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Beekeeping and Bee Conservation

Advances in Research

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BEEKEEPING AND BEE CONSERVATION - ADVANCES IN RESEARCH

Edited by **Emerson Dechechi Chambó**

Beekeeping and Bee Conservation - Advances in Research
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Meet the editor

Dr. Emerson Dechechi Chambó has a Bachelor of Biological Science, a Specialist degree in Ecology and a Master's degree in Animal Science from Universidade Estadual do Oeste do Paraná (UNIOESTE), Brazil. He holds a Doctorate degree in Animal Science from the Universidade Estadual de Maringá (UEM), Brazil. He is presently a researcher with PNPD/CAPES, linked to UNIOESTE and Universidade Federal do Recôncavo da Bahia (UFRB), where he works in the graduate program in Sustainable Rural Development and Agricultural Sciences, respectively. He is a member of two renowned bee research groups in Brazil, namely the Bee Research Group at the UEM and the Group Insecta at UFRB. Top among his interests are the study of beekeeping and experimental statistics.

Contents

Preface

Many societies have engaged in beekeeping through the ages. The practice, which began as a purely extractive activity, has become an "art," in which, for many, conservation precedes exploitation. Beekeeping was born at some point in prehistory through the convergence of bees and mankind but, until recently, the vital importance of bees to life on our planet was not widely known. Presently, researchers agree that the extinction of these insects could lead to a drastic decline in food production, affecting the world's population. A scenario without bees—although unknown—is postulated to include catastrophes such as the collapse of the food chain due to insufficient pollination and a landscape no longer colored by the beautiful flowers that adorn our planet. Moreover, many other consequences may occur, but I will let them reside in the reader's imagination.

Nonetheless, because of the importance of bees both from an economic and environmental standpoint, many researchers in various branches of the field seek improvements in the beekeeping process, as well as in the development of consumer products generated from bees. Moreover, due to a growing global concern about the disappearance of bees, either due to diseases or due to the use of pesticides in agricultural crops, research in the field has become increasingly important.

"Beekeeping and Bee Conservation — Advances in Research" presents current issues in the field of bees in multiple contexts and ties together experiments conducted by some of the world's most renowned researchers. The authors' point-of-view and own research results or current review of knowledge are described in a clear and objective way, which is very useful for beginners in the study of the subject and is likewise valuable for the more experienced on the subject, who may find new hypotheses to be tested and broaden their future prospects in the field.

"Beekeeping and Bee Conservation — Advances in Research" is wide in scope, focusing largely on *Apis mellifera*. Topics range from genetics, to pollination studies, to the preservation of bees. It includes a chapter dedicated to stingless bees and another for bumble bees. The main objective of this book is to offer the scientific community an indispensable source of information for research and to assist in further investigations, both in relation to the productive aspects of the beekeeping chain as well as the conservation of bees.

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Chapter 1

A Comprehensive Characterization of the Honeybees in Siberia (Russia)

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Additional information is available at the end of the chapter

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Abstract

A comprehensive study of some populations of honeybee (332 colonies) in Siberia (Tomsk region, Krasnoyarsk Krai (Yenisei population), Altai) using morphometric and molecular genetic methods was conducted. Infestation of bees (132 colonies) by *Nosema* has also been studied. Three variants of the COI-COII mtDNA locus were registered: PQQ, PQQQ (typical for *Apis m. mellifera*), and Q (specific for southern races). It was established that 64% of bee colonies from the Tomsk region and all colonies studied from the Krasnoyarsk and the Altai territories originate from *Apis m. mellifera* on the maternal line. According to the morphometric study, the majority of bee colonies of the Tomsk region are hybrids; in some colonies the mismatch of morphometric and mtDNA data was observed. Moreover, the majority of bee colonies infected by *Nosema* were hybrids. Yenisei population may be considered as a unique *Apis m. mellifera* population. Microsatellite analysis (loci A008, Ap049, AC117, AC216, Ap243, H110, A024, A113) showed the specific distribution of genotypes and alleles for some loci in the bees, which differ by geographical location. Loci A024 and Ap049 are of considerable interest for further study as candidate markers for differentiation of subspecies; locus A008 can be considered informative for determining of different ecotypes of *Apis m. mellifera*.

Keywords: honeybee, COI-COII locus, microsatellites, *Nosema*, Siberia

1. Introduction

In Siberia, the honeybee was introduced about 230 years ago. It was the dark-colored forest bee *Apis mellifera mellifera* L., or the Middle Russian race (a term adopted in Russia), that was cultivated

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in Siberia as the most adapted to the harsh climatic conditions of the region. At the end of the last century, bees of southern races, such as the Carpathian race or *Apis mellifera carpatica* (a derivative of *A. m. carnica*) and the Caucasian gray mountain race (*Apis mellifera caucasica* Gorb.), have been actively imported to Siberia. This process had become widespread and almost uncontrollable, which leads to a high level of crossbreeding of bees.

At present, one of the beekeeping problems in different countries is a massive bee hybridization, which leads to the reduction of the range of native subspecies, the formation of hybrids, and "deterioration" of the genotypic composition of honeybees. Hybrid populations are less adapted to environmental conditions that rapidly change during the year and are characterized by the higher morbidity and low immunity [1–3].

Introgressive hybridization modifies the genetic pool of local honeybee populations leading to the loss of their genetic identity [4]. The process of hybridization of different subspecies of honeybee can cause the destruction of the established gene complexes, leading to decrease in adaptive properties of organisms and populations and the change in biological and economically significant indicators of bees. The observed widespread hybridization of honeybees and the formation of hybrid bees can certainly contribute to the spread of disease. The extent of hybridization, characteristics of hybrid bees, the study of genetic processes that occur during hybridization, and evaluation of the effects of hybridization are of considerable interest.

The goal of this study is the morphometric and molecular genetic (mtDNA and microsatellite analysis) characterization of honeybees in Siberia and the assessment of the infestation of bee colonies by *Nosema*.

2. Materials and methods

2.1. Region

Bees and bee colonies were investigated in three regions of Siberia: the Tomsk region, the Krasnoyarsk Krai, and the Altai Krai (**Figure 1**).

The Tomsk region is located in the geographic center of Siberia, in the southeastern part of the West Siberian Plain. The distance between the northern and southern boundaries of the meridian is about 600 kilometers; therefore, the climate of the southern and northern regions is markedly different. A climatic characteristic of the northern region is a more severe and prolonged winter season. Almost the entire territory of the region is within the taiga zone, where forests cover about 60% of the territory. The climate is temperate continental with considerable daily and annual amplitudes and long winters (5–6 months). The average annual temperature is –0.6 °C, while the average temperature in July is +18.1 °C and in January is 19.2 °C. The frost-free period is 100–105 days. Precipitation is 435 mm.

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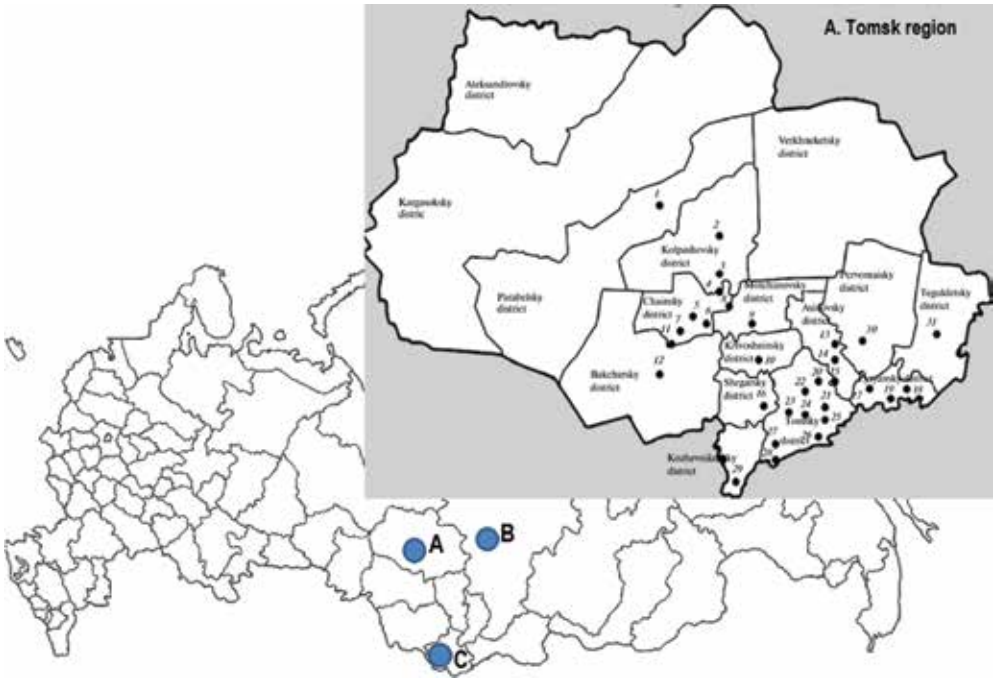

Figure 1. Map of localization of studied areas of Siberia (dots A–C) and apiaries of the Tomsk region (dots 1–31): A, the Tomsk region; B, the Krasnoyarsk Krai; C, the Altai Krai. *1*, s. Parabel; *2*, vicinity of g. Kolpashevo; *3*, d. Novoabramkino; *4*, s. Leboter; *5*, s. Podgornoe; *6*, d. Strelnikovo; *7*, s. Gorelovka; *8*, d. Sarafanovka; *9*, s. Sokolovka, s. Mogochino; *10*, s. Krivosheino; *11*, s. Vysoky Yar, d. Krylovka; *12*, s. Bakchar, s. Parbig; *13*, d. Tihomirovka; *14*, ur. Kuzherbak; *15*, s. Novikovka; *16*, s. Kargala; *17*, s. Dubrovka; *18*, s. Okuneevo; *19*, s. Zyryanskoe; *20*, d. Kuskovo; *21*, p. Zarechnyi (Mezheninovskoe rural settlement); *22*, d. Bodazhkovo, s. Semiluzhki, p. Zarechnyi (Malinovskoe rural settlement); *23*, d. Nizhne-Sechenovo, d. Berezkino, s. Zorkaltsevo, s. Rybalovo, d. Kudrinsky uchastok, d. Gubino; *24*, p. Sinii Utes, d. Magadaevo, d. Prosekino, s. Kolarovo, vicinity of Tomsk; *25*, d. Bolshoe Protopopovo; *26*, s. Mezheninovka; *27*, d. Kandinka, s. Kurlek; *28*, s.Yar; *29*, d. Elovka; *30*, d. Krutolozhnoe; *31*, s. Teguldet. Apiaries located at a distance less than 15 km from each other are marked as a single point.

The Krasnoyarsk Krai is located in the Eastern Siberia. The climate is sharply continental, where 70% of the territory is occupied by forests.

The Altai Krai is located in the south-east of Western Siberia. The region contains almost all natural zones of Russia—the steppe and forest steppe, taiga, and mountains. The climate of the Altai Territory is highly heterogeneous because of various geographical conditions. Foothills have a temperate climate, the transition to continental.

2.2. Samples

The samples are obtained from different geographic parts (ecologically and climatically different districts) of the Tomsk region, including districts with a high beekeeping activity (the southern districts) or districts with a low apicultural activity (the northern districts), according to the local knowledge of specialists from the Society of Beekeepers. Honeybees from the apiaries of the Krasnoyarsk Krai and the Altai Krai were also investigated for comparison.

A total of 332 bee colonies (60 apiaries) from Siberia were investigated by morphometric (3043 honey bee workers) and molecular genetic methods (2073 bees by mtDNA analysis and from 252 to 515 bees by microsatellite analysis): 318 bee colonies from the Tomsk region; 10 colonies from the Krasnoyarsk Krai, and 5 colonies from the Altai Krai (**Figure 1**).

Bee colonies from the Krasnoyarsk Krai were collected from the unique isolated Old Believers population, which existed for more than 60 years in forest without the importation of new honeybees.

Bee colonies from the Altai Krai have been collected in the apiary, located in the foothills.

Infestation of bee colonies by *Nosema* infections were studied in 1983 samples obtained from 132 bee colonies from 68 apiaries of Siberia during 2012–2015.

2.3. Morphometric method

Morphometric parameters (wing venation), including the cubital index, the hantel index, and the discoidal shift, were studied (**Figure 2**).

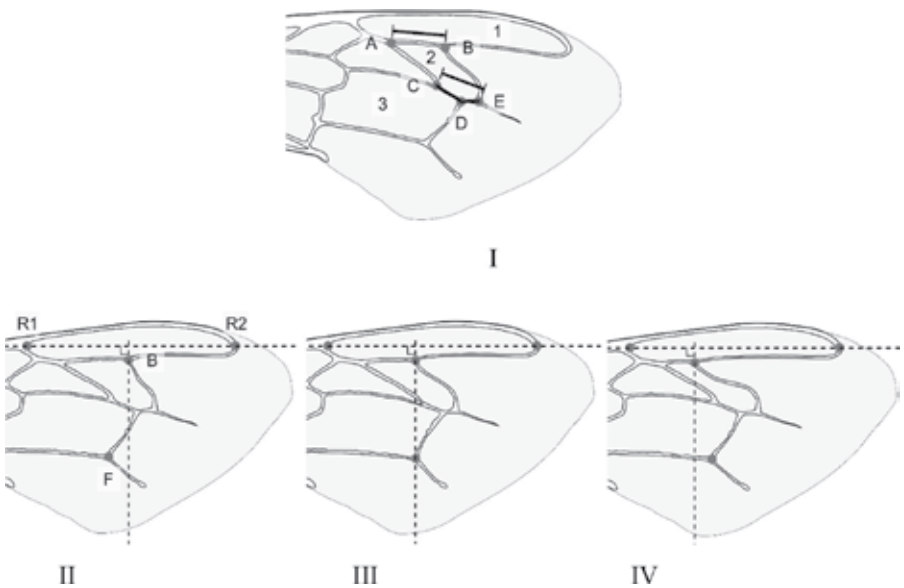

Figure 2. Scheme of the front wing venation of honeybee (I) and discoidal shift (II, III, and IV), showing the position of the horizontal and vertical lines (dashed lines). A, B, C, D, and E—the key points and segments that are used in determining the wing index (cubital index: CD/DE; hantel index: CE/AB). Options of discoidal shift: II—negative (point *F* is located to the left of the perpendicular line); III—zero (point *F* located on a perpendicular line); IV—positive (point *F* is located to the right of the perpendicular line). Designation of sells: 1, radial; 2, cubital; 3, discoidal.

2.4. mtDNA analysis

DNA isolation and polymerase chain reaction (PCR) was carried out according to standard techniques with some modifications [5,6]. To amplify the COI–COII mtDNA locus, the

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following sequences of primers were used: 3′-CACATTTAGAAATTCCATTA, 5′-ATAAA-TATGAATCATGTGGA [5]. Amplification products were fractionated in 1.5% agarose gel, and the results were documented with the use of Gel-Doc XR+.

2.5. Microsatellite analysis

Variability of eight microsatellite loci was studied: A008 (=A8), Ap049, AC117, AC216, Ap243, H110, A024, and A113. PCR was performed using specific primers and reaction conditions according to Solignac et al. [7]. Amplification products were analyzed with ABI Prism 3730 Genetic Analyser (Applied Biosystems, Inc., Foster City, CA) and GeneMapper Software (Applied Biosystems, Inc.). Two microliters of PCR products were mixed with GeneScan500-ROX size standards (Applied Biosystems, Inc.) and deionized formamide. Samples were run according to the manufacturer's recommendations. These genetic parameters were calculated: allelic frequencies and standard error.

2.6. Infestation of honeybees by *Nosema*

From 10 to 70 bees were randomly selected from each bee colony and were examined for the presence of *Nosema*. Bee samples were stored in 70% (v/v) ethanol at room temperature prior to testing. The analysis was performed separately for each bee. The midgut of each sample was isolated, and one part of the midgut was used for the detection of *Nosema* spores under a light microscope, while the other part was used for DNA extraction. The midgut was suspended in 200 μL of distilled water and examined by dark-field microscopy for the presence of *Nosema* spores [8]. DNA was extracted from the midgut using a DNA purification kit, PureLink™ Mini (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol.

After extraction, the samples were submitted to duplex-PCR [9,10]. The primer sequences utilized to amplify the 218-bp fragment corresponding to the 16S ribosomal gene of *N. ceranae* were 218MITOC–FOR 5′–CGGCGACGATGTGATATGAAAATATTAA–3′ and 218MITOC–REV 5′–CCCGGTCATTCTCAAACAAAAAACCG–3′[9]. The primer sequences used to amplify the 321 bp fragment corresponding to the 16S ribosomal gene of *N. apis* were 321APIS–FOR 5′–GGGGGCATGTCTTTGACGTACTATGTA–3′ and 321APIS–REV 5′–GGGGGGCGTTTAAAATGTGAAACAACTATG–3′[9]. PCR was performed using specific primers and reaction conditions according to Hamiduzzaman et al. [10]. PCR products were analyzed on 1.5 % (m/v) agarose gels and visualized using UV illumination (Gel Doc XR+, BioRad, Foster City, CA, USA). All analyses were carried out in duplicate, positive and negative controls were used, and identical results were obtained.

In addition to the use of specific primers and fragment size to identify the species present, a selection of fragments (both *N. ceranae* and *N. apis)* was verified by DNA sequencing. Sequencing was done in both directions using forward or reverse primer (BigDye Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems, Foster City, CA). DNA sequencing was performed using ABI Genetic Analyzer 3730 (Applied Biosystems) according to the manufacturer's protocol.

3. Results and discussion

Using the mtDNA analysis (locus COI-COII), we performed molecular genetic analysis of bee colonies (5–6 samples from each bee colony) to determine the origin of bee colony on the maternal line.

3.1. Genetic diversity of COI-COII mtDNA locus

An assessment of the genetic diversity of the COI-COII mtDNA locus in honeybee populations from the Tomsk region was conducted (see details in reference [11]). Three variants of the COI-COII mtDNA locus were registered: PQQ, PQQQ (typical for Middle Russian race), and Q

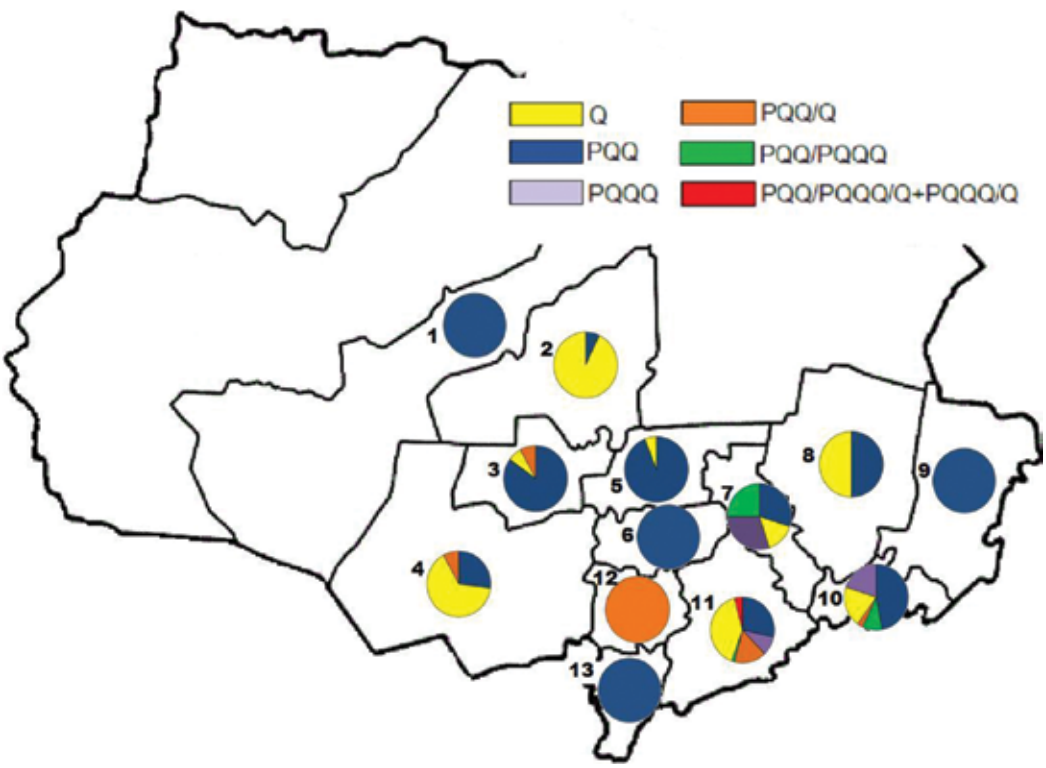

Figure 3. Distribution of COI-COII mtDNA locus variants for the districts (numbers 1–13) of the Tomsk region. Northern districts: 1, Parabelsky; 2, Kolpashevsky; 3, Chainsky; 4, Bakcharsky; 5, Molchanovsky; 6, Krivosheinsky; and southern districts: 7, Asinovsky; 8, Pervomaisky; 9, Teguldetsky; 10, Zyryansky; 11, Tomsky; 12, Shegarsky; 13, Kozhevnikovsky. Variants PQQ/PQQQ/Q (1%) and PQQQ/Q (3%), which are found only in the Tomsk district, are combined.

(typical for southern races). We established that 64% of bee colonies on the maternal line originate from the Middle Russian race, 28% of colonies originate from southern subspecies, and 8% are mixed bee colonies. The southern parts of the Tomsk region (with a high beekeeping activity) show a higher genetic diversity of honeybees as compared with the northern regions, which are dominated by bee colonies (96%) and apiaries (73%) that are homogeneous for the genetic variant of locus COI-COII. The bee colonies derived from the Middle Russian breed were genetically heterogeneous for the COI-COII locus: the PQQ variant was registered in 86.1% of the total number of bee colonies of the Middle Russian race, PQQQ was registered in 9.4%, and another 4.5% of bee colonies showed the presence of individuals with both allele PQQ and allele PQQQ.

Based on the analysis of mtDNA (locus COI-COII), assessment of the genetic diversity of the honeybee in apiaries of the Tomsk region has shown that the genetic structure of bee populations in the Tomsk region is complex and mosaic, especially in the southern parts of the region (**Figure 3**). No large areas with an array of bees having a homogeneous genetic (race) composition and maternally originating from the Middle Russian race have been found; a few apiaries were revealed, in which all bees originated from the Middle Russian breed.

In the study of variability of the COI-COII mtDNA locus in honeybees from apiaries of the Krasnoyarsk Krai and the Altai Krai, two variants of the COI-COII locus specific for Middle Russian race were identified: only variant PQQ was registered in honeybees of Krasnoyarsk Krai (Yenisei population) and two variants (PQQ and PQQQ) were found in honeybees from the Altai Krai. No a variant Q specific for southern races of bee was detected.

Due to the fact that mtDNA analysis allows assessing only the maternal component in the genome of the honeybee, bee colonies were investigated by the morphometric analysis to identify the characteristics of both the maternal and paternal lines, and to assess the level of hybridization.

3.2. Morphometric study of honeybees

The results of the morphometric study of honeybees from examined regions of Siberia (the Tomsk region, the Krasnoyarsk Krai, and the Altai Krai) were different.

According to the morphometric study, the majority of the studied bee colonies of the Tomsk region are hybrids between the Middle Russian race of bees and bees of southern origin (predominantly Carpathian race). Data on the distribution of subspecies and hybrids in the apiaries of the Tomsk region on the basis of cubital index are shown in **Figure 4**. Some of the apiaries, which cultivate the Middle Russian bees, were found in the northern and southern parts of the Tomsk region.

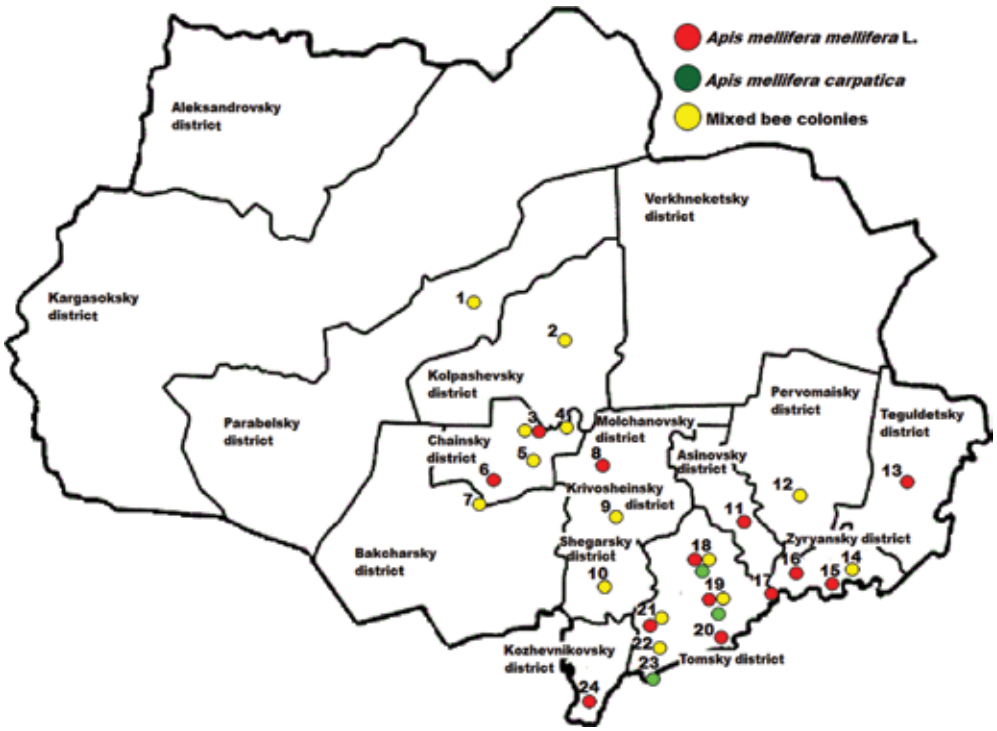

Figure 4. Distribution of subspecies and hybrids in the apiaries of the Tomsk region on the basis of cubital index of bee workers. Studied settlement are indicated by numbers: 1, s. Parabel; 2, vicinity of g. Kolpashevo; 3, Podgornoe; 4, s. Leboter; 5, d. Strelnikovo; 6, s. Gorelovka; 7, s. Vysoky Yar, d. Krylovka; 8, s. Mogochino; 9, s. Krivosheino, Sokolovka; 10, s. Kargala; 11, ur. Kuzherbak; 12, d. Krutolozhnoe; 13, s. Teguldet; 14, s. Okuneevo; 15, s. Zyryanskoe; 16, s. Dubrovka; 17, s. Novorozhdestvenskoe; 18, s. Kornilovo, s. Semiluzhki, p. Zarechnyi (Malinovskoe rural settlement); 19, p. Sinii Utes, d. Magadaevo, d.6 Prosekino, s. Kolarovo, vicinity of Tomsk; p. Zarechnyi (Mezheninovskoe rural settlement); 20, s. Mezheninovka; d. Arkashovo; 21, s. Zorkaltsevo, s. Rybalovo, d. Kudrinsky uchastok, d. Gubino; 22, s. Kurlek; 23, s.Yar; 24, d. Elovka. Apiaries located at a distance less than 15 km from each other are marked as a single point.

