of protein was detected by enhancing chemiluminescence (ECL) using x-ray film and ECL reagents (Amersham, UK).

Reverse-transcription –polymerase chain reaction (RT- PCR)

Total RNA was extracted from RAW264.7 cells with RNAzol B (TEL-TEST, Friendwood, USA) reagent, and the total RNA was prepared by acid guanidinium thiocyanate-phenol-chloroform

extraction procedure according to the manufacturer's instructions. RT-PCR was performed in a single reaction tube with an Access RT-PCR system (Promega, WI, USA). Amplified PCR product was confirmed in a 1.5 % agarose gel and quantification was done with a gel calculation program (Optima 6.5, MD, USA). The sequences of sense and anti-sense primers (Bioneer, Korea) were shown in Table 1.

Statistical analysis

Nitrate and TNF-α production is expressed as a mean ± S.D. of 3 to 5 independent experiments. The statistical significance was determined using Student's t-tests (SAS Institute, 2004).

Results

Analysis of fractionated ABV by gel filtration

Proteins from ABV were separated by size exclusion gel chromatography (Fig. 1B). The determination of main components of ABV resulted by comparison with venom protein standards (Sigma, USA; Fig. 1A). Identification of each peak was performed by SDS-PAGE (Fig. 1C). Hyaluroniase, phospholipase A_2, mellitin, and apamin could be detected in peaks I, II, III and IV. The comparison between peaks of honeybee venom standards and whole AVB showed that all major venom proteins and peptides were found similar in terms of compositions but a slight difference in proportions.

Inhibition of NO and *i*NOS by ABV

As shown in Fig 2, the marcrophages, RAW 264.7 cells were stimulated by 100 ng/ml LPS at 22 h and that the highest rate of NO_2- production occurred within the concentration ranges of 50 mM. The accumulated nitrite in the culture medium, estimated by the Griess method, was used as a reference for NO synthesis from RAW 264.7 cells. When various ABV concentrations were treated with culture media, ABV significantly inhibited LPS induced NO_2-production in a dose dependence. In these experiments, the concentrations of bee venom that inhibited production by 50 % (IC_{50}) after incubation of RAW 264.7 cells for 22 h was at 20 ng/ml. No effects on cell viability was observed in used concentrations of ABV. The viability of RAW264.7 cells as measured by MTT cytotoxicity was unaffected at various concentrations for ABV. However, over 1 μg/ml cell viability was decreased by 60% (Fig. 3). With the assumption that the inhibition of NO production by ABV in RAW264.7 cells could be caused by a decrease in the *i*NOS protein expression, the effect of ABV on the *i*NOS protein expression was examined in cell treated with LPS for 22 h. As treatments with ABV inhibited *i*NOS protein expression induced by LPS without internal control, ,βactin, the specific inhibition of *i*NOS protein expression by ABV (Fig. 5). In addition RT-PCR analysis was performed in order to examine the effects of ABV on the transcriptional expression of *i*NOS in the inflammatory reactions of RAW 264.7 cells. The expression of iNOS mRNA was inhibited by 10 and 100 ng/ml dose of ABV (Fig. 6). The results suggest that ABV contributes to the decreased *i*NOS protein, *i*NOS mRNA and NO production in RAW 264.7 cells.

Decrease of TNF-α production by ABV

Although RAW 264.7 cells contained about 58 pg/ml of TNF-α as a basal level, when RAW 264.7 cells were stimulated with 100 ng/ml of LPS for 22 h, TNF-α produced about 3280 pg g/ml (Fig. 4). Therefore, this condition was applied to analyze suppressive effect on TNF-α production by ABV. Production of TNF-α was significantly decreased by ABV dose-dependently (Fig. 4). To determine whether ABV had a direct effect on TNF-α production, TNF-αprotein was measured using western blot analysis. RAW 264.7 cells were treated with LPS and ABV for the various concentrations. Production of TNF-α was significantly decreased by ABV (Fig. 5). Treatment with ABV decreased secretion on TNF-α at 10 ng/ml or more. TNF-α mRNA, as measured by RT-PCR, was decreased by AVB (Fig. 6). The results suggest that ABV contributes to the decreased TNF-αprotein and mRNA in RAW 264.7 cells.

Discussion

Acute inflammation can cause septic shock, rheumatoid arthritis or other disease in humans and experimental animals (Higgs *et al.,* 1984). It has been reported that the excessive production of NO and TNF-α could cause various inflammatory diseases (Higgs *et al*., 1984; Vince *et al.,* 2005). NO is a small radical molecule that is synthesized from L-arginine by nitric oxide synthase (NOS) in various cells and tissues (Ryu, e*t al.,* 1998). Among the isoforms identified, the inducible form (*i*NOS) is primarily expressed in macrophages and is regulated by various modulators, including different cytokines (Zhang & Morrison, 1993; Barua, *et al.,* 2001). TNF-α, which are produced by activated macrophages and other cells. This has a multifunctional biological action on the activities of target cells, both immune and non-immune cells (Tracey *et al.,* 1988; Beutler & Cerami, 1989; Barua, *et al.,* 2001). Thus, TNF-α is considered a major inflammatory mediator with systemic inflammatory properties, as well as anti-tumor activity (Mohler *et al.,* 1994).

Honeybee venom has been widely used to relieve the pain and the treatment of inflammatory diseases in Korea (Kwon *et al.,* 2002; Kim *et al.,* 2005). The general component and pharmacological effects of bee venom had been reported (Piek, 1986; Kim, 1990). Honeybee venom contains several peptides including melittin, apamin, adolapin, mast cell degranulating peptide, enzymes, biologically activity amines and non-peptide components (Lariviere & Melzack, 1996; Kwon *et al.,* 2002). Enzymes are composed of phospholipase A_2, hyaluonidase, acid phosphominoesterase, αD-glucosidase and lypophospholipase (Banks & Shipolini, 1986). Each honeybee venom substance has been investigated to prove their anti-inflammatory effects (Somerfield *et al.,* 1984; Saini *et al.,* 1997). Honeybee venom showed an anti-inflammatory effect by inhibiting iNOS and COX-e expression possibly through the suppression of NF-κB and MARK activities (Jang *et al.,* 2005). However, Asian honeybees are widely dispersed in the Asian region, yet, ABV substance and anti-inflammatory effects have never been explained.

By comparison between standard Western honeybee venom and ABV with the Sephadex TM200 column and SDS-PAGE profiles, we found out that major venom components from two species were of similar proportions (Piek, 1986; Kim, 1990). Although the rate of melittin and apamin content of whole ABV is lower than that of Western honeybee venom, the most

abundant venom components have equal compositions in both venoms. We have tested bee venom from Asian honeybee and have shown the demonstrated effects of ABV on the production of cytokine in LPS-activated macrophages. ABV suppressed the production of NO, *i*NOS and TNF-α in activated macrophages, which suggested that ABV might contain a potential anti-inflammation compound. NO was significantly inhibited by ABV. NO synthesis by iNOS is increased in inflammatory diseases and leads to cellular injury. The present studies demonstrate that the ABV markedly decrease *i*NOS protein and mRNA expression in RAW 264.7 cells. The inhibitory activity of NO production by ABV may come from the inhibition enzyme expression of nitric oxide synthase.

Also, both TNF-α production and TNF-α mRNA were significantly decreased by ABV. The viability of RAW264.7 cells were assessed to be above 90% by MTT method at the sample concentrations for the nitrite and TNF-α assay.

In conclusion, the compositions of ABV are similar to Western honeybee venom but the melittin and apamin content of Koran ABV is exceptionally lower than that of Western honeybee venom. We have demonstrated that ABV inhibits the production of TNF-α in LPS-stimulated macrophages and the inhibition is closely associated with the suppressed NO. These findings suggest that ABV can be useful as a potential immunotherapeutic agent, which is potentially applicable for inflammation.

Table 1. Sequence of primers and sizes of the investigated genes in RT-PCR analysis

Gene	v	Primer sequences	% seed increase/relative to no visits
iNOS	Sense	5'-CCCCTTCCGAAGTTTCTGGCAGCAGC -3'	496
	Antisense	5'-GGCTGTCAGAGCCTCGTGGCTTTGG -3'	
TNF-α	Sense	5'-TTGACCTCAGCGCTGAGTTG-3'	364
	Antisense	5'-CCTGTAGCCCACGTCGTAGC-3'	
β-actin	Sense	5'-GTGGGCCGCCCTAGGCACCAG-3'	603
	Antisense	5'-GGAGGAAGAGGATGCGGCAGT-3'	

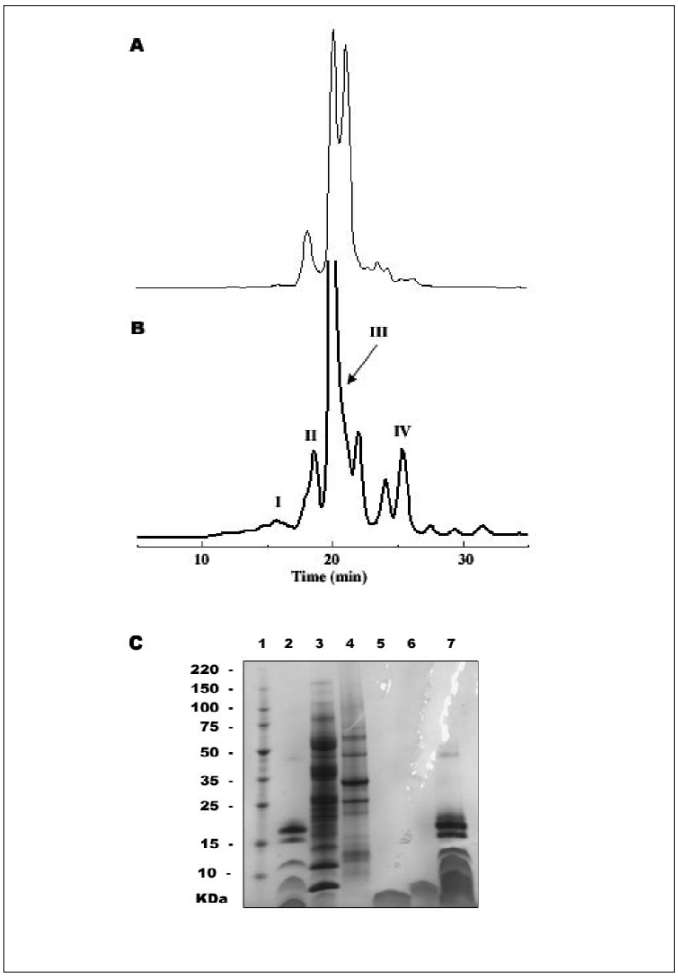

Fig. 1. Gel filtration of 100 mg freeze-dried whole ABV on Sepadex TM200 10/300. Elution with 0.1 M ammonium formate buffer, pH 4.5. Determination of main components were compared with standard proteins by the optical density at 280 nm (A, Sigma whole honeybee venom; B, whole ABV; peak I, hyaluronidase; peak II, phospholipase A_2; peak III, melittin; peak IV, apamin) and SDS-PAGE analysis (C). SDS-PAGE is 4–20 % gradient gel. C was stained with Coomassie blue R-250. Lane 1, Sigma molecular weight standard; lane 2, Sigma whole honeybee venom; lane 3, hyaluronidase (peak I); lane 4, phospholipase A2 (peak II); lane 5, melittin (peak III); lane 6, apamin (peak IV); lane 7, whole ABV.

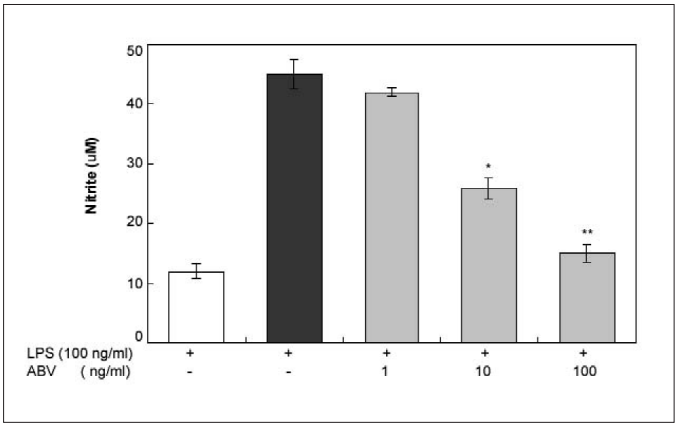

Fig. 2. Inhibitory effect of the ABV on LPS-induced NO production in RAW 264.7 cells were incubated for 22 h with LPS (100 ng/ml) in the presence or absence of indicated concentration of ABV. Accumulated nitrite in the culture medium was determined by the Griess reaction. The values are mean ± S.D. of three independent experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$ by Student's t-test.

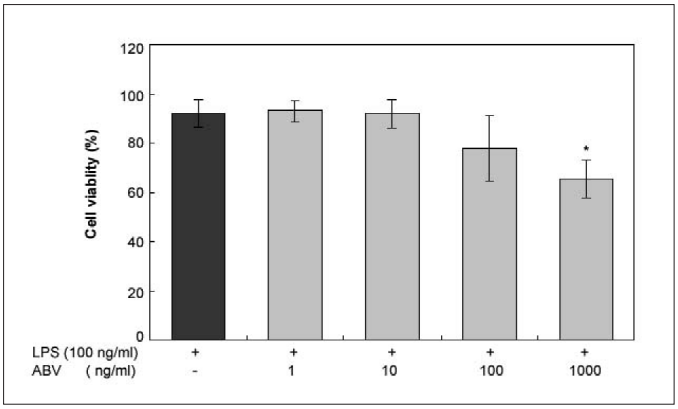

Fig. 3. Cell viability (%) after 22 h of incubation with ABV at the respective concentrations. RAW 264.7 cells were treated with LPS (100 ng/ml) or ABV either alone or in combination. Viability was evaluated by MTT assays at 22 h after treatment. Values are given as percent (mean ± S.D.). $^{*}p < 0.05$ by Student's t-test.

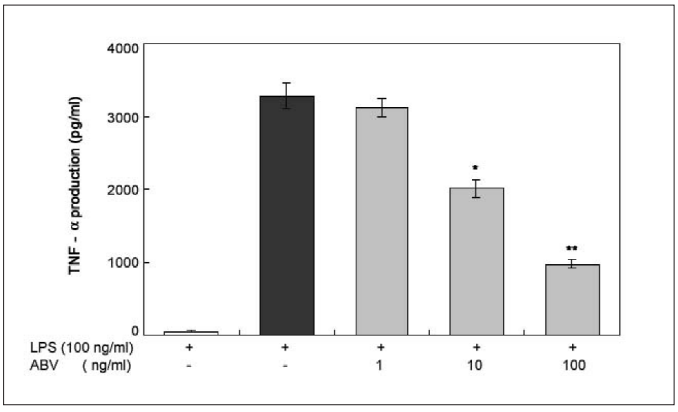

Fig. 4. ABV inhibited LPS (100 ng/ml)-induced TNF- · production from RAW 264.7 cells. ABV was added to the cell culture with final concentration of 1, 10 and100 ng/ml. 22 h culture supernatants were assayed for TNF- · by an ELISA kit. The values are mean ± S.D. of three independent experiments. $^{*}p < 0.05$ and $^{**}p < 0.005$ by Student's t-test.

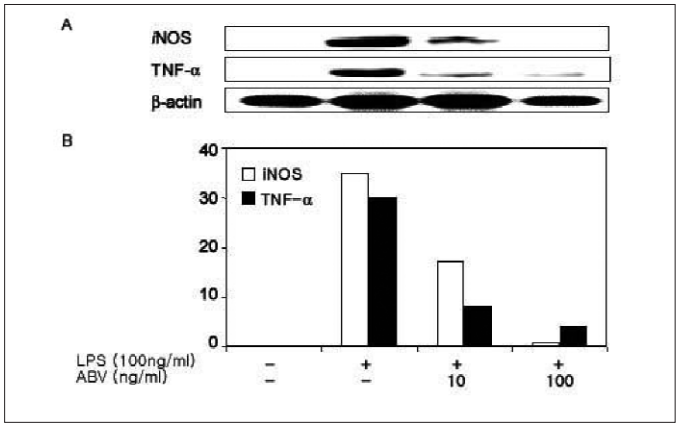

Fig. 5. Inhibitory effect of the crude ABV on LPS-induced expression of iNOS and TNF- · protein. RAW 264.7 cells were incubated with LPS (100 ng/ml) in the presence or absence of indicated concentration of ABV. After 22 h incubation, cell lysates were separated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane and blotted with a mouse monoclonal anti –*i*NOS or TNF-α antibody. β–actin was used as an internal control (A) and relative densitogram of western blot (B). This blot is a representative of three independent experiments. Results are shown for cells that were either unactivated (lane 1), activated with LPS (lane 2) and activated in the presence of 10 and 100 ng of ABV (lanes 3 and 4).