Bee colonies obtained from isolated apiaries of the Krasnoyarsk Krai are of considerable interest. The area with these isolated apiaries was not influenced by other subspecies of honeybee for many years, and all studied bees had only variant PQQ of the locus COI-COII mtDNA. However, when comparing the data of the morphometric study of bees from isolated apiaries with the Russian and European standards of the *Apis. m. mellifera*, the decrease of the lower limit values of cubital index was observed in the studied bees, and, as a result, for most bee colonies the deviation from the mean values of cubital index was shown (**Table 1**). There are several possible explanations for the results. First, this may be the result of genetic drift, the effect of which may be because of the fact that these apiaries are isolated and there are a limited number of bees. Second, the large scale of variability of the cubital index is the result of adaptation to the environment in more severe climatic conditions. Nevertheless, these isolated apiaries in the Krasnoyarsk Krai may be considered as a unique population of the Middle Russian bee that exists for a long time without affecting other subspecies of honeybee.

Geographical location: settlement	Bee colony, №	Cubital index, standard units		Hantel index, standard units		Discoidal shift, %		
		Lim: *min* *max*	*M* ± *m*	*Lim:* *min* *max*	*M* ± *m*	–	0	+
Ostyatskoe	1	1.24 2.00	1.61±0.04	0.675 0.892	0.795±0.011	100.0	0	0
	2	1.39 1.74	1.51±0.02	0.743 0.912	0.849±0.012	83.3	16.7	
	3	1.23 1.74	1.51±0.03	0.736 0.883	0.837±0.008	83.3	16.7	0
	4	1.20 1.67	1.45±0.02	0.723 0.900	0.837±0.009	97.0	3.0	0
	5	1.24 1.79	1.46±0.03	0.735 0.923	0.842±0.010	87.0	13.0	0
Kolmogorovo	1	1.32 2.10	1.60±0.05	0.724 0.900	0.820±0.009	97.0	3.0	0
	2	1.12 1.76	1.51±0.03	0.758 0.919	0.845±0.008	93.0	7.0	0
	3	1.28 1.86	1.56±0.04	0.746 0.985	0.810±0.011	97.0	3.0	0
	4	1.07 1.76	1.45±0.04	0.716 0.945	0.830±0.011	97.0	3.0	0
Yaksha	1	1.31 1.85	1.59±0.02	0.711 0.846	0.775±0.008	100.0	0	0
	Standard for *Apis mellifera mellifera*							
I		1.30 2.10	1.70	0.600 0.923	No data	No data		
II		1.30 1.90	1.5 to 1.7	0.600 0.923		91–100	5–10	0.00

Minimum 30 samples from each bee colony were studied.
Lim, limits of value of the sing; *M* ± *m*, average value of the sign ± the standard error of the mean; I, European breed standard based on values of cubital and hantel indexes [12]; II, Russian breed standard.

Table 1. Morphometric parameters (wing venation) of honeybee workers from 10 bee colonies of the Krasnoyarsk Krai (Yenisei population).

The results of morphometric analysis confirmed the origin of bee colonies of Altai population from the Middle Russian race, but some influence of the southern races have been shown. For example, the parameter "Discoidal shift" deviates from the Russian breed standard: individuals with a positive value and zero of discoidal shift were found in bee colony No. 7 (**Table 2**).

If bee colonies from the Krasnoyarsk Krai were obtained from the territory distant from the center and located in sparsely populated areas, in the taiga, the bee colonies from the Altai Krai inhabit the territory, characterized by high development of beekeeping and a constant active importation of bees of different origins.

3.3. The accordance of morphometric parameters and data of mtDNA analysis in honeybees in Siberia

The results of the outward morphological characters-based diagnostics of honeybees (the cubital index, the hantel index, and the discoidal shift) received from 11 bee colonies differing in the variants of the COI-COII mtDNA locus are presented (**Table 2**). Only for 4 of the 11 bee colonies, a full compliance with the criteria of the breed according to the morphometric and mtDNA analysis (the three *Apis mellifera mellifera* colonies and one family of *Apis mellifera carpatica*) was shown. The remaining seven colonies are hybrid, and for three colonies a significant imbalance between genetic and morphometric parameters was shown. Hence, in order to determine the breeds in the conditions of mass bee hybridization, it is important to consider not only the features of mtDNA, but morphometric parameters as well, among which the discoidal shift is probably the most important.

These data are consistent with the results of the research of hybrid apiary, where for many years (over 30) the Middle Russian bee was bred, but the last 10 years, the southern races have been actively imported [6]. More than 50% of individuals refer to the southern races according to mtDNA analysis (variant Q of the locus COI-COII; "southern" mitotype). But none of these individuals corresponded to the southern race according to morphometric analysis (**Table 3**). In 33% of cases, individuals with "southern" mitotype had two morphometric features characteristic to the Middle Russian race.

For bees, originating from the Middle Russian race (variant PQQ of the locus COI-COII), full compliance between mitotype and morphometric parameters was found in approximately 6% of the individuals. 18% of bees had mitotype and two morphometric parameters which specific to the Middle Russian bees.

This indicates a process of cross-breeding of Middle Russian and southern races on this apiary. However, the process of "ousting of genes" is derived differently for bees of different origin: for bees of Middle Russian race the process of "ousting of genes" is smaller in scale, as among individuals with variant PQQ a smaller percentage of bees with "southern" morphometric characters was registered in comparison with the same data shown for bees with "southern" mitotypes.

Geographical location			Bee colony, №	Number of studied bees	Sequence composition of the COI-COII mtDNA locus	Cubital index, standard units			Hantel index, standard units		
region	District	Settlement				*Lim: min max*	*M*	*sd*	*Lim: min max*	*M*	*sd*
					Apis mellifera mellifera[*]						
Tomsk region	Tomsky	p. Zarechnyi	1	30	PQQQ	1.39 2.23	1.66	0.216	0.712 0.932	0.826	0.052
		s. Kurlek	2	28	PQQQ	1.74 3.29	2.14	0.376	0.857 1.053	0.937	0.055
	Zyryansky	s. Dubrovka	3	30	PQQ	1.43 2.47	1.69	0.232	0.672 0.933	0.849	0.060
	Molchanovsky	s. Mogochino	4	30	PQQ	1.26 2.56	1.92	0.290	0.806 1.000	0.879	0.055
			5	43	PQQ	1.36 2.00	1.73	0.181	0.693 0.926	0.821	0.038
Altai Krai	Zmeinogorsky	Vicinity of c. Zmeinogorsk	6	29	PQQ	1.19 2.00	1.55	0.232	0.758 0.967	0.858	0.062
			7	30	PQQQ	1.50 2.50	1.80	0.245	0.722 0.984	0.845	0.059
Krasno-yarsk Krai	Yeniseisky	p. Yaksha	8	30	PQQ	1.31 1.85	1.59	0.132	0.711 0.846	0.775	0.044
					Southern breeds[*]						
Tomsk region	Tomsky	s. Semiluzhki	9	50	Q	1.68 3.64	2.51	0.374	0.867 1.210	1.050	0.047
		s. Kurlek	10	29	Q	1.30 2.29	1.66	0.220	0.735 0.965	0.878	0.060
		p. Sinii Utes	11	30	Q	1.83 2.87	2.37	0.334	0.815 1.053	0.931	0.065
Standart of breeds	*A. m. mellifera*[**]		PQQ, PQQQ and other			1.30 2.10	1.70	–	0.600 0.923	–	–
	A. m. mellifera[***]					1.30 1.90	1.6	–	0.600 0.923	–	–
	A. m. carnica[**]		Q			2.40 3.00	2.7	–	≥ 0.925	–	–
	A. m. caucasica[**]		Q			1.70 2.30	2.0	–	No data	–	–

Lim, limits of values; *M,* arithmetic mean; *sd,* standard deviation.
[*]Breed indicated according to the data of mtDNA analysis.
[**]European breed standard based on values of cubital and hantel indexes [12].
[***]Russian breed standard. Discoidal shift are given according to Russian standards.

Table 2. Morphometric parameters (wing venation) of honeybee workers of 11 bee colonies from apiaries of Siberia.

Geographical location			Bee colony, №	Number of studied bees	Sequence composition of the COI-COII mtDNA locus	Discoidal shift, %		
region	District	Settlement				–	0	+
			Apis mellifera mellifera[*]					
Tomsk region	Tomsky	p. Zarechnyi	1	30	PQQQ	73.30	26.70	0.00
		s. Kurlek	2	28	PQQQ	32.10	53.60	10.70
	Zyryansky	s. Dubrovka	3	30	PQQ	73.33	26.67	0.00
	Molchanovsky	s. Mogochino	4	30	PQQ	70.00	30.00	0.00
			5	43	PQQ	100.0	0.00	0.00
Altai Krai	Zmeinogorsky	Vicinity of c. Zmeinogorsk	6	29	PQQ	94.00	6.00	0.00
			7	30	PQQQ	46.70	46.70	6.60
Krasnoyarsk Krai	Yeniseisky	p. Yaksha	8	30	PQQ	100.0	0.00	0.00
			Southern breeds[*]					
Tomsk region	Tomsky	s. Semiluzhki	9	50	Q	4.00	20.00	76.00
		s. Kurlek	10	29	Q	72.40	27.60	0.00
		p. Sinii Utes	11	30	Q	6.70	76.70	16.70
Standart of breeds	*A. m. mellifera*[**]		PQQ, PQQQ and other			–	–	–
	A. m. mellifera[***]					91–100	5–10	0.00
	A. m. carnica[**]		Q			0–5	0–20	80–100
	A. m. caucasica[**]		Q			60–70	20–30	3–5

Lim, limits of values; *M,* arithmetic mean; *sd,* standard deviation.
[*]Breed indicated according to the data of mtDNA analysis.
[**]European breed standard based on values of cubital and hantel indexes [12].
[***]Russian breed standard. Discoidal shift are given according to Russian standards.

Table 2. Continued.

mtDNA		Variant PQQ		Variant Q	
Number of studied bees, %		44.44		55.56	
Race		*Apis mellifera mellifera*	Southern race	*Apis mellifera mellifera*	Southern race
The combination of features characteristic for different races	3 parameters $x^1 + x^2 + x^3$	5.6	7.4	7.4	0.0
	2 parameters, total, including	18.5	13.0	33.3	14.8
		1.9	1.9	1.9	11.1

mtDNA		Variant PQQ		Variant Q	
	$x^1 + x^2$	3.7	11.1	0	3.7
	$x^1 + x^3$	13.0	0	31.5	0
	$x^2 + x^3$				
	1 parameter, total	13.0	18.5	14.8	33.3

x^1, x^2, x^3 are parameters of cubital index, hantel index, and discoidal shift, respectively.

Table 3. The accordance of morphometric parameters in individuals with different genetic variants of the COI-COII mtDNA locus (see details in reference [6]).

Thus, the result of study of hybrid apiaries and bee colonies indicate, on the one hand, the importance and the necessity of a comprehensive approach to the exact characterization of honeybee races. On the other hand, the results are of scientific interest for the study of genetic processes during hybridization of different subspecies of honeybee and for analyzing the process of "ousting of genes" of one race by genes of other race. For example, hybridization between the Middle Russian bee and Carpathian bee is of interest because the races belong to different evolutionary branches.

For such studies, microsatellite loci are the most informative molecular genetic markers. Microsatellite markers can be useful for the study of genetic structure of different honeybee populations and bee colonies, evaluation of genetic diversity and introgressive hybridization, differentiation of different subspecies (ecotypes), the establishment of evolutionary relationships and adaptive features of four evolutionary branches (A, M, C, and O), mapping quantitative trait loci (QTL), and search of genetic markers associated with economically significant characteristics [3,7,13–46].

Characterization of the allele spectrum of microsatellite loci and analysis of their variability in subspecies, colonies, and individuals in the honeybee populations is the initial stage of any of the above research.

3.4. Microsatellite analysis

Variability of eight microsatellite loci (A008 (=A8), Ap049, AC117, AC216, Ap243, H110, A024, and A113) in honeybee from Siberian region was studied. Seven loci were polymorphic and only for AC216 locus one homozygous genotype was registered in all the studied bees (allele 91 bp). For each locus, the range and frequency of genotypes and alleles were determined (**Table 4**).

Locus	Genotype	Frequency of genotype	Allelic frequency with an error
A008	152–152	0.006	P_{152}=0.0311±0.0054
	152–162	0.049	P_{162}=0.8049±0.0123

Locus	Genotype	Frequency of genotype	Allelic frequency with an error
	152–170	0.002	P_{166}=0.0010±0.0031
	162–162	0.736	P_{168}=0.0010±0.0031
	162–168	0.002	P_{170}=0.0213±0.0045
	162–170	0.016	P_{172}=0.0243±0.0048
	162–172	0.039	P_{174}=0.0825±0.0086
	162–174	0.033	P_{176}=0.0029±0.0017
	166–172	0.002	P_{178}=0.0262±0.0050
	170–170	0.006	P_{180}=0.0039±0.0019
	170–174	0.016	
	172–172	0.004	
	174–174	0.037	
	174–176	0.004	
	174–178	0.031	
	174–180	0.008	
	176–178	0.002	
	178–178	0.010	
	n=515		
Ap049	118–127	0.002	P_{118}=0.0010±0.0001
	121–127	0.002	P_{121}=0.0069±0.0025
	121–130	0.006	P_{127}=0.6581±0.0149
	121–139	0.006	P_{130}=0.1759±0.0120
	127–127	0.529	P_{139}=0.1403±0.0109
	127–130	0.187	P_{142}=0.0010±0.0001
	127–139	0.053	P_{152}=0.0168±0.0040
	127–152	0.019	
	130–130	0.055	
	130–139	0.045	
	130–152	0.002	
	139–139	0.081	
	139–152	0.013	
	142–152	0.002	
	152–152	0.002	
	n=506		

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Locus	Genotype	Frequency of genotype	Allelic frequency with an error
AC117	175–175	0.008	P_{175}=0.0910±0.0092
	175–179	0.020	P_{179}=0.0879±0.0090
	175–183	0.145	P_{183}=0.8211±0.0123
	179–179	0.012	
	179–183	0.131	
	183–183	0.683	
	n=489		
H110	162–162	0.567	P_{162}=0.7522±0.0167
	162-166	0.116	P_{166}=0.0627±0.0093
	162–170	0.254	P_{170}=0.1851±0.0150
	166–166	0.003	
	166–170	0.003	
	170–170	0.057	
	n=335		

n, number of studied samples is indicated in bold.

Table 4. Characterization of variability of seven microsatellite loci in honeybees from Siberia.

Locus	Genotype	Frequency of genotype	Allelic frequency with an error
Ap243	255–255	0.401	P_{255}=0.5278±0.0222
	255–263	0.167	P_{263}=0.3175±0.0207
	255–269	0.056	P_{269}=0.0833±0.0123
	255–272	0.028	P_{272}=0.0635±0.0109
	255–275	0.004	P_{275}=0.0079±0.0039
	263–263	0.175	
	263–269	0.075	
	263–272	0.040	
	263–275	0.004	
	269–269	0.004	
	269–272	0.028	
	272–272	0.012	
	272–275	0.008	

Locus	Genotype	Frequency of genotype	Allelic frequency with an error
	n=252		
A024	94–94	0.344	P_{94}=0.4736±0.0186
	94–98	0.036	P_{96}=0.1014±0.0112
	94–100	0.033	P_{98}=0.0375±0.0070
	94–102	0.175	P_{100}=0.0194±0.0051
	94–104	0.014	P_{102}=0.2097±0.0152
	96–96	0.067	P_{104}=0.1528±0.0134
	96–104	0.058	P_{106}=0.0056±0.0028
	96–106	0.011	
	98–98	0.019	
	100–100	0.003	
	102–102	0.089	
	102–104	0.067	
	104–104	0.083	
	n=360		
A113	208–212	0.003	P_{208}=0.0013±0.0013
	210–210	0.003	P_{210}=0.0144±0.0043
	210–218	0.021	P_{212}=0.2350±0.0153
	210–220	0.003	P_{214}=0.0026±0.0018
	212–212	0.177	P_{218}=0.5953±0.0177
	212–214	0.005	P_{220}=0.1084±0.0112
	212–218	0.078	P_{222}=0.0013±0.0013
	212–220	0.013	P_{224}=0.0013±0.0013
	212–222	0.003	P_{226}=0.0183±0.0048
	212–226	0.005	P_{228}=0.0196±0.0050
	212–228	0.003	P_{232}=0.0026±0.0018
	212–232	0.005	
	218–218	0.475	
	218–220	0.117	
	218–226	0.021	
	218–228	0.003	
	220–220	0.018	
	220–224	0.003	

Locus	Genotype	Frequency of genotype	Allelic frequency with an error
	220–226	0.010	
	220–228	0.034	
	n=383		

Table 4. Continued.

Microsatellite loci differed in variability: the minimum number of alleles was detected for loci AC117 and H110 (3 alleles) and the maximum number of alleles was registered for loci A008 (10 alleles) and A113 (11 alleles). At the same time, for six of the seven polymorphic loci (except locus A024), one major allele with a frequency of more than 0.5 (from 0.5278 for allele "255"of locus Ap243 to 0.8211 for allele "183" of locus AC117) was registered regardless of the number of detected alleles.

To identify the features of honeybee from different geographical areas, the comparative analysis of the variability of the studied loci was carried out for the bees of *Apis mellifera mellifera* (= dark-colored forest bee, Middle Russian race) of four populations (Siberia, the Urals, and Europe) using our own data (Tomsk region and Krasnoyarsk Krai) and literature data [15,16,47] (**Tables 5 and 6**). The Ural population (Bashkir population) located in the nature reserve is a unique population of the dark-colored forest bee (Burzyan bee).

Locus	Alleles (pb)	Allelic frequency					
		Russia			Europe**		
		Krasnoyarsk Krai	Tomsk region	Ural* (Bashkortostan)	Belgium (Chimay)	Sweden (Umea)	France (eight geographic areas)
A008	148				0.783	0.727	0.267–0.969
	152		0.006				
	154			0.897			0–0.083
	155						0–0.033
	156			0.053	0.133	0.227	0.017–0.300
	157						0–0.050
	158			0.053		0.023	0–0.117
	159						0–0.017
	160				0.050		0–0.100
	162	1.000	0.912		0.033		0–0.034
	164					0.023	0–0.020
	166		0.003				0–0.017
	170		0.003				

Locus	Alleles (pb)	Allelic frequency					
		Russia			Europe**		
		Krasnoyarsk Krai	Tomsk region	Ural* (Bashkortostan)	Belgium (Chimay)	Sweden (Umea)	France (eight geographic areas)
	172		0.032				
	174		0.044				
N		***120***	***170***	***48***	***60***	***44***	***634***
A024	94	0.216	0.741		No data	No data	
	96	0.358					
	98	0.132		0.896			0.804
	100	0.034	0.020				
	102	0.025	0.227				
	104	0.216	0.012				
	106	0.020		0.104			0.130
	108						0.065
N		***102***	***172***	***48***			***46***
A113	202				0.083	0.024	0.017–0.267
	204						
	208						0–0.017
	210	0.021	0.009				
	212		0.174				0–0.030
	214		0.006		0.033		0.010–0.500
	216			0.063			0–0.017
	218	0.898	0.540	0.865			0–0.020
	220	0.081	0.183	0.042	0.833	0.857	0.433–0.810
	222		0.003	0.032		0.024	0–0.041
	224		0.003		0.017	0.048	0–0.060
	226		0.040			0.048	0–0.034
	228		0.043		0.017		0.017–0.071
	230						0–0.052
	232						0–0.017
	234				0.017		0–0.017
	236						0–0.020
	238						0–0.017

Locus	Alleles (pb)	Allelic frequency					
		Russia			Europe**		
		Krasnoyarsk Krai	Tomsk region	Ural* (Bashkortostan)	Belgium (Chimay)	Sweden (Umea)	France (eight geographic areas)
	240						0–0.010
N		***118***	***175***	***48***	***60***	***44***	***634***

N, number of studied samples.
*data from reference [47].
**data from references [15,16].
The minimum and maximum values of allelic frequencies represented for loci A008 and A113 in honeybees of France populations; allelic frequencies for locus A024 are given for bees of only Northern France population.

Table 5. Allele frequency at three loci in honeybees from different geographic areas of Russia and Europe.

Locus	Alleles (pb)	Allelic frequency		
		Siberia		Ural
		Krasnoyarsk Krai	Tomsk region	Bashkortostan
Ap049	118	0.005		
	121	0.005	0.003	
	123			0.917
	127	0.810	0.711	
	130	0.138	0.249	0.063
	138			0.021
	139	0.014	0.037	
	152	0.029		
Number of studied samples		***105***	***175***	***48***
Ap243	254			0.646
	255	0.280	0.524	
	257			0.354
	263	0.542	0.254	
	269	0.140	0.056	
	272	0.037	0.143	
	275		0.024	
Number of studied samples		***107***	***63***	***48***
H110	160			0.615
	162	0.624	0.837	
	163			0.302

Locus	Alleles (pb)	Allelic frequency		
		Siberia		Ural
		Krasnoyarsk Krai	Tomsk region	Bashkortostan
	166	0.376	0.056	
	168			0.083
	170		0.107	
Number of studied samples		***117***	***135***	***48***

N, number of studied samples.

Table 6. Allele frequency at three loci in honeybees from different populations of Russia.

Siberian populations (Tomsk region and Krasnoyarsk Krai) are closest in spectrum and allele frequencies of most studied loci (A008, Ap049, A113, Ap243, H110). The Ural population located to the west of Siberian region differs from Siberia for some loci: for locus A008 differences were registered in the spectrum of alleles, for the locus A024—in the frequency of alleles, for the loci Ap049 and Ap243—in both the spectrum and frequency of alleles. It is remarkable that the Ural population has a greater similarity in the spectrum of alleles of loci A024 and A008 to European populations.

The differences in the spectrum of alleles and the frequency of allele registration for locus A008 were revealed in honeybees of Siberia, the Ural, and European populations. For honeybees of the Ural and Europe, shorter alleles of locus A008 were predominant (154 bp and 148 bp, respectively), whereas for bees from Siberia allele "162" was the most specific. Probably this locus should be considered as a marker related to geographic and environmental conditions (specific adaptation to local conditions) [1,3,48,49] because the different populations of dark-colored forest bee (European, Ural, and Siberian populations) were compared in this study.

For some loci, for example A113, allelic spectrum overlaps, but the frequency of the alleles was different in honeybees of different populations. Different factors of population dynamics (such as founder effect, genetic drift, natural selection) can be causes of this phenomenon.

Thus, it is shown that for some loci the specific distribution of genotypes and alleles were detected in the bees, which differ by geographical location. Further research is needed and the expansion of gene-geographic studies of honeybee is relevant.

To assess the informativeness of studied loci for the differentiation of different subspecies of honeybee, the comparison of the spectrum of predominant alleles in bees of different evolutional branches (M and C) and from different geographical localization was conducted (**Table 7**). Comparison of the data on the variability of microsatellite loci studied in bees of different origin and different geographical location allows making some conclusions and adjustments with respect to informativeness of these loci as markers for differentiation of subspecies of honeybee.

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For locus A008, the differences in the spectrum of the most common alleles are registered between the *Apis m. mellifera* living in different geographical regions (as shown above), and between the two southern races (*Apis m. caucasica* and *Apis m. carpatica*).

For locus A113 clear differences in length of the most frequently detected allele were not detected both among bees of a common origin and between bees belonging to different races. Probably this locus cannot be considered informative for determining of the subspecies.

Loci A024 and Ap049 are of considerable interest for further study as candidate markers for inclusion in the diagnostic panel, differentiating subspecies. So, in general, for the locus A024 the majority of bees and bee colonies *Apis m. mellifera*, regardless of their habitat, are characterized by shorter length of alleles. Perhaps, for locus Ap049 the differences exist in the allelic spectrum between bees belonging to different races.

	Geographical location	**Sequence composition of the COI-COII mtDNA locus (breed)**	**Predominant allele**	**Allelic frequency**
Locus A008	Tomsk region	PQQ/PQQQ	162	0.71–1.00
	Krasnoyarsky Krai	PQQ	162	1.00
	Ural (Bashkir population)[1]	PQQ	154	0.63–1.00
	Tomsk region[2]	Q	174	0.58–0.61
	Sochi area[3]	Q	158	0.88–1.00
	Europe[4]	*A.m.mellifera*	148	0.27–0.97
Locus A113	Tomsk region	PQQ/PQQQ	218	0.67–0.82
			212	0.61
			220	0.50
	Krasnoyarsky Krai	PQQ	218	0.85–0.95
	Ural (Bashkir population)[1]	PQQ	218	0.50–1.00
			220	0.50
	Tomsk region[2]	Q	212	0.94–1.00
	Sochi area[3]	Q	222	0.50
	Europe[4]	*A.m.mellifera*	220	0.433–0.857
Locus A024	Tomsk region	PQQ/PQQQ	94	0.60–0.90
			102	0.54
	Krasnoyarsky Krai	PQQ	98	0.50
			96	0.50–0.71
	Ural (Bashkir population)[1]	PQQ	98	0.50–1.00
			106	0.50
	Tomsk region[2]	Q	104	0.65

	Geographical location	Sequence composition of the COI-COII mtDNA locus (breed)	Predominant allele	Allelic frequency
	Sochi area[3]	Q	106	0.88–1.00
	Europe[4]	*A.m.mellifera*	98	>0.80
Locus AP049	Tomsk region	PQQ/PQQQ	127	0.62–0.92
			130	0.77
	Krasnoyarsky Krai	PQQ	127	0.50–0.96
	Ural (Bashkir population)[1]	PQQ	129	0.50–1.00
			130	1.00
	Tomsk region[2]	Q	139	0.66–1.00
	Sochi area[3]	Q	139	1.00

*Data on allelic frequencies, the frequency of which = or > 0.5 are shown.
[1]Data on the Ural (Bashkir population) are taken from reference [47].
[2]Our own data for the Carpathian breed (*Apis m. carpatica*) imported into the territory of the Tomsk region from Carpathian breed nursery (d. Mukachevo, Ukraine).
[3]Data on the Caucasian honeybee (*Apis m. caucasica*) from the Sochi area are taken from reference [47].
[4]Data on the European population are taken from references [15,16].

Table 7. Comparative analysis of the frequency of the most common alleles* of microsatellite loci in honeybees of different maternal origins and geographic localization.

In order to determine the subspecies status of an individual honeybee, a honeybee colony, or a honeybee population, it is important to compare allelic counts and genotypes across different studies. However, no standard reference material, such as a standard allelic ladder, is available for honeybees [3]. In addition, the spectrums of analyzed microsatellite markers often do not overlap and primary data on the allele spectrum and allele frequencies are not always presented in publications. In general, the present stage of the study of variability of microsatellite loci in *Apis mellifera* can be considered as a period of accumulation of information. At this stage of the study of honeybee it should be with caution relate to the use autosomal loci to determine the subspecies of honeybee.

3.5. Infestation of honeybees by *Nosema* in Siberia

Importation of races of southern origin to the territory of Siberia, where the Middle Russian breed for a long time lived, on the one hand, led to a massive hybridization of bees, a loss of purebred, decreased immunity, and increased incidence of bees. On the other hand, the import of bee families from other areas (the European part of Russia, Uzbekistan), disadvantageous in the epidemiological situation, led to the spread of diseases that have not previously registered in the territory of Siberia.

This situation was evaluated for nosemosis: the distribution *Nosema* infection throughout Siberia was studied, the species of microsporidia were determined, and the origin of bee colonies infected with *Nosema* was investigated.

Nosemosis is a parasitic disease of adult honeybees (*Apis mellifera* L.) caused by two described species of microsporidia, *Nosema apis* [50] and *Nosema ceranae* [51]. The disease occurs throughout the world, causes significant detriment to honey production, and results in economic losses. The original assumption was that *N. apis* specifically infects the European honeybee *A. mellifera*, causing nosemosis, and that *N. ceranae* is a specific pathogen of the Asian honeybee, *A. cerana*. Recently, it became evident that *N. ceranae* is also widespread in the *A. mellifera* population throughout the world and is already found in North and South America, across Europe and Asia [52–58]. It has been subsequently detected across Canada and the United States [59,60] and has been confirmed in Central America [61], Australia [62], and North Africa [63].

The geographical distribution of *Nosema* in Russia is not well known [64,65]. In addition, information on the prevalence of *N. ceranae* in Russia, including Siberia, is not complete [66]. Previously, nosemosis in honeybees in Siberia was attributed exclusively to *N. apis*. The problem of the distribution of *Nosema* and the consequences of infection for honeybees has not yet been resolved. The effects of the *Nosema* infection on survival and productivity of honeybees are not well studied.

For the period of 2012–2015, a screening study of 132 bee colonies from 68 apiaries of Siberia for the presence of *Nosema* spores was carried out [65]. For an objective evaluation, the different methods were used: microscopy and PCR. We found that honeybees of 33 colonies from 132 studied (25.0%) and 21 apiaries from 68 studied (30.9%) had spores detectable by light microscopy. As it is difficult to distinguish *N. ceranae* and *N. apis* morphologically, a PCR assay based on 16S ribosomal RNA has been used to differentiate *N. apis* and *N. ceranae*. To characterize further the identity of which species of *Nosema* was present, we performed PCR using primers specific for either *N. apis* or *N. ceranae. Nosema* positive samples (determined from light microscopy of spores) of adult worker bees from 33 bee colonies (21 apiaries) were tested to determine *Nosema* species using PCR primers of the 16S rRNA gene specific for *N. ceranae* or *N. apis*.

The samples of 28 bee colonies from 33 infected colonies (84.8%) from 19 apiaries were positive by PCR using *N. apis* specific primers, and the samples from three colonies (3/33, 9.1%) were positive for *N. ceranae* (only two of apiaries). Samples co-infected with both *N. ceranae* and *N. apis* were registered in two bee colonies (2/33, 6.0%) from two apiaries. To confirm the PCR findings, the DNA fragments were sequenced. Sequence analysis revealed a complete sequence identity for *N. apis* (GenBank Accession No U97150) and *N. ceranae* (GenBank Accession No DQ486027).

Nosema-infected bees were found in samples collected from five districts and mainly in the southern climatic areas (temperate continental parts of Siberia) (**Figure 5**). In the northern district (**Figure 5**, C – Chainsky) bees infected by *Nosema apis* are imported from Uzbekistan. It was established that *Nosema ceranae* revealed in bees from the southern districts of the Tomsk region (**Figure 5**, Shegarsky and Tomsky districts) was introduced with infected bees from southern regions of Russia.

The studied bees from apiaries of Krasnoyarsk Krai and Altai Krai were not infected with *Nosema*.

Reports on the impact of *N. ceranae* infections on honeybee health and colony survival are contradictory, and various symptoms of the disease have been described [4,48,52,54–56,59,60,67–76]. Adult bees become infected by ingesting *Nosema* spores, which germinate in the midgut and infect cells of the midgut epithelium. *Nosema* infection caused by *N. apis* is characterized mainly by dysentery, whereas *N. ceranae* is described as causing death of individuals and colonies not preceded by any visible symptoms [9,68]. *Nosema apis* infection is restricted to the midgut epithelium [77], whereas *N. ceranae* has also been detected by molecular methods in other bee tissues such as malpighian tubules and hypopharyngeal glands [78].

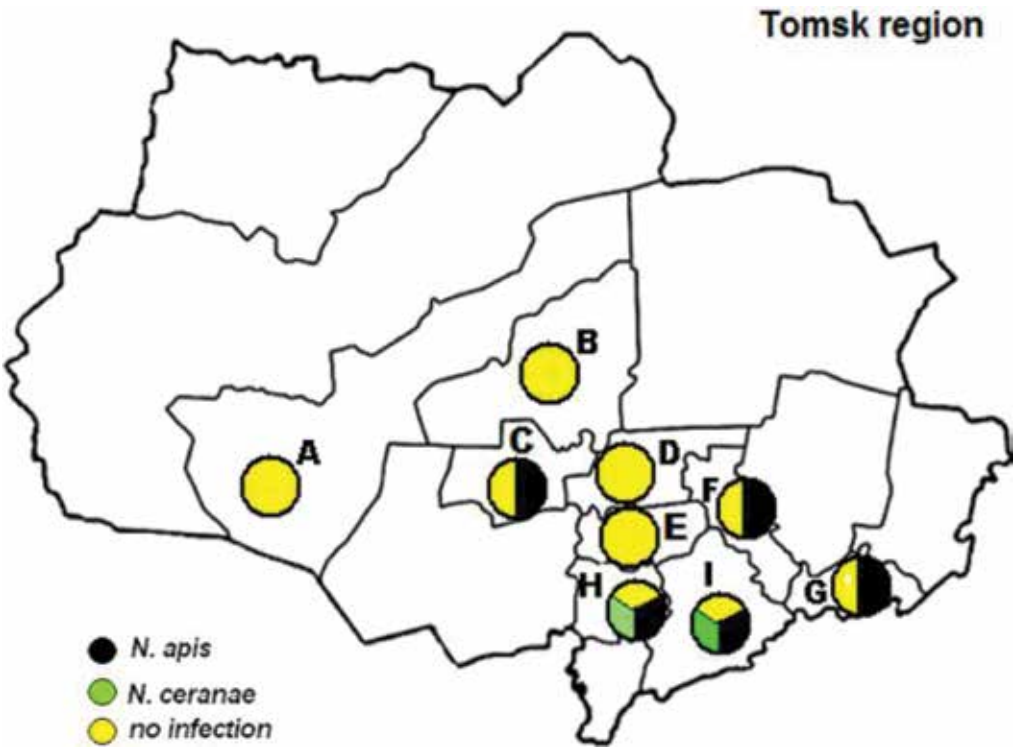

Figure 5. Distribution of *Nosema* in the honeybee colonies (*Apis mellifera* L.) throughout the Tomsk region (Western Siberia) (dots *A*–*I*). Bee colonies not infected by *Nosema* are indicated in yellow; bee colonies corresponding to infection by *N. apis* or *N. ceranae* are indicated in black and green, respectively. Sectors in circles indicate representation cases (existence/absence) of an infection without frequency. Literas (A–I) indicate the districts of the Tomsk region: A, Parabelsky; B, Kolpashevsky; C, Chainsky; D, Molchanovsky; E, Krivosheinsky; F, Asinovsky; G, Zyryansky; H, Shegarsky; I, Tomsky.

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Perhaps, *N. ceranae* is the most aggressive of the two *Nosema* species in relation to the host and appears to be replacing *N. apis* in some populations of honeybees.

Currently, several reasons for the widespread presence of the parasite *N. ceranae* in the world and its displacement of *N. apis* are discussed in the literature. On the one hand, nosemosis produced by *N. ceranae* is considered a global problem because this parasite has wide prevalence in multiple hosts [79]. *Nosema ceranae* is a more aggressive parasite compared with *N. apis,* and consequently, it is more widespread than *N. apis*. On the other hand, the killing of honeybee colonies by *N. ceranae* could be a regional problem rather than a global phenomenon [80], and the virulence of *N. ceranae* could be influenced by climatic conditions [81–85] or might actually depend on honeybee race and honeybee genetic diversity [4,48,74,75,86–88].

It is assumed that the level of infestation in honeybees can be associated with the race and the origin (local or non-local) of the bees. Some differences in the resistance to *Nosema* have been shown in Russian bee breeds [86]. Levels of *N. ceranae* infestation differed significantly between lineages and colonies for both Russian and Italian workers [87]. Unlike genetically homogeneous Italian lines [87], Russian bee lineages have a high genetic diversity and are characterized by high resistance to disease. Differences in infection levels were significant between local and introduced bee colonies [4,74]. The use of local honeybees provides a higher chance of colony survival because of their adaptation to regional environmental factors such as climate and vegetation [48,75].

To determine if the infection incidence of bees by *Nosema* is associated with the races of bees, we analyzed breeds of bee derived from *Nosema*-positive colonies using morphometric (wing venation) and molecular-genetic (mtDNA) analyses (**Table 8**). The results of molecular genetic analysis (COI-COII locus) of honeybees have been published in reference [11]. According to the mtDNA analysis, PQQ and PQQQ variants of the locus COI-COII (*A. m. mellifera*, evolutionary branch M) were detected in two colonies (families No. 2 and 4), and Q variant (evolutionary branch C) was registered in four colonies (families No. 1, 3, 5, and 7). Family No. 6 had bees with different variants of the COI-COII locus (PQQ and Q) and apparently was formed by mixing two colonies having different origins. As a result, morphometric studies have shown that colonies No. 1, 3, 4, and 6 can be considered as subspecies of *A. m. mellifera* and that colonies No. 2, 5, and 7 are hybrids. However, according to the combined morphometric and mtDNA analysis, only family No. 4 can be considered as *A. m. mellifera*, whereas six *Nosema*-infected bee colonies did not correspond to any of the standards but were honeybee hybrids (**Table 8**). Furthermore, some colonies that were observed not only differed in morphometric parameters compared with the standards but in a mismatch of morphometric data and results of the mtDNA analysis for the two honeybee colonies. Honeybees infected with *N. apis* (colony No. 3) and bees infected with *N. ceranae* (colony No. 1) correspond to the *A. m. mellifera* race (branch M) according to the morphometric analysis, whereas the results of the mtDNA analysis confirmed the origin of these bees from branch C. Thus, our results indicate that examined honeybees infected with *Nosema* could be of hybrids of the two races (*Apis m. mellifera* and *Apis m. carpatica*).

№ colonies	*Nosema* species	Sequence composition of the COI-COII mtDNA locus	Morphometric parameters			
			Cubital index, standard units		Hantel index, standard units	
			Lim: min max	M ± m	Lim: min max	M ± m
1	*N. ceranae*	Q	1.30 2.29	1.66 ± 0.04	0.735 0.965	0.878 ± 0.011
2	*N. ceranae*	PQQQ	1.74 3.29	2.14 ± 0.07	0.857 1.053	0.937 ± 0.010
3	*N. apis*	Q	1.35 2.11	1.70 ± 0.03	0.667 0.917	0.804 ± 0.011
4	*N. apis*	PQQ	1.45 2.80	1.78 ± 0.06	0.754 1.0	0.846 ± 0.013
5	*N. apis*	Q	1.41 2.82	1.90 ± 0.06	0.656 1.176	0.880 ± 0.018
6	*N. apis*	PQQ/Q	1.28 2.80	1.73 ± 0.06	0.707 1.0	0.834 ± 0.015
7	*N. apis*	Q	1.43 2.35	1.86 ± 0.04	0.733 1.057	0.885 ± 0.011
Standard breeds (subspecies)**						
A. m. mellifera		PQQ, PQQQ and other	1.3 2.1	1.7	0.600 0.923	No data
A. m. carpatica		Q	2.3 3.0	2.65	≥0.925	No data

M ± *m*, average value of the sign ± the standard error of the mean.
*Thirty samples of bees were examined in each family.
**Definition of subspecies was carried out based on European standard honeybee [12].

Table 8. Characterization honeybee colonies infested by *Nosema*.

For comparison, the assessment of the origin of the bee colonies not infected with *Nosema* (24 families from 38 analyzed) was carried out using morphometric and mtDNA analysis. Among the 24 bee colonies not infected with *Nosema,* 18 bee colonies were identified as *A. m. mellifera* (75.0 %), 3 colonies were identified as *A. m. carpatica* (12.5 %), while 3 colonies were identified as hybrids (12.5 %).

At present, the cold climate is considered as one of the limiting factors of *N. ceranae* distribution. It appears that the spread of *N. ceranae* across the globe is reduced in colder climates [81,82], as *N. ceranae* spores are capable of surviving high temperatures (60 °C) and desiccation, but they are intolerant of cold (4 °C) [81,82,89]. The marked decrease in *N. ceranae* spore germina-

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tion was observed after even a short exposure to low temperatures (4 °C) [82]. In warmer climates, *N. ceranae* is more competitive than *N. apis* [48,82], but the spores of *N. ceranae* appear to be much more vulnerable than the spores of *N. apis*, in particular, to freezing, and the apparent replacement of *N. apis* for *N. ceranae* remains enigmatic [83].

The different prevalence of *N. ceranae* may simply reflect its time of arrival, by natural spread or by the importation of infected honeybees, and mobility of bees within a country. Reduced or inhibited *N. ceranae* spore germination at low temperatures should hamper the infectivity and spread of this pathogen in climatic regions characterized by a rather cold winter season [82]. The presence of *N. ceranae* in the Tomsk region (Western Siberia, Russian) was reported previously by us [65,66] confirms the fact of a widespread *N. ceranae* infection in honeybee population throughout the world. However, we found *N. ceranae*-infected bee colonies in cold climate with long winters and humid summers, and this parasite is not associated with colony depopulation or honeybee collapse. We established that these previously infected colonies had been imported from other areas of Russia. The fact that *N. ceranae* is registered in the territory of Siberia with its severe climatic conditions does not agree with data on a weak survival of spores at low temperatures. At the same time, the colonies infected with *Nosema* (*N. apis* or *N. ceranae*) are found predominantly in the southern areas of the Tomsk region, which is characterized by more developed beekeeping and active delivery of breeds of southern origin (*A. m. caucasica* and *A. m. carpatica*) that leads to massive honeybee hybridization. Introgressive hybridization modifies the genetic pool of local honeybee populations, leading to the loss of their genetic identity [4]. The process of hybridization of different subspecies of honeybees can cause a destruction of evolutionarily developed gene complexes, leading to a decrease in the adaptive properties of organisms and populations and to a change in biological and economically significant characteristics of honeybees. The observed widespread hybridization of honeybees and the formation of hybrid bees will certainly contribute to the spread of disease.

In our research, the majority of bee colonies infected by *Nosema* were hybrids. This finding is consistent with the view that hybrid forms are poorly adapted to changing environmental conditions and less resistant to the disease. Therefore, our results on the *Nosema* infestation of bee colonies are not surprising. At the same time, it is impossible to make a conclusion about the pathogenicity of a parasite based on our data. Perhaps, hybrids are characterized by other developmental conditions of the parasite in comparison with pure breeds that do not realize the pathogenicity of *N. ceranae* in the host. Also, there is an open question about the distribution of a *Nosema* in the northern part of the Tomsk region (influence of a cold climate, insignificant number of hybrids, etc.) where the colonies infected with *Nosema* were not detected except Chainsky district (*N. apis*-infected bees were imported from Uzbekistan). Siberia can be an ideal location to study how the spread of this disease correlates with climatic conditions and how the disease moves to particularly remote areas. This is an especially intriguing thought since changes in disease prevalence and pathogen virulence because of climatic change are widely discussed [80]. Obviously, more research is needed to elucidate the full effect of *N. ceranae* infection in *A. mellifera* colonies in different geographical areas and to understand if individual virulence levels and colony virulence levels differ between the two parasites.

4. Conclusion

This study of honeybees in Siberia shows the need for a comprehensive approach to the study of various aspects of the honeybee, such as differentiation of subspecies, the role of environmental (geographical) factors in the formation of the genetic diversity of bees, and the incidence of bees.

The primary task of the study of the genetic diversity of honeybees is to determine their subspecies composition. When performing gene-geographical research, it is important to consider the assessment of adaptive and selective significance of genetic markers. This is also important for the planning and conducting of works having applied nature.

Along with exterior characters used for a long time to identify the breed of honeybees, molecular genetic techniques are actively applied. However, in connection with the high level of hybridization of bees, when about one-third of bee colonies show an imbalance between genetic and morphometric parameters, and in some cases, their complete mismatch occurs, a comprehensive analysis of the bees is necessary.

The presence of hybrid forms in an area where the genetic diversity is studied, on the one hand, creates unfavorable background for conservation of gene pools of unique subspecies (for example, dark-colored forest bee), on the other hand, makes it difficult to search for adaptively significant and economically valuable traits (possible distortion of results and their interpretation). Therefore, it should be taken into account in conducting such studies. The above data also indicate that only the exterior or just genetic traits may be insufficient to determine the origin of bees and only the simultaneous analysis of morphometric parameters and data on the variability of locus COI-COII of mtDNA allow to evaluate the breed and cases of hybridization objectively.

In the conditions of widespread crossbreeding of bees, genetic methods to control the purity of bee colonies must also be improved. Research in this direction is carried out by international and Russian researchers [43,47,90]. Therefore, on the basis of extensive research carried out on the territory of Eastern Europe (search of informative markers was conducted among more than 1,000 SNP using five different analytical methods), five panels, consisting of 48, 96, 144, 192, and 284 markers informative for determining the ancestral origin of species have been developed. The authors propose to use the results of this study to identify and evaluate the impurity of C-lines (in particular, *Apis m. ligustica* and *Apis m. carnica*) to the M-line (*Apis m. mellifera*) [43]. Russian researchers have only begun such studies, but the results obtained at this stage suggest that populations of honeybees living on the territory of Russia are characterized by wide genetic variability, and it is unlikely to develop a uniform panel of markers for the entire territory of the Russia for differentiation of the various breeds of bees. It is necessary to integrate the scientific achievements and results of the various laboratories and scientific groups of all over the world to establish general regularities of the genetic variability of the bees and to assess the adaptive and selective potential of honeybees.

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Chapter 2

Breeding Program Design Principles for Royal Jelly

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Additional information is available at the end of the chapter

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Abstract

This research was carried out to infer the genetic value to produce royal jelly in Africanized *Apis mellifera* L. honeybees with the compilation of data collected from 2006 to 2011. Genetic information of the selected and accessed colonies was obtained using the total DNA extraction techniques of nurse honeybees' thorax with molecular markers for MRJP3 protein and characterized in *Apis mellifera* L. From the information on the colonies and genealogical structure were predicted genetic values of the colonies and queens for the larvae acceptance trait (%), royal jelly per colony (g), and royal jelly per cup (mg). Animal model with Bayesian Inference was used from Multiple Trait Gibbs Sampling software in Animal Models, Gibbs chains 58,500 cycles resulting from 650,000 cycles with intervals and disposal of 65,000 and 10 withdraw, respectively. From the predicted values, the colonies were classified into upper and lower. To compare the average of the genetic values according to the genotypes, the average multiple comparison tests were proceeded and implemented in routine PROC GENMOD from the Statistical Analysis System. Environmental effects were considered, time and hive type (standard Langstroth) as having flat distribution and collection as chi-square distribution. The studies presented an increase in the alleles C and D and the alleles D and E—referring to MRJPs—found in the highest genetic value for royal jelly production. Alleles D, E, and C are important when evaluating the parameters larvae acceptance, royal jelly per colony, and royal jelly per cup and, occasionally, it was the DE genotype that stood out royal jelly production. Genotypes DE, DC, and EC are those that should be kept in this evaluation system for royal jelly production, and the other genotypes should be discarded because they had the worst performance for the parameters evaluated.

Keywords: *Apis mellifera* L., MRJP3, Bayesian inference, genotypes, genetic evaluation

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1. Introduction

Despite the growing number in honey export figures in recent years, beekeeping in developing countries goes through a period of technological stagnation; that is, the genetic quality of the honeybees just has not shown significant progress and also has not developed new effective management techniques with increased productivity. The great demand for high performance of honeybee colonies with desirable behavioral characteristics contributes to changing the natural biodiversity through mass importation of queens [1]. From this point, Almeida and Carvalho [2] reported that to enter the increasingly competitive market of bee products, it is important that beekeepers innovate in management and use of technologies, going to observe beekeeping by a business vision, and contribute to maintaining the genetic biodiversity of honeybees. In Brazil, beekeeping activity has not received major financial support from the federal government or the service companies have technical training and knowledge to pass on technologies, new or traditional, to beekeepers. However, with the devaluation of the country's currency, the real against the dollar in recent years, every day beekeeping has become more rewarding and competitive in relation to other agricultural activities; however, we note that there has been an increase in the number of managed hives, but there is no significant increase in the number of beekeepers.

One way to improve production is through genetic breeding. Animal breeding programs select the best individuals to be used as breeding to the next generation [3] and evolved over the past decades [4] because science and technology have come to assist in better identification of genetic information available. Breeding programs have calculations, scientific principles, biotechnology, and advances in computing and information technology, which together enable the almost total of process efficiency. The honeybee improvement is very important for beekeepers, but for this improvement, it is necessary join honeybee adaptation to the environment, be productive, and be economically sustainable for beekeepers [4]. However, Kinghorn et al. [5] reported that the adoption of applied techniques to the breeding depends on the balance between what is possible from a technological point of view and what is acceptable in socio-economic context of the production system.

The Africanization process led to significant changes in the rearing and production, which combined with the research, led advances in instrumental insemination, queen production, genetic breeding with determination of strains for the production of honey, royal jelly, and hygienic behavior. Thus, the need for greater professionalism among beekeepers spurred the search for information and the inclusion of genetic breeding programs.

Among several factors that provide improvements in production, we can highlight the role of animal breeding. Many are the work of initiatives in this area in our country, all focused on strategies that will generate genetic progress proven to beekeeping. For instance, the continuous production of selected queens allows the beekeeper to immediately substitute dead or old queens by young queens, with desirable genetic and phenotypic characteristics. Moreover, the perfect development and productivity of the colony depends mainly on the age and quality of its queen [6], once the progeny inherits half of characteristics from the mother queen. The

quality of the queens is affected by the genotype, nutrition, production methods, time of production, and the age of the larvae, among other factors [7].

The structuring of a breeding program involves the planning issues that are critical to its success [5], especially in honeybees [8]. Definition of the strategies is shown in **Figure 1**, and the training of personal for their development is a key factor to the success of the program [8].