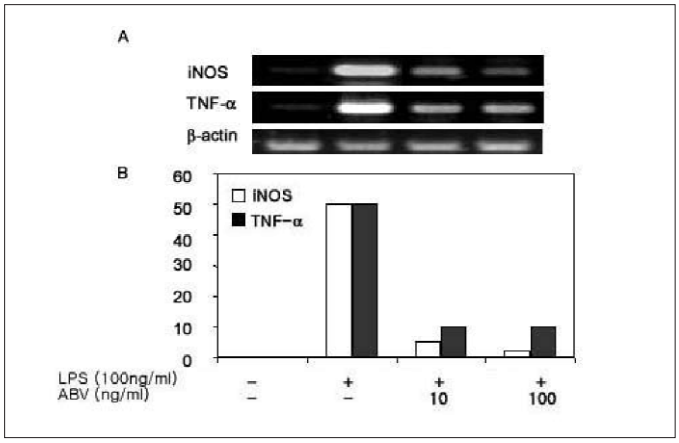

Fig. 6. RT-PCR analysis of regulation of *i*NOS and TNF-α mRNA by ABV in LPS activated RAW 264.7 cells for 22 h (A) and relative densitogram of RT-PCR (B). Total RNA fractions were prepared form activated cells treated with of without 10 and 100 ng of ABV. Results are shown for cells that were either unactivated (lane 1), activated with LPS (lane 2) and activated in the presence of 10 and 100 ng of ABV (lanes 3 and 4). Similar results were obtained in three additional independent experiments.

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ORIGINAL RESEARCH ARTICLE

Effect of the maceration time on chemical composition of extracts of Brazilian propolis

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Recieved 5 January 2005, accepted subject to revision 18 February 2005, accepted for publication 28 June 2006.

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Summary

It is believed that longer maceration times change significantly the ethanolic extract composition of propolis, improving its pharmacological, nutritional and antimicrobial properties. To probe whether such superior composition is indeed obtained, 10 samples of propolis from several regions in Brazil and one sample from North America were left to macerate in 70% ethanol for periods between 20 days and one year. The resultant extracts were evaluated in terms of the yield of extracted material and also analyzed by electrospray ionization mass spectrometry (ESI-MS) fingerprinting. A small increase in the yield of the extracted material over the period analyzed was observed. ESI-MS fingerprints indicate qualitatively the same composition but with a greater incidence of the high-mass components after six months. The extracts of one sample of green Brazilian propolis were also quantified using both gas chromatography mass spectrometry and high performance liquid chromatography. No new components were identified after longer maceration times and the changes in the relative concentrations of the identified components were in line with the results of the yield and ESI-MS fingerprints. Therefore, prolonged extraction periods do not necessarily result in richer propolis extracts.

Keywords: Brazilian propolis, electrospray ionization – mass spectrometry, chemical composition of propolis, maceration, high performance liquid chromatography, gas chromatography – mass spectrometry.

Introduction

Propolis is the name given to a resinous substance collected by bees from various plant exudates and mixed with the wax bees secrete [Marcucci and Bankova, 1999]. After maceration with ethanol, crude propolis is separated into three distinct fractions: one containing insoluble components such as inorganic compounds, pollen grains, earth and others; another containing wax; and a third containing the ethanol-soluble compounds. This third fraction, known as ethanolic extract of propolis (EEP), contains compounds that are greatly studied throughout the world for their many pharmacological activities [Marcucci, 1995; Burdock, 1998].

The chemical composition of EEP is quite complex and depends on the plant sources of the resins. Greenaway *et al.* [1990] analyzed propolis from temperate zones and *Populus* bud exudates by gas chromatography mass spectrometry (GC/MS), determining these to be the plant sources for propolis from these regions. Flavonoids, which are pharmacologically active, are the principal components of propolis from temperate zones such as Europe, Asia and North America [Bankova *et al.,* 1982]. Chromatographic studies have determined different chemical classes in propolis of Brazilian and European origin [Bankova *et al.,* 1995]. Only small quantities of flavonoids are found in green Brazilian propolis from the states of São Paulo and Minas Gerais [Marcucci et al., 1999; Bankova *et al.,* 2000]. Green Brazilian propolis presents, however, large quantities of phenolic acids (caffeic, ferulic and p-coumaric acids), their esters and several prenylated derivatives [Aga *et al.,* 1994; Tasawa *et al.,* 1998; Marcucci *et al.,* 2001]. These substances are frequently found in South American species of *Baccharis* [Zdero *et al.,* 1989]. Differently, propolis from the south of Brazil contains triterpenic acids derived from species of *Araucaria* trees [Bankova *et al.,* 1999], whereas the plant origins and chemical composition of red

propolis from the northeast of Brazil have not yet been determined.

Brazilian propolis is exported to several countries and is consumed in food and beverages to improve human health [Marcucci *et al.,* 2001]. It accounts for 10 to 15% of the world propolis production but in Japan for instance, Brazilian propolis accounts for 80% of the imported propolis where green propolis from the southeast of Brazil is considered to be the best [Pereira *et al.,* 2002]. Brazilian beekeepers frequently allow propolis to macerate for periods between 30 days and six months, with the objective of obtaining more concentrated EEP with a more diverse, richer composition.

Previous studies to determine the factors which influence the yield and composition of Brazilian propolis extracts have been effected; maceration for ten days with solvents containing between 30% and 100% ethanol showed that extracts with the highest yields and the greatest number of components were obtained using solvents containing 70% of ethanol or more [Sawaya *et al.,* 2002, 2004 a]. A slight increase in yield of EEP between extracts macerated for 10, 20 and 30 days was observed, although the composition appeared to be qualitatively the same [Cunha *et al.,* 2004].

The purpose of the present study was to observe, in greater depth, the effect of the length of maceration on the composition of the EEP. Samples of Brazilian propolis from the several regions in Brazil and one sample from North America were extracted for periods of 20 days, 30 days, 6 months and one year. The resultant extracts were evaluated in terms of the yield of extracted material and also analyzed by direct infusion electrospray ionization mass spectrometry fingerprinting in the negative ion mode: ESI(-)-MS. These fingerprint mass spectra have been used as an efficient, quick (less than 1 min analysis time) and relatively detailed appraisal of the qualitative chemical composition of samples of complex chemical composition such as whisky [Müller *et al.,* 2005], wine [Catharino *et al.,* 2006], beer [Araújo *et al.,* 2005] and edible oils [Catharino *et al.,* 2005], in their most polar and acidic or basic components. ESI(-)MS fingerprinting has also been used to characterize propolis samples from different geographical origins [Sawaya *et al.,* 2004 b] and to indicate plants that are the sources of propolis resins [Sawaya *et al.,* 2006].

The extracts of one sample of green Brazilian propolis were also analyzed using the more traditional methods of gas chromatography mass spectrometry (GC/MS) and high performance liquid chromatography (HPLC) to observe if the results of these quantitative analyses were in accordance with the results of ESI(-)-MS and yield.

Material and Methods

Propolis samples and extraction

Eleven samples of crude propolis were obtained from hives of *Apis mellifera* honeybees. The place of collection of each sample can be found in Table 1. The samples were ground to a particle size of 2 mm or less in order to have more homogenous samples and to increase the surface of contact with the solvent. In an Erlenmeyer flask, 20 g of ground propolis were put together with 100 mL of a solvent containing 70% absolute ethanol and 30% distilled water (v/v). The flasks were sealed, to avoid the evaporation of the solvent, and kept in a dark room, at room temperature, with sporadic shaking, for the following periods: 20 days, 30 days, 6 months and 1 year. Therefore, for each propolis sample, four flasks, one for each period of maceration, were prepared from the same ground propolis. At the end of each period the solutions were filtered and the liquid extracts evaporated in a rotary evaporator until constant weight. This weight was used to determine the yield (% w/w) of EEP for each extraction period.

Electrospray Ionization-Mass Spectrometry (ESI-MS)

The EEP were dissolved in a solution of 70 % chromatographic grade methanol (Tedia, Fairfield, OH, USA) and 30% deionized water (v/v). The solutions used for analysis contained approximately 50 ng of dry propolis extract for every 1mL of methanolic solution, plus 5 μL of ammonium hydroxide (Merck, Darmstadt, Germany).

These solutions were analyzed by direct infusion by means of a syringe pump (Harvard Apparatus) at a flow rate of 10 μL/min. ESI-MS mass spectra were acquired in the negative mode using a hybrid high resolution Micromass Q-TOF mass spectrometer, under the following conditions: Capillary -3000 V, cone -40 V, dessolvation temperature 100°C.

Gas Chromatography / Mass Spectrometry (GC/MS)

Exactly 2.0 mg of each extract were weighed and dissolved in 50 μL of dry pyridine, 50 ÌL of N, O bis(trimethylsilyl)trifluoroacetamide (BSTFA) were added and the mixture heated at 80° C for 20 minutes in a screw-cap vial, before analysis by GC/MS.

The constituents of the sample were separated on a Hewlett Packard 5890 gas chromatographer coupled with a quadrupole mass analyzer. An Ultra-2 HP column was used (25 m x 0.2 mm) with a 0.33 μm coating of Phenylmethyl-siloxane. The samples were introduced using a microsyringe (1μl) with He as carrier gas, linear velocity of 0.6 mL/min. Temperature program used was: 80 – 240° C at 8°/min, 240 – 300° C at 12 °/min and a 20 min. hold at 300° C. The quadrupole mass analyzer contained an EI ion source set at a temperature of 100° C with filament potential of 70 eV.

The compounds were identified by computer searches in commercial libraries or injection of acquired standards and compounds isolated from other samples propolis in previous studies [Marcucci *et al.,* 2001]

High Performance Liquid Chromatography (HPLC)

Analytical HPLC was run on HPLC (Merck-Hitachi, Germany), equipped with a pump (model L-7100, Merck-Hitachi, Germany) and a diode array detector (L-7455, Merck-Hitachi, Germany). Separation was achieved on a Lichrochart 125-4 column (Merck, Darmstadt, Germany) (RP-18, 12.5 x 0.4cm, 5Ìm particle size) using water: formic acid (95:5, v/v) (solvent A) and methanol (solvent B). The elution was carried out with a linear gradient and a flow rate of 1 ml min-1. The detection was monitored at 280 and 340 nm and the components identified by comparison with standards or previously isolated compounds.

Results

Yield

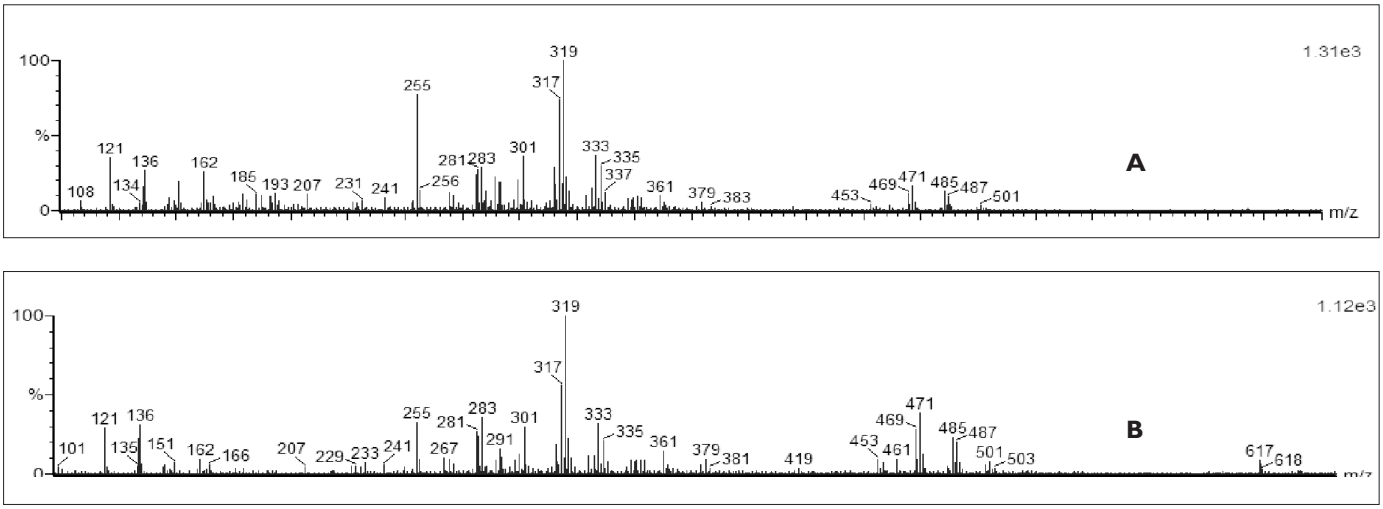
The yield of the extracts obtained can be observed in Table 1. Although variation can be observed between samples due to the amount of ethanol extractible resin in each sample the results follow the same trend for the different types of Brazilian propolis as well as for the North American sample. The average results show a 11% increase in yield between 20 days and one year.

Table 1. Percentage of extracted matter obtained from the propolis samples macerated in 70 % ethanol for 20 days to one year.

		YIELD (%W/W) AFTER MACERATION FOR:			
Sample	**State and country of origin**	**20days**	**30 days**	**6 months**	**1 year**
Brown 1	Rio Grande do Sul, Brazil	69.0	69.2	73.8	74.4
Green 2	São Paulo, Brazil	60.5	61.1	70.6	71.1
Green 3	Minas Gerais, Brazil	64.7	65.8	67.6	69.3
Green 4	São Paulo, Brazil	55.3	56.8	60.1	63.2
Green 5	São Paulo, Brazil	55.1	55.9	57.9	59.8
Green 6	Minas Gerais, Brazil	49.6	51.1	53.5	56.9
Red 7	Bahia, Brazil	61.0	63.8	69.4	71.1
Red 8	Bahia, Brazil	73.9	74.1	75.3	76.7
Red 9	Maranhão, Brazil	60.1	61.3	64.1	68.7
Red 10	Bahia, Brazil	40.7	41.5	45.6	49.9
North America	New York, USA	71.6	72.5	75.3	76.0
Average		60.1	61.2	64.8	67.0

ESI(-)-MS.

Overall, all the extracts of the same samples provided very similar ESI(-)-MS. Those in Figure 1 show the same set of components (as far as only their m/z are concerned) for the same samples after 20 days and 1 year of maceration. An increase in the relative concentration of some high mass components is however clearly noted.