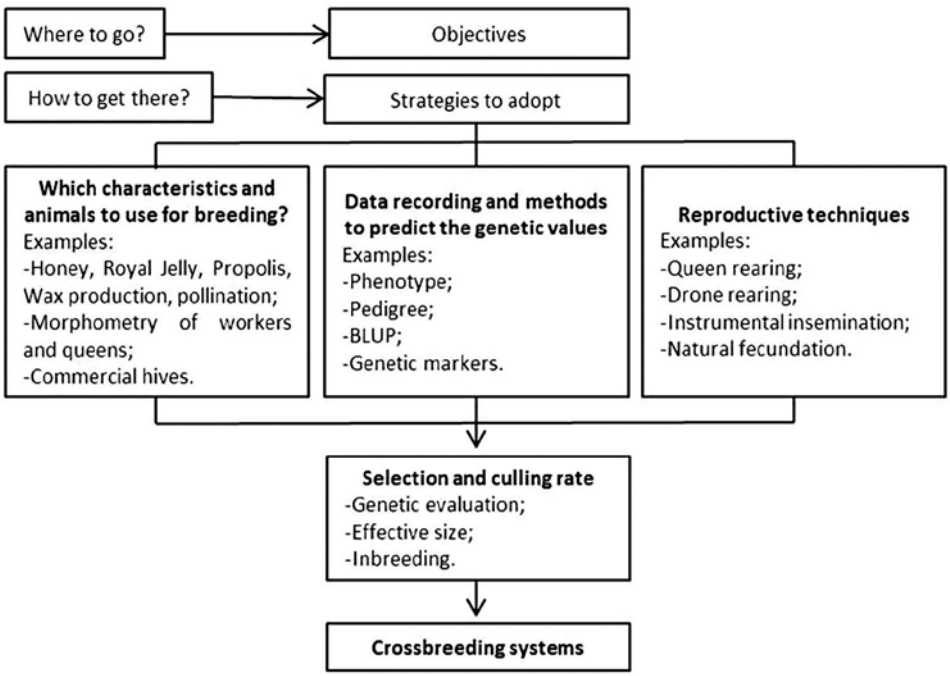

Figure 1. Strategies for honeybee breeding programs. Source: Ref. [8] adapted from Ref. [5].

The interest in establishing a breeding program with Africanized honeybees in the country is growing as well as the search for adequate and accurate methods for estimating genetic parameters for economic interest. Studies such as Costa-Maia et al. [9], Faquinello et al. [10], Wielewski et al. [11], Garcia et al. [12], and Padilla et al. [13] currently make up part of the framework of publications related to estimate genetic parameters of Africanized honeybees in Brazil, through the method of mixed models and Bayesian inference.

Faquinello et al. [10] estimated parameters of variance and covariance genetic, heritability, and genetic correlations for royal jelly production in *Apis mellifera* L. Africanized through Bayesian inference and concluded that royal jelly production is greatly influenced by external environment and possibly by internal environment by promoting low values of heritability to the analyzed characteristics. Moreover, these factors to be identified should be controlled by the beekeeper as much as possible; otherwise, the acquisition of queens with fitness for royal jelly production no longer will ensure maximum production. The authors [10] found values of positive genetic correlations between royal jelly production by colony and per cup of 0.29, for

larvae acceptance and royal jelly production by colony were 0.42, and low 0.06 for larvae acceptance and royal jelly production per cup. Selection based on the characteristic of royal jelly production per colony presented a positive trend of genetic gain for the larvae acceptance and royal jelly production per cup.

The research of genetic parameters in a given population to establish a selection process becomes a possibility for the characteristics of economic interest. Therefore, there is an increase in the frequency of desirable genes of the locus of economic importance. Therefore, the genetic progress is due to increasingly correct use of information on individuals applying for selection, resulting from the growing momentum in the methodological knowledge of genetic evaluation and the use of molecular genetics using molecular markers.

To further improve the quality of information, molecular genetics plays a key role because it locates and explores genes that have the greatest effect on the expression of quantitative traits. Lino-Lourenço and Costa-Maia [8] reported that the main livestock goal of genomic analysis in domestic animal species has been the dissection of the genetic architecture of the characteristics of economic interest, determining the number of genes and the contribution of each one for the expression of the phenotype and enabling the understanding that beyond the various genes of small effect, there are those few who print large effect.

Selection based on phenotype was complemented by information based on the genetic value, and then the influence of molecular genetics came to add and improve the quality of information in animal breeding programs. It is expected in future, an increase in gain per selection because the genetic merit of the animal can be obtained directly in its genome in a genetic evaluation program featuring all and any existing polymorphism. Therefore, studies on royal jelly production in the sphere of quantitative genetics associated with molecular genetics have great importance for the establishment of breeding programs of *Apis mellifera* L. This research line demands beyond the laboratory work, it requires knowledge of management production and the royal jelly quality.

Royal jelly is a heterogeneous substance secreted by cephalic glands (hypopharyngeal and mandibular glands by nurse honeybees of *Apis mellifera* L.), which feed the larvae in the first 3 days of life, while the queen is fed with royal jelly throughout all its existence. Royal jelly is yellowish product, viscous, and creamy consistency, having a characteristic odor and slightly spicy flavor [14]. Its secretion is promoted by pollen intake with added regurgitated solutions of the nursing workers, consisting primarily of sugars [15].

Fert [16] reported that royal jelly is a product that regenerates cells and tissues, normalizes blood pressure, and stimulates the production of red blood cells. Royal jelly has numerous functional properties and has been widely used in medicines, cosmetics, and healthy food in many countries [17]. In general, it consists of 60% water, proteins (41–42% dry matter), carbohydrates (30% dry matter), and small amounts of minerals, polyphenols, and vitamins [18]. Due to its composition, royal jelly is a key factor in the development of queen honeybees by the potential to increase in fertility, longevity, and body size [19].

For royal jelly production, especially during natural food shortage season, honeybees will need more protein provided by pollen, which can also be obtained from dairy products or brewer's

yeast. Royal jelly production is induced with artificial cells for queen rearing, containing newly hatched larvae, so the worker honeybees are encouraged to deposit royal jelly to feed the developing larvae [20].

Italian honeybees were introduced in China at the beginning of twentieth century [21], and royal jelly is one of the most important products to the Chinese beekeepers, producing between 200 and 3500 tons/year corresponding to 90% of total world production [22]. Initially, royal jelly production in China was only 0.2–0.3 kg/colony/year. Beekeepers of Zhejiang province began the process of selecting honeybees, and after 20 years, the production increased to 2.0–3.0 kg/colony/year. Since 1980, the Chinese government noticed the importance of this product to the country and started to invest in honeybee selection for royal jelly production in the same province and the other regions of the country, with production reaching from 6.0 to 8.0 kg/colony/year in the 2000s, and currently this value exceeds 10 kg/colony/year [23]. To reach such high production values, Chen et al. [24] developed a production system, which involves eight steps to obtain these high yields, including dietary supplementation, the adaptation or adequacy of equipment, manipulation abilities, and high number of cups per colony.

The main protein found in royal jelly—known as Major Royal Jelly Proteins (MRJPs) account for over 90% of total soluble protein composition [25]. The genes encoding key proteins of royal jelly began to be identified in studies made by Klaudiny et al. [26] and Albert et al. [27]. After these pioneering studies, several researches aim to characterize new genes encoding the MRJPs [25, 28, 29]. The locus Mrjp is part of an arrangement of nine genes encoded on an array of 65 kb. The MRJP family appears to have evolved from a single ancestral gene encoding a member of the yellow protein. Five genes encoding the proteins of yellow family are located in the genomic region containing the genes encoding MRJPs [30].

The five main representatives of this family are MRJP1, MRJP2, MRJP3, MRJP4, and MRJP5 [25, 31]. Although it has been proposed that the royal jelly has substances that induce differentiation of the queen, little is known about the function of its components, especially the protein portion [32]. The MRJPs were characterized in *Apis mellifera* L. [29, 30]. However, Baitala et al. [33] reported that data in the literature on the use of MRJPs as molecular markers in genetic structure of population studies and as selection markers associated with the improvement of royal jelly production are still scarce.

1.1. Factors influencing royal jelly production

Among the factors that affect royal jelly production are the internal factors of the colony, such as posture and acceptance of larvae, and external factors, such as nutrition, climate conditions, temperature, precipitation, humidity, and even genetic factors [10, 34]. Toledo and Mouro [35] observed the same environmental variables and reported that the maximum external temperature and relative humidity interfered positively, i.e. increased royal jelly production, while the rainfall had a negative influence.

Studying the effect of environmental variables in the royal jelly production, Toledo et al. [34] stated that precipitation did not affect the royal jelly production as well as the addition of protein supplement (35%). Minimum relative humidity and maximum temperature negatively

affected the number of accepted larvae and maximum relative humidity positively influenced the amount of accepted larvae [34]. Garcia and Nogueira-Couto [36] reported that there are differences in the larvae acceptance for the royal jelly production performed at different times during the year.

Royal jelly production is influenced by the number of collections per season, geographical location of the apiary, the experience of the beekeeper, and the genetic origin of honeybees [37]. Albarracín et al. [38] reported that larvae acceptance between genetic groups of Italian and Africanized origin and found no significant differences between them; the average production of royal jelly per colony was similar for both.

Over a month, the time collection 48 h after the larvae grafting presented higher income than the time collection 72 h after the grafting. van Toor [20] recommended a technique of two collections, the first being after 72 h after the larvae grafting and the second performed 48 h later; colonies producing 180 mg or less of royal jelly per cup should be replaced. However, Zheng et al. [21] recommended that the new standard assessments for royal jelly quality in China consider the collection time, because they found significant differences in the sample quality after 24, 48, and 72 h the larvae grafting. Sereia et al. [39] concluded that the average amount of royal jelly deposited every 68 h per cup was higher when supplemented with isolated soy protein, brewer's yeast, palm oil, and linseed oil. Some studies reported variation in royal jelly production of *Apis mellifera* L. Africanized honeybees from 188 to 234 mg/cup [10, 35, 40]. Toledo and Mouro [35] stated that the amount of royal jelly obtained per cup varies with the time that is left within the colony and obtained average of 253 mg/cup for Africanized and 198 mg/cup for Carniolian honeybees. Sereia et al. [39] observed differences for the percentage of total acceptance of grafted larvae with a mixture of linseed oil + palm oil and isolated soy protein + brewer's yeast that had, respectively, 63.45% and 63.75% of accepted cups when compared with palm, linseed, isolated soy protein, yeast, and controls I and II (45.80%, 49.71%, 50.32%, 50.95%, 49.60%, and 52.17%, respectively).

Sereia et al. (2010a) [41] studied the supplements with different nutrients in honeybee diet and found that by having a glandular origin, royal jelly production varies with the nutritional quality of the available sources and recommended protein supplementation to royal jelly production in honeybee colonies, still being economically viable the beekeeper [42]. Furthermore, the use of dietary supplementation on the royal jelly production is a matter that is part of the quality specifications of this product in France [37]. Sereia et al. [41] evaluated the nutritional quality supplements containing six different sources of oil and protein and concluded that the use of linseed and palm oils combined with brewer's yeast to prolong the longevity of Africanized honeybee workers, and the sunflower oil increases the royal jelly production [43]. Royal jelly production involves biological and behavioral interactions intrinsic to the honeybees, and its variability has important genetic and environmental components internal and external to the colonies [34, 36].

1.2. Chronology of selection for royal jelly production

As an agribusiness activity in development in Brazil [34], beekeeping has great potential for marketing products in addition to honey, such as royal jelly, for example, although it is not

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considered a product of the conventional beekeeping [42]. Besides these products, the Brazilian beekeepers are increasing the production of propolis mainly and pollen because their prices are rising too.

Currently, in Brazil, there is a poly-hybrid that emerged from the crossing of four subspecies of European (*Apis mellifera mellifera*; *A. m. caucasica*; *A. m. carnica*; *and A. m. ligustica*) with African honeybee *Apis mellifera scutellata* [44], who went through natural selection processes and adapted, resulting in what is called today of *Apis mellifera* L. Africanized, with great potential for selection by their genetic diversity [35]. Garcia and Nogueira-Couto [36] reported that African honeybees have adapted excellently in Brazil by the similarity of environmental conditions of the Brazilian territory with its homeland, the high adaptive capacity and to print this trait in their offspring crossings with other subspecies, ensured the expansion of Africanized honeybees throughout the Americas. Africanized honeybees present many differences from European honeybees, such as size and shape of the nest, life cycle (shorter for Africanized honeybees), colony growth and reproduction, production of males, swarming, defensiveness [45], and finally honey production, in tropical climate conditions.

Since 2006, at Maringa State University, studies intensified the evaluation system in Africanized honeybee colonies started in 1996, by joint operation of quantitative genetics with molecular genetics, aiming to provide grants to start a breeding program to select characteristics of economic interest, such as increased production of royal jelly. The utilization and production of technical evaluations in Beekeeping Division of the Experimental Farm of Iguatemi at the University, receiving support from the Laboratory of Genetics and Cellular Biology, started more effectively compiling data for the selection of *Apis mellifera* L. Africanized honeybees.

Mouro and Toledo [40] had higher royal jelly productions in hybrid Carniolan in relation to Africanized honeybee colonies. However, royal jelly production increased 109.19% in the first generation after performing the selection in Africanized honeybees. Therefore, it is essential to select the queens and recommended that the criteria to be adopted for the selection should be the production per colony for not occur losses in adaptive traits like disease resistance [40].

Africanized honeybees when selected, for royal jelly or honey production, were more effective in royal jelly production than Carniolian honeybees [35]. In addition, as an efficient measure to increase production, Toledo and Mouro [35] recommended selection of Africanized colonies because of the genetic diversity of wild swarms or natural colonies of these honeybees in Brazil, by presenting significant results and are applicable to the field reality. Toledo and Mouro [35] used Africanized honeybee colonies collected in nature for the royal jelly production, from August 1996 to March 1998. After initial results, the five most productive colonies were selected and compared with five Carniolan hybrid colonies with daughter queens from queens who came from Germany and observed that the Africanized honeybees produced more royal jelly when compared with Carniolan honeybees.

From August 2002 to February 2003, Toledo et al. [43] evaluated the royal jelly production in honeybees that received supplementation isoproteic (30%) and isolipid (5%) and concluded that honeybee food containing sunflower oil increased royal jelly production by colony at

28.79%. van Toor [20] reported that they should select the colonies with royal jelly production above average, considering the size of the colonies, high production of nurse honeybees, and favorable genetic predisposition.

Bayesian inference is a tool that contributes to efficient selection programs and has been used in the evaluation of animals to obtain more accurate estimates. The distribution of data allows the analysis of sets with varying sizes, providing accurate estimates of the variance components, breeding values, and credibility intervals [10, 46]. Interest in establishing breeding programs in Africanized honeybees in the country is increasing as well as the search for adequate and accurate methods for estimating the genetic parameters for economic interest.

Faquinello et al. [10] used Bayesian inference to estimate the variance components, covariance, and genetic parameters for royal jelly production in Africanized honeybees through the animal model. During the experimental period, these authors [10] evaluated several parameters of royal jelly production in colonies with daughter queen of matrix colonies. These parameters were larvae acceptance, royal jelly production per colony, and royal jelly production per cup in overlapped nucs (five frames each) and overlapped Langstroth hives (10 frames each). Selection based on genetic evaluation of queens contributed to the increase royal jelly production choosing parameters like larvae acceptance, royal jelly production by cup, and royal jelly production per colony [10]. Having a quality queen also means greater production of eggs which in turn will strengthen the colony optimizing production [47]. Sereia and Toledo [48] reported that there was genetic effect in the royal jelly production and concluded that descendants of other best royal jelly producing colonies presented higher number of accepted larvae and larger amount of royal jelly deposited by cup.

The number of nurse honeybees in the colony can directly influence the amount of royal jelly produced. Several surveys presented increased expression of *mrjp* levels or increased the amount of royal jelly protein in hypo pharyngeal glands of nurse honeybees, which is not developed in forager honeybees [49].

Baitala et al. [33] using microsatellite markers identified seven alleles mrjp3 in the Africanized honeybees producing royal jelly and also confirmed the increased frequency of alleles C, D, and E of Mrjp3 in selected colonies. Production results from the genetic evaluations indicated that the analyzed queens had similar royal jelly production, suggesting that there was no difference to the alleles under selection for this feature, but the genotypes chosen for the matrix colonies with queens were being held in daughter queens and drones. Parpinelli et al. [50] verified the genetic variability of locus Mrjp3, Mrjp5, and Mrjp8 from colonies *Apis mellifera* L. Africanized, selected for royal jelly production since 2006 to identify molecular markers associated with the royal jelly production, and observed the fixing of these alleles during the reproduction process selection. Three sites were polymorphic and produced a total of 16 alleles and have been identified four new alleles for the locus Mrjp5 [50]. The effective number of alleles for the locus Mrjp3 was 3.81. The average observed heterozygosity was 0.4905, indicating a high degree of genetic variability for the locus analyzed. High values of the inbreeding coefficient (Fis) for locus Mrjp3, Mrjp5, and Mrjp8 indicated excess of homozygotes, i.e. the

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selection of *Apis mellifera* Africanized queens for royal jelly production is keeping alleles mrjp3 C, D, and E, despite the C allele has occurred with low frequency. However, the genetic variability of the queens is decreasing for the analyzed locus, with excess of homozygotes, but the large number of drones to fertilize the queens hampers the production of homozygous genotypes for the locus Mrjp3 [50].

Researches conducted at Maringa State University to get the best yields in the royal jelly production are based on assessments of honeybees over several years in colonies fertilized naturally [10], quantitative and molecular analysis, with selection based on genetic markers for protein MRJP3 [33] that relate to the colonies that have the most significant quantitative production of royal jelly [41, 42, 50].

Researchers, such as Mouro and Toledo [40], Toledo and Mouro [35], Baitala et al. [33], Toledo et al. [34], Faquinello et al. [10], Toledo et al. [43], and Parpinelli et al. [50], with our research contribute to the continuity of this research line. The selection of honeybees must occurs using controlled crossings with instrumental insemination techniques to achieve the homozygous individuals to define the allele that contributes most to the largest increase in production. However, it is very important to include sometimes a different queen with different genetic for keeping the heterosis.

Based on the above, this research was carried out to predict the genetic value of Africanized *Apis mellifera* L. honeybee colonies producing royal jelly, based on genetic information through production characteristics (larvae acceptance and royal jelly production per colony and per cup) with compilation of data collected from 2006 to 2011.

2. Material and methods

The experiment was conducted at the Experimental Farm of Iguatemi at Maringa State University, Brazil, from January to April 2011, and the data that contributed to the Africanized honeybees assessment system for prediction of breeding values in the parameters evaluated for royal jelly production were collected from 2006 to 2011, concurrent with the genomic DNA extractions of the same selected colonies with molecular genetic markers.

2.1. Identification of genomic DNA

Genetic information of selected and evaluated colonies was obtained from the Laboratory of Genetics and Cellular Biology at the University by Baitala et al. [33] and Parpinelli et al. [50]. All these authors followed the method for extracting the total DNA from nurse honeybee thorax, described by Bardakci and Skibiński [51] and adapted to be used in *Apis mellifera* L.

Polymerase chain reaction (PCR) was performed using specific primers synthesized to amplify the repetitive regions of the locus Mrjp3 [28], amplification reactions being carried out in a Techne thermal cycler TC-512, and amplification conditions for primer MRJP3 based on the method described by Albert and Schmitz [52].

2.2. Queen rearing and royal jelly production

Queen rearing was carried out in specialized laboratory to develop such activity in air-conditioned environment for larvae grafting, with an average temperature of 33 ± 2°C and relative humidity of 60 ± 10%. Colonies were provided by beekeepers from different regions of States of Parana, São Paulo, Mato Grosso, Mato Grosso do Sul, and Sergipe, as well as Paraguay and Colombia. Whenever a colony or swarm died, it was replaced by another one. All colonies were identified, and queens were marked with numbered plates on the thorax, located in a five frame hive or in a Langstroth hive, depending on the colony size and the season of the year. From this, we started to rear the first generation of daughters from those queens. The method used for queen production was adapted from Doolittle [47]. The grafting was simple with larvae aged between 0 and 24 h and controlled genealogies. Periodically, all colonies were genotyped to know their genealogy.

The starting–finishing colonies were mini-hives [53]. For queen rearing, 10 queens from each selected colonies were produced in each generation. Each colony was settled from two overlapped nucs with a queen excluder between them. In this, mini-hive colony was a cup bar frame with 30 acrylic cups, 15 in the upper bar, and 15 in the lower with different genealogies, identified and randomly distributed (**Figure 2**). After 10 days, the queens' cells were removed from the starting–finishing colonies and were placed in incubators until the queens emerged in glass vial of 20 mL with a piece of paper, identifying the genealogy and hive number. Newly emerged queens were anesthetized with carbonic gas (CO_2), identified with a numbered label in the upper thorax, placed in a plastic cage, and brought stored until their introduction in the colonies, for royal jelly production.

Figure 2. Withdrawn of frame with queen cells for queen production.

After emergency, the queens were anesthetized with CO_2 for the measurement of body weight (mg) in a precision digital scale 0.001 g and length and abdomen width (mm) through digital precision caliper 0.01 mm–0.0005". Queens with body weight above 180 mg were allocated in JZsBZs™ type cages and kept in an incubator with nurse honeybees and introduced in five-frame hives to be mated naturally. This introduction occurred after at least 24 h after the supersedure to avoid risk of plunder and being a period of greater acceptance [47]. As the queens were inseminated naturally, the information regarding paternal genealogy was considered as unknown.

The beginning of oviposition was monitored to start royal jelly production evaluations after 50 days as this ensured that all the worker honeybees were daughters from the new queen. Terada et al. [54] verified that the average longevity of an Africanized worker is 26.3 days.

For royal jelly production, in each colony was introduced a frame containing three bars and 100 artificial cups in total. After 66–72 h frames were removed (**Figure 3**), larvae were discarded and royal jelly collected with suction device. Larvae grafting were scheduled, based on the schedule followed by Wielewski et al. [11].

Figure 3. Bar with cups showing the larvae acceptance and royal jelly produced.

2.3. Statistical analysis

Data of weight, length, and width of the abdomen in all generations of selection of the newly emerged queens were subjected to analysis of variance (ANOVA) using SAS software [55] and the averages in every generation for each evaluated trait were compared by Tukey's test at 5%.

After obtaining the data of body weight, length, and width of the abdomen of the newly emerged queens, proceeded to the genetic evaluation using the software *Multiple Trait Gibbs Sampling in Animal Models* (MTGSAM), developed by Van Tassel & Van Vleck [56], making the Bayesian estimation using the Gibbs sampling method.

The animal model used was in the following:

$$y = X\beta + Za + e$$

where y is the vector of observations; X is the incidence matrix of fixed effects, contained in the vector β; β is the vector of fixed effects; Z is an incidence matrix of additive genetic effects; a is the vector of additive genetic effects; and e is the vector of random errors associated to each observation.

where y, a, and e have normal multivariate joint distribution, as follows:

$$\begin{bmatrix} y \\ a \\ e \end{bmatrix} \sim NMV\left\{\begin{bmatrix} X\beta \\ 0 \\ 0 \end{bmatrix}; \begin{bmatrix} ZGZ'+R & ZG & R \\ GZ' & G & 0 \\ R & 0 & R \end{bmatrix}\right\}$$

In unicaracter analysis, G is the genetic variance and covariance matrix as $A\sigma_a^2$, A being relationship matrix, and σ_a^2 is the additive genetic variance; R is the residual variance matrix given by $I\sigma_e^2$, I being identity matrix, and σ_e^2 is the residual variance of the trait.

For bicaracter analysis, the G matrix is $G_0 \otimes A$, A being the relationship matrix, and G_0 is the additive genetic covariance matrix as follows:

$$G_0 = \begin{bmatrix} \sigma_{a_1}^2 & \sigma_{a_1 a_2} \\ \sigma_{a_2 a_1} & \sigma_{a_1}^2 \end{bmatrix}$$

The matrix R is given by $R_0 \otimes I$, I being identity matrix by equal order to drone number, and R_0 is the residual covariance matrix, as below:

$$R_0 = \begin{bmatrix} \sigma_{e_1}^2 & \sigma_{e_1 e_2} \\ \sigma_{e_2 e_1} & \sigma_{e_1}^2 \end{bmatrix}$$

In analysis strategy, it was used Gibbs chains of 58,500 cycles resulting from 650,000 cycles were generated, respectively, with initial disposal of 65,000 iterations and sampling intervals for every 10 iterations. The convergence of chains was tested by Heidelberger and Welch [57]

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test, implemented in Convergence Diagnosis and Output Analysis (CODA)—R software—Version 2.12.1. [58].

Colonies were classified into upper and lower, with the predicted values, considering the average genetic values of the parameter used as classification criteria. Each genotype was estimated probability rating in the higher and lower classes, from the PROC GENMOD routine from Statistical Analysis System [55] in which it was considered the data binomial distribution with logarithmic linkage. Classification probabilities for each genotype were tested by *T* test, using PROC GENMOD routine with 5% of significance.

Comparing the averages of genetic values in function of genotypes, proceeded averages multiple compilation test implemented in PROC GENMOD routine. To reduce interference of environmental effects in royal jelly production, the model used for prediction of breeding values, the environmental effects of year were considered—2006–2011, time—the four seasons of the year and type—colony model, as having flat distribution, and collection and distribution of chi square.