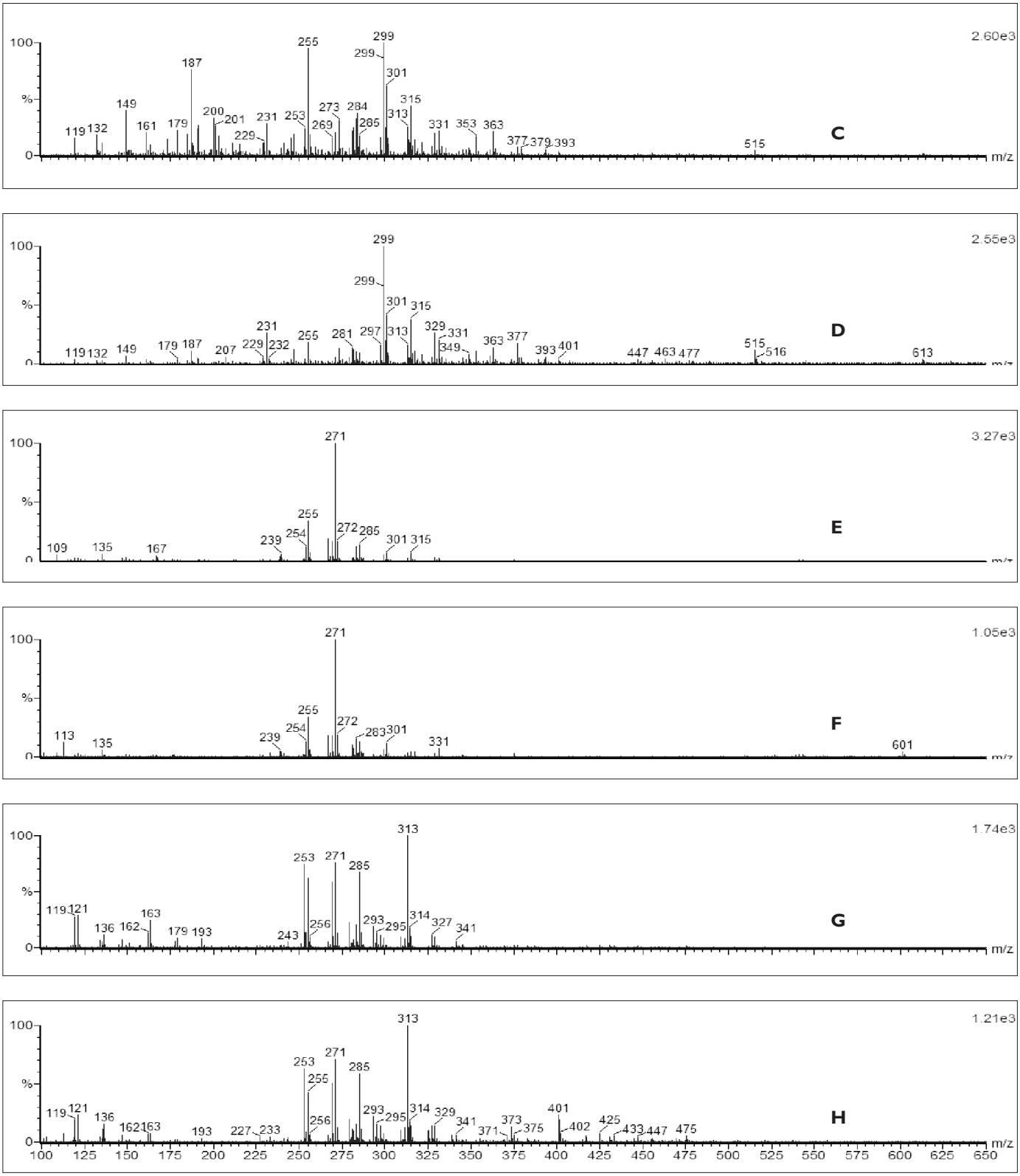


Fig. 1. ESI(-)-MS fingerprints of samples representative of the different types of propolis extracts after 20 days (A, C, E, G) and one year (B, D, F, H) extraction for brown Brazilian propolis (A, B), green Brazilian propolis (C, D), red Brazilian propolis (E, F), and North American propolis (G, H).

GC/MS

The GC/MS analysis of a sample of green Brazilian propolis is in full accordance with the ESI-MS data since it shows qualitatively for all extracts a very similar composition, but an increase in the concentration of some common components (as can be seen by the comparison of the area of selected peaks). Figure 2 displays the chromatograms obtained for different periods of maceration, whereas Table 2 lists the identified compounds and the area of their corresponding chromatographic peaks.

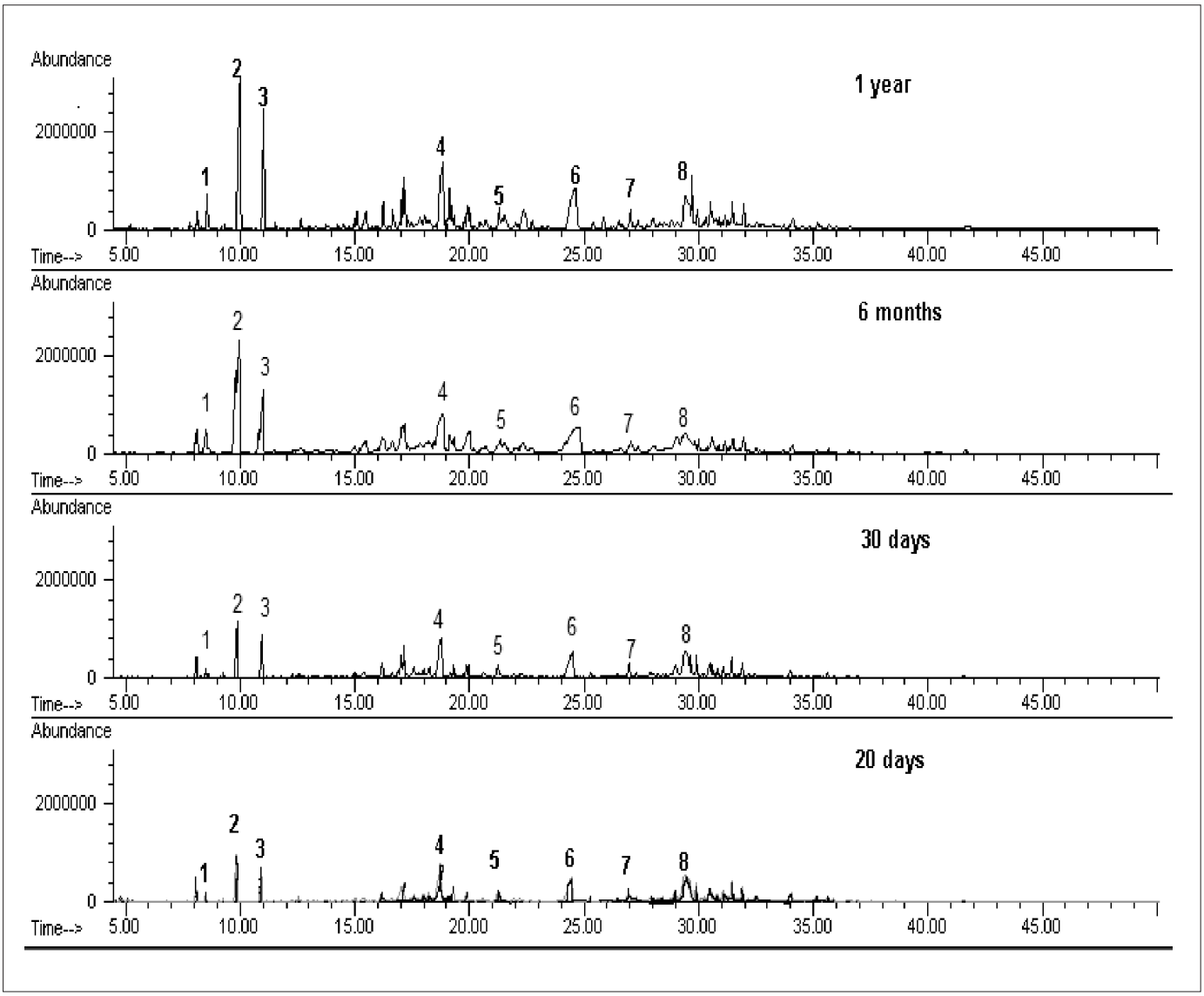


Fig. 2. GC/MS chromatograms of ethanolic extracts of a sample of green Brazilian propolis macerated for 20 days to one year. Numbers 1–8 correspond to the identified compounds seen in Table 2.

Table 2. Retention time and area of the peaks of selected compounds, identified by GC/MS after trimethylsilyl derivatization, in the ethanolic extracts of sample of green Brazilian propolis macerated for 20 days to one year.

		AREA OF PEAKS IN EXTRACTS MACERATED FOR:			
Name	**Rt (min)**	**20days**	**30 days**	**6 months**	**1 year**
1. Trimethylsilyl ether of glycerol	8.5	1.06	1.22	6.54	6.37
2. Hydrocinnamic acid ethyl ester	9.8	7.78	11.64	48.63	51.74
3. Trimethylsilyl ester of hydrocinnamic acid	10.9	5.69	9.29	25.84	25.91
4. Trimethylsilyl ester of p-trimethylsiloxy cinnamic acid	18.8	10.14	16.18	27.34	28.18
5. Trimethylsilyl 3,4-bis(trimethylsiloxy) cinnamate	21.3	1.08	3.75	4.34	4.99
6. 3-prenyl -4-hydroxycinnamic acid x 2 TMS	24.5	11.21	16.65	35.49	34.43
7. 3,4 dihydroxi-5-prenyl cinnamic acid x 3 TMS	27.0	2.44	4.03	7.28	5.72
8. 3,5-diprenyl- 4- hydroxycinnamic acid x 2 TMS	29.4	14.66	21.46	23.11	25.24

HPLC

Figure 3 shows the HPLC-DAD chromatograms obtained for the sample of green Brazilian propolis extracted for 20 days and for one year. Table 3 displays the results for the quantification of selected components of these propolis extracts by HPLC. The identified compounds were present in all the extractions, and the relative concentration of some components change slightly with maceration time.

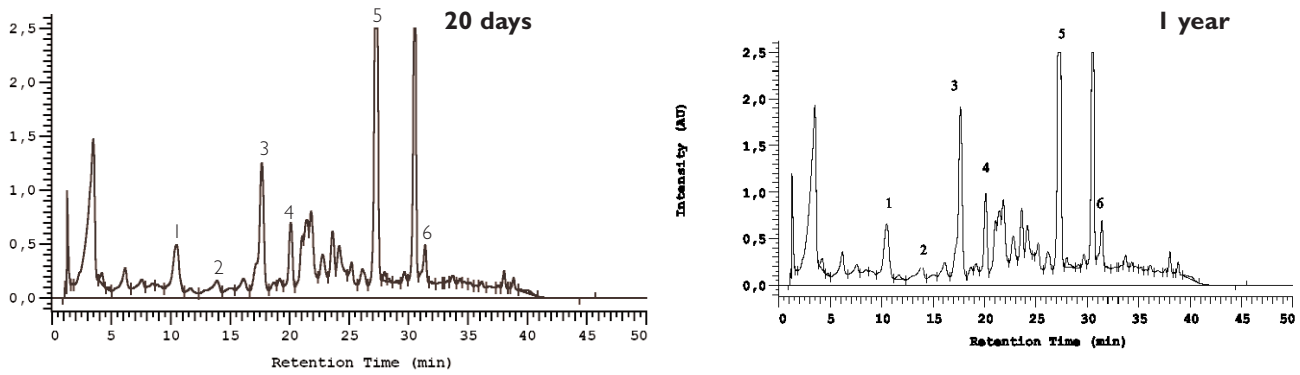


Fig. 3. HPLC/DAD chromatograms of ethanolic extracts of a sample of green Brazilian propolis macerated for 20 days to one year. Numbers1–6 correspond to the identified compounds found in Table 3.

Table 3. Concentration of components of the ethanolic extracts of a sample of green Brazilian propolis macerated for 20 days to one year, analyzed by HPLC.

	YIELD (%W/W) AFTER MACERATION FOR:			
Compound	**20days**	**30 days**	**6 months**	**1 year**
1. Dihydrokaempferide	30.53	36.95	35.93	35.20
2. Kaempferol	1.09	6.42	6.83	6.45
3. 3-prenyl-4-hydroxycinnamic acid	7.11	7.78	7.17	8.04
4. Kaempferide	27.62	29.18	29.43	30.81
5. 3,5-diprenyl-4-hydroxcinnamic acid	29.69	30.75	30.87	31.27
6. 2,2-dimethyl-8-prenyl-2H-1-benzopyran-6-propenoic acid	10.72	11.99	11.20	12.72

Discussion and Conclusions

Contrary to previous suggestions, ESI(-)-MS fingerprints of five types of EEP show consistently that their qualitative composition remains practically the same over the very broad period of maceration time investigated (20 days up to 1 year), with just a slight increase in the high mass components. The results of this qualitative analysis were further supported by HPLC-DAD and GC/MS quantification for a sample of green Brazilian. Green propolis was chosen for quantification since it is a well studied type of Brazilian propolis, therefore many of its pharmacologically important components have been identified [Marcucci and Bankova, 1999; Marcucci *et al.,* 2001] and HPLC methods have been developed for their identification and quantification [Marcucci *et al.,* 2000; Midorikawa *et al.,* 2001]. The HPLC results show an increase in the concentration of the selected components between 20 days and 30 days. After this period, fluctuations were observed in the HPLC concentrations of some compounds, which may indicate their decomposition after extended maceration periods. After one year, GC/MS also indicates decomposition since it shows a slight decrease in the measured area of the TMS derivatives of some important components (3-prenyl -4-hydroxycinnamic acid and 3,4 dihydroxi-5-prenyl cinnamic acid).

The yield (% w/w) of EEP for each extraction period shows an increase in the percent of extracted matter over the period of time investigated for all the samples analyzed. These results follow the same trend for the different types of Brazilian propolis as well as for the North American sample. Some variation can be observed between individual samples, but this is probably due to the amount of ethanol extractible resin in each sample. The average results show a total increase in yield of 11% between 20 days and one year, with the greatest increase observed between 30 days and 6 months. Indeed very little is gained by prolonging the maceration time from 6 months to one year.

The extra cost due to a prolonged maceration time would only be justified if the pharmacological, nutritional and antimicrobial properties of propolis extracts were improved. As ESI-MS, HPLC and CG/MS show no qualitative difference in the extracts obtained after 20 days and after one year, the 11% increase in yield hardly justifies the extra length of time that beekeepers must wait to commercialize this product. The three techniques show no richer composition but rather just a slight variation in the relative concentration of some major common components.

Finally, this is the first time, to the best of our knowledge, that prenylated cinnamic acids have been identified by GC/MS (as their TMS derivatives). As green propolis contains mainly non-volatile polar components, derivatization is required for GC/MS and the 70 eV EI mass spectra of such compounds are not available in commercial MS libraries. These limitations may explain why prenylated cinnamic acids have been not reported yet. These compounds are not exclusive, but are characteristic of the green Brazilian propolis, and can be clearly isolated by HPLC and detected by DAD [Marcucci *et al.,* 2000, 2001] and MS [Midorikawa *et al.,* 2001].

Acknowledgments

The authors acknowledge financial support from the following Brazilian foundations: PROPEP-USF, CNPq and FAPESP.

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ORIGINAL RESEARCH ARTICLE

Tissue distribution and properties of glutathione S-transferases in *Apis cerana cerana* Fabricius and *Apis mellifera ligustica* Spinola

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Received 27 October 2005, Revision 20 June 2006, accepted 3 August 2006.

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Summary

The glutathione S-transferases (GSTs) have been recognized to play an important role in the detoxification of xenobiotics. The tissue distribution and activities of GSTs in *Apis cerana cerana* **Fabricius** and *Apis mellifera ligustica* **Spinola** were assayed using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. GSTs were present at all the developmental stages of honeybee and widely distributed in different organs. GSTs from the midgut of both species showed the highest activity among all the detected organs. The enzymes were preferentially present in cytosol fraction of midgut. GSTs activities of each organ in A. cerana were lower than those in *A. mellifera.*

In *A. cerana,* the activity and Michaelis Menten (K_m) value of GSTs increased along with the development, no significant difference of activity and K_m was observed among different tissues. The enzymes activities increased rapidly and reached a maximum at the pupal stage to 0.081μmol.min^{-1}.mg^{-1} protein.

A. mellifera samples represented a different pattern. Significant differences of activity and K_m value were observed in different tissues. The GSTs in the midgut had the highest K_m value. However, the highest activity was found in the larvae and the lowest in the adult. The GSTs activities in drone were higher than those in worker bee. During the life span of *A. mellifera* worker bee, enzymes activities fluctuated between 0.2 and 0.4 μmol.min^{-1}.mg^{-1}pro. from the first day to 20th day and reached a peak on day 21 of adulthood.

Significant differences of specific activity and kinetic parameters of GSTs were observed among five honeybee subspecies. The highest activity was in *A. mellifera caucasica* and the lowest in *A. cerana cerana.* The GSTs from *A. mellifera anatolica* had the highest K_m and those from *caucasica* had the lowest. In *A. mellifera*, it was GSTs from anatolica that had the highest V_{max} and those from *caucasica* had the lowest. The GSTs from *A. cerana* had a higher affinity for CDNB than those from *A. mellifera.*

Keywords: *Apis cerana cerana, Apis mellifera ligustica,* glutathione S-transferases, kinetic characteristics, developmental stages

Introduction

Apis cerana and *A. mellifera* are two main honey bee species in China, adapted to a variety of environmental conditions. The fact that the two species have the similar regional distribution suggests that they probably use the genetic strategies of adaptation. Different enzymes are known to be responsible for adaptation mechanisms, among these enzymes are glutathione S-transferases(EC 2.5.1.18). The GST represents a multigene family of multifunctional proteins, widely distributed in almost all animals and plant kingdoms (Mannervik *et al.,* 1985). They are mainly cytosolic enzymes and catalyse the conjugation of reduced glutathione (GSH) to a wide variety of electrophilic compounds (Boyland and Chasseaud, 1969). The enzymes have been recognized to play an important role in the detoxification of xenobiotics, in both vertebrates and invertebrates (Grant and

Matsumura, 1989; Brophy *et al.,* 1989). They represent a very interesting detoxification mechanism due to their involvement in tolerance to insecticides (Motoyama and Dauterman, 1980; Clark *et al.,* 1985; Fournier *et al.,* 1992; Kostaropoulos *et al.,* 2001). Several authors have noted that increases in both GSH content and GST activity are associated with resistance to organophosphorus insecticides (Reidy *et al.,* 1990; Clark, 1989).

The presence of GSTs activity in *A. mellifera* L. was reported in 1984 (Yu *et al.,* 1984). In a more extended investigation, Smirle and Wisnton (1988) confirmed the presence of GSTs and mixed function oxidase activity in the adult worker bee and reported that as the worker bee foraged the specific activity of GSTs activity increased and reached its highest levels in the adult organisms.

For *A. mellifera macedonica,* GST activity is present at all developmental stages. The highest activity towards the substrate CDNB is found in the adult stage and the lowest is in the egg. The kinetic characteristics of the whole enzyme change along with the insect development. The number of isoenzymes and their rate of expression vary as the insect develops (Papadopoulos *et al.,* 2004a). The enzyme is heterodimeric with subunit molecular masses of 29 and 25 kDa, respectively. Two main isoenzymes with distinct kinetic properties are present, with isoelectric points of 7.40 for the alkaline and 4.58 for the acidic forms, respectively. The two enzymes are induced independently by factors such as insecticide treatments and environmental conditions, including low temperatures or starvation (Papadopoulos *et al.,* 2004b).But the investigation of GSTs in *A. cerana cerana* has not been launched so far.

In the present work, the results of some of the physical and catalytic properties of this enzyme are reported. The tissue distribution and activities of GSTs from *A. cerana cerana* are reported for the first time in the paper, compared with *A. mellifera* L. The result may contribute to the understanding of the sensitivity of the honeybees to pesticides and deal with a view to determining eventually their role in the metabolism and excretion of pesticides.

Materials and Methods

Chemicals and reagents

CDNB and GSH were bought from Sigma Chemicals (USA). Ethylenediaminetetraacetic acid (EDTA) was obtained from Fisher (Fair Lawn, NJ).Bovine serum albumin and other chemicals were purchased from Beijing Tongzheng Bio. Com..

Biological samples

A. mellifera caucasica, A. mellifera anatolica, A. mellifera carnica, A. mellifera ligustica were supplied by the apiary of the Institute of Apicultural Research, Chinese Academy of Agricultural Science. *A. cerana cerana* was supplied by the apiary of Department of Entomology, China Agricultural University. All colonies were healthy. Samples of bees were shaken off frames at random and kept in the incubator at 34.3°C and 70% relative humidity for 4h to empty their intestinal contents before being used for enzyme assays. Samples were always collected in the late morning to control possible diurnal variations in enzyme activity that could obscure differences between colonies.

Enzyme preparation

Adult *A. cerana* and *A. mellifera* worker bee or isolated tissues or body sections, were homogenized in phosphate buffer at pH 6.5(*Yu et al.,* 1984; Smirle, 1990), and then centrifuged at 10,000g for 20min (4°C)(Wood *et al.,*1986). The supernatant was collected for determination of enzyme activities and K_m

The enzyme during postembryonic period of *A. cerana* and *A. mellifera* was prepared from whole bodies of 8–10 individuals.

The midguts for determining subcellular distribution of GSTs activities were prepared from 30 individuals and homogenized in an ice-cold modified medium [100 mM potassium phosphate buffer, pH 6.5(Yu *et al.,* 1984; Smirle, 1990), 1 mM EDTA].The crude homogenate was filtered through filter paper and then centrifuged at 600g for 10 min in a Himac CP 80ß rotator. The cell debris was collected and the supernatant was centrifuged at 10,000g for 20 min again to obtain the soluble fraction and mitochondrial fraction (pellet). The soluble fraction was centrifuged at 105,000g for 90 min again to obtain supernatant and microsome (pellet) (Wood *et al.,* 1986; Zhang *et al.,* 2001). The cell debris, mitochondrial fraction and microsome were suspended in 100 mM sodium phosphate buffer, pH 6.5 and stored at -83°C for further analysis.

Determination of enzyme activities

GSTs activity was measured with CDNB as an electrophilic substrate (Grant *et al.,* 1989) according to Habig *et al.* (1974) with some modifications. The reaction mixture (900μL) consisted of 790μL sodium phosphate (0.1M, PH6.5), 30μL 30mM GSH, 30μL 30mM CDNB and 50μL enzyme extract. The enzyme reaction was conduced at 25°C for 2 minutes. Absorbency of the formed conjugate was continuously monitored at 340 nm against a control sample lacking enzyme (i.e. blank) using a Beckman spectrophotometer, and GST activity was expressed in nmoles CDNB conjugated/min/well using the published extinction coefficient ($e=9.6mM^{-1}.cm^{-1}$).

All assays were performed at room temperature and were run in triplicate. Each experiment was repeated at least three times.

Developmental changes of GSTs activity in *A. cerana* and *A. mellifera* L.

Homogenates of nymph, larvae and adult of *A. cerana* and *A. mellifera* L. worker bee were prepared at 2.5 insects per ml, those of nymph of *A. mellifera* L. drone at 2 nymphs/ ml, and those of larva of *A. mellifera* L. drone at 2.5 larvae/ ml. The homogenate was centrifuged at 10,000 g for 20 min then the supernatants were used to determine the enzyme activity.

The method of determination of the enzyme activities was as same as described above. Each assay contained 30μL 30 mM CDNB, 30μL 30 mM GSH in 0.1 M phosphate buffer pH 6.5 at 25°C.

Tissue distribution of GSTs activity in *A. cerana* and *A. mellifera*

All the designated tissues were excised from the worker bee of *A. cerana* and *A. mellifera*. Head, thorax and abdomen without midgut were homogenated in 200 μL of phosphate buffer pH 6.5, and midgut in 400μL buffer and then centrifuged at 10,000 g for 20 min. The supernatant was used to determine the enzyme activity with the foregoing method.

Daily changes of GSTs activity in the adult worker bee of *A. mellifera*

To obtain worker honeybee with known age, a single frame of upcoming emergent brood without adult bees clung was removed from a hive and incubated in darkness for 24h at 34.3°C and 70% relative humidity to get newly emerged worker bees. Emerged worker bee were transferred to a honey frame and collected randomly at designed times using a pair of tweezers. The abdomen was used for enzyme preparation. The enzyme activity was determined with the method mentioned above.

Determination of protein content

The protein was determined according to the method of Bradford (1976). Bovine serum albumin served as a standard.

Data Analysis

The results were analyzed by analysis of variance (ANOVA), considering the significance at a level of $P < 0.05$.

Results

Subcellular distribution of GSTs in *A. cerana* and *A. mellifera*

The spectrophotometric assay for the GSTs activity from *A. cerana* and *A. mellifera* using GSH and CDNB as substrates demonstrated that GSTs were preferentially present in cytosol fraction (Fig.1). The occurrence of a microsomal GSTs activity lower than that in cytosol was also demonstrated in *A. cerana* and *A. mellifera* homogenates.

There was a difference in subcellular distribution between *A. cerana* and *A. mellifera*. In *A. cerana,* the specific activity of GSTs in microsomes was 50% of that in mitochondrial fraction and only 40% of that in cytosol. In *A. mellifera,* the specific activity of GSTs in microsomes was 1.54-fold higher than that in mitochondrial fraction but only 0.43-fold of that in cytosol.

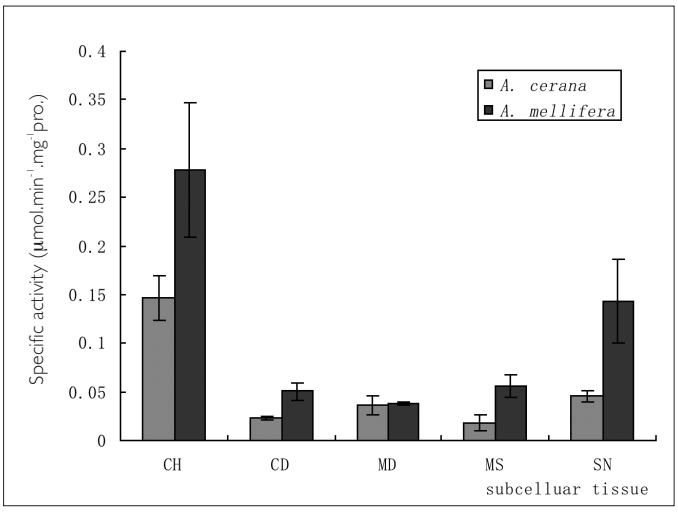

Fig. 1. Subcellular distribution of GSTs in *A. cerana* and *A. mellifera* worker bee

Note: CH = Crude homogenate; CD = Cell debris; MD = Mitochondrion; MS = Microsome; SN = Supernatant

Tissue distribution of GSTs activity in the adult worker bee of *A. cerana* and *A. mellifera*

Enzyme preparations of different tissues (10,000g supernatants) from *A. cerana* and *A. mellifera* were assayed using GSH and CDNB as substrates, and the results were showed in Fig.2.

Midgut represented the highest GSTs activity among the tissues determined in both species. Besides the midgut, the activity of GSTs in abdomen without midgut, head and thorax showed a descending order in *A. mellifera,* and significant differences were observed among them. However, there were no significant differences of GSTs activity among different tissues in *A. cerana.* Fig. 2 also showed that the GSTs activities of different tissues in *A. cerana* were significant lower than those in *A. mellifera.*

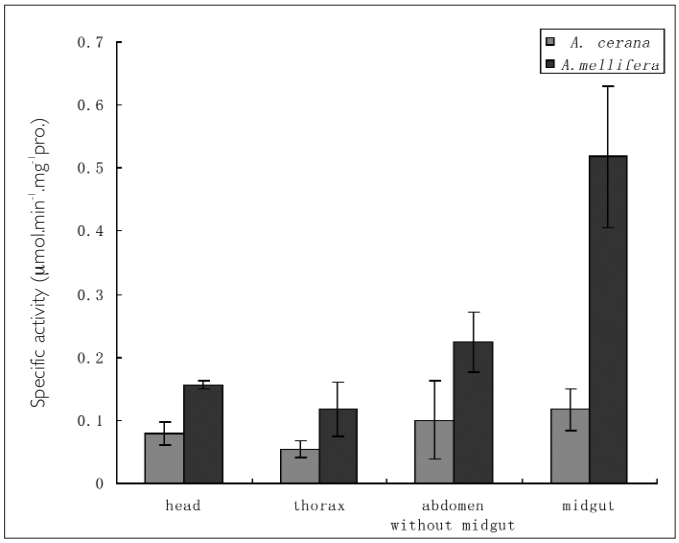

Fig. 2. Tissue distribution of GSTs in *A. cerana* and *A. mellifera* worker bee

The K_m and V_{max} values of GSTs for GSH and CDNB were also determined in different tissues in both species. As showed in Table 1, there was no difference in the K_m values of GSTs from different tissues in *A. cerana* ($P>0.05$). In *A. mellifera,* however, significant differences were observed among tissues. The GSTs from the midgut had the highest K_m value, subsequently was that from the abdomen without midgut, head and thorax.

The rank orders of the V_{max} values of different tissues of the two species both were midgut> thorax> abdomen without midgut> head. Significant differences were observed in tissues between the two species.

There was no statistical difference between the K_m values of GSTs of the two species in the four tissues. No difference was found in V_{max} level of the two bees in thorax and abdomen without midgut. However, significant differences ($P< 0.05$) were observed between the V_{max} level of the two bees in head and midgut.

Table 1. The K_m and V_{max} of GSTs in different tissues of *A. cerana* and *A. mellifera* worker bee.

Tissues	*A. cerana*		*A. mellifera*	
	K_m (mmol/L)	V_{max} (μmol.min^{-1}.mg^{-1}pro.)	K_m (mmol/L)	V_{max} (μmol.min^{-1} .mg^{-1}pro)
head	0.648±0.208a	0.146±0.046a	0.549±0.066a	0.245±0.005a
thorax	0.870±0.269a	0.207±0.053b	0.515±0.140b	0.283±0.048b
abdomen without midgut	1.030±0.724a	0.151±0.0598c	0.120±0.045c	0.184±0.041c
midgut	1.730±0.856a	0.269±0.078d	1.240±0.131d	0.704±0.009d

Note: Values are means ± standard deviations of the mean of three separate preparations. Within a column, values with different letters indicate a difference significantly at $P < 0.05$.

Developmental changes of GSTs activity in the worker bee of *A. cerana* and *A. mellifera*

Data in Fig.3 showed that different developmental stages of the worker bee had different GSTs activities in both *A. cerana* and *A. mellifera.* The GSTs activities of *A. cerana* in different developmental stage were lower than those of *A. mellifera.* An increase in enzyme activity as development proceeded was readily seen in *A. cerana.* A 119% increase in specific activity was detected from larvae to pupae, while no significant difference was observed thereafter.

But in *A. mellifera,* the larvae showed the highest GSTs activity, and the adult showed the lowest activity. For *A. mellifera,* the activity of GSTs in various stages of drone was higher than that in worker bee.

The K_m and V_{max} values of two species for CDNB were determined in the three developmental stages (Table 2). The results indicated that they did not change significantly in the transition from larval to adult stage. The changes of K_m values did not show the same trend as that of the enzyme activities. For *A. cerana,* a 40% decrease of K_m values was detected as the bee developed from larva to pupa. In contrast, as the bee developed from pupa to adult, an 85% increase was detected.

For *A. mellifera,* the changes of K_m values had the same trend as that of the enzyme activities.

Compared with those of the worker bee, the K_m values of drone were relatively lower but V_{max} were higher in *A. mellifera.*

The changes of GSTs V_{max} in the different developmental stages of worker bee of both species had the same trend , i.e. the pupa> the adult> the larva.

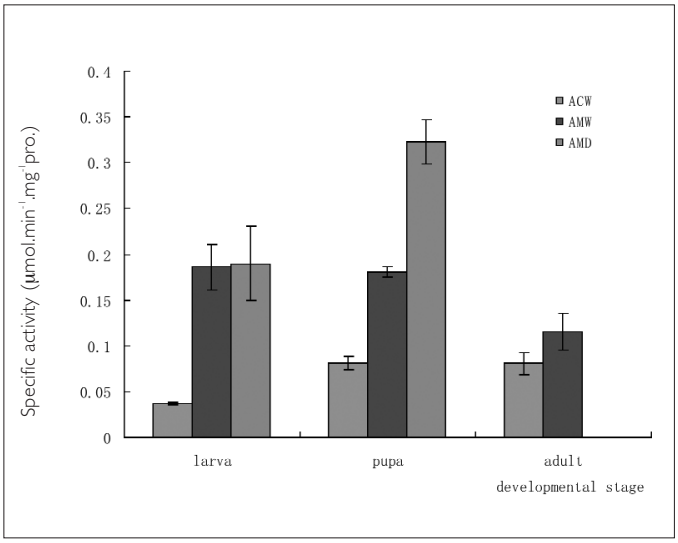

Fig. 3. The specific activity of GSTs in *Apis cerana* and *A. mellifera* in three developmental stages (ACW: *Apis cerana* worker bee; AMW: *Apis mellifera* worker bee; AMD: *Apis mellifera* drone)

Table 2. Developmental changes of Glutathione S-transferase in *A. cerana* and *A. mellifera.*

Developmental stage	*A. cerana*		*A. mellifera*	
	K_m (mmol/L)	V_{max} (μmol. min^{-1}.mg^{-1}pro.)	K_m (mmol/L)	V_{max} (μmol.min^{-1} .mg^{-1}pro)
LW	0.615±0.235a	0.067±0.025a	0.547±0125a	0.224±0.017a
PW	0.369±0.207a	0.140±0.050a	0.542±0.120a	0.231±0.034a
AW	0.684±0.203a	0.120±0.025a	0.505±0.077a	0.205±0.075a
ND	Not Done	Not Done	0.439±0.090a	0.315±0.012a
PD	Not Done	Not Done	0.473±0.160a	0.332±0.038a

Note: LW: larvae of worker bee; PW: Pupae of worker bee; AW: Adults of worker bee; ND: Nymph of drone; PD: Pupae of drone. Values are means ± standard deviations of the mean of three separate preparations. Within a column, values with different letters indicate a difference at $P < 0.05$.