Bayesian inference was used by MTGSAM software as a tool to define the genealogy and predict breeding values for each colony in the traits, as larvae acceptance per grafting (%); royal jelly production per colony (g); and royal jelly production per cup (mg). The use of this type of analysis is appropriate to raise the accuracy of the dataset that follows this kind of beekeeping analysis protocol.

SAS software was used to determine the differences between production parameters in relation to the predicted values for the evaluated colonies. Considering that each colony is a superorganism [59], it was worked for analyzes concerned with different individuals—78 units of repetitions, a corresponding period to 6 years from 2006 to 2011. The genotypes identified that appear more frequently over the years of selection in the colonies submitted for royal jelly production were as follows: DE, DC, CE, EF, and FG [50].

3. Results

3.1. Performance rating

Table 1 represents the statistical differences found for the selected colonies evaluated by the production parameters, among the high and low classes of genetic value, considering the genotypes. It was observed that there were no statistical differences for the total larvae acceptance per grafting (%) among the categories of DE and EF genotypes, being FG the genotype ranked as the one that had the worst performance for this parameter with total chances of appearing below the average obtained. In this case, the DC genotype stands out for having 71% chance of being ranked above the average of the values found for acceptance. For total royal jelly production per colony (g), it repeated practically the same performance conditions between genotypes, and although there was no statistical difference, the highlight was DE genotype with 48% of chance to be classified as being high genetic value for this parameter. When performed royal jelly production per cup, DE genotype achieved the highest

rating, presenting 60% of probability to be above the average, significantly differentiating from EF genotype and not differing from the others. The EF genotype had the worst rating, there was 78% chance of this genotype be classified as low genetic value for this parameter.

Genotype	Class	Evaluated parameters		
		Total larvae acceptance per grafting (%)	Royal jelly per colony (g)	Royal jelly per cup (mg)
DE	High-genetic value	0.50a	0.48a	0.60a
	Low-genetic value	0.50	0.52	0.40
DC	High-genetic value	0.71a	0.43a	0.43ab
	Low-genetic value	0.29	0.57	0.57
CE	High-genetic value	0.50a	0.25a	0.25ab
	Low-genetic value	0.50	0.75	0.75
EF	High-genetic value	0.44a	0.33a	0.22b
	Low-genetic value	0.56	0.67	0.78
FG	High-genetic value	0b	0b	0.33ab
	Low-genetic value	1	1	0.67

Means followed by the same letters within the classes, in the same column, are not statistically different from each other by *T* test ($P > 0.05$).

Table 1. Probability of classification of high and low genetic value of different genotypes for total larvae acceptance per grafting, royal jelly production per colony, and royal jelly production per cup.

3.2. Prediction of genetic values

Averages of predicted genetic values for each genotype are presented in **Table 2**. To larvae acceptance, the genetic values of DE and DC genotypes were higher in relation to the EC, EF, and FG genotypes. There was no difference between genotype DE and all others. The DC genotype differed significantly from FG genotype, which presented the worst performance in this parameter. For total royal jelly production per colony, DE genotype was superior not only differentiating from DC and CE genotypes and did not differ significantly from EF and FG genotypes.

	Genetic values		
Genotype	**Total larvae acceptance per grafting (%)**	**Royal jelly per colony (g)**	**Royal jelly per cup (mg)**
DE	0.8043ab	0.4505a	0.0234a
DC	2.3118a	−0.0955ab	−0.007b
CE	−6.7991ab	−1.1013b	−0.0388b
EF	−4.2494ab	−0.612b	−0.0109ab
FG	−9.2538b	−1.2472b	−0.0283ab

Means followed by the same letter, in the same column, do not differ statistically from each other by T test ($P > 0.05$).

Table 2. Average genetic values for the characteristics evaluated in terms of different genotypes.

4. Discussion

For more accurate estimates, taking into account data distribution and the possibility of working with a small sample size, Bayesian inference is being increasingly used in honeybee husbandry trials because it produces accurate estimates of the variance components, genetic values [10], and credibility intervals, contributing to an efficient selection program [60]. However, studies using these methods have not been conducted for the royal jelly production in honeybees until not long ago [10, 11]. Metorima et al. [61] recommend this method for data without restriction of this nature, and it should be used as a tool in obtaining more accurate estimates for research in honeybees.

Genetic value is part of the genotypic value transmitted from parents to offspring. The prediction of genetic values helps evaluations between statistical analyzes on quantitative genetics with molecular genetics. Costa-Maia et al. [9] reported that accurate genetic parameter estimation allows prediction of the genetic value of the animal and, therefore, identification of genetically superior individuals.

All laboratory studies indicated that certain alleles are disappearing while others are settling, which can be observed by increase in the frequency of the relevant alleles in the royal jelly production. After DNA extraction from nurse honeybees, Baitala et al. [33] and Parpinelli et al. [50] noted an increase in the frequency of alleles C, D, and E on bees forming part of phenotypic and molecular genetic evaluation system for royal jelly production.

The reduction of heterozygosity contributes to the fact that the alleles C, D, and E have appeared more frequently and reducing the frequency for the F and G alleles. Observing the parameter royal jelly production per colony, we can see that the D allele has an important contribution, as were the DE and DC genotypes that stood out. During the time that genetic evaluations were conducted for the royal jelly production, alleles that most closely related to the increased production were maintained throughout the selection process, agreeing to Parpinelli et al. [50]. Parpinelli et al. [50] reported that the selection based on royal jelly production for MRJPs is leading to homozygosity of these loci, especially MRJP3, which presented the lowest value of observed heterozygosity.

To total larvae acceptance after the grafting, only the FG genotype was classified as inferior, while other were all high, highlighting the DC genotype with 71% chance of being so classified as superior. In a survey that compared the Doolittle and the "Starter" methods for royal jelly production, Baumgratz et al. [62] found larvae acceptance percentages of 63% for the first method and 50% for the second, values greater than 29.20%, observed by Toledo et al. [34]. Faquinello et al. [10] reported average larvae acceptance of 52.13%, which agrees with the performance of almost all genotypes in this study. Sereia et al. [39] observed differences for the grafted larvae acceptance with a mixture of linseed oil + palm oil and isolated soy protein + brewer's yeast that had, respectively, 63.45% and 63.75% when compared with palm, linseed, isolated soy protein, yeasts and controls I and II (45.80%, 49.71%, 50.32%, 50.95%, 49.60%, and 52.17%, respectively).

The same classification was repeated for the parameters royal jelly production per colony and royal jelly production per cup, whose average for Faquinello et al. [10] was 6.26 g and 190.07 mg, respectively. This was due to the fact the DE genotype has differed statistically only from EF genotype, with no difference between the other. Baumgratz et al. [62] observed productions per cup of 268 and 269 mg. Faquinello et al. [10] found positive correlations between royal jelly production per colony and royal jelly production per cup, total larvae acceptance rate after the grafting with royal jelly production per colony, and low correlation between total larvae acceptance after the grafting rate and royal jelly per cup. Muli et al. [63] when evaluating the royal jelly production potential between two subspecies of African *Apis mellifera* L., they found that the collection after 3 days had higher yields of royal jelly per cup—349.5 mg than gathering after 2 days—236.3 mg.

Royal jelly production is greatly influenced by the environment. The genetic correlation indicated that the selection increased royal jelly production per colony, the larvae acceptance, and royal jelly production per cup [10]. Li et al. [64] concluded that the hypo pharyngeal glands of nurse honeybees selected for royal jelly production were significantly higher than in non-selected honeybees. By microscope images, it could be seen that the royal jelly secretion period on selected honeybees was higher than in non-selected honeybees [65]. This highlights the importance of selection, genetic evaluation of individuals, and the starting of a breeding program of honeybees, mainly Africanized.

The average obtained for genetic values, DE genotype obtained positive values for the three parameters, followed by DC genotype also presented that the average value of larvae acceptance in a positive way, i.e. classified as superior. All other genotypes presented negative values for the average genetic value, and that means were rated lower. For total larvae acceptance after the grafting, there were only differences between DC and FG genotypes, being the DC genotype higher than the others. When total larvae acceptance is small can increase the royal jelly production per colony by increasing in the amount of royal jelly deposited per cup [66]. Genetic values were predicted royal jelly production and the use of genetic evaluation techniques presented that the alleles D and E—referring to MRJP—is the most genetic value to produce royal jelly.

The environmental influences and genetic differences in mating level hampering honeybee breeding [67]. Royal jelly production is a controllable genetic trait [68] and for its high

commercial value, there is a need for tools for the establishment of breeding programs [4, 33, 34, 40, 42]. Harbo and Rinderer [69] reported that the selection of superior genotypes with honeybees involves the use of improved queen replacement techniques, instrumental insemination, and assisted selection with molecular marker. However, in the literature, there is little data available for molecular markers associating to royal jelly production [4, 10, 11, 33, 34, 40, 42, 70]. The selection of more productive queens benefits everyone interested in beekeeping and in breeding control and selection [71], for this is necessary evaluate thousands of colonies in several apiaries, accurate record keeping, and if possible, a insemination laboratory [72].

Studies, such as Mouro and Toledo [40], Toledo and Mouro [35], Baitala et al. [33], Toledo et al. 34, Faquinello et al. [10], Toledo et al. [43], Parpinelli et al. [50], and this present research with predicted values are important and should be taken into account for the implementation of breeding programs. These data allow the continuation of this research line with selection based on the prediction of genetic values of the selected colonies. However, with controlled crosses using instrumental insemination techniques, it is possible to obtain homozygous for the allele that contributes to the largest increase to the royal jelly production. Moreover, the high allelic polymorphism for MRJP3 protein is an indicator that this biomolecular marker can be used in studies of the genetic structure of Africanized honeybees and so, after the selection process, establishes a breeding program [33, 70] that should be adopted by the government so that this technology would be widespread in the country and among beekeepers.

As a result of the analyzes, it is concluded that alleles D, E, and C are most important when the production parameters evaluated are larvae acceptance after the grafting and royal jelly production per colony and per cup. Thus, DE, DC, and EC genotypes should be kept in the evaluation system for royal jelly production, while the others should be discarded or replaced as it had the worst performance for these important parameters in production.

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Chapter 3

Improvement and Selection of Honeybees Assisted by Molecular Markers

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Additional information is available at the end of the chapter

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Abstract

Royal jelly is an important apiarian product for honeybees and has been used as an important ingredient to human health and healthy life style. Because of its wide use, there is great demand in their production. As royal jelly is a secretion of the cephalic glands of bees and it is produced at a certain age of the workers, it is necessary to perform the selection of producing queens to increase the amount produced. The employment of molecular markers is a tool that can be used to identify the genotypes of the best producers. Among the molecular markers, one of them called MRJP3 (Major Royal Jelly Protein 3) has been used in the Program of Improvement of *Apis mellifera* Royal Jelly Producing (PIAMRJP), State University of Maringá, Brazil. This molecular marker has been efficient in genotyping queens' royal jelly producers. Combined with classical breeding studies, the selection of queens assisted by MRJP3 marker has allowed to keep the selected genotypes for royal jelly production since 2006 (10 years). In this chapter, we present the main aspects of royal jelly, the hypopharyngeal glands, the major proteins of royal jelly and how it can be used as molecular markers.

Keywords: *Apis mellifera*, MRJP3, microsatellite, royal jelly, honeybee queen

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1. Introduction

Genetic improvement in any organism has the objective of increasing the gene frequencies of the economic importance of loci to be selected in the population. In relation to bees, this means increasing the frequency of the number of colonies that produce above the average generation from which the selection was made.

The production of royal jelly and honey production are the result of the combined work of the workers [1], and therefore the entire colony becomes a unit of selection, where the assessment of improved queens is carried out by production workers' progeny [1,2]. Royal jelly production studies allowed to observe considerable variation in its production by Africanized honeybees [3,4]. These results show the need for selection of queens [5].

Selection of bees with genotypes involves the use of improved queens' replacement techniques and instrumental insemination and for molecular marker-assisted selection. There are few data in the literature linking molecular markers for the production of royal jelly. The identification and characterization of several loci of the major royal jelly proteins (MRJPs) allowed using one of the loci *Mrjp3* as a molecular marker for selection of *Apis mellifera* queens Africanized. Early studies by selecting queens and genotyping the best producers began in 2006, in the apiary of the State University of Maringá, Brazil. The first study associating the MRJP3 marker with royal jelly production was realized by [6].

High variability in major royal jelly proteins (MRJPs), especially MRJP3 to contain microsatellite regions, indicated a great potential of using these proteins, particularly microsatellite regions occurring in the *Mrjp3* gene as a marker for selecting studies for the improvement of the production of royal jelly. Subsequently, other researches were conducted using classic improvement parameters such as MRJP3 marker. The results to date have shown that this marker is important to genotype producing arrays of royal jelly.

Thus, this chapter shows the importance of royal jelly to honey bees and to human health, the importance of improving assisted by molecular markers and the results obtained with the selection of royal jelly producing queens and genotyped for MRJP3.

2. Royal jelly

Royal jelly is secreted by the mandibular and hypopharyngeal glands located at the head of honeybees [7]. Hypopharyngeal gland secretion has a clear, water-like consistency and is rich in protein, while the mandibular gland produces a white secretion with milky consistency [7, 8]. Royal jelly can be described as a viscous substance, white-yellowish or grayish white, slightly opalescent with a characteristic pungent odor, although not unpleasant or rancid (**Figure 1**) [9–10]. These glands have the highest growth rate and activity of worker nurses between days 10 and 14 [11–15]. The development of the glands can be influenced by internal factors of the colony such as offspring and population density and external factors such as foraging and enabling bees to adapt quickly to the colony [13,16–18].

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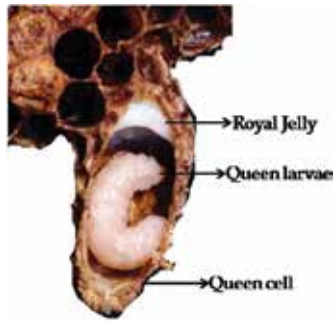

Figure 1 Queen cell with queen larvae of *A. mellifera* and royal jelly.

Royal jelly is a glandular secretion recognized for complex composition, containing minerals, proteins, amino acids, steroids, phenols, carbohydrates, vitamins, lipids, acetylcholine and other unknown substances [9,19]; it is important too in reproduction and development. Royal jelly is the larval food until the third day of development when it becomes the exclusive food of the queen throughout her life, guaranteeing fertility and increased longevity. From third day, worker larvae are fed a mixture of honey, pollen and water, known as brood food; drones receive food brood and royal jelly [20].

The average lifespan of queens of *A. mellifera* live is 1 to 2 years [21]; they become sexually mature 6 days after emergence, mate about 17 drones and store all of the sperm needed to fertilize eggs for the duration of their lifespan [22]. Few drones rear in the summer, but a slight rise in drone rearing occurs during swarming [20]. Queens can lay 1500–2000 eggs per day throughout their lives [23,24], depending on the needs of the hive and environmental factors, while a large number of workers (sterile females) are responsible for maintaining the hive.

Due to the fertility and longevity of queens, related to the exclusive feeding with royal jelly, studies have been conducted considering similar effects in humans. Some beneficial effects have been attributed to consumption of royal jelly, as elimination of physical and mental fatigue, appetite normalization activation of brain function, improved vision, increased resistance against viral infections and skin rejuvenation [10].

Owing to considerable amount of proteins, free amino acids, lipids, vitamins, sugars and bioactive substances such as 10-hydroxy-trans-2-decenoic acid and antibacterial protein 350 KDa proteins, royal jelly becomes an ingredient for various healthy foods [25]. Review carried out by [25] shows several studies have reported that the royal jelly exhibits beneficial physiological and pharmacological effects in mammals, including vasodilative and hypotensive activities, antihypercholesterolemic activity and antitumor activity.

3. Molecular marker Major Royal Jelly Protein 3

Royal jelly contains from 12 to 15% crude protein consisting of soluble proteins in water and water-insoluble proteins. The fraction of soluble proteins of royal jelly produced by the hypopharyngeal and mandibular glands contains several major proteins with molecular

weight between 47 and 80 kDa [26] besides a small amount of minor proteins such as antibiotics and peptides [27,28]. Those proteins constitute the main group of major royal jelly proteins. The MRJPs represent between 82 and 90% of the total proteins of larval jelly [19]. Some regions of MRJPs can be focused on amino acids rich in nitrogen, thus high levels of nitrogen would be stored in MRJPs. The availability of nitrogen can be critical to the rapid growth of young larvae, as well as for the development of the queen [29]. These observations support the hypothesis that MRJPs have an important role in the nutrition of bees [19].

Major royal jelly protein-3 can be visualized on denaturing SDS-PAGE electrophoresis in head extracts of worker nurses (10–14 days old) or royal jelly (**Figure 2**). The polymorphism was estimated by [30].

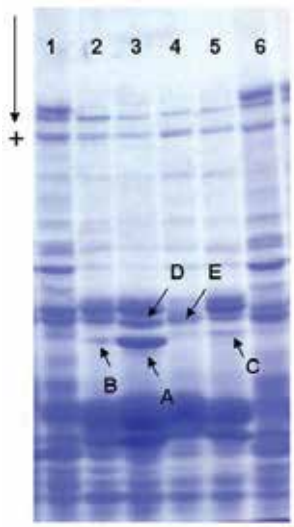

Figure 2 Denaturating SDS-PAGE electrophoresis showing MRJP3 polymorphism in extracts of head of *A. mellifera* nurse. A, B, C, D, E = alleles. Source: Baitala et al. (2013).

MRJPs genes encoding a group of proteins that have a common evolutionary origin with Yellow proteins of *Drosophila melanogaster* [31]. Genome of *Drosophila* encodes at least seven family members of Yellow proteins [32], whose loci are involved in the larval pigmentation [33], unlike the MRJPs that have nutritional function of larvae.

The genes encoding key proteins of royal jelly began to be identified in studies [34] and [35]. After these pioneering studies, several studies have been published in order to identify and characterize new genes encoding the MRJPs proteins [19,31,36,37]. The availability of the complete genome of *Apis mellifera* [38] made it possible to identify new genes encoding proteins of the family MRJP [29].

Since the first study were identified nine proteins in MRJPs *A. mellifera* (MRJP1, MRJP2, MRJP3, MRJP4, MRJP5, MRJP6, MRJP7, MRJP8, and MRJP9) besides an incomplete polypeptide, MRJPψ, encoded by a pseudogene. The genes encoding these proteins are located on chromo-

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some 11 [29]. Classification of *A. mellifera* MRJPs has been performed based on the N-terminal sequences of purified protein and cDNA sequences available in the cDNA library.

Analysis using PCR and DNA sequencing showed that the different alleles of the gene encoding MRJP3 protein differ in length as a result of a varying number of repeating basic units in a region of the *Mrjp3* gene [31,36]. The authors attributed the polymorphism of these proteins is a consequence of the presence of a region with varied number of repetitive sequences in tandem (microsatellite). These markers are comprised of a variable number of identical sequences having from 15 to 100 base pairs, in tandem and repeated up to 50 times. The molecular differences in four types of MRJP3 have shown that the polymorphism of these proteins is linked to the size variability, which is determined genetically by bees from the same colony [19].

The *Mrjp3* is a polymorphic locus that has been identified by DNA sequencing five alleles and PCR analysis identified at least 10 alleles of different sizes [36]. This study also revealed a Mendelian inheritance and high variability of the genomic locus of MRJP3.

Although *A. mellifera* [19,29,31,35–37] and other bees of the genus *Apis* [39–43] having the MRJPs are characterized, data in the literature on the use of MRJPs as molecular markers for selection associated with the improvement of royal jelly production are still scarce.

4. *Apis mellifera* queens' selection using MRJP3 marker

The genetic improvement has the aim to increase the frequencies of desirable genes of the loci of economic importance to be selected in a population [44]. Thus, the genetic breeding of bees has the goal to increase the frequency of the number of colonies that produce above the average generation from which the selection was made. Selection of honeybees with superior genotypes involves the use of improved queens replacement techniques, instrumental insemination and molecular marker-assisted selection [45].

Selection of queens is carried out by genetic evaluation, which depends on the estimation of the components of (co)variance and genetic parameters for identification of genetically higher bees. Royal jelly production evaluated by Bayesian inference had a heritability estimate of 0.27% acceptance, 0.10 for the production of royal jelly per colony and 0.55 per dome [5]. The analyses performed by these authors showed that selection of queens can increase the production of royal jelly by colony, larval acceptance and production of royal jelly by the dome, and the external factors can modify the gene expression of individuals.

However, there are few data in the literature associating molecular markers for the production of royal jelly. One of the first molecular studies carried out to obtain DNA markers related to production of royal jelly was performed by [46]. These authors reviewed a total of 96 alleles produced for 10 microsatellite loci and according to the observed allele frequency for some alleles, it was possible to identify seven alleles that can be used as markers bees producing large quantities of royal jelly. The use of molecular markers, particularly microsatellites, can

contribute to detect polymorphisms that might be useful to identify colonies of bees with high productivity of royal jelly.

High variability of MRJPs proteins and especially the MRJP3 to contain microsatellite region shows a great potential to use MRJP family proteins as markers for selection of producing queens for improving the production of royal jelly. Use of MRJPs as molecular markers in studies of population genetics and as selection markers associated with the improvement of royal jelly production is still scarce. Some researches have shown that this molecular marker is efficient to be used in the selection of royal jelly-producing queens.

Africanized honeybees selected for royal jelly production showed high allelic variability for the locus *Mrjp3* (**Figure 3**), showing the potential of this marker for selection [6]. In this research analyses of multiple linear regressions with EPD (expected progeny differences) values for royal jelly production were performed. The variance analyses indicated that the *Mrjp3* repetitive region influenced the genetic value of queen's offspring for royal jelly production. The determination coefficient (R2) for the significant alleles of the repetitive region of *Mrjp3* indicated that 36.85% of the EPD variation is explained by the variation of *C*, *D* and *E* alleles. Authors concluded that the three alleles present a considerable genetic effect on the variation of royal jelly production.

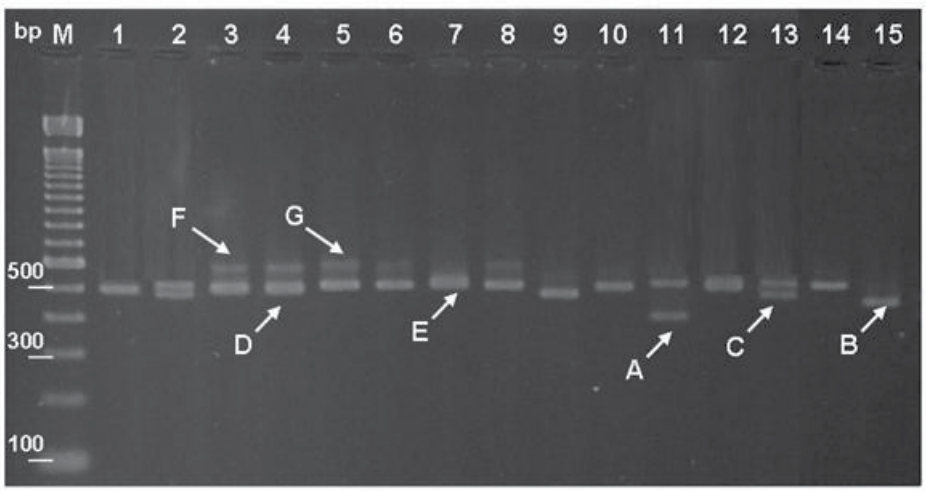

Figure 3 Molecular marker MRJP3. Number = *A. mellifera* DNA. A, B, C, D, E, F, G = *Mrjp3* alleles. M = molecular weight marker. Source: Baitala et al (2010).

Continuing the process of selection and the Program of Improvement of *Apis mellifera* Royal Jelly Producing (PIAMRJP), State University of Maringá, Brazil, alleles of the locus *Mrjp3* descendants queens, those selected by [6], were evaluated in 2011 [47]. Results showed that the royal jelly-producing queens had a high degree of genetic diversity and excess homozygous alleles. The highest frequencies were estimated for *Mrjp3 D* and *E* alleles 0.3357 and 0.3107,

respectively, showing that the selection process of queens royal jelly producing these alleles are being maintained and only the *C* allele had a low frequency of 0.0321.

Results obtained by [47] confirm those obtained by [6], the locus *Mrjp3* and their alleles *C*, *D* and *E* influence the genetic value for producing royal jelly; however, the real role of MRJP3 these bees has not yet been identified. The sequencing of *Mrjp3* of *A. mellifera* Africanized alleles in PIAMRJP was performed [48]. Homology and identity of these sequences were compared with the sequences deposited in the database for *A. mellifera* (**Figure 4**). Alleles *Mrjp3* detected showed high identities with alleles deposited in BLAST system. Alleles *Mrjp3 C, D* and *E* are being maintained in the genome of the selected matrices queens.

High similarity among the *Mrjp*3 alleles analyzed and those described in other studies show that the *Mrjp3* locus is conserved among species and subspecies of *Apis*. Similar results were obtained by [40]. These authors found that there are high similarity sequences and intron-exon have the same structure between four species *A. mellifera, A. cerana, A. dorsata* and *A. florea.*

The selection of royal jelly-producing queens may be promoting a selection of these reproduction bees, can alter the genetic characteristics of a given population, can be influenced by the process of transmission of these genes generation to generation [49]. However, it is important to maintain a degree of genetic variation, which results in a larger potential response to selective improvement [50].

In addition to the continuous genotyping of royal jelly–producing queens to locus *Mrjp3*, we developed a study to see if the mitochondrial DNA (mtDNA) of Africanized bees *A. mellifera* maintained in the breeding program have African or European origin. This research was performed by [51], using matrices producing royal jelly.