Daily changes of GSTs activity in the adult worker bee of *A. mellifera*

The GSTs activities of the adult worker bee of *A. mellifera* were measured during their life span (24 days on average). The GSTs activities fluctuated between 0.2 and 0.4μmol.min^{-1}.mg^{-1}pro. from day 1 to day 20, and reached a peak on day 21, which was about 0.75 μmol.min^{-1}.mg^{-1} pro.

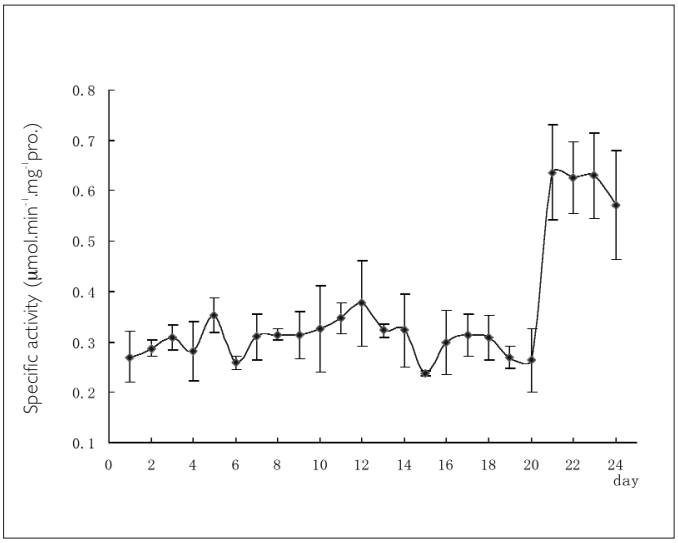

Fig. 4. Daily changes of GSTs activity in the adult worker bee of *Apis mellifera.*

Comparison of GSTs activities in the adult worker bee of different subspecies of *A. mellifera* and *A. cerana*

Data in Table 3 showed significant differences of GST activities among the five honeybee species. The enzyme activity in *A. mellifera caucasica* was the highest and that in *A. cerana cerana* was the lowest.

Significant differences of GSTs properties were also observed by kinetic parameters. At a fixed GSH concentration, the K_m values were 0.50 mM, 0.20 mM, 0.26 mM, 0.13 mM and 3.83 mM, respectively. In *A. mellifera,* the GSTs from *carnica* had the highest K_m value and those from *caucasica* had the lowest, those from anatolica had the highest V_{max} and those from *caucasica* had the lowest. The apparent K_m value for *A. cerana* was higher than that for *A. mellifera* and V_{max} value for *A. cerana* was lower than that for *A. mellifera.* This suggested that GSTs from *A. cerana* had a higher affinity for CDNB than those from *A. mellifera.* The enzyme's catalytic efficiencies (K_{cat} /K_m) were 0.62, 1.65, 1.54, 1.85 and 0.09, respectively.

Discussion

As to our knowledge, this study represents the first report on the GSTs activities and biochemical characteristics such as K_m, V_{max} in *A. cerana* in comparison with *A. mellifera.*

The ontogenetic pattern of total GSTs activity towards CDNB was studied in the honeybee, *A. cerana* and *A. mellifera.* The distribution and biochemical characteristics such as K_m, V_{max}, specific activity of total GSTs were investigated. We conducted this investigation because the honeybee is holometamorphosis insect with four well-defined developmental stages and the detoxification status of each stage is unknown. The results of the present investigation showed that the activity and distribution of GST were different in tissues, sex, subcellular and strains.

GSTs belong to phase II detoxification system involved in conjugation reactions and may also detoxify a number of toxic ligands by acting as a non-catalytic intracellular binding protein (Kostaropoulos *et al.,* 2001). It is believed that these enzymes play essential roles in the survival of insects exposed to endogenous or exogenous xenobiotics. In a previous investigation, Smirle (1993) found a positive correlation between the activity of the detoxification enzymes GSTs and the resistance to certain insecticides.

The GSTs exist in both cytosolic and microsomal in the honeybee according to our investigation. Majority of GSTs in insect are homo-or hetero-dimers of subunits of 26 kDa, and are found mainly in the cytosol(Yu, 2002). The significance of microsomal GSTs, however, cannot be overlooked, since these enzymes, which are associated with microsomal membrances, may be more important than those in cytosolic in the detoxification of harmful metabolites formed by cytochrome P450. Furthermore, many substrates of GSTs are lipophilic and would tend to accumulate in the endoplasmic reticulum of the cell. Midgut microsomal GSTs and cytosolic GSTs are different qualitatively in the fall armyworm(Yu, 2002).

Table 3. Comparison of GSTs activities in the adult worker bee of different subspecies of *A. mellifera* and *A. cerana*

Subspecies	**Specific activity (μmol.min^{-1}.mg^{-1} pro.)**	**K_m (mmol/L)**	**V_{max} (μmol.min^{-1}.mg^{-1} pro.)**
A. mellifera carnica	0.33±0.05a	0.50±0.18a	0.31±0.14a
A. mellifera ligustica	0.40±0.08b	0.20±0.09b	0.33±0.03b
A. mellifera anatolica	0.42±0.05c	0.26±0.13c	0.40±0.07c
A. mellifera caucasica	0.45±0.08d	0.13±0.01d	0.24±0.02d
A. cerana cerana	0.21±0.07e	3.83±0.02e	0.36±0.03e

Values are means ± standard deviations of the mean of three separate preparations. Within a column, values with different letters indicate a difference at $P < 0.05$.

Our results had shown that GSTs commonly exist in different tissues in *A. cerana* and *A. mellifera* (Fig. 2), which resembles those report on vertebrate and insect enzymes. In locust, the GSTs activities were found in many different tissues, such as fat-body, Malpighian tubes, gastric caecae, mid-gut, hind-gut, integument, fore-gut, blood and so on, especially in digestive system (Cohen *et al.,* 1964). In *Triatoma infestans* tissues, the genitalia were the richest source of glutathione transferases in insect organ (Wood *et al.,* 1986). The fat body contained 50% of GSTs activity in the *Lucilia cuprina* (Wiedemann), with 25% being in the cuticle, 15% in the gut, and 10% in the blood (Kotze and Rose,1987). In both *A. cerana* and *A. mellifera,* midgut is the richest source, which accounts for approximately 35% and 51% of the GSTs activity in *A. cerana* and *A. mellifera*. Because the midgut is the place where insects digest food and take up chemical substances, it is reasonable to assume that the midgut has the maximum GSTs activities. Like other insects, the abdomen is the main resource of GST activity in honeybee.

Several ontogenetic studies have been reported for mammals (Gregus *et al.,* 1985). Four insects (*Lucilia cuprina, Aedes aegypti, Plutella xylostella* and *Tenebrio molitor*) exhibit a similar model as the one we found in *A. cerana,* i.e. the highest GSTs activity appearing in the pupal stage (Table 3). Low GSTs activity in the adult stage appears to be quite common in mammals and insects (Gregus *et al.,* 1985; Kotze and Rose, 1987). This may be explained in three respects: firstly, the fact that the pupae are immobile and consequently are more vulnerable to unfavorable environmental conditions (Gillott,1980) including the presence of toxic substances; secondly, the elevated biosynthesis and construction of adult tissues in the pupal stage (Doctor and Fristrom,1985), and therefore high specific activity could mean high ability of detoxification and as a consequence protection of important and crucial biosynthetic pathways from inhibition by toxic substances. Thirdly, adult honeybees do not contain a large amount of fat body in the abdomen. Because the fat body is known to play an important role in the detoxification as well as storage of insecticide, the extremely low fat body content in honeybee may be, in part, responsible for their high susceptibility to insecticides.

There was an increase of V_{max} from larvae to pupae followed by a decrease in the adults in *A. cerana.* This suggested an increase in the total amount of GSTs in the pupal stage. The pupal stage in many insects has evolved to resist unfavorable conditions, and is a stage immediately preceding the reproductive stage of insect. Thus, the increase of total GSTs may offer greater protection to that stage and compensate for the simultaneous decrease of GST affinity for GSH as judged by the observed increase in K_m value. In the adults, both the affinity and the amount of the enzyme were low as indicated by the high K_m and low V_{max} values. This implies that the GSTs detoxification system is of potentially low importance in this stage.

The data indicated that the larvae of *A. mellifera* contained a much higher level of GSTs than the pupae and adults. Analysis of variance carried out for the GSTs specific activities of various developmental stages showed significant differences between the different stages. Pupae do not feed and therefore they may contain fewer plant chemicals and xenobiotics than the larvae that are actively feeding. Larvae that are actively feeding have the possiblity to ingest contaminating toxic chemicals along with the diet and therefore one would expect them to have a higher level of GSTs.

Our results about the K_m and V_{max} values of GSTs in *A. cerana* and *A. mellifera* showed no significant changes in the developmental stages. It suggested that the production of the present GSTs increased during the developmental stages rather than altered or new GSTs formed. Indeed, while the V_{max} values of GSTs in *A. mellifera* are always higher than those in *A. cerana.*

A. mellifera macedonica (Papadopoulos *et al*., 2004a) and *Triatoma infestans* (Wood *et al.,* 1986) exhibited maximum GSTs activity in the adult stage. However, the K_m and V_{max} values were similar in larvae, pupae and adults. This suggested that the amount of the enzyme present in the adult stage was higher than that in the previous stages.

In *A. mellifera,* GSTs reached a maximum levels on day 21 of adulthood, and lowest in 6-day-old worker bee (Fig. 4). In a honey bee colony, apart from laying fertile eggs, all the tasks which are necessary for the growth and survival of the colony are accomplished by the workers. These tasks include feeding and caring for the brood produced by the queen, building new comb, defending the colony and foraging for food, water and propolis. During the latter part of their lives, usually 20 days old worker bees become foragers. Since GSTs has been linked to detoxifying ingested chemicals that are toxic, they may actually be needed in the adult stage because this is the only stage that is exposed to the external environment. Our results are in line with the report of Smirle and Wisnton (1988) that the specific activity of glutathione S-transferase reaches its maximum value as the adult worker bee forages and play a significant role as the enzyme in the adult stage.

The presence of varying GSTs levels in different developmental stages has already been reported for insects as a consequence of environmental or genetic effect. The GSTs activity of *Tetranychus urticae* is highest in 2-4-day-old female adults and drops considerably with progressing age. Age also influences the amount of GSTs in animals like the mouse (Shoemaker *et al*., 1981), rat (Spearman and Lerbman,1984) and mosquito (Hazelton and Lang ,1983), although these changes are not very dramatic.

Our results that the GSTs activities in drone of *A. mellifera* were higher than those in worker bee resemble those reported on vertebrate and insect enzymes. *Limande limande* collected in spring was pronounced sexual differences in the activities of GSTs. Being in prespawning condition, males exhibite higher activities than females from the same location (Dirk and Lange,1995). In *T. infestans,* activity of GSTs in male genitalia is much higher than that in female (Wood *et al.,* 1986).

The significant differences in GSTs activity and kinetic characteristic (including different species) observed among five species, implied that the GSTs were different in nature between species. Interspecies variations of GSTs activity were observed in freshwater and marine fish species (Förlin *et al.,* 1995). Different GSTs activity was also observed in three genetic lines of rice (Deng and Kriton, 2002). Inter-strain variations exist in mouse liver GSTs activity (Wheldrake *et al*.,1981). The multiple isoenzymes hypotheses can be suggested to explain our results. Multiple GSTs isoenzymes are a common phenomenon in vertebrates, like fish and mammals. The presence of multiple isoenzymes is quite usual in insects and has been reported in a number of species. In *Tenebrio molitor* larvae, the existence of four isoenzymes was reported, as well as alternations in some GST characteristics during development (Kostaropoulos *et al.,*

1996).Drosophila melanogaster has two major classes of well-characterized GST proteins, the GST D isoenzyme (a family of eight intronless genes) and GST 2. *Drosophila simulans* has three forms GST: GST D, GST2 and third major form (Gaëlle *et al.,* 2001).In fall armyworm larvae, the midgut possesses five isoenzymes, namely MG GST-1, MG GST-2, MG GST-3, MG GST-4 and MG GST-5, all of which are heterodimers with subunit molecular weights of 26,700 to 30,000. No qualitative difference in isoenzyme composition is observed during larval development. The fat body contains three isoenzymes, namely, FB GST-1, FB GST-2 and FB GST-3, all of which are homodimers with subunit molecular weights of 20,100 to 29,000(Yu,1995).

The distribution of GST isoenzymes was found to be tissue- and region-specific in rats (Haider *et al.,* 2004). GST isoenzyme profile can be altered by several physiological factors such as age (Tee *et al.,* 1992; Carrillo *et al.,* 1991) and sex (Singhal *et al.,* 1992; McLellan and Hayes, 1987).

Two main isoenzymes were confirmed to present in all developmental stages of *A. mellifera macedonica,* one in the alkaline area and the other in the acidic (Papadopoulos *et al.,* 2004b). This was deduced by the elution profile of unpurified cytosolic fraction when it was subjected to chromatofocusing at pH range 7 to 4. While in the larval stage only two main isoenzymes appeared to be present in the elution profile, in the pupal stage more isoenzymes appeared to be present in the acidic area. However, these isoenzymes were not expressed in the adult stage.

In conclusion, our findings reveal a very interesting developmental model of GSTs that is followed by the bee and which, to our knowledge, is unique. The comparative investigation indicates the usefulness of establishing baseline data of GSTs enzyme sets for honeybee species. The multiple GSTs isoenzymes in honey bee are required to be confirmed.

Summary

The glutathione S-transferases (GSTs; EC 2.5.2.18) are a multigene family of enzymes which play an important role in metabolism of both endogenous compounds and xenobiotics and have also been implicated in development of pesticide and drug tolerance. Very little is known about the distribution and properties of GSTs in A. cerana and *A. mellifera* L. In this paper we first report on the activities of GSTs activities and characteristics such as K_m ,V_{max} using CDNB and GSH as substrates.

Glutathione S-transferases were present in all the developmental stages of *A. cerana* and *A. mellifera* L. and were widely distributed in all the determined organs. Midgut represented the highest activity among all the determined organs in both honey bee species. The enzymes were preferentially present in cytosol fraction of midgut. The GSTs activity in each organ from *A. cerana* was lower than that from *A. mellifera.*

In *A. cerana,* the activity and K_m value increased as development proceeding, no significant differences of activity and Km was observed among different tissues. The enzymes level increased rapidly and reached a maximum at the pupal stage.

A. mellifera samples represented a different pattern, in which significant differences of activity and K_m were observed among different tissues. The GST in the midgut had the highest K_m. The highest activity was found in the larva, and the lowest in the adult. The activity in drone was higher than that in worker bee. During the life span of *A. mellifera* worker bee, enzyme activity fluctuated between 0.2 and 0.4 $\mu mol.min^{-1}.mg^{-1}$ pro. from day 1 to day 20 and reached a peak on day 21 of adulthood.

The significant GST differences of specific activity and kinetic parameters were observed among the five honeybee subspecies. The highest activity of GST was in *A. mellifera caucasica* and the lowest in *A. cerana cerana.* The GST from *A. mellifera anatolica* had the highest K_m and that from *A. mellifera caucasica* had the lowest. The highest V_{max} of GST in *A. mellifera* was from *anatolica* and the lowest from *caucasica.*

The GST from *A. cerana* had a higher affinity for CDNB than that from *A. mellifera.*

Our results are redound to the future toxicity research on *A. cerana* and *A. mellifera.* The comparative investigation indicates the usefulness of establishing baseline data of GST enzyme for honeybee species. This study deals with a view to determining eventually GSTs role in the metabolism and excretion of pesticides in two species. In this paper, the results of some of the physical and catalytic properties of this enzyme are reported.

Acknowledgments

This work was financially supported in part by The National Natural Science Foundation of China (30471314). We thank Professor Liu Xianshu, Doctor Shi Wei, Master li Xiu, Master Yang Lu, Mr. Peng Wenjun and Mr. Liu Fuxiu for giving honeybee. Thanks are also due to Doctor Li Jianke in preparation of this manuscript. The technical assistance of Doctor Shi Xueyan is appreciated.

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NOTES AND COMMENTS

Reliability of an island mating apiary under routine management

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Received 11 August 2005, accepted unconditionally for publication 8 December 2005.

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Keywords: *Apis mellifera,* honeybee, DNA-microsatellite, mating apiary, polyandry

Introduction

Islands are widely used to achieve controlled matings of virgin queens because queens and drones are not expected to cross open water during mating flights. However, uncontrolled matings on island mating areas have repeatedly been reported for many islands in close proximity to mainland shorelines (c.f. Neumann et al., 1999b). With increasing distance between island and mainland one would expect increasing reliability of mating control. Moreover, uncontrolled matings are more likely when the number of drone producing colonies [DPCs] is low. Under routine mating apiary management conditions several strong DPCs are to be placed on the island in order to provide enough drones to saturate local drone congregation areas [DCAs] and prevent matings with undesired drones from the mainland (c.f. Neumann et al., 1999b). Here we test the reliability of a mating apiary on the island of Neuwerk under local routine conditions with an ample supply of drone producing colonies typical for managed island mating apiaries.

Eight strong DPCs (22 frames) and 99 mating nuclei with virgin sister queens were placed on the North Sea island of Neuwerk. Six of the 80 nuclei with mated queens were used for the analysis (Table 1). Neuwerk is 9.3 km of open water away from the mainland with other drones. The virgin queens were allowed to mate freely for a three-week period during the local reproductive season (July). After the queens initiated oviposition, sealed worker brood was sampled from each mating nucleus colony and sealed drone brood from each DPC. DNA was extracted from flight muscle tissue of pupae and genotyped at nine polymorphic DNA microsatellite loci (B124, A7, A24, A35, A43, A76, A88, A107 and A113; Estoup et al., 1994) using routine protocols (Moritz et al., 2003). DNA was amplified with fluorescent dye labeled primers (FAM, HEX and TET). Allelic DNA fragment sizes were scored in two electrophoresis runs in a DNA sequencer (ABI 310) using Genotyper© software, an internal size standard (GeneScan 500) and the protocols of the supplier. The putative queen genotypes of the DPCs were derived from pooled drone samples (n=20 drone offspring for each DPC queen, Moritz et al., 2003). Likewise, the putative genotypes of the island-mated queens and of their drone mates (=number of observed matings) were inferred from the worker offspring using Mendelian rules (n=20 workers each queen). The number of estimated matings was calculated as in Neumann et al. (1999b). To detect uncontrolled matings, the possible genotypes of the drones derived from the DPC queens were compared with the actual detected father genotypes.

A total number of 54 observed matings on the island were detected (Table 1), which could all be assigned to genotypes of DPC drones (data not shown but available on request from PN). The non-detection error (probability for not identifying a foreign drone because it has the same allele combination at all loci as a drone from a DPC) was <1% over all loci and colonies.

Although the mud flats around Neuwerk fall dry at low tide, we were unable to detect uncontrolled matings. This confirms earlier studies (see Neumann et al., 1999a and references therein) that a distance of 9.3 km to the mainland is sufficient to ensure controlled matings under the given supply of DPC. Likewise, our data also confirm earlier studies (Neumann et al. 1999a,b) showing that queens mate less frequently under such island conditions compared to the European mainland where over 15 matings are common.

Thus, reliable matings can be achieved on islands if they are sufficiently far from the mainland and an adequate drone supply is provided. Nevertheless, mating control through island apiaries is achieved at the cost of reduced mating frequencies that may have negative effects on the number of spermatozoa in the spermatheca (Schlüns et al. 2005).

Table 1. Number of observed and estimated matings for the queens mated on Neuwerk (n = sample size; k_{obs} = number of observed matings, k_{est} = number of estimated matings; n.d. = non-detection error).

Colony	***n***	$\mathbf{k_{obs}}$	$\mathbf{k_{est}}$	**n.d.**
w76	20	8	8.79	0.021
w77	20	9	10.37	0.002
w93	20	11	14.44	0.004
w94	20	8	8.79	0.011
w99	20	9	10.37	0.011
w100	20	9	10.37	0.004
		9.0±0.45	**10.52±0.85**	**0.009±0.003**
		E=54		

Acknowledgements

We wish to thank P Leibe for technical assistance. Financial support was granted by the European Commission [RFAM, BABE network] and an Emmy Noether fellowship of the DFG [PN].

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NOTES AND COMMENTS

Drift of *Varroa destructor*-infested worker honey bees to neighbouring colonies

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Received 20 November 2005, accepted subject to revision 25 January 2006, accepted for publication 1June 2006.

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Keywords: *Apis mellifera, Varroa destructor,* drift

Introduction

Varroa destructor-infested honey bee (*Apis mellifera*) colonies usually die if left untreated. However, colonies that have been treated with miticides are usually reinfested soon after treatment (Greatti *et al.* 1992). It has been suggested that this reinfestation may occur because of bees drifting between infested and treated hives or from bees robbing heavily infested colonies. Excluding drifting drones from hives did not reduce the rate of reinvasion (Greatti *et al.* 1992). The literature cites two views regarding correlation between worker bee drift and varroa infestation. Neumann *et al.* (2000) did not find a correlation between worker bee drift and *varroa* infestation. They state this may be because the overall level of worker drift was low. This result contrasts with Sakofski (1990) who found that drift of worker bees was higher if the levels of *varroa* infestation were higher.

The purpose of this investigation was to determine whether the reinfestation of colonies is due to *varroa* increasing the rate with which workers drift from infested colonies into neighbouring colonies.

On 7 December 2004, sixteen honey bee colonies from the same apiary were randomly allocated among four apiary sites (4 colonies per apiary), located at least 5 km apart, in the Waikato region of New Zealand. It is expected that there were unknown apiaries within the foraging range of test colonies. The colonies were housed in Langstroth hives with two full-depth brood boxes. These had at least twelve full-depth frames of bees and four frames of brood at the start of the trial. Honey supers were added to the hives when required and not removed until after the trial was completed. The colonies were treated with Bayvarol® strips in March 2004 and then not treated until 7 December 2004. This was to ensure that the *varroa* populations in the untreated colonies would reach critical levels during the trial. Two randomly selected colonies from each site were treated with Bayvarol® strips according to label instructions, while the other two remained untreated. The Bayvarol® strips were replaced every eight weeks until the end of the trial. It was assumed that Bayvarol® does not influence drift. Each *varroa*-infested hive was placed ca 300 mm from a hive containing Bayvarol® strips. Two pairs were placed ca 900mm apart in a straight line with entrances facing the same direction so as to maximise drift.

Between one and three frames of emerging bees were removed from each hive every four weeks from 18 January to 12 April 2005. These were placed into separate boxes to collect the emerging bees. The boxes were placed in an incubator at 35°C with a tray of water for 24 h. The following day, 200 newly-emerged bees from each box were marked with a spot of coloured paint (Testor™) on the thorax. A different colour was used to mark the bees from different hives in the same apiary. The marked bees and frames were returned to their parent hive the same day that they were marked. The frames were inserted first and then the bees were placed on the top bars of the top super. When less than 200 bees emerged in a box, these bees were painted and returned to their hive. The frames however, were returned to the incubator for a second 24 hours. The required number of additional emerging bees, totalling 200, were then collected, marked and returned to their parent hive the next day.

Two weeks after the bees had been returned to their parent colonies, the number of painted bees in each colony was recorded by checking all bees on each frame. It is probable that some marked bees were foraging when the checks were carried out as they were all conducted on the same day during foraging. Any painted bees that had drifted into a neighbouring colony were recorded and killed.

The trial was discontinued on 26 May by which time six of the eight *varroa*-infested colonies had died. When the colonies were inspected shortly before they died it was noted that the colony strengths had declined to less than one frame covered with bees. In every case the queen, a few bees and a small patch of brood were present. The size of the patch varied from 5cm to one frame of capped brood without enough workers to care for it. All honey from colonies that died was robbed out.

For each hive, the percentage of marked bees that drifted from that hive to the neighbouring colonies in the other

treatment was calculated for each sampling episode. This percentage (and arcsin square root of proportion) for each episode was analysed by analysis of variance allowing for apiary and treatment. The average percentage of bees drifting from untreated hives infested with *varroa* into neighbouring treated hives did not exceed 3% (Table 1). There were no significant differences ($P > 0.1$) between the percentage of drifting bees from the *varroa*-infested colonies and the treated colonies at any stage throughout the trial. This suggests that the reinfestation of treated colonies does not predominantly result from heavy *varroa* infestations causing worker bees to drift to other colonies, more than they generally do in practically *varroa*-free colines. This supports Neumann *et al.* (2000). The normal level of drift in apiaries will, however, spread *varroa* from untreated to treated colonies. This observation supports the practice of treating all colonies in an apiary simultaneously.

The presence of the queen and a small number of workers in the dying colonies suggests that the reinfestation was not due to colonies absconding and flying into colonies in the apiary. The alternative cause of reinfestation of treated colonies may be that the bees from treated colonies rob dying colonies and transport *varroa* back to their colonies.

As a large number of the painted bees did not drift into other hives in the apiary or die in their own hive, we cannot surmise where the marked or unmarked bees from the dying colonies went. The low levels of drift from all colonies support the initial assumption that Bayvarol® treatments do not influence drift.

Table 1. The mean number of marked bees recovered in live colonies (SE) and the average percentage (SE) of marked bees that drifted from one treatment to neighbouring colonies in the other treatment.

	3 Feb 2005	**3 Mar 2005**	**11 Apr 2005**	**28 Apr 2005**
Marked bees recovered from live colonies	90.3 (6.1)	111.1 (10.4)	72.5 (11.7)	117.2 (19.6)
Number of live Bayvarol® colonies	8	8	8	8
Number of live untreated colonies	8	7	5	5
% drift from Bayvarol® into untreated	2.2 (0.89)	1.1 (0.37)	0.0 (0)	0.1 (0.06)
% drift from untreated into Bayvarol®	3.0 (1.48)	0.6 (0.29)	1.3 (1.1)	0.2 (0.21)
SED	1.68	0.52	0.82	0.18
P	0.6	0.3	0.13	0.4
***P* (arcsin transformed)**	0.7	0.4	0.1	0.6

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Journal of Apicultural Research 45(3): 157–158 (2006)

NOTES AND COMMENTS

Electronic monitoring of feeding behaviour of *Varroa* mites on honey bees

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Received 10 December 2004, accepted subject to revision 18 February 2005, accepted for publication 16 June 2006.

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Keywords: electronic penetration graph, monitor, feeding behaviour, *Varroa destructor*

Introduction

The *Varroa* mite (*Varroa destructor* Anderson and Trueman) is an ecto-parasite and the most devastating pest of the honey bee (*Apis mellifera* L) (Sammataro *et al,* 2000). Because *Varroa* mites are small and feed on adult bees between body segments, or on larvae and pupae inside brood cells, it is difficult to observe their feeding behaviour. Donzé and Guerin (1994) reported that *Varroa* mites fed 0.81–1.49 hr on honey bee worker pupae during a 24-hr period. Their criterion for identifying feeding bouts was based on observing mites on the pupa (the feeding site) rather than on actual feeding activity. We decided to test whether it is possible to monitor the feeding behaviour of *Varroa* mites using electronic means. The Electronic Monitor of Insect Feeding (EMIF) has been widely used to study feeding behaviour of leafhoppers and aphids on plants (Zehnder *et al,* 2001). In a typical setting, an insect and the potting soil in which a plant is cultured are both attached with electrodes. When the insect touches the plant in any fashion, the circuit is completed and a current flows through the insect and the plant. This current can be amplified and recorded as voltage changes. When the insect inserts its mouthparts into the plant, the resistance becomes smaller and hence results an elevation of voltage in the recorder. We thought it might be possible to use the same machine to monitor *Varroa* mite feeding behaviour because when a mite sucks heamolymph from a bee, resistance might be lower than when the mite is walking or resting on a pupa. In our study, we used an alternate current Insect Feeding Monitor (IFM) manufactured by the Elaine Backus laboratory, University of Missouri (Columbia, MO, USA). The IFM output voltage was 80 mV and frequency 4,000 Hz. The IFM was connected to a personal computer which ran WINDAQ (Dataq, Akron, OH, USA). The IFM can monitor four channels (mites) simultaneously.

Combs containing brood cells of bees were taken out of colonies infested with *Varroa* mites. After several adult *Varroa* mites were collected from drone brood cells, each mite was attached to a 1.27 nm diameter gold wire that was 30–60 mm long using a silver conducting paint (#60805, Ladd Research, USA). Mites were cold anaesthetized on ice to reduce their movement and facilitate adherence. The gold wire was then attached to the input electrode that was connected to the IFM. A pupa at purple-eye stage was removed from a cell and placed on a thin plastic sheet that was bent so that it has both a horizontal and a vertical surface. The plastic sheet was made from a re-closable plastic (Ziploc) bag (Inteplast Group, Ltd, USA). A horizontal slit was cut in the vertical surface and the gold wire with the mite attached passed through the slit so that the movement of the mite was restricted. This was done to prevent the mite from touching the other electrode directly, causing a "short-circuit" (circuit formed but bypassing the bee pupae). The electrodes were made of copper. Three electrodes were used for each mite; one was attached to the gold wire, another attached to the bee pupa (by having the pupae lying next to the electrode), and the third was a reference electrode to reduce interference (hanging in the air, not contacting bees or mites). The experiment was done June to August 2004 under natural room light conditions and ambient temperatures of ca 26° C.

After comparing the waveforms on the computer and mite behaviours under the microscope or on a television monitor, four types of waveforms were identified: resting, walking, feeding and probing (Fig. 1). The "resting" waveform is a relatively straight line without spike, exhibiting no voltage changes compared to the baseline. The "walking" waveform is spiky and characterized by median frequency (5.51 ± 1.7 Hz, mean ± SD) spikes with the highest voltage changes (0.03 to 0.17 volt) among all three non-resting waveforms. The feeding waveform is characterized by a sudden increase in voltage (about 0.06 volt), indicating that the mite has pierced the pupa and electrical resistance is rapidly reduced. This low resistance state was maintained until the mouthparts were withdrawn, after which the voltage returned to the baseline level. During the feeding, the waveform is characterized by symmetrical spikes in both directions (increase or decrease in resistance) that are of the highest frequency (10.67 ± 1.59 Hz) among all waveforms. Finally, the probing

waveform resembles many short feeding spurts, with voltages reaching the same level as the feeding wave, but then returning quickly to the baseline This pattern is repeated at a frequency of 1.45 ± 0.40 Hz.

After identifying the waveforms, we attempted to record 12 *Varroa* mites feeding on worker pupae continuously for 24 hr and to analyze their time distribution in the four behaviours using these waveforms. Most of the mites escaped, but the remaining 3 mites showed that they fed only during the daytime (6:00 – 21:00 hr) and not at night (21:00 – 6 hr), and the total feeding time was short (0.35 ± 0.12 hr per 24 hr). These results should be viewed cautiously as the feeding monitoring occurred under artificial conditions (outside brood cells, at room temperature and florescent lighting during daytime) and might not represent those under natural conditions. We also compared the feeding behaviour of mites when they fed on worker (n=7) or drone pupae (n=6) using an average recording duration of 8 hr. Mites feeding on drone pupae were more active than those on worker pupae and spent significantly less time resting than those on worker pupae (6.55 ± 1.09 [drones] *vs* 7.54 ± 0.35 hr [workers], t = 2.23, P = 0.047). They also spent more time feeding on drone pupae, but the difference was not significant (0.59 ± 1.13 [drones] *vs* 0.079 ± 0.13 [workers], t = -1.20, P = 0.25). Walking and probing times were not statistically different on the two types of hosts. It is not clear whether the difference represents naturally occurring differential feeding behaviours toward drone and worker pupae or represents an artifact due to the fact that all mites were obtained from drone brood and then monitored on different hosts.

Because this is the first time an EMIF was used to study the feeding behaviour of *Varroa* mites, there are still many problems to be solved. For example, the gold wire was not insulated which required us to restrict mite movement so that no short circuit would occur. It was also weak and many mites escaped after breaking the wires. However, our data show that it is feasible to use an EMIF device to study *Varroa* mite feeding behaviour. With this device, it may be possible to perform long-term monitoring of mites without human observation. It also may be possible to study mite host choice preferences (*eg,* a young bee *vs* a forager) or subtle behavioural changes due to sublethal pesticide effects. We anticipate many uses for the EMIF system in studies of *Varroa* mite feeding behaviour.