Mitochondrial DNA was analyzed using the molecular marker PCR-RFLP with specific primers and restriction enzymes to European and African honey bees. Analyses were performed with workers' daughters of royal jelly-producing queens in 2013, seven years after the beginning of the PIAMRJP started in 2006. After this period of selection and analysis of genetic parameters, alleles *C, D and E* are being maintained in queens, evidencing the role in royal jelly production. Queens selected for royal jelly production showed predominance of African mtDNA; therefore, genes of maternal origin are African. Use of microsatellite markers and mtDNA can be used in bee improvement programs to ensure the genetic origin of queens and verify the efficiency of Program of Improvement of *Apis mellifera* Royal Jelly Producing [51].

The employment of molecular markers in selection programs and improvement of honey bees for royal jelly production is efficient because it allows keeping genotypes of interest to ensure the highest productivity of the hives. The microsatellite marker MRJP3 has shown good results as a tool to verify the genotypes of producing matrices, facilitating identification and maintenance of the hives in the apiary of the Program of Improvement of *Apis mellifera* Royal Jelly Producing.

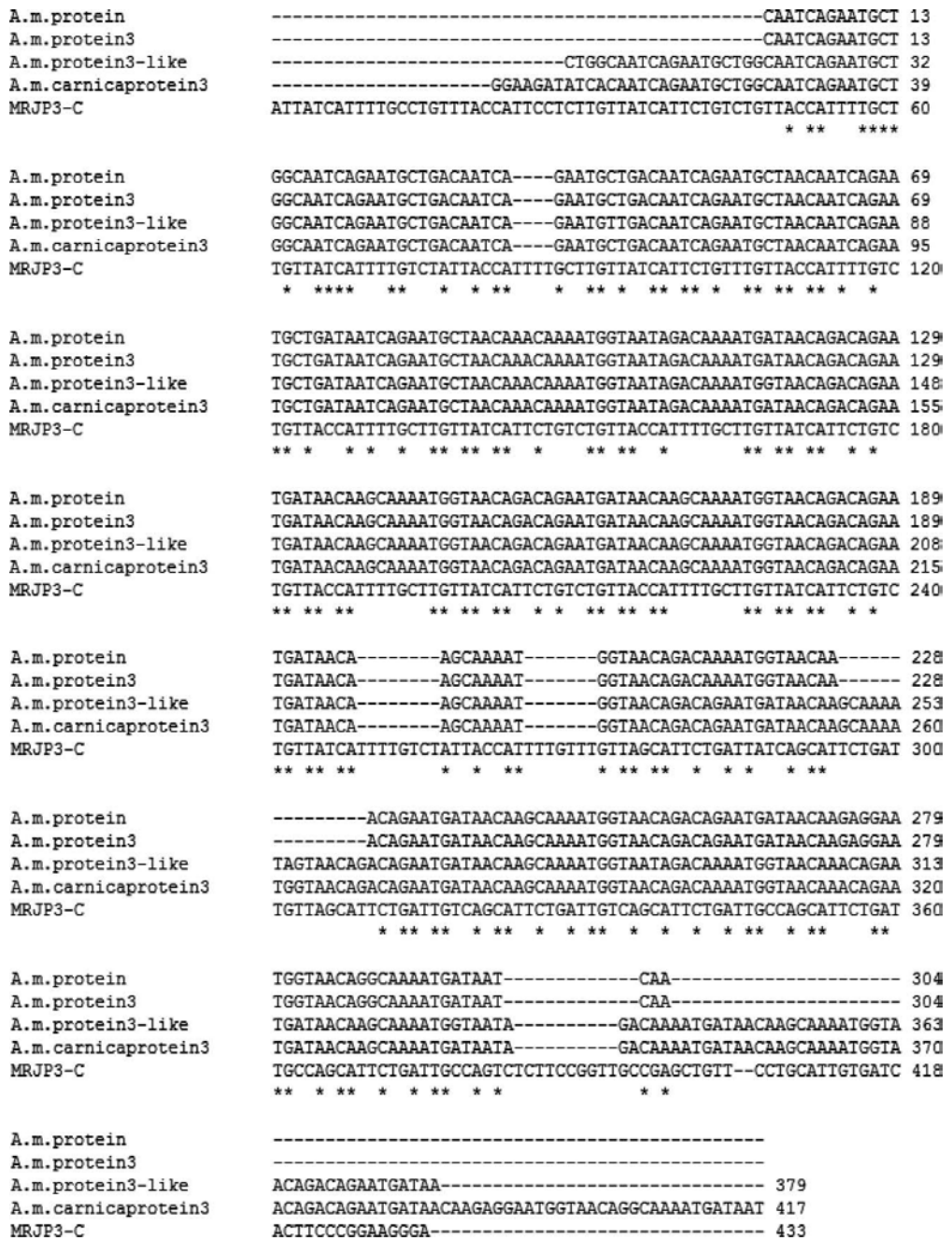

Figure 4 Alignment of sequences similar to the *Mrjp3 C* allele performed using ClustalW2 (EMBL-EBI); sequences include *A. mellifera* major royal jelly protein mRNA, complete cds (GU434675.1); *A. mellifera* major royal jelly protein 3 (*Mrjp3*), mRNA (NM_001011601.1); *A. mellifera carnica* major royal jelly protein 3 *(Mrjp3)* gene, complete cds (AY663104.1); and PREDICTED: *A. mellifera* major royal jelly protein 3-like (LOC727045), partial mRNA **(**XM_001122757.2). "*" = nucleotides identical in all of the aligned sequences. Source: Casagrande-Pozza (2011).

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Chapter 4

Impacts of Pesticides on Honey Bees

Francisco Sanchez-Bayo and Koichi Goka

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/62487

Abstract

This chapter focuses on the detrimental effects that pesticides have on managed honey bee colonies and their productivity. We examine first the routes of exposure of bees to agrochemicals used for crop protection and their application to crops, fate and contamination of water and plants around the fields. Most of the time, the exposure of bees to pesticides is through ingestion of residues found in the pollen and nectar of plants and in water. Honey bees are also exposed to pesticides used for the treatment of *Varroa* and other parasites. The basic concepts about the toxicity of the different kinds of pesticides are explained next. Various degrees of toxicity are found among agrochemicals, and emphasis is given to the classic tenet of toxicology, "the dose makes the poison," and its modern version "the dose and the time of exposure makes the poison." These two factors, dose and time, help us understand the severity of the impacts that pesticides may have on bees and their risk, which are analysed in the third section. Sublethal effects are also considered. The final section is devoted to some practical advice for avoiding adverse impacts of pesticides in beekeeping.

Keywords: residues, toxicity, exposure, sublethal effects, risk management

1. Introduction

For centuries, beekeepers have been aware of the environmental conditions that help prosper their honey bee colonies: a diversity of flowers from trees, shrubs, the so-called weeds and even crop plants. A healthy, diverse floral environment has always been the recipe for a healthy, bumper honey production. Perhaps the only problems they faced were the occasional infection by microorganisms, diseases and parasites that could kill the bees and their colonies [1] or the unpredictable vagaries of weather that could affect flower production on particular bad years.

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In the past few decades, however, beekeepers have had to cope with a new threat to their business: agrochemical pesticides, which are scattered over large areas of crops, fruit groves, forests and other environments for the control of insect pests, weeds, vermin and plant diseases. There was no doubt, from the beginning, that chemical insecticides could represent a serious threat to bees for the simple reason that bees are insects and, therefore, susceptible to any poison designed to kill insect pests. Consequently, strict toxicity testing was and still is required before such chemicals can be registered for use in crop protection [2, 3], at least in developed countries. Despite these regulations, the number of managed honey bee (*Apis mellifera* L.) colonies in the United States declined from 6 million in 1947, when DDT was introduced in agriculture, to less than 3 million in 2010 [4]. Similar trends have been observed in Europe, where the number of apiaries declined 14% in Scandinavia and 25% in central Europe between 1985 and 2005, although they increased 13% in the Mediterranean countries in order to counteract the lower production in the north [5].

But, what about other pesticides, such as herbicides and fungicides? Could they also affect honey bee productivity? If the target of such chemicals is not the insects, many argued, they are probably safe to the bees. Research conducted in the past few years in countries with a long history of pesticide usage suggests differently. It is now acknowledged that the extensive and prolonged used of herbicides leads to a reduced diversity of flowering plants [6, 7] that inevitably affect the bees' colonies [8] and their productivity. Moreover, the combination of some fungicides with insecticides has been revealed more deadly to the bees than either chemical alone [9]. Lately, the indiscriminate use of acaricides in apiaries for the control of parasites, such as *Varroa destructor*, has added one more threat for the beekeepers, as these chemicals are also toxic—although to a lesser degree—to the honey bees. Not surprisingly, the colony collapse disorder (CCD) has been linked by some authors not only to parasites and diseases but also to pesticide usage [10].

In these circumstances, a new management approach is needed for successful beekeeping. Production of honey and wax is no longer dependent on the availability of flowers in the surrounding environment, but rather appears to be intimately linked to the quality of food that the bees collect. It is now clear that pesticide-contaminated flowers affect the health of the honey bee colonies to the extent that their productivity declines [11]. In order to better manage this situation, we must first understand how bees are exposed to pesticides and what are the consequences of such exposure for the health of the individual bees, the colony and their overall productivity.

2. Exposure of bees to agrochemicals

Most insecticides are applied as sprays over the crop canopy, but sprays of herbicides and fungicides are usually applied directly on the soil before the planting of crops. In all these cases, droplets and dust from the applications can fall directly on the bees that fly across the treated fields or nearby because wind can carry the tiny droplets and dust particles hundreds of metres away from the crop [12]. A single droplet of insecticide may be sufficient to kill a bee

because the spray solutions contain concentrated doses of these chemicals—this is the most common cause behind the bee incidents reported in the literature [13, 14]. Granular pesticides that are incorporated into soil (e.g., herbicides) have no direct exposure to bees.

The so-called systemic insecticides are usually applied as seed coatings. The treated seeds are introduced into the soil using pneumatic drilling planters, and the friction of the seeds in the machinery produces dust particles that are heavily loaded with the insecticides. These poisonous particles can also cause a great deal of mortality among bees, if they happen to be in the surroundings [15]. Systemic insecticides applied this way are taken up by the crop plants as they grow and their residues are present in all parts of the treated plant, including the flowers, pollen and nectar [16]. Not only the crop plants but also the weeds and bushes that grow in the vicinity are affected [17, 18] because they also take up small amounts of residues that spread through the soil through lateral water flow [19] or are contaminated through dust/spray drift. In addition, some plants can produce guttation drops in the early hours of the morning (e.g. maize, strawberries), and systemic insecticides appear in such drops in elevated concentrations [20] that are capable of killing the bees.

Most of the time, the exposure of bees to pesticides is through ingestion of residues found in the pollen and nectar of contaminated plants, whether from the crop plants or from the weeds around the fields [21]. It is important to realise that bees forage everywhere they can and search for the most suitable flowers that produce pollen and nectar in abundance. Thus, some crops are more attractive than others; for example, the yellow flowers of canola (rape seed oil), sunflowers and many weeds that grow in and around the crops are more attractive to bees than the flowers of potato plants. Pesticide residues in pollen and nectar are taken by the forager bees to their colonies and remain in the beebread and honey for quite some time [22, 23]. These residues are then fed to the larvae and the queen, which are affected in similar ways as the forager bees.

In addition to food, bees also drink water to keep their body temperature under control [24]. Pesticide residues in soil eventually move into the water and appear in the streams, creeks and ponds of agricultural areas and beyond, which are thus contaminated with a mixture of agrochemicals [25]. Some water contamination is also due to drift from spray applications, particularly from insecticides [26, 27]. Honey bees, bumblebees and wild bees like to drink from puddles, irrigation ditches, ponds and streams, and if these waters are contaminated with pesticide residues, the forager bees ingest them as well [28].

Apart from the pesticides used in agricultural production, honey bees are also exposed to the acaricides used for the control of *Varroa* and other parasites. In this case, bees come in contact with the high residue levels present on the waxy cells of the comb [29], affecting mainly the developing larvae [30] and presumably the adult honey bees and the queen.

Given the enormous variety of agrochemicals used in crop production, it is not surprising that, to date, residues of 173 different compounds have been found in apiaries [21]. It should be realised that through the various routes of exposure to pesticides in the environment (**Figure 1**), bees are not threaten by one or two chemicals alone but by cocktails of many agricultural compounds.

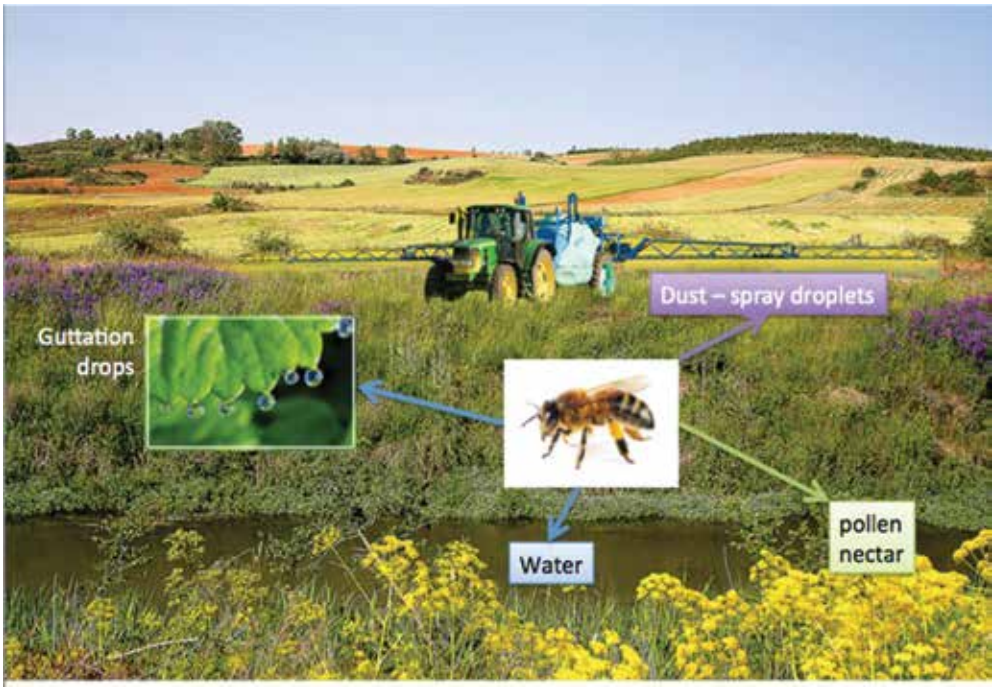

Figure 1. Routes of exposure of bees to agricultural pesticides.

3. Toxicology of pesticides

Pesticides are toxic chemicals with specific mode of action, meaning they are designed to specifically control a target group of organisms by interfering with particular metabolic pathways. Thus, insecticides and acaricides kill insects and mites by disrupting their neuronal activity, their moulting process or other specific metabolism of these arthropods; herbicides and algicides kill plants and algae by disrupting their photosynthetic capacities or the synthesis of essential organic compounds and fungicides kill fungi by inhibiting the formation of their cell membranes or another metabolism specific of these organisms. There are other kinds as well, like rodenticides that kill small mammals, bird repellents, etc. The term biocide is reserved for broad-spectrum poisons that kill any organism, mainly microbes, but also large animals.

The toxicity of each kind of pesticide, however, is not exclusive to the target group of organisms: other species that share similar metabolism are affected as well, although usually to a lesser degree. The potency of a pesticide to any species is defined by the dose of toxic chemical that is lethal to 50% of individuals of that species (LD50), and such dose varies from species to species. Doses lower than the LD50 are considered 'sublethal', but they can also cause mortality on a certain proportion of the species population, i.e., 20 or 30% of individuals may die. In general, sublethal doses cause toxic effects that do not kill the organisms but still affect their

normal functioning and health. For example, exposure of bees to sublethal doses of neurotoxic insecticides may cause stress [31], paralysis or abnormal behaviours without killing the bees [32].

By their very nature, insecticides are the most toxic compounds to bees, whereas herbicides are largely innocuous (**Table 1**). Beekeepers should be wary of any insecticide application in the vicinity of their hives because spray drift could certainly inflict a heavy toll on the bees. Pesticide applicators are aware of this danger and, in many countries, are required to inform beekeepers before they apply insecticides to a crop [33]. Also, while acaricides are less toxic to bees than to the target parasites, excessive amounts of their residues in the combs may have unpleasant consequences for the health of the bees [34].

Pesticide type	Chemical name	Contact* LD50(μg/bee)	Oral* LD50 (μg/bee)	Half-life† (days)
Acaricides	Acrinathrin	0.17	0.12	22
	Amitraz	50	-	1
	Coumaphos	20	4.6	-
	Fenpyroximate	11	-	49
	Tau-fluvalinate	8.7	45	4
	Tetradifon	1250	-	112
Fungicides	Azoxystrobin	200	25	78
	Boscalid	200	166	118
	Captan	215	91	4
	Carbendazim	50	-	22
	Chlorothalonil	135	63	44
	Myclobutanil	40	34	35
	Propiconazole	50	77	214
	Quintozene	71	-	210
	Tebuconazole	200	83	47
Herbicides	Metolachlor	-	110	90
	Norflurazon	1485	-	225
	Simazine	879	-	90
Insecticides	Beta-cyfluthrin	0.031	0.050	13
	Bifenthrin	0.015	0.20	87
	Carbofuran	0.16	-	14
	Chlorpyrifos	0.072	0.24	50
	Clothianidin	0.039	0.004	121
	Cypermethrin	0.034	0.064	69

Pesticide type	Chemical name	Contact* LD50(μg/bee)	Oral* LD50 (μg/bee)	Half-life† (days)
	DDT	8.8	5.1	6200
	Diazinon	0.38	0.21	18
	Endosulfan	6.4	21	86
	Fenthion	0.22	-	22
	Fipronil	0.007	0.001	142
	Imidacloprid	0.061	0.013	174
	Malathion	0.47	9.2	1
	Mevinphos	0.094	-	1
	Pyrethrum	0.18	0.057	-

*Source: ECOTOX and AGRITOX databases, compiled in Sánchez-Bayo and Goka [21].
†Source: Footprint database (IUPAC). http://sitem.herts.ac.uk/aeru/iupac/

Table 1. Toxicity of common pesticides to bees (LD50 at 48 hours) by contact or oral exposure and their persistence in soil (half-life)

All animals, including bees, are endowed with detoxification mechanisms that transform and eliminate most toxic chemicals. Currently, the majority of organic pesticides are degradable either in the organisms themselves or in the environment. The exception is the organochlorine pesticides (e.g. insecticides like DDT and lindane), which are very persistent and recalcitrant. Because they were applied in large quantities in the past decades, their residues are still present —although at low levels—in the soils of many countries, even if nowadays are banned from use in agriculture. Due to their low solubility in water, organochlorine residues are not taken up by the plants growing in contaminated soils, and so they do not appear in the pollen or nectar of the flowers.

The persistence of pesticides is evaluated by their half-life ($t_{1/2}$), which is defined as the time required for half the amount of a chemical to disappear from a medium, that is, water, soil, air or biological tissues. Half-lives longer than 90 days indicate that the pesticide may accumulate, since more than 5% of the amount applied will remain in the environment after 1 year [35]. Residues of persistent pesticides found in pollen or nectar (Table 1) will, therefore, remain in the beebread throughout the entire season of honey production.

Systemic insecticides, such as neonicotinoids (e.g. imidacloprid) and fipronil, are more toxic and persistent than the majority of organophosphorus (e.g. malathion), carbamates (e.g. carbofuran) and pyrethroids (e.g. cypermethrin) (Table 1). Given their high solubility in water, their residues also appear in water bodies of agricultural areas and the rivers they drain into [36, 37]. As they are applied consistently as seed dressings, their residues may remain in the soil for years and are taken up by the crop and weeds, ending up in the nectar and pollen of all plants in the treated landscape [16]. This poses a risk to bees, not only because of their high toxicity and availability but also due to their particular mode of action. For example, neonicotinoids show delayed toxicity at low doses, so apart from various sublethal effects

they cause [38], they end up killing the bees if they are exposed to the residues for a long period [39]. Both neonicotinoids and fipronil also produce immune suppression on honey bees [40, 41] and, consequently, they predispose bees to *Nosema* infections [42] and outbreaks of viral diseases that are commonly transmitted by *Varroa* mites [43, 44]. As a result, colonies feeding on honey and pollen contaminated with these neurotoxic insecticides may succumb to the combined effects of chemicals and diseases [45].

The toxicity of certain insecticides can be enhanced in the presence of ergosterol-inhibiting fungicides (e.g. propiconazole, myclobutanil), which act as synergists. Indeed, this type of compounds inhibits the detoxification system in bees [46, 47], so the insecticide and acaricide residues are not metabolised or eliminated as fast as they should. Furthermore, the toxicity of insecticides and acaricides used for *Varroa* control is often additive or synergistic [9]. Since the food that forager bees collect is usually contaminated with a mixture of both insecticides and fungicides, and because most managed apiaries are treated with acaricides, the combined toxicity and synergism of all these chemicals pose a real threat to the health and survival of honey bee colonies and all other species of wild bees.

Sublethal exposure to pesticides, including fungicides and some herbicides, often produce stress in animals, because the organisms try to metabolise and get rid of the toxic chemicals quickly using large amounts of energy. Apart from stress, bees experience other negative effects when exposed to sublethal doses of pesticides. For example, under conditions of chronic exposure, honey bee larvae fed on pollen contaminated with chlorpyrifos produced very few queens [48]. Wild bees (*Osmia bicornis*) exposed to sublethal levels of thiamethoxam and clothianidin had their reproductive success reduced by 50% [49], while honey bee queens experienced unusually high rates (60%) of supersedure [50]; bumble bees (*Bombus terrestris*) colonies exposed to sublethal levels of thiamethoxam failed to perform and produced 85% less queens than normal [51]. Sublethal doses of neonicotinoid insecticides also cause disorientation and memory loss in forager bees [38], contributing to less efficiency in the collection of pollen by bumble bees [52]. Sublethal doses of the acaricide coumaphos also produce abnormal mobility in the exposed honey bees [53]. Undoubtedly, all these effects disturb the performance of the individual bees and that of the colony [54].

Finally, the indirect effects caused by herbicides cannot be ignored. Herbicides are not toxic to bees, but they disturb the environment in which bees and other pollinators live. Plant biodiversity, and its associated arthropod communities, have certainly decreased in areas that have been treated with herbicides for many years [55, 56]. The lack of certain plant species, mainly weeds, implies an impoverishment of the natural environment that sustains pollinators, including honey bees. Consequently, bees find more difficult to collect the variety of pollen that is required for a healthy bee diet [57]. Poor bee nutrition due to scarcity of flowers is the indirect result of continuous herbicide applications in crops and forestry areas over many decades.

4. Risk of pesticides to bees

Having explained above the routes of exposure to pesticides and their various impacts on bees, an evaluation of the actual risks that current pest control products and acaricides used for treating hives pose to honey bees is needed. The main risk derives from the acute toxicity of the chemicals to the bees, which produce their mortality in the short or middle term. Other risks include sublethal effects that may harm the performance of hives and the long-term viability of the colonies, as mentioned above.

Risks are typically estimated as probabilities of harm and are based on the acute toxicity and the frequency with which a chemical may affect the bees. Three scenarios can be considered: (1) risks from spraying of pesticides over agricultural fields; (2) risks posed by ingestion of agrochemical residues found in pollen, honey and water, which are collected and ingested by the forager bees and transported to the hive, where they are processed into honey and beebread and fed to the other bees, the larvae and the queen; and (3) risks from exposure to combs treated with acaricide products.

4.1. Risk from exposure to sprays

For the first scenario, the only data required are the concentrations of the active ingredients in the spray solutions applied and their acute toxicity, i.e., LD50 values for each chemical, since the probability of a bee being sprayed on can be considered 100% if the bee flies directly through the spray cloud in the field, or if a hive is placed downwind and within the normal range of spray drift by aerial or ground-rig applications, i.e., less than 1 km. This kind of risk is estimated using the typical hazard quotient HQ

$$HQ = \frac{Exposure(\mu g)}{LD50(\mu g / bee)} \tag{1}$$

where the exposure term can be determined by the concentration of active ingredient in the spray droplets and the volume received by the bees, according to the following expression

$$HQ = \frac{Concentration(\mu g / ml) \times vol.droplets(ml)}{LD50(\mu g / bee)} \tag{2}$$

In this case, the HQ can be indicative of high risk when its value is 1 or more, since 50% or more bees exposed would die; moderate risk is when HQ values are between 0.1 and 1 and low risk when it is less than 0.1, as fewer than 10% (similar to a natural mortality rate) of bees would be threaten.

Estimates of risks are typically done by considering the spray drift [58, 59] and the exposure to the flying bees [60]. For example, to compare the risk posed by different products under the same conditions, the spray drift volume can be fixed, e.g. 500 droplets for a bee crossing the spray cloud in a few minutes, at 5×10^{-4} μl for a standard droplet would result in 0.25 μl/

bee. **Table 2** shows a comparison of the risk that commonly applied pesticides would have in such situations.

Pesticide type	Chemical name	Droplet concentration (μg/ml)	LD50 (μg/bee)	HQ	Risk evaluation
Acaricide	Amitraz	200	50.0	0.001	Low
	Dicofol	240	19.0	0.003	Low
	Propargite	600	62.1	0.002	Low
Fungicide	Azoxystrobin	75	200.0	<0.001	Negligible
	Fludioxonil	12.5	50.3	<0.001	Negligible
	Mancozeb	750	226.2	<0.001	Negligible
	Tolclofos-methyl	500	100.0	0.001	Low
Insecticide	Abamectin	18	0.03	0.15	Moderate
	Acetamiprid	225	7.9	0.007	Low
	Beta-cyfluthrin	25	0.031	0.20	Moderate
	Bifenthrin	100	0.015	1.70	High
	Carbaryl	500	0.84	0.15	Moderate
	Chlorantraniliprole	350	4.0	0.022	Low
	Chlorpyrifos	300	0.072	1.04	High
	Difenthiuron	500	1.5	0.083	Low
	Dimethoate	400	0.12	0.85	Moderate
	Endosulfan	350	6.35	0.014	Low
	Esfenvalerate	50	0.026	0.48	Moderate
	Fipronil	200	0.007	6.8	High
	Imidacloprid (spray)	200	0.061	0.81	Moderate
	Imidacloprid (dust)	24*	0.061	0.1	Moderate
	Indoxacarb	150	0.58	0.064	Low
	Lambda-cyhalothrin	250	0.048	1.3	High
	Methidathion	400	0.27	0.37	Moderate
	Methomyl	225	0.50	0.11	Moderate
	Pririmicarb	500	35.7	0.004	Low
	Spirotetramat	240	242	<0.001	Negligible
	Thiamethoxam (spray)	250	0.025	2.5	High
	Thiamethoxam (dust)	36.8*	0.025	0.37	Moderate

*Data for dust particles from conventional pneumatic planters [61, 62].

Table 2. Risk of common agricultural pesticides to honey bees that fly across a spray cloud (ppm) and receive a total dose of 0.25 μl/bee

The example in Table 2 reveals that the insecticides fipronil, thiamethoxam, bifenthrin, lambda-cyhalothrin and chlorpyrifos are the most dangerous to bees when sprayed to agricultural crops. The microencapsulated formulation of lambda-cyhalothrin is particularly hazardous because bees can carry the microcapsules containing the concentrated chemical to the hive. In general, dust particles of neonicotinoid-treated seeds and spray droplets of pyrethroids, organophosphorus and carbamate insecticides pose moderate or high risks, whereas other insecticides and acaricides present low risks in comparison. The fungicides shown here, and possibly most others applied as foliar sprays, pose low or negligible risks to bees by direct contact with spray droplets. This evaluation is in agreement with the reported incidents of pesticides on bees in the United Kingdom [63] and Canada [64]. Obviously, the most toxic insecticides are the most dangerous to bees.

4.2. Risks by oral exposure

For the second scenario, ingestion of contaminated food, data on the concentration and frequency of residues in each media are essential, as well as information on the dietary intake of pollen, honey and water by each caste of bees, that is, foragers, nurses, larvae and queen. Oral exposure to contaminated food is considered the typical exposure of bees in the hive. The risk expression in this case would take the form

$$Risk = \frac{Frequency(\%) \times residue\,dose(\mu g)}{LD50(\mu g / bee)} \tag{3}$$

where the residue dose of a given pesticide can be estimated for different bees as the product of the concentration of residues in pollen, honey or water by the total intake of a particular caste of bee [21]. In turn, total intakes are estimated from daily intakes and the life span of bees, which vary from 5–6.5 days for larvae, 8–10 days for brood attendants and nurses, to 30 or more days for foragers [65]. The food intake by queens is hard to estimate, as they are fed royal jelly (a particular combination of pollen and honey), can live several years and vary their intake —which is unknown—throughout the reproductive and winter seasons. For the toxicity, oral LD50s are used in this case. The risk estimated by expression (3) can be interpreted as the probability that a given pesticide residue has of causing 50% mortality among the bees that ingest the contaminated pollen or nectar.

In recent years, a number of studies have reported the residue levels of agricultural pesticides found in pollen [66, 67] and nectar of flowers [68, 69], in water bodies of agricultural areas [28], as well as in beehive matrices, such as beebread, honey and wax [70, 71]. Based on these reports, we estimated the average and maximum residues for each pesticide as well as their frequency of appearance in those matrices. This information allowed us to calculate the risks that bees encounter when feeding on such contaminated food or drinking sources. A summary of results for the compounds that pose the highest risks by oral exposure of combined food and drink is shown in **Table 3**.

Chemical name	Residues (μg/kg)			Larvae		Nurses		Foragers	
	Pollen	Honey	Water*	Risk (%)	T50 (days)	Risk (%)	T50 (days)	Risk (%)	T50 (days)
Thiamethoxam	28.9	6.4	4.1	2.77	23	4.80	27	276	7
Gamma-HCH (lindane)	7.6	176.5	-	0.62	9	0.01	979	200	3
Clothianidin	9.4	1.9	2.6	1.02	54	1.91	58	39.5	13
Imidacloprid (total)	19.7	6.0	0.9	1.19	68	1.57	103	25.4	25
Cypermethrin	13.9	18.1	-	0.13	119	0.04	711	4.00	44
Coumaphos (total)	128.3	105.5	-	0.11	1444	0.06	5524	2.62	545
Dinotefuran	45.3	13.7	-	0.10	49	0.13	74	1.50	20
Quinalphos	-	9.6	-	<0.01	253	-	-	1.29	91
Methiocarb	1.4	15.0	-	<0.01	1080	<0.01	>5000	1.08	391
Chlorpyrifos	32.6	3.9	-	0.04	1605	0.13	1118	0.86	764
Carbaryl	58.9	23.4	-	0.41	202	0.42	392	0.54	80
Beta-cyfluthrin	2.2	9.0	-	0.10	190	0.01	3497	0.43	69
Dimethoate	2.3	4.8	-	0.01	1198	<0.01	>5000	0.40	440
DDT (total)	31.2	44.2	-	<0.01	3871	<0.01	>5000	0.29	1432
Pirimiphos ethyl	-	19.0	-	<0.01	401	-	-	0.21	144
Diazinon	8.5	17.0	-	0.04	426	0.01	3869	0.19	156
Malathion	17.1	98.0	-	<0.01	3218	<0.01	>5000	0.15	1167
Pirimicarb	-	38.0	-	<0.01	3500	-	-	0.10	1261
Phosmet	339.3	-	-	0.07	991	0.79	168	-	-
Fipronil (total)	1.6	-	-	0.02	596	0.27	101	-	-
Acrinathrin (total)	146.8	-	-	0.01	719	0.17	122	-	-

*From the survey by Samson-Robert et al. [28].

Table 3. Average pesticide residue levels in food and water (ppb) and their risk by oral exposure to worker honey bees and larvae. The time to reach the oral lethal dose (T50, days) is also shown for a comparison

Despite the high risk of some chemicals, namely neonicotinoids, most insecticide residues in pollen and honey present a moderate risk to bees (1 to 5%), especially those of pyrethroid and organophosphorus compounds. Overall, 21 of the 113 pesticide residues in food for which toxicity data are available pose some kind of risk to honey bees, but only 8% of the chemicals are of concern. Residues in water are more variable from place to place: the data shown in

Table 3 are from one survey in Canada where only neonicotinoids, fungicides and herbicides were found—the risks posed by the latter two groups were negligible nonetheless, so they are not shown in the table.

4.3. Risks by contact exposure

Apart from oral exposure, bee larvae may also be in contact with residues deposited on the walls of the comb cells, in particular, the acaricides used for controlling *Varroa*. Although the highest loads of pesticide residues in a hive are found in the wax [23], the availability of such chemicals is thought to be minimal except for the fumigated acaricides. The risk of the latter products to bee larvae should be estimated not as oral intake, as some authors do [30], but rather as contact exposure. The expression (3) can be used, with the maximum residue dose in this case estimated as 5 mg of active compound per cell for a single larva and the contact LD50 instead of the oral one. The results of the risk analysis for a number of acaricides to honey bee larvae are shown in **Table 4**.

Pesticide	Residues in wax (μg/kg)	Risk by contact (%)	T50 (days)
Acrinathrin (total)	139.0	0.03	247
Amitraz (total)	585.5	<0.01	>5000
Bromopropylate	16.4	<0.01	>5000
Carbofuran (total)	19.4	<0.01	1649
Chlorfenvinphos	1156	0.14	709
Coumaphos (total)	1352	0.02	3003
Dicofol	6.8	<0.01	>5000
Pyridaben	5.4	<0.01	1957
Spirodiclofen	28.5	<0.01	>5000
Tau-fluvalinate	3144	0.15	551
Tau-fluvalinate+amitraz	3730	21.25	11
Tau-fluvalinate+coumaphos	4496	122.6	11
Tetradifon	7.9	<0.01	>5000

Table 4. Average acaricide residue levels in comb wax (ppb) and their risk to larvae of honey bees. The time to reach the lethal dose (T50, days) is also shown for a comparison

As it can be seen, the risks of acaricides to bee larvae are below 1% for all individual chemicals, but increases dramatically for synergistic mixtures, such as tau-fluvalinate with amitraz or coumaphos. Except for the latter mixtures, the overall risk to bee larvae of the individual products is very low or negligible compared to that of the same compounds by oral ingestion of contaminated food and water (Table 3).

4.4. Novel approaches to risk assessment

Another way of estimating risks, particularly for oral exposures, is by calculating the time that would take for a bee to reach the LD50 of a given pesticide, based on the daily intake of contaminated food and water. This estimate is made using the expression

$$T50(days) = \frac{LD50(\mu g / bee)}{Daily\,\text{intake}(\mu g)} \tag{4}$$

where T50 is the time to reach the median lethal dose (LD50), also termed median time to death. As it can be expected, there is a good correlation between the T50 values estimated using equation (4) and the risk values calculated using equation (3)—see Tables 3 and 4.

Neonicotinoid insecticides, however, can cause delayed mortality due to their agonistic mode of action [39]. This particularity means that their acute oral LD50s, which are usually estimated for exposures of 48 hours, are insufficient to estimate accurate risks of these insecticides, because the actual dose that causes the death of the bees decreases as the time of exposure increases [72]. Consequently, the mathematical function that relates the median time to death (T50) with the median lethal dose (LD50) is used to estimate the risk, as follows

$$Ln\,T50(days) = a + b\,Ln\,LD50(\mu g / bee) \tag{5}$$

where *a* (intercept) and *b* (slope) are empirical parameters specific to each chemical and species tested (in this case honey bees). The approach estimates the cumulative mortality with exposure time with more precision than the standard equation (4), as explained in a previous study [21].

4.5. Risk from synergistic mixtures of pesticides

The above tables help determine the pesticides that pose the greatest danger to bees, whether by exposure to spray droplets or dust, oral ingestion of contaminated food and water or contact with chemicals used for mite control in the hives. It is clear that the majority (92%) of pesticides registered for agricultural production do not pose significant or measurable risks to honey bees, but this is only when considering the exposure to individual compounds.

Recent developments, however, indicate that combination of certain chemicals, in particular insecticides and acaricides with fungicides or mixtures of acaricides, is more toxic to bees than the individual compounds on their own. The additive and synergistic effects of those mixtures have already been mentioned above, and estimation of the risks they pose needs to be calculated using the same approaches but modifying the toxicity of the insecticide or acaricide by a synergistic factor [21]. These factors are calculated experimentally for several combinations of fungicides with insecticides and/or acaricides [73], and some examples are shown in **Table 5**.

Insecticide or acaricide	Fungicide	Synergistic factor	Risk to larvae (%)		Risk to nurses (%)	Risk to foragers (%)
			Wax	Food	Food	Food
Acetamiprid	Propiconazole	104.7	<0.01	<0.01	<0.01	<0.01
Acetamiprid	Fenbuconazole	4.5	<0.01	<0.01	<0.01	<0.01
Coumaphos	Fenpyroximate	20.0	<0.01	0.77	<0.01	<0.01
Cyhalothrin	Propiconazole	16.2	2.16	<0.01	<0.01	<0.01
Cyhalothrin	Myclobutanil	10.9	<0.01	<0.01	<0.01	<0.01
Cyhalothrin	Penconazole	4.4	<0.01	<0.01	<0.01	<0.01
Tau-fluvalinate	Myclobutanil	50.0	<0.01	0.01	<0.01	<0.01
Thiacloprid	Propiconazole	559.4	0.89	0.08	0.30	<0.01

Table 5. The synergistic effect of some fungicides with insecticides or acaricides and their risks to honey bees

Although the increases in risk are obvious, only the interaction of the pyrethroid insecticide cyhalothrin with propiconazole points to a moderate concern for bee larvae; even the risk of thiacloprid appears to be low under these circumstances. However, the risk of certain acaricide mixtures, such as tau-fluvalinate with amitraz or coumaphos, used in *Varroa* treatments, can be very high for the larvae (see Table 4).

5. Management in order to avoid pesticide impacts

The various risks estimated above give us some clues about the type of exposure most dangerous to the different castes of bees in the hives. Spray drift is the main cause of incidents involving mortality of forager worker bees [63, 74], whereas ingestion of contaminated pollen, nectar and water is at the root of the CCD malady that affects many apiaries of the world [45], affecting mainly the nurse workers and the queen in particular [49, 51]. In addition, the acaricides used in *Varroa* treatment pose a significant risk mainly to the bee larvae, and consequently to the long-term sustainability of the colonies. Awareness of these threats can help beekeepers and farmers draw specific management plans to avoid pesticide impacts.

Beekeepers should be aware of the landscape environment on which their managed bees forage, bearing in mind that a large proportion of the land in developed and developing countries is used for agricultural production where pesticides of all kinds are used on a regular basis. Since usage of these plant protection products cannot be stopped, as they are necessary for agricultural production, a rational approach must look at minimising the risks of such agrochemicals to bees.

Chemical companies are obliged by law to state on the labels whether their products are dangerous to bees or not. If so, they must specify the risks they pose and the specific actions to take, such as "DO NOT spray any plants in flower while they [the bees] are foraging."

However, label warnings are ineffective unless there is proper communication among the applicators, farmers and beekeepers. It is the responsibility of the former to ensure that beekeepers are informed of any spraying operations, so that hives are moved to a safe location during the spraying season. Moving hives usually takes more than 24 hours, so farmers must notify their neighbouring beekeepers with sufficient time in advance. Only thus damage by drift to the hives can be avoided.

Bees are generally active between sunrise and an hour or two before sunset, and most honey bees forage within a 2–4 km radius of their hive, although may travel as far as 7 km or more in search of pollen and nectar when their local sources are scarce [75]. Therefore, pesticide risk to bees can be reduced by spraying the crops in the evening, when bees are not foraging.

Despite all precautions, if an area in which the crop or weeds were in flower has been sprayed inadvertently, the farmer should notify the affected beekeepers in order for them to take appropriate action. This should ensure the managed bees are kept out of that sprayed area for a while. As well as the cropping areas, damage may occur when pesticides drift over the neighbouring vegetation that is foraged by bees, including hedges, road-side weeds and trees, such as fruit trees, eucalypts, etc. For example, coolibah trees (*Eucalyptus microtheca*) grow on plains along many river courses in the cotton growing areas of Australia and are a primary source of nectar and pollen for wild and honey bees. The Australian cotton industry has produced a best management practices manual in which, among other recommendations, indicates to the cotton growers how to deal with this issue and be aware of the possible damage to beekeepers. "With good communication and good will," says the manual, "it is possible for apiarists and cotton growers to work together to minimize risks to bees, as both the honey industry and cotton industry are important to regional development." [33].

In summary, awareness of the problems that pesticides have for bees should prompt appropriate actions by all parties involved in order to minimise the chemical impacts on bees and the productivity of the apiarist industry. Such actions must aim, first of all at managing the use of agrochemicals in ways that do not harm other producers of the land. In addition, farmers should minimize the contamination of the surrounding landscapes, including water bodies, with pesticides, because not only honey bees but a large array of pollinator species (e.g. butterflies, bumblebees, hoverflies, etc.) may also be affected.

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Chapter 5

Advances in Pharmacological Activities and Chemical Composition of Propolis Produced in Americas

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Additional information is available at the end of the chapter

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Abstract

Propolis is a resinous material produced by bees from the selective collection of plant exudates that are subsequently mixed with beeswax and salivary bee secretions. Propolis has been used in folk medicine, and certainly, several studies have validated its biological properties. The chemical composition and pharmacological activities of propolis collected through North (including Central America and Caribbean) and South America have been studied in the last years, and several papers have reported differences and similarities among the analysed geographical samples. Propolis has been classified according to its aspect and plant source; however, the ecological diversity present along the Americas provides a plethora of botanical resins. Herein, we summarize and discuss most of the studies performed at present on this profitable product for apiculture, attempting to compare the bioactivity, phytochemical diversity and botanical sources of honeybee propolis produced in Americas.

Keywords: Propolis from Americas, biological properties, chemical constitution, *Apis mellifera*, botanical sources

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1. Introduction

Bees are the most ecologically important pollinators for flowering plants, a coevolutive activity they have been performing for more than 100 millions of years. In particular, eusocial bees have reached an evolutionary success by living in perennial colonies (approximately 50,000 individuals) and developing sophisticated recruitment communication mechanisms to foraging and profit the chemistry of plants via manufacture and application beehive products for their own benefit [1–5]. According to their significant role as vectors of pollen in agricultural crops and the impact of beehive products for human societies, bees have earned an important position in different civilizations through history and geographies. Indeed, the bee management practice has been described since ancient times, including two types of beekeeping: apiculture (Apinae) and meliponiculture (Meliponinae). The first includes the Asian honeybees (*Apis cerana*) and western European honeybees (*Apis mellifera*), while the second refers to the native tropical and subtropical stingless bees (*Melipona* sp., *Oxytrigona* sp., *Scaptotrigona* sp., *Tetragonisca* sp. and *Trigona* sp., among others). At present, the beekeeping practice with western European honeybee is geographically widespread as a consequence of human migrations; thus, *A. mellifera* have settled down in all the lands that men have done, reaching a wide phytogeographical distribution range, including almost every vegetated place on earth [2, 3, 6–10].

Inside the hive, the cooperative behaviour of eusocial bees is reflected by the contribution of each individual into colony maintenance, resulting in a suitable community health termed social immunity, which is characterized by hygienic practices, accompanied with the removal of diseased brood to avoid the persistence of pathogens and parasites inside the hive. However, one of the expensive consequences of social living is the disease transmission due to high interaction among individuals, and in order to supply additional immunological benefits, bees collect antimicrobial natural resins to produce a substance called propolis [4, 11–13]. Propolis, such as honey, beeswax and royal jelly, is one of the beehive products that have been valuable for human societies through the ages. In general, honeybees (*A. mellifera*) produce propolis on the basis of a selective harvesting of resins present in leaves, buds, sap flows, trichomes and other actively exuding plant structures that are subsequently mixed with beeswax and salivary gland secretions, yielding a chemically complex resinous material [1, 14–18]. The gathered exudates are mostly incorporated into propolis without chemical modifications; however, some glycosides are subjected to enzymatic action by salivary hydrolases from bees [19, 20]. Unlike honeybees, stingless bees produce different resinous materials by adding soil and clay particles to the final mixture of plant exudates and beeswax, resulting in a particular matrix often called geopropolis, which differs from propolis of *A. mellifera* by the presence of minerals in addition to the absence of plant trichomes [6, 20–22].

Propolis varieties produced by honeybee and stingless bee possess mechanical and biological properties, and these materials are used inside the hive in order to seal cracks, to prevent structural damage and to act as a thermoregulatory resource; in addition, those products are used as chemical weapons to protect the colony from diseases, acting as antimicrobial and as embalming substances that avoid putrefaction of killed intruders [1, 14–18, 23, 24]. Moreover,

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in several human traditional medicine systems, propolis has been used as a remedy due to its properties. In fact, Ancient Egyptians, Greeks and Romans employed this sticky material mainly as a wound healing and as an antiseptic agent. In addition, in Central and South America, Maya and Inca civilizations used cerumen and geopropolis produced by stingless bees as a folk remedy [15, 20, 25–28]. Nowadays, propolis is used in alternative medicine in Japan, and as a remedy to treat wounds, burns, sore throat and stomach ulcer in the Balkan states, meanwhile geopropolis is employed by the population of some tropical countries in Americas as empirical remedy for wound healing, gastritis, infections among others. In this context, propolis and geopropolis represent a promising source of bioactive compounds for pharmacological research [1, 14–18, 21, 28, 29].

Propolis has been extensively studied, and in the last decades, propolis has aroused scientific attention and many reports have been published concerning its broad spectrum of pharmacological activities and its bioactive components [14, 15, 18, 28]. At present, the biological activities reported for propolis include antibacterial [30–32], antioxidant [31, 33], antiparasitic [34–38], antifungal [39–41], antiviral [42], local anaesthetic [43], anti-inflammatory [44, 45], immunomodulatory [46, 47], antitumor [48–50], and antiproliferative activity on cancer cell growth [50–54], among others. There are remarkable differences in the biological activities of propolis from dissimilar geographical origin, and those mainly depend on the qualitative and quantitative variations of its characteristic chemical constituents, which are provided by botanical sources. Thus, the chemical diversity of propolis is dictated by the phytogeographical conditions and the climatic characteristics, and finally by the honeybee species involved in its production [55–57]. In that sense, the chemical composition of propolis from temperate zones (Europe, North America, Southern South America, and West Asia) differs from those of tropical zones (Central and South America, South and Southern Asia and Africa), as several studies have reported it in recent years.

Exudates from poplar buds (*Populus* spp.) are described as the main botanical source of propolis from temperate regions, as well as birch (*Betula alba* L.), horse chestnut (*Aesculus hippocastanum* L.), alder (*Alnus glutinosa* Medik), beech (*Fagus sylvatica* L.), and some conifers. At present, over 300 chemical compounds have been identified in different temperate propolis, including phenolic acids and esters, flavonoids, terpenes, lignans, aromatic aldehydes and alcohols, fatty acids, stilbenes and steroids [14, 15, 18, 58]. Otherwise, over 250 compounds have been identified in propolis samples from different tropical regions, including prenylated benzophenones, organic acids, prenylated organic acids, terpenes, alcohols and isoflavonoids [58, 59], where the main plant origins are *Baccharis dracunculifolia, Araucaria angustifolia, Clusia minor*, *Clusia rosea*, *Dalbergia ecastophyllum*, *Macaranga tanarius*, *Hyptis divaricata* and *Eucalyptus citriodora,* among others [1, 58–61]. Nevertheless, it has been reported that despite the plant origin of propolis produced by *A. Mellifera*, its overall percent composition remains at certain point preserved, comprising 50% botanical resins, 30% waxes, 10% essential and aromatic oils, 5% pollen, and 5% other organic substances. However, as a result of chemical diversity present in propolis, its organoleptic properties may vary considerably, including its physical aspect,

consistency, aromatic smell, and its colour that fluctuates from dark-brown to yellow [22, 58, 62–64].

Propolis must be purified in order to proceed into pharmacological and chemical investigations. The removal of inert material, which is mainly wax, is generally performed by preparing alcoholic or hydro-alcoholic extracts of the macerated raw material, where ethanol, ethanol 70% and methanol are often used. Several analytical techniques have been used to identify and characterize the chemical constituents of propolis samples from different geographical origins, including chromatographic and spectroscopic methods, such as thin-layer chromatography (TLC), gas chromatography (GC), high-performance liquid chromatography (HPLC) and their coupled techniques to mass spectrometry (MS) [1, 58]. GC–MS analysis has been used for the identification of volatile and semi-volatile components of propolis; however, many propolis constituents are not sufficiently volatile for GC–MS analysis, even with derivatization procedures. In that sense, electrospray ionization (ESI) has been extensively used to ionize nonvolatile, thermally unstable, heavy and polar molecules; therefore, ESI-MS and its tandem version ESI-MS/MS have been applied for analyse propolis. ESI ionizes more efficiently polar compounds with acid [negative ion mode (−)] or basic sites [positive ion mode (+)], an important characteristic for the chemical study of propolis, since most of the pharmacologically active constituents identified in propolis possess certain polarity, commonly acidic of phenolic moieties [65, 66]. In addition, nuclear magnetic resonance (NMR) techniques have been used to fully characterize the isolated compounds from propolis [1, 58].

The chemical composition and pharmacological activities of propolis samples collected throughout North America (including Central America and Caribbean) and South America have been studied in the last years, and several papers have reported differences and similarities among the analysed geographical samples. At present, propolis from Americas has been classified according to its aspect and plant source, due to the ecological diversity present along the continent that provides different botanical resins. The major classes include the North and Southern South American propolis described as 'poplar' type, as well as Cuban, Brazilian and Mexican 'red' propolis type, in addition to the Brazilian "green" type, and finally the Cuban and Venezuelan "Clusia" type, reflecting a propolis diversity produced as consequence of adaptive responses of European honeybees to the beehive necessities and vegetation present in Americas, both in tropics and in temperate zones. Moreover, propolis produced by the stingless bees in Americas has gain attention and different samples have been chemically and biologically analysed in recent years, exhibiting in some cases similarities in bioactivity and composition with propolis of honeybees from the tropical areas. However, in this chapter, we were exclusively focused on honeybee propolis.

Some authors have reviewed and presented an extensive number of reports in propolis research with different approaches [1, 14, 15, 18, 21, 28, 55, 58, 62], although the discussion of analyses carried out in propolis from Americas in an attempting to compare the bioactivity, phytochemical diversity and botanical sources has not been approached. Herein, we summarize and discuss for the first time; to the best of our knowledge, most of the studies on honeybee

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propolis produced in Americas, including recent advances in research for this profitable product for apiculture. Most of the studies performed on propolis from Americas are depicted in **Figure 1**.

Figure 1. Chemical and pharmacological studies on propolis produced by *Apis mellifera* on Americas. The black dots on the Map indicate the geographical origin of studied propolis.