Acknowledgement

We thank Michael Langenberger, George Ayers, and two anonymous reviewers for comments that improved this manuscript. This manuscript is based in part on a project by JZ for the High School Honors Science Program (HSHSP) at Michigan State University.

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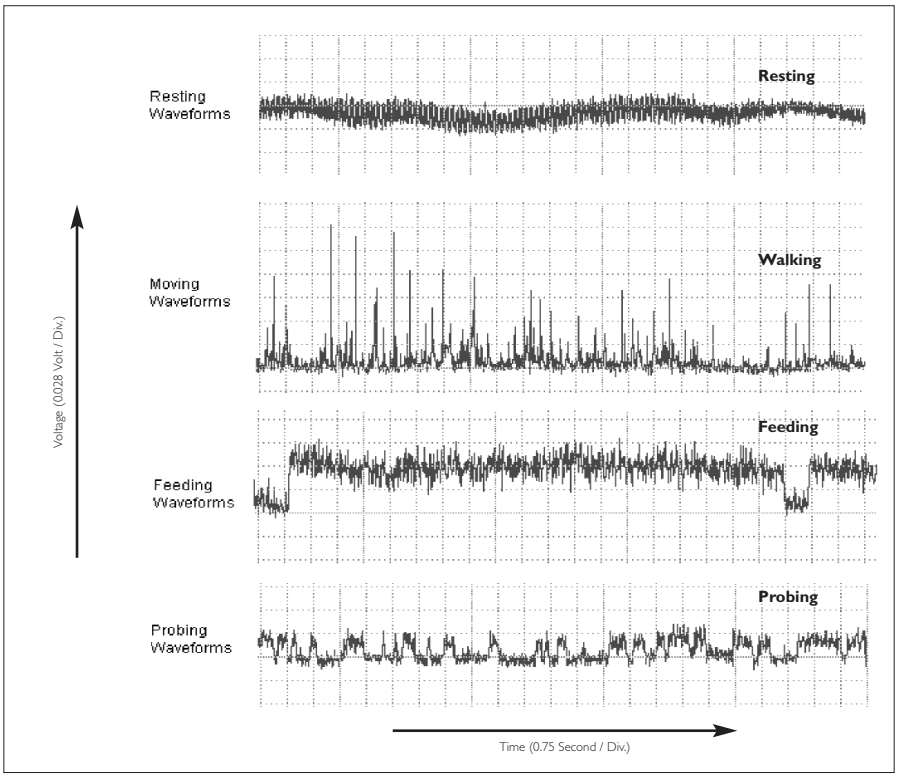

Fig. 1. Different waveforms produced by the Insect Feeding Mornitor (IFM) resulting from different mite behaviours on honey bee drone pupae. Arrows indicate the position of baseline on each wave form, which has a voltage value of zero.

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NOTES AND COMMENTS

Nest odour changes following queen loss in *Apis mellifera*

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Received 10 December 2004, accepted subject to revision 18 February 2005, accepted for publication 16 June 2006.

Keywords: volatile semiochemicals; SPME, GC/MS, Africanized honey bee, nest usurpation

Introduction

The nest odour of a honey bee colony is a mixture of volatile compounds emitted by the nectar, pollen, resin and wax, and by the bees themselves. This odour is used by returning foragers to locate the nest entrance (Butler et al., 1969), but does not seem to be used to determine whether a nest is a bee's native nest (Free, 1958; however, workers can discriminate their native nest odour in at least one context, Ferguson & Free, 1981). Here, I investigate the effects of queen loss on the nest odour of honey bee colonies. Loss of the queen is expected to change the nest odour because the queen herself produces volatile compounds (Gilley et al., in press) that undoubtedly contribute to a colony's volatile bouquet, and because volatile compounds are likely produced by the events such as queen-rearing that such loss triggers.

Demonstration of a change in nest odour following queen loss would be interesting for at least two reasons. First, nest odour may be used by swarms of Africanized honey bees to locate European honey bee colonies, which they then usurp by eliminating the European queen and installing their own queen in her place. Usurpation occurs more often in colonies that have a failing queen, no queen, or a new queen (Schneider et al., 2004). If we are able to detect changes in nest odour as a result of queen loss, then it's likely that usurpation swarms may be using them as a cue to detect colonies susceptible to usurpation. Second, and more generally, if we can detect changes in nest odour as a result of queen loss, then we could use nest odour to determine the presence, absence, or perhaps condition of a colony's queen. Because samples of nest odour can be taken from outside the hive with little disturbance to the colony, these methods could with some development provide beekeepers with a useful diagnostic of queen status.

I sampled nest odour by inserting for 30 min a solid-phase microextraction (SPME) fiber (65μm polydimethylsiloxane-divinylbenzene, Supleco, fiber 57326U) into each of five five-frame nucleus colonies. The colonies were of known European origin and had been dequeened one hour before sampling. The fibre was held in place, approximately 5cm from the hive bottom, by a wire support that hung from the middle of the top bars of frames two and three. A small wire cage, approximately 1.5cm in diameter, surrounded the fibre to prevent contact with bees or nest materials. Smoke was not used during fibre insertion or removal to avoid unwanted noise in the signal. After sampling, the fibre was immediately injected into a gas chromatograph coupled to a mass spectrometer (Varian CP-3800 GC/Saturn 2200 MS) and desorbed at 250°C with a 10:1 split ratio. The compounds were separated on a Varian FactorFour VF-5ms 30m x 0.25mm column with the following programmed parameters: column temperature 40°C for 1 min, then ramped to 100°C at 6 degrees per minute, then ramped to 200°C at 12.5 degrees per minute, then ramped to 250°C at 20 degrees per minute; helium flow rate 1 ml/min. The MS was operated in EI mode at 150 eV. Peak areas were calculated using each peak's three most abundant ions to reduce noise and thus enable accurate quantification of low-concentration compounds (Saturn Workstation v.5.52, integration parameters: peak width = 2.0 sec, slope sensitivity = 5 s/n, tangent = 5%). Kovats retention indices were calculated using the formula $I_x = 100n + 100(t_x-t_n) / (t_{n+1} - t_n)$, where I_x is the retention index of the compound of interest, t_x is the retention time of the compound of interest, t_n and t_{n+1} are the retention times of the n-alkanes eluting immediately before and after the compound of interest, and n is the number of carbon atoms in the n-alkane eluting immediately before the compound of interest. Colonies were sampled again using identical methods ten days later, at which time the number of sealed queen cells was also noted. This entire procedure was performed three times (beginning September 9, 16, and 23, 2005), each time using five different colonies, for a total of fifteen colonies.

From these samples, I detected twenty-three common compounds (i.e., peaks that were present in at least 75% of the samples from either Day 0 or Day 10), as described in Table 1. Relative amounts of peaks which were not normally distributed were transformed using the Box-Cox procedure before further analysis (Minitab Release 14; Peak 2, $\lambda = 0.337$; Peak 3, $\lambda = 0.337$;

Table 1. Properties of the 23 common peaks found in samples of air within honey bee hives, and the change in their relative amounts following queen loss. RT is the retention time, RI is the retention index. Major ions are the five ions present in greatest amounts that were also larger than 10% of the base peak; ions 1 – 50 were not included because of high variation in this portion of the spectrum. Change in mean amount refers to the difference between the mean amount of each peak on Day 0 versus Day 10, expressed as a percentage increase or decrease from the Day-0 amount. P_{Day}-value refers to the significance of the effect of Day by a two-way ANOVA. P-values are shown only for those peaks where trial was not a significant factor in the ANOVA (i.e., $P_{trial} > 0.050$).

Peak	Mean RT	RI	Relative Amounts of Major Ions (ion = % of base peak)					Change in Mean Amount	P_{Day}-value
1	5.99	870	91=100%,	105=13%,	106=48%			+ 115.8%	0.023
2	6.50	893	51=32%,	77=45%,	78=79%,	103=53%,	104=100%	+ 64.9%	0.605
3	7.53	935	77=58%,	79=35%,	91=100%,	92=37%,	93=90%	+ 114.9%	
4	8.32	967	51=27%,	77=77%,	105=100%,	106=20%		- 61.6%	0.010
5	8.66	980	69=17%,	77=53%,	79=32%,	91=77%,	93=100%	+ 81.5%	
6	8.81	986	69=52%,	77=35%,	79=28%,	91=64%,	93=100%	+ 7.6%	0.780
7	8.91	990	69=57%,	77=38%,	79=25%,	91=68%,	93=100%	+ 51.9%	0.849
8	9.29	1006	56=82%,	57=64%,	67=100%,	69=67%,	95=56%	- 49.5%	0.005
9	9.45	1012	77=60%,	79=45%,	91=100%,	92=35%,	93=100%	+ 55.2%	0.740
10	9.65	1020	74=22%,	75=38%,	111=51%,	146=100%,	148=60%	- 8.8%	0.758
11	9.84	1028	91=38%,	117=18%,	119=100%,	117=18%,	134=22%	- 17.5%	0.453
12	9.96	1033	67=100%,	77=37%,	79=46%,	91=41%,	93=63%	+ 55.4%	
13	10.37	1049	77=50%,	79=59%,	80=31%,	91=100%,	93=73%	- 74.6%	
14	11.37	1089	77=38,	91=95%,	93=100%,	105=41%,	121=89%	- 13.1%	0.719
15	11.61	1099	50=18%,	51=33%,	77=72%,	105=100%,	136=19%	+ 40.0%	0.083
16	11.79	1108	55=64%,	56=61%,	57=100%,	67=72%,	81=72%	- 60.6%	0.001
17	13.65	1210	55=100%,	56=33%,	67=84%,	81=53%,	82=38%	- 26.6%	0.123
18	14.84	1291	73=100%,	147=20%				- 78.2%	
19	14.96	1300	56=28%,	57=93%,	70=29%,	71=100%,	85=48%	- 38.1%	0.050
20	16.81	1456	73=100%,	147=41%,	149=20%			- 56.3%	
21	17.29	1500	55=35%,	57=100%,	69=24%,	71=77%,	85=50%	- 60.3%	0.001
22	19.22	unknown	55=19%,	57=100%,	69=21%,	71=71%,	85=40%	- 71.3%	
23	20.70	unknown	55=47%,	57=100%,	71=82%,	85=47%,	97=29%	- 70.5%	

Peak 5, $\lambda = 0.225$; Peak 7, $\lambda = 0.337$; Peak 9, $\lambda = 0.001$; Peak 12, $\lambda = 0.337$; Peak 18, $\lambda = 0.225$; Peak 20, $\lambda = -0.450$; Peak 21, $\lambda = -0.113$; Peak 23, $\lambda = 0.224$). For six compounds, Day (0 or 10) explained a significant portion of the variance in amount ($F \geq 4.22$, d.f. = 1,26, $P \leq 0.050$ from two-way analysis of variance) while Trial (1, 2, or 3) did not ($F \leq 3.16$, d.f. = 2,26, $P \geq 0.05$) (Table 1). For three of these compounds (Peaks 1, 4, and 21), the effect of Day was consistent across trials (i.e., $F \leq 2.95$, d.f. = 2,24, $P \geq 0.072$ for tests of Day x Trial). For three other compounds (Peaks 8, 16, and 19), the effect of Day was not consistent ($F \geq 13.76$, d.f. = 2,24, $P < 0.001$ for tests of Day x Trial). The change in relative amounts of these three compounds between Day 0 and Day 10 was greater during Trial 1 than Trial 3 (one-way analysis of variance with Tukey's pairwise comparisons; $F \geq 12.81$, d.f. = 2,12, $P \leq 0.001$; all family error rates = 0.05). For nine other compounds, neither Day nor Trial explained a significant portion of the variance in amount ($F \leq 3.25$, d.f. = 1,26, $P \geq 0.083$ for Day; $F \leq 3.22$, d.f. = 2,26, $P > 0.056$ for Trial). For eight compounds, Trial explained a significant portion of the variance in amount (i.e., $P_{Trial} < 0.050$); these peaks were eliminated from the analysis because changing environmental conditions between trials, rather than queen loss, may have been responsible for any observed differences in amounts between Day 0 and Day 10 (the P values for these eight peaks are not shown in Table 1).

My experimental design does not allow me to rule out the possibility that the differences shown in Table 1 are the result of a seasonal change in nest odour (e.g., a decreasing supply of a particular variety of pollen, or a trend of decreasing temperature), but this is unlikely for two reasons. First, as described above, varying

levels of at least three compounds could not be explained by the trial in which the measurements were made, as would be expected if there were seasonal or environmental effects. Second, in a small pilot study of two queen-right nucleus colonies, there was no consistent decrease in the amounts of these compounds over 8 days.

Therefore, I conclude from these results that queen loss does affect nest odour in a way that can be detected using the methods described above. This provides a basis for testing hypotheses concerning the role of nest odour in attracting usurpation swarms and could be the first step toward a non-invasive diagnostic of queen status.

The source and identity of the observed compounds remains to be determined. Five compounds, of the six where the effect of Day was significant, decreased in relative amounts following queen removal. This suggests that they are volatile components of queen semiochemicals that had been distributed to the workers or nest materials prior to queen removal. One of the six compounds (Peak 1) increased in amount, which suggests that it is a volatile product of the queen rearing process. However, there were no correlations between the number of sealed queen cells and the Day 10 relative amounts of Peak 1 (or of any of the 23 peaks). This compound may therefore be associated with another element of queen loss, such as the development of worker ovaries.

Acknowledgement

The author gratefully acknowledges Judith Hooper for technical assistance in operating the GC/MS and the Almond Board of California for funding this research.

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Journal of Apicultural Research 45(3): 162–164 (2006)

REVIEWS

World-Famous Polish Beekeeper – Dr. Jan Dzierżon (1811–1906) and his work in the centenary year of his death

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Received 28 April 2006, accepted subject to revision 11 May 2006, accepted for publication 23 May 2006.

"Truth, truth over everything. Lies and mistakes will pass by, but truth will remain".

"Prawda, prawda ponad wszystko. Kłam i błąd Dzierżon przeminą, a prawda pozostanie".

– Jan Dzierżon

Introduction

Jan Dzierżon was born on 16th January 1811 into a Polish peasant's family, in the small village of Łowkowice (Lowkowitz) in the Kluczbork (Kreuzburg) district in Silesia. After his attendance at schools in Łowkowice, Byczyna (Pitschen) and Wrocław (Breslau), he studied in the Theological Faculty of Wrocław University. After completion in 1833, he was appointed curate in the parish of Siołkowice (Schalkowitz) and then parish-priest in Karłowice (Karlovitz, Carlsmarkt). Soon after his arrival in the parish he went in for farming and beekeeping in his newly established apiary, where he conducted his famous experiments and observations of bees. Long-lasting conflicts with the German administration (official letters to government offices, sharp polemic articles to papers standing up for the rights of local population etc.) and the ecclesiastical authorities (e.g. criticism of dogma concerning papal infallibility). Resulted, ultimately, in his excommunication (excommunica maior, 30 X 1873). He ceased ecclesiastical work and went from Karłowice parish to his native home Łowkowice, where he settled permanently and engaged in apicultural work and experiments on bees (ca. 400 bee-colonies in 12 apiaries), as well as welfare work among beekeepers and the local community.

Among the great Dzierżon's discoveries, which are very significant for development and the shape of modern beekeeping, the most important seem to be:

- The discovery (1835; published in 1845) of parthenogenesis of bees and the connection of this phenomenon with the occurrence of 3 forms of their adult specimens (queens, drones, workers), which has fundamental importance in the recognition of the biology of these insects and the life of bee colony (Woyke 1961).
- He recognized that royal jelly is produced as a secretion of the bee's pharyngeal glands and is used as food for all bee larvae during the first days of their life after hatching from the eggs. Later it is significantly differentiated; queen larvae are fed substantially with royal jelly all the time of their development; other bees being feed royal jelly in the beginning only, and later a mixture of pollen, honey and royal jelly, so they finish their development as bee workers and drones.
- His view concerning the origin of bee wax, its production (wax gland 's secretion) by bees and observing the association of this process with intensive feeding of bees.
- His observation of the influence of intensive pollen feeding on the fat body accumulation of young bees preparing themselves for the winter season.
- His experiments and observations on pollen-substitute collection and its use, e.g. flour and other powdery substances (dust, mushroom spores) by bees.
- A new type construction of bee-hive (Dzierżon's side-opening hive) using mobile top bars, i.e. upper lifts to which bees fixed the honeycombs (so called "snozy", in Polish), which make possible bee colony observations inside hives and the taking apart of the bee nests without damage to the combs.
- Experiments on the crossing of Italian and national bees for confirmation of some working hypotheses (among others the parthenogenesis phenomenon).

- The idea of a queen-mating station – separation of drone-rearing colonies in isolated places, distant from apiaries, and used for controlled insemination of queens.
- The recognition of honeydew production by scale insects, which have a part in honeydew flow, and are next to aphids as its producers.
- Remarks and observations on: honeydew honey as harmful provisions when stored in honeycombs for wintering bee colonies, foul brood as a dangerous bee disease and necessity for its control, etc.
- Important observations of the biology and behaviour of bees. Amongst others, the copulation of the queen; longevity of queens, dances of swarming bees; oviposition of nonfertilised eggs, drone-laying queens and laying workers; drone wintering in queenless colonies.
- Observations of mutual relationship of bees – plants and remarks concerning the continuity ("faith") of bees in working plants, visiting flowers and on the benefits from pollination of orchards and agricultural cultivars, such as grown fruits, crops and harvest abundance.

Dzierżon's experiments and discoveries, during the whole his life, evoked a lot of long-lasting discussion and controversy among scientists and beekeepers. There were a lot of friends and adherents, but also opponents too, especially on the parthenogenesis question (K. Krasicki, F.v.Berlepsch, F. Dickel, M. Kuckuck, J. Pérez). Finally, the validity of the parthenogenesis theory was confirmed and acknowledged by scientific authorities and specialists from zoology, physiology and genetics (C.T.E. Siebold, R. Leuckart, E. Bresslau); it was accepted also by famous beekeepers (e.g. W. Langstroth, B. Ambrozy, J. Lubieniecki). Contemporary apiarists used Dzierżon's observations in practice. While some of the results of his research are also currently very useful and have become the inspiration for further investigations (Bornus 1961).

In acknowledgement of his experiments and his contribution to the development of apiculture and advanced apidology, heads and representatives of many countries awarded him medals and martial orders, scientific associations awarded him honorary memberships, and Munich University granted him the title of Doktor Honoris Causa.

Dzierżon died on the 26th October 1906 in Łowkowice. Throughout his hard working life he had a great passion and love of bees, and his principles were expressed in the following sentence, which he wrote: "Truth, truth over everything. Lies and mistakes will pass but truth will remain". A version of this maxim was also contained in the following words: "Wahrheit, Wahrheit über alles", which were written on his tomb in Lowkowice cemetery.

In these times, when there is so much discussion on the importance of "rights and values" in our life, the lowering of moral principles, and complaints of lack of respect for authorities, Dzierżon should be an example for beekeepers, scientists and above all for the younger generation of society. Sadly, his work, which is still insufficiently known and not appreciated in some circles, one can even suspect that at times a conscious silence has been maintained,

Among the great number of publications on Dzierżon, the monograph study (Brożek et al. 1978) is the most important. The

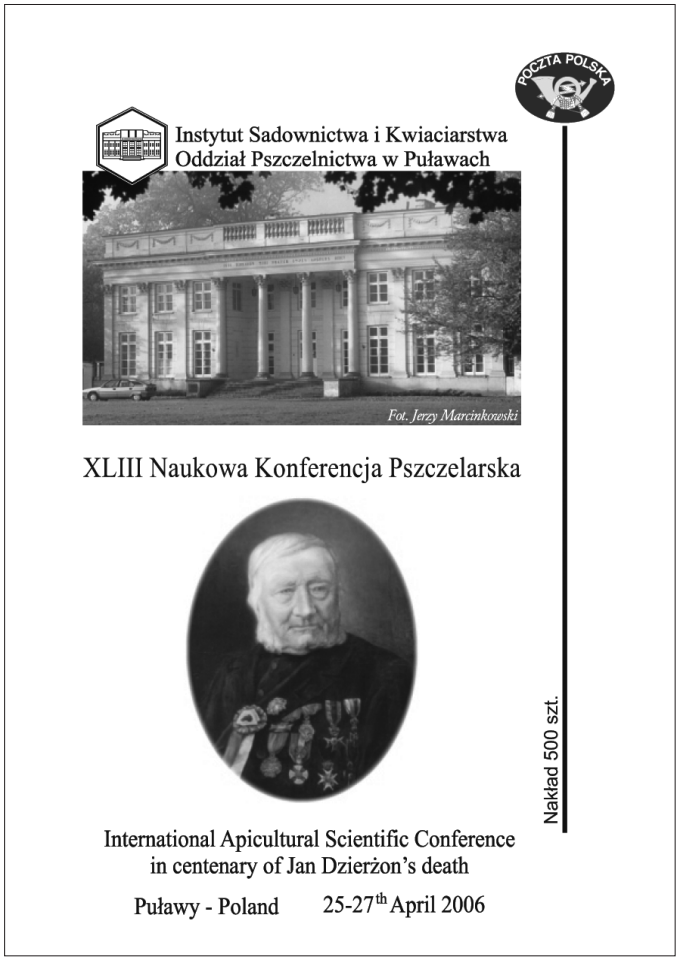

Fig. 1. Part of a postcard: Jan Dzierżon (1811–1906). Oil portrait by Stefan Dylewski. Museal Collection in Kluczbork (photo: J.Wę clewski).

Fig 2. Stamp series issued by Polish Philatelic Post Centre entitled: "50th Anniversary of the death of Dr Jan Dzierżon 1811–1906", showing portrait of the world famous apiarist and a worker honeybee (*Apis mellifera* L.) on blooming clover against the background of a beehive.

monograph edited by the Silesia Institute in Opole was prepared on the grounds of a rich archive of documents and materials, both Dzierżon's publications and articles by other authors. The richness of Dzierżon's curriculum vitae seems to have sufficient material for the preparation of a comprehensive and interesting film. However, the realization of such a film doesn't seem to be a simple enterprise.

Worthy of approval and imitation are the initiatives of people and institutions whose aim has been the popularization of Dzierżon's work in apiculture, e.g. some Polish Philatelic Post Centre issues were dedicated to the commemoration of important Dzierżon's anniversaries and jubilee celebrations (a total of over 23 postage stamps, post marks, date-markers, postcards etc.) including the present 100 year anniversary of Dzierżon's death and celebration of 2006 as Dzierżon's Year (Figs 1, and 2).

These celebrations and issues serve to remind us and to popularize the famous beekeeper's silhouette but above all to mark his significant contribution to apidology and the development of European and world apiculture.

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IBRA AGM

Notice is hereby given that the Fifty-seventh Annual General Meeting of IBRA will be held at:

University Chaplaincy, 22 North Road, Cardiff, Wales
2.00 pm, Saturday 7 October 2006

Agenda

1. To approve the minutes of the 56th Annual General Meeting
2. To receive the Chairman's Report
3. To receive the Report of Council and the Accounts for 2005.
4. To elect Members of Council.

The following members of Council retire by rotation and, being eligible, offer themselves for re-election:

Dr D. Griffiths
Dr W. D. J. Kirk
H. Kjaersgaard
Prof M. Matsuka
Dr. R. Paxton
T. J. K. Showler

Council proposes that the number of Members of Council shall remain at 20 for the time being.

5. Messrs Huw J. Edmund, Chartered Accountants will continue as Reporting Accountants.

By order of the Council
David Smith
Secretary IBRA

Proxy voting and nominations for Council

Two parts of IBRA's formal charter are printed here as they relate to the nomination of people to Council and proxy voting.

1. Article 43 of the Memorandum of Association provides:

No person not being a member of the Council retiring at the meeting shall, unless recommended by the Council for election, be eligible for office on the Council at any General meeting, unless within the prescribed time before the day appointed for the meeting, there shall have been given to the Secretary notice in writing, by some member duly qualified to be present and to vote at the meeting for which such notice is given, of his/her intention to propose such a person for election, and also notice in writing, signed by the person to be proposed, of his/her willingness to be elected. The prescribed time above mentioned shall be such that, between the date when the notice is served, and the day appointed for the meeting there shall not be less than seven nor more than 28 intervening days.

2. A member entitled to attend and vote at this meeting may appoint a proxy to attend and, on a poll, vote in his/her stead.

A proxy need not also be a member of the company. Proxies must be lodged at least 48 hours before the meeting.

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OBITUARY

David Francis (1928–2006)

David Francis occupies a unique position in the fifty eight year history of IBRA in that he is the only person to have served both as Director of the organization and Chairman of its Governing Council. He took on each of these roles at difficult times and handled them supremely professionally yet always with a concerned and warm personal touch.

During his life he travelled extensively and to many he would have appeared as the archetypical English gentleman. However, you did not have scratch the surface much, particularly on a rugby football international day, to realise that David was a Welshman born on St Patrick's Day in Brecon mid Wales. Intelligent and diligent in his attitude to work he attended the local public school, Christ College, where the educational standards and demands on the pupils were high and there was little time for leisure.

Nevertheless, at this time he did lay down the foundations of three interests that were to stay with him for the rest of his life – photography, aeroplanes and bees. Around the age of 12 David took up beekeeping with passion and learned the craft in a slightly unconventional way compared with the traditions found in the UK. His teacher was a German and kept his bees in the conventional German bee house not often seen in the UK! David became an expert beekeeper but probably needed some of his hard won honey crop to placate the neighbours after the backyard of the terraced family home was occasionally filled with angry bees.

Following a successful school career David went to Bangor University in North Wales where he obtained his BSc in Forestry and Botany. Then he managed to combine his academic qualifications and scientific interest in the natural world with his teenage passions for planes and photography when he took up a job in Canada conducting aerial and ground forest surveys. He went on to do similar work in what was then the British Colonial Service including a period of secondment to undertake a two-year postgraduate course at Oxford University at the end of which he was truly a specialist in aerial surveying.

In 1954, after several years in the Gold Coast (Ghana) he left the Colonial Service, becoming as he would later recount with much amusement the Service's "youngest pensioner", and joined Huntings Aero Surveys. His brief was now much broader and he was involved in air, land and marine surveys of natural resources worldwide for development purposes. He eventually became Managing Director of Huntings and remained with the company for 33 years. This was no office-bound, fixed-location life for in addition to work for the firm he undertook secondment and consultancies with the United Nations in New York and the Food and Agriculture Organization based in Rome. In all, he visited 99 countries around the world on his assignments.

David was a friendly and approachable man endowed with boundless common sense but above all he cared about people. He firmly believed in the founding ideals of the UN and was saddened by the corruption and indifference he often encountered in high places within national administrations around the world. He was dedicated to doing whatever he could to help humanity from his involvement in the "Freedom from Hunger Campaign" of the 1960's all the way through to various developmental projects to promote beekeeping as a micro economic sustainable enterprise when he was Director and then Chairman of IBRA.

In this busy life he still found time to undertake the time consuming training necessary to obtain a private pilot's licence. Again he took a slightly unconventional approach his instructor being an ex US Navy flyer. David worked out that a man who could find, and then put the plane down safely, on a bucking aircraft carrier in a huge ocean would give added value to the lessons. Coupled with his meticulous approach to all he undertook the lessons obviously paid off and were put to the test on more than one occasion. While Director of IBRA he visited Somalia and was driven for many hours across dangerous and inhospitable country eventually arriving at a forlorn airstrip that had one small plane. A local tribesman was squatting near by: David approached and asked where he could find the pilot. "That is you" was the succinct reply, "I am your navigator"! The mission was very successful.

In 1988 IBRA was in a depressed state. In the proceeding 3 years Dr Crane had stepped down as director, her successor resigned for personal reasons and the next director died within three months of appointment. In addition there were radical changes in the way NGO's such as IBRA were being funded so the job called for a person of remarkable skills and a steadying hand on the controls. David agreed to do the job for 2 years. In that time his managerial abilities brought the finances under control so that when he left in 1990 the books actually balanced. More than that he steadied the ship and re-established IBRA's place in the world of apiculture. In the 1990's he put his knowledge of beekeeping and geography to good use acting as an agent and consultant in the world trade of beeswax and honey.

Before long he was IBRA's Chairman and again fighting to secure the survival of the Association. His chairman's report, made at the 1998 AGM (*Bee World* Volume 79, Number 4, Page 165), is a tribute to the way in which he handled the difficult situation.

His three and a half year tenure of office bore witness to his dedication and the high level of support and personal input he gave through some difficult times. He stepped down as chairman but was prepared to put his years of business experience and financial acumen to good use by continuing to Chair the Finance Committee.

David died, after a short illness, on Sunday 4 June. To his children, Diana, Alison and David, of whom he was justly proud, his much loved grandchildren but especially to his wife of over 50 years, Jackie, IBRA extends its most sincere condolences. IBRA has lost a true and loyal supporter: we appreciate what he did for the Association but above all it was a privilege to have him as a friend.

CONFERENCE CALENDAR

1st International Forum on Apitherapy APIMEDICA 2006

Athens, Greece
12 –15 October 2006

In response to the growing interest in this subject Apimondia and the Greek Scientific Apitherapy Centre have organized a Forum which will take place in Athens between 12–15 October 2006.

More details from:
www.apimedica2006.gr
www.apimondia.org

XVth Conference of Improvers and Inventors in Apiculture and International Beekeeping Exhibition

Prague, Czechoslovakia
17–19 November 2006

Organised by the Czech Beekeeping Union in cooperation with the Slovak Beekeepers Union.

More details from:
Contact Ladislav Baxa
email: apiculturefair@email.cz
www.vcelarstvi.cz

29th General Assembly of the International Union of Biological Sciences (IUBS)

Washington, USA
9–13 May 2007

At the invitation of the National Academy of Sciences, USA, a three day symposium "Biological Sciences for the 21st Century: Meeting the Challenges of Sustainable Development in an Era of Global Change" will be held on the occasion of this Assembly.

For more information on the General Assembly and the Symposium
www.iubs.org
www7.nationalacademies.org/IUBS/

Apimondia 2007 Beekeeping Down Under

Melbourne, Australia
9–14 September 2007

This is the world's foremost beekeeping gathering held every two-year. The next event is Melbourne, Australia and will be held from 9–14 September 2007.

More details from:
www.apimondia2007melbourne.com

GUIDELINES FOR AUTHORS

Journal of Apicultural Research, incorporating ***Bee World,*** is a peer-reviewed journal covering the biology, ecology, natural history and culture of all types of bees (superfamily Apoidea). The journal welcomes the submission of three types of article:

Original Research Articles
These describe original scientific research on the above topics. Theoretical papers are also published. Articles on melissopalynology or bee hive products must be hypothesis-driven or cover one or more significantly large geographic region. Articles on the biological activity of hive products must identify the active agents.

Notes and Comments
These describe techniques or original research that is too narrow in scope to justify a longer article. Constructive criticisms of research articles that have appeared in this journal or elsewhere are also published.

Reviews
These are authoritative reviews of a clearly delimited topic in bee biology or the economics, technology, sociology or history of beekeeping. The breadth of coverage and inclusion of references should be complete enough to provide a practising scholar with sufficient background knowledge to begin working in that field.

This journal is the official journal of IBRA, the International Bee Research Association. For information on IBRA's services visit the web site (www.ibra.org.uk) or contact

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A Colour Guide to Pollen Loads of the Honey Bee (2nd edition)

William Kirk

Beekeepers everywhere are fascinated by the sources of pollen brought back to the hive by their bees. Being able to identify the plants producing this pollen is the key to unlocking important information about bee forage in your area. Dr Kirk and his team have painstakingly collected pollen loads from bees from Britain and Germany, giving us surprising evidence of the natural variation in the colours seen at the hive entrance.

The guide contains a key of 500 colours, and describes the pollen loads of 268 plant species found in Europe. Many of the species, particularly the crops, are grown more widely, so the book will also be useful in temperate areas worldwide, such as in the USA or Australia. There is also an account of how this book is useful as a practical guide to pollen identification. The text is given in English, French and German, because of the wide demand for such a book throughout Europe.

The new second edition includes updates and corrections of the first edition. It also benefits from the many advances in colour printing technology that have occurred over the twelve years since the first edition came out in 1994. The new edition is printed more accurately and should replace copies of the first edition, which may have changed colour through fading and paper aging over the intervening period.

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Volume 45 | Number 3 | 2006

Journal of Apicultural Research

Journal of Apicultural Research inc. Bee World 45 (3) 2006

INTERNATIONAL BEE RESEARCH ASSOCIATION

Design www.stazikerjones.co.uk