2. North American propolis

North America comprises the geographical region occupied by the countries of Greenland, Canada, United States and Mexico, in addition to the countries included in Central America and the Caribbean Islands. As a continent, North America is a terrestrial portion of the

Americas, which is considered rich in biodiversity and a land with a plethora of natural resources that are distributed along its extension. Moreover, this landmass has many topographical variations, from valleys to extensive chains of tall mountains, and is characterized by possessing a wide range of climates and biomes starting from tundra, subarctic forest, followed by temperate forest, plains, deserts and tropical forests [67, 68].

Although the honeybee is not native from North and South American continents, it is wide distributed throughout the Americas. European colonists have brought *A. mellifera* to the Western Hemisphere at the beginning of seventeenth century, by virtue of being a very adaptable bee species and for its management for human benefit [9, 69]. Afterwards, the African honeybee (*A. mellifera scutellata*) was initially introduced by scientists into Southeastern Brazil at mid-1950s, and since the accidental escape of African queen bees, a gradual process of Africanization of feral and managed *A. mellifera* colonies has reached the tropical and subtropical areas of North and South America [70–72]. Prior and after the Africanization process, propolis collected from Canada, United States, Mexico, Cuba, Honduras and El Salvador has been chemically and pharmacologically analysed, and here, we summarized the main results.

2.1. Canada

Chemical analysis on Canadian propolis by chromatographic technics, such as GC–MS and HPLC–MS, has identified the presence of poplar characteristic compounds. To the best of our knowledge, one of the initial studies on chemical composition of Canadian propolis was performed on a sample collected from Sydenham region in Ontario State [73]. Fifteen flavonoids, including pinobanksin-3-*O*-alkanoates and methyl ether derivatives of quercetin were detected. The major constituents in this Canadian propolis were the flavanones pinocembrin and pinobanksin-3-*O*-acetate and the flavones chrysin and galangin, and according to the presence of high amounts of these compounds, the botanical origin was associated to American poplars *P. deltoides*, *P. fremontii* and *P. maximowiczii* from section *Aigeiros*, which are characterized by the biosynthesis of these compounds [73].

In North America, poplars from section *Tacamahaca* and *Leuce* are as wide distributed as poplars from *Aigeiros* section. With the aim to determine the chemical composition of propolis from Canada collected outside the geographic zone of distribution of *Aigeiros* poplars, two samples from different regions the Boreal forest (Richmond, Québec) and Pacific coastal forest (Victoria, British Columbia) have been analysed by GC–MS. The major constituent in Victorian propolis was *p*-hydroxyacetophenone, followed by benzyl hydroxybenzoate, cinnamic acid and significant amounts of five dihydrochalcones. The presence of these compounds suggested that a poplar characteristic from the section of *Tacamahaca*, *P. trichocarpa* Torr. et Gray, is the plant source of propolis from the Canadian Pacific coast [74]. Otherwise, the main constituents found in propolis from Richmond region were *p*-coumaric and cinnamic acids and their derivatives, wherein the high presence of these compounds and the low amount of flavonoids are characteristic of resins of *Populus* spp. from section *Leuce*, which suggested that *P. tremuloides*, a widespread poplar in the Canadian Boreal forest, could be the botanical source of Richmond propolis. The antioxidant or antiradical activity of Canadian propolis collected from

British Columbia and Québec was determined by DPPH assays. Both samples presented a potent-free radical scavenging (FRS) activity (at ~26 μg/mL = 79 ± 5 and 65 ± 7%, respectively), and those results were related to the presence of aforementioned diverse phenolic compounds [74].

More studies have been done regarding the chemical composition of commercialized extract formulations of Canadian propolis, such as Herstat® propolis extract ACF® (antiviral complex of flavonoids), which is patented and manufactured with propolis (at 3% concentration) collected from a specific area in Western Canada (probably Manitoba), which is rich in poplar trees. Bankova et al. [75] have found by GC–MS that the chemical composition of the marketed ointment formulation was mainly comprised by benzoic and *p*-coumaric acid, benzyl p-coumarate and a group of dihydrochalcones, in addition to pinocembrin chalcone and pinostrobin chalcone. Interestingly, the presence of all these compounds suggested a mixed botanical origin, the exudates of both *P. balsamifera* and *P. tremuloides* [75]. The antiviral activity of Herstat® propolis extract, specifically against both types of Herpes simplex infection: HSV-1 and HSV-2, was determined by clinical studies conducted with the application of this ointment, which resulted to be more effective than acyclovir treatment by presenting a significant shortening of healing time and a reduction of the local symptoms of cold sores [76, 77]. Additionally, with the aim to understand the basis of the antiviral activity of Herstat® Canadian propolis extract, Bankova et al. [75] determined by *in vitro* studies the virucidal effect of this ointment on HSV-1 and HSV-2 and the adsorption suppression of virus HSV-1 on MDBK bovine kidney cells. The *in vitro* results were in accordance with those obtained by structured clinical studies with the topical ointment, supporting the usefulness of propolis extract against herpes virus lesions [75].

Furthermore, another commercial Canadian propolis acquired from Trophic™ products was analysed with the aim to determine the composition and to assess its antioxidant properties. Raw commercial propolis was extracted using a two-step sequential process with ethanol and water, wherein the higher antioxidant activity (by FRAP and DPPH methods) was exhibited by the ethanolic extracts, and a higher polyphenol and flavonoid content. Furthermore, the ESI-MS fingerprints revealed a resemblance with Brazilian brown propolis, in addition to the presence of chrysin and pinocembrin, among other flavonoids [78]. All these studies concerning to Canadian propolis provide enough phytochemical evidence about the botanical origin of those bioactive resins, which are mainly collected from different poplars species along the country, confirming the chemical composition of a propolis characteristic of temperate zones.

2.2. United States

The arrival of *A. mellifera* to the United States from England has been described circa 1622, initially in the Colony of Virginia and later in other Colonies in the Eastern region of North America [69]. In recent years, a significant number of studies have been done in regard to propolis collected throughout the United States, and one of the first studies was carried out with the aim to evaluate the inhibitory activity of propolis (collected in Illinois state) against *Paenibacillus larvae* ssp. *larvae* (formerly *Bacillus larvae*), the etiological agent of American foulbrood. A propolis solution exhibited an *in vitro* toxic effect on *P. larvae* (at 10 μg/mL);

however, on later *in vivo* experiments, propolis administered to infected colonies only showed a short-term bacteriostatic effect that allowed *P. larvae* proliferation on the hive after treatment [79, 80].

To our knowledge, the subsequent analyses performed in propolis from the United States were focused on the identification of main chemical constituents of samples from Western Ohio, and both North and South Georgia (Athens and Claxton, respectively), where some similarities to the flavonoid profile of European propolis were found in Ohio and North Georgia samples, in addition to the identification of kaempferol, galangin, 3,3′-dimethoxyquercetin and 3-methoxykaempferol in Ohioan propolis [81]. Those flavonoids occur naturally in some species of Populus, Pinus, Betula, Alnus and Aesculus (horse chestnut), plant sources that are visited by honeybees for resins [16] and were proposed by the authors as possible botanical origin of Ohioan propolis. Interestingly, none of the aromatic acids (ferulic, caffeic and cinnamic acid) commonly present in European propolis were found in Ohioan sample, which established a difference among this propolis [81].

Moreover, in a comparative study with different geographical samples around the world, Kumazawa et al. [82] determined by HPLC analyses that a propolis sample from United States (supplied by Api corporation and Tamagawa University, Japan) was mainly composed by aromatic acids, flavonoids, and their ester and methyl ether derivatives. In particular, the high amounts of p-coumaric acid, pinobanksin-5-methyl ether, pinobanksin, chrysin, pinocembrin, galangin, pinobanksin-3-*O*-acetate and tectochrysin suggested a mixed botanical origin of exudates from *P. tremuloides* and poplars from section *Aigeiros* (Central and Eastern America): *P. fremontii* or *P. deltoides* or *P. maximowiczii* [18, 74, 75, 82]. In addition, this United States propolis presented a moderate FRS activity (DPPH assay: ≥50% at 20.0 μg/mL and by β-carotene–linoleic acid system: ≥30% at 10.0 μg/mL) in comparison with samples from China Australia, New Zealand and Hungary (≥70% at 20.0 μg/mL and ≥60% at 10.0 μg/mL, respectively) [82]. In another comparative study, the effective antimicrobial concentration of propolis from United States (at 20% concentration; the site collection is not specified), Turkey and Australia against oral pathogen microorganisms (*P. gingivalis*, *P. intermedia*, *C. rectus*, *F. nucleatum*, *C. albicans*, *C. parapsilosis*, *C. krusei*) without cytotoxicity induction on human gingival fibroblasts was determined. United States propolis, and as well as Australian, exhibited an effective growth-inhibitory activity against the tested microorganisms; however, at the same concentrations (dil. 1:256 for bacteria and 1:2048 for Candida species), a cytotoxic effect to gingival fibroblasts was observed [83].

In another comparative analysis, propolis from Eastern United States (Indiana and New York), Europe (Bulgaria, Finland and England) and Brazil was studied by ESI-MS/MS. Chrysin, pinocembrin and *p*-coumaric acid were identified in all those propolis samples, suggesting a similar botanical origin in poplar species in Eastern United States, South Brazil and Europe propolis [84]. Furthermore, propolis samples collected from the states of Oregon and Northern California (three samples from each one) were studied by GC–MS and HPLC analyses, displaying these samples a high content of *p*-hydroxyacetophenone, *p*-coumaric acid, *t*-cinnamic acid and flavonoids, such as galangin, chrysin, pinocembrinin and pinobanksin derivatives, in addition to the presence of terpenes, terpenoids, chalcones and dihydrochal-

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cones, which are chemical compounds characteristic of resins of balsam poplars of *Tacamahaca* section in the western part of the North American continent [85]. Interestingly, ferulic acid and caffeic acid esters were detected in samples from Oregon and only in one specimen from California; these compounds typically occur in resins from poplars of *Aigeiros* section (cottonwoods) and are absent in resins from poplars of the *Tacamahaca* section [86]. Taken together, these results indicated that two samples from California possessed a pure plant origin on *Tacamahaca* poplars, while the other propolis specimen from California and the three samples from Oregon have a mixed plant origin from both *Tacamahaca* and *Aigeiros* poplars due to the presence of caffeic acid esters and ferulic acid [85].

Recently, a comprehensive analysis by GC–MS of ten geographically distinct propolis samples collected throughout the United States, including the cold North, the wet Southeast and the dry Southwest Regions, has provided a classification system by applying a chemometric approach principal component analysis (PCA) based on the relative amounts of main chemical classes found in the samples. Propolis from New York (NY-2,3,8,10), Pennsylvania (PN-6), Louisiana (LA-1), Minnesota (MN-9), Nebraska (NE-4), Nevada (NV-5) and North Carolina (NC-7) was analysed, and over 60 chemical constituents grouped in main compound types (benzoic and cinnamic acid derivatives, chalcones, flavanones and dihydroflavonols, flavones and flavonols, phenolic glycerides, and terpenes) were identified [87]. As expected, different geographical samples presented distinct chemical profiles. In all samples, poplar-type propolis compounds were found (aromatic acids and their esters, flavonoids and chalcones). Three main groups were obtained: group I) propolis rich in cinnamic acid derivatives (samples NY-2, NY-3, MN-9 and NY-10), such as benzoic, cinnamic, p-coumaric, and ferulic acids and benzyl-*p*-coumarate, is characteristic of *P. tremuloides* Michx. (American aspen) exudates, which would be the botanical source of these samples [87].

Group II) propolis with high concentrations of flavonoids (NE-4, PA-6 and NY-8), such as pinocembrin, pinobanksin, pinobanksin-3-*O*-acetate, chrysin, galangin and pinocembrin chalcone, chemical profile typical for poplar bud resins from section *Aigeiros* (*P. fremontii* resins were considered the main plan source). Group III) propolis rich in triterpenes (LA-1, NV-5, and NC-7), such as 3-oxo-6β-hydroxy-lup-20(29)-en-28-oic acid [87], a triterpenic acid previously identified in Honduran propolis, which suggested the participation of additional botanical resins to poplar exudates, essentially the tree *Liquidambar styraciflua* L. (main botanical source of Honduran propolis) [88], a distributed plant in east and southeast regions of the United States and characterized by the occurrence of this triterpene, among benzyl *p*-coumarate, and cinnamic acid derivatives. The presence of triterpenes is a new finding for North American propolis, since triterpenes have been only reported in samples from tropical and subtropical regions [58, 87, 88]. Moreover, the quorum sensing inhibitory (QSI) activity of the ten United States propolises was evaluated using the acyl-homoserine lactone (AHL)-dependent *Chromobacterium violaceum* strain CV026, with the aim to identify potential antivirulence capacity in those samples. The group II exhibited the highest QSI effect. This classification provided and insight on the mixed plant origin of some United States propolis, in addition to the already-known poplar-type propolis [87].

In another study, with the aim to characterize the antimicrobial activity of propolis against beehive pathogens, 12 samples were collected from different geographical regions of the United States (Chaska, MN; Baton Rouge, LA; Ithaca, NY; Jamestown, ND; Lincoln, NE; Raleigh, NC; Wakinsville, GA; Tucson, AZ; Aspen, CO; Vacaville, CA; Beaumont, TX; Fallon, NV) and were evaluated against the bee pathogens *P. larvae* and *Ascosphaera apis*. The chemical composition profile of propolis samples was analysed by LC–MS-based metabolomic methods, revealing differences on chemical patterns and, as well, different ability of propolis samples to inhibit the growth of both pathogens. The highest activity on *P. larvae* and *A. apis* was exhibited by propolis from Nevada (IC_{50}: 41.6 and 8.6 μg/mL, respectively), followed by Texas (IC_{50}: 46.9 and 10.0 μg/mL, respectively) and California samples (IC_{50}: 74.1 and 7.4 μg/mL, respectively) [13].

In order to track the botanical origin of antimicrobial resins (against *P. larvae*) gathered by *A. mellifera* in Northeastern United States, an analysis by HPLC and UPLC-TOF-MS of plant material collected by individual honeybees from an apiary located in Minnesota was carried out. Afterwards, using metabolomic methods (principal component analysis), the phytochemical patterns were analyzed and compared to those of resinous material collected from 6 North American *Populus* spp. and 5 hybrids, in addition to other plants in the surrounding areas. The results showed that honeybees only foraged resins from *P. deltoides* and *P. balsamifera* among many other plant sources available, including the chemotaxonomically related ones. From 26 individual resin foraging bees, 10 resulted to transport resin from *P. deltoides* and 15 from *P. balsamifera*. Moreover, phytochemicals present on *P. deltoides* and *P. balsamifera* resins did not showed to be influenced by regional or seasonal effects. These data suggested that honeybees discern among closely related *Populus* species to collect resinous material, in addition to a foraging behaviour maintained by individuals inside the hive to exclusively one plant source. Finally, the antimicrobial effect of *Populus* spp. resins against *P. larvae* presented differential inhibition as consequence of variations in secondary metabolites present in those resins [4].

Further chemical studies have been done in propolis collected outside the temperate poplar zone of North America, specifically in the Sonoran Desert (Southwestern United States). Wollenweber and Buchmann [89] have analysed several propolis samples from managed and feral honeybee colonies located in Arizona State with the aim to determine the botanical origin of propolis from desert zones. The widespread vegetation in Sonoran Desert is comprised by xeromorphic shrubs and cacti rather than poplars, which are scarcely found in some watercourse zones. Wollenweber and Buchmann determined by TLC and GC–MS that some propolis samples presented the fingerprint pattern of *P. fremontii*, and as well, some others exhibited a mixed plant origin of *P. fremontii* and *Ambrosia deltoidea* (Torrey) Payne; meanwhile, samples out of flight reach of poplars contained flavonoid and other phenolic compounds characteristic to specific plants in this area, such as *Ambrosia deltoidea* and *Encelia farinosa* A. Gray [89].

In addition to the mainland United States, the Hawaiian Islands chain is included in the political territory of this country, and it represents a totally different ecological scenario with tropical characteristics. In that sense, poplars are not available for propolis production in Hawaii; thus, honeybees must use other botanical sources. In a recent study, Inui et al. [90]

have chemically investigated Hawaiian propolis by HPLC–ESI-MS in order to identify its botanical origin. A family of prenylflavonoids was identified in this propolis sample [90], chemical compounds that are additionally present in the Pacific propolis type (Okinawa, Taiwan, Indonesia and Myanmar propolis) [61]. By comparison of the chemical profile *Macaranga tanarius* resulted to be the main plant origin [90]. Previous studies suggested that exudates from buds and bark of *Plumeria acuminata*, *P. acutifolia*, *Schinus terebinthifolius* and *Psidium guajava* could be gathered by honeybees [18].

All these studies on United States propolis revealed that *A. mellifera* visits mainly *Populus* species in order to gather their prized antimicrobial resins. Although the phytochemical evidence additionally established that different complementary plants provide attractive resins to honeybees or even they represent the mainly chemical source of propolis collected from diverse climatic regions of the continental United States, plants such as *L. styraciflua*, *A. deltoidea* and *E. farinosa* and even *M. tanarius* in the tropical Hawaiian islands. Moreover, the great input provided by Wilson et al. [4] regarding to the foraging fidelity of one single individual of the colony to collect resins from exclusively one plant source, which suggests organization and specialization of individuals to particular plants in order to provide chemical diversity inside the hive. All these studies emphasize the variety of propolis types produced in the ecological regions of United States, suggesting mixed botanical origins for particular samples, yielding in a wide spectrum of pharmacological activities.

2.3. Mexico

Mexico is included among the five countries of the world with a great richness of endemic species, and this consideration is mainly related to the wide range of topographical diversity and the variety of climatic zones that lie between North American deserts and Mesoamerican forests [91]. Propolis collected from different ecological regions of Mexico has been analysed, including samples from North American deserts (Sonoran Desert), tropical forests, southern semi-arid highlands and temperate sierras.

One of the most investigated Mexican propolis types is Sonoran Desert propolis. Since almost a decade, several studies have been reporting the biological activities and main chemical constitution of samples collected from arid and semi-arid lands in the Sonora State [Ures (UP), Pueblo de Alamos (PAP) and Caborca (CP)]. Hernandez et al. [51] reported that the chemical composition of Sonoran propolis was mostly comprised by phenolic acids, flavonoids and their ester derivatives; moreover, pinocembrin, chrysin and pinobanksin-3-*O*-acetate were the main constituents in these three samples [51]. In particular, the presence of rutin, naringenin and hesperetin was exclusively found in propolis from PAP; meanwhile, xanthomicrol was found in the samples of PAP and CP, and 3′-desmethoxysudachitin compound was only detected in CP [51]. According to Wollenweber and Buchmann [89], the presence of xanthomicrol and 3′-desmethoxysudachitin is characteristic of *A. deltoidea* exudates, a plant that could be implied in the botanical source of CP and PAP since its widespread distribution along the Sonoran Desert. Otherwise, the presence of caffeic acid phenetyl ester (CAPE) was restricted only to UP, which is a chemical compound found in propolis from temperate zones, in addition to the higher amounts of pinocembrin, chrysin and pinobanksin-3-*O*-acetate found in this sample

that supported this resemblance to temperate propolis, suggesting that poplars from section *Aigeiros*, such as *P. fremontii* could be the botanical source of this propolis [14, 55, 73].

Additionally, the chemical constitution of propolis from CP was further investigated in another study carried out by Li et al. [54], which resulted in the NMR characterization of three new flavonoids: (2*R*,3*R*)-3,5-dihydroxy-7-methoxyflavanone 3-(2-methyl)butyrate, (7″*R*)-8-[1-(4′-hydroxy-3′-methoxyphenyl)prop-2-en-1-yl]chrysin, and (7″*R*)-8-[1-(4′-hydroxy-3′-methoxyphenyl)prop-2-en-1-yl]galangin, and as well other 41 isolated chemical compounds characteristic of exudates from the genus *Populus*, including aromatic acids, flavonoids and its esters. In addition, the *in vitro* cytotoxicity of the 44 chemical compounds was evaluated on PANC-1 human pancreatic cell line, and (7′*R*)-8-[1-(4′-hydroxy-3′-methoxyphenyl)prop-2-en-1-yl]galangin showed to possess the most potent preferential cytotoxicity (PC_{50}: 4.6 μM) [54]. Lately, two phenylallylflavanones, (2*R*,3*R*)-6-[1-(4′-hydroxy-3′-methoxyphenyl)prop-2-en-1-yl]pinobanksin and (2*R*,3*R*)-6-[1-(4′-hydroxy-3′-methoxyphenyl)prop-2-en-1-yl]pinobanksin 3-acetate were identified in CP by first time and were new for propolis in general. These phenylallylflavanones additionally displayed a cytotoxic effect against PANC-1 (PC_{50}: 17.9 and 9.1 μM, respectively) [92]. Moreover, the cytotoxic evaluation of 39 of those 44 compounds, isolated from CP, was carried out on a panel of six different cancer cell lines: murine colon carcinoma (colon 26-L5), murine melanoma (B16-BL6), murine Lewis lung carcinoma (LLC), human lung adenocarcinoma (A549), human cervix adenocarcinoma (HeLa) and human HT-1080 fibrosarcoma (HT-1080). The compounds (2*R*,3*S*)-8-[4-Phenylprop-2-en-1-one]-4′,7-dihydroxy-3′,5-dimethoxyflavan-3-ol, cinnamyl *p*-coumarate and 2-acetyl-3-caffeoyl-1-*p*-coumaroylglycerol exhibited the most potent cytotoxic effect in comparison with the tested flavonoids, phenolic acid derivatives and glycerides from CP [53].

More studies have been done in order to determine the biological activities of Mexican propolis samples collected in semi-arid and arid zones (UP, PAP and CP). Sonoran propolis showed a strong antiproliferative effect on human and murine cancer cell lines A549 (IC50: 58.6 μg/mL), HeLa (IC50: 31.7–49.8 μg/mL), LS-180 (IC_{50}: 53.3–84.9 μg/mL), RAW 264.7 (Abelson murine leukemia virus transformed macrophages; IC_{50}: 0.8–5.2 μg/mL) and M12.C3.F6 (murine B-cell lymphoma cells; IC_{50}: 3.1–6.8 μg/mL). Moreover, CAPE, galangin, xanthomicrol and chrysin induced a significant antiproliferative effect on most of the cancer cell lines evaluated (IC_{50}: 3.2–95.4 μM) [51]. Since DNA harvested from cancer cells treated with UP exhibited a ladder of internucleosomal DNA cleavage pattern characteristic of apoptosis, in addition to the morphological changes observed in treated cells, a study conducted with the aim to determine biochemical events produced at earlier stages of apoptosis has been done. By annexin V-FITC/Propidium iodide double labelling, it has been demonstrated that Sonoran propolis treatment induced antiproliferative effect on M12.C3.F6 cells through apoptosis induction, and this apoptotic effect resulted to be mediated by modulations in the loss of mitochondrial membrane potential and through activation of caspases signalling pathway (3, 8 and 9). Additionally, some of the constituents of Sonoran propolis that induce apoptosis in cancer cells were characterized by an HPLC–PDA–ESI-MS/MS analysis, followed by isolation procedures and NMR spectroscopy that yield eighteen flavonoids, commonly described in poplar-type propolis, including two esters of pinobanksin, pinobanksin-5-methylether-3-*O*-propanoate

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and pinobanksin-5-methylether-3-*O*-butyrate were described by first time in propolis samples in general. Moreover, pinobanksin, pinobanksin-3-*O*-propanoate, pinobanksin-3-*O*-butyrate, pinobanksin-3-*O*-pentanoate, galangin, chrysin and CAPE induced antiproliferative activity on M12.C3.F6 cells through apoptosis induction [52].

The antibacterial and FRS activities of Sonoran propolis (UP, PAP and CP) have been tested by broth microdilution method and by DPPH assay, respectively. Sonoran propolis exhibited antibacterial activity against only Gram-positive bacteria, and UP presented the highest inhibition against *Staphylococcus aureus* (MIC: 100 μg/mL), followed by CP. CAPE, an exclusive constituent of UP, showed high growth inhibitory activity towards Gram-positive bacteria, particularly against *S. aureus* (MIC: 0.1 mM). CP presented the highest FRS activity (86% at μg/mL). The chemical constituents CAPE and rutin presented a high antioxidant activity (90.4 and 88.5% at 70 μM, respectively) in comparison with ascorbic acid control (95.0% at 70 μM). These results suggested that the presence of CAPE and rutin could be implied in the biological activities induced by Sonoran propolis [31]. Furthermore, the anti-*Vibrio* activity of those propolis samples collected in North-western Mexico was evaluated by broth microdilution method. UP presented the highest antibacterial effect against *Vibrio cholerae* O1 serotype Inaba, *V. cholerae* non-O1, *V. vulnificus* (MIC_{50}: <50 μg/mL), and *V. coholerae* O1 serotype Ogawa (MIC_{50}: 100 μg/mL). The constituents CAPE and galangin presented a potent growth inhibitory activity (MIC_{50}: 0.05–0.1 mM) against *V. cholerae* strains (non-O1 and serotype Ogawa) [93]. Additionally, the *in vitro* antiparasitic activity of Sonoran propolis against *Giardia lamblia* has been tested. UP showed the highest growth inhibitory effect (IC_{50}: 63.8 μg/mL) in comparison with CP and PAP (IC_{50}: >200 μg/mL). Among the chemical constituents of Sonoran propolis evaluated, CAPE had the highest growth inhibitory activity (IC_{50}: 222.1 μM), followed by naringenin (IC_{50}: 461.8 μM), hesperetin (IC_{50}: 494.9 μM) and pinocembrin activity (IC_{50}: 680.6 μM) [34].

Since UP showed to be one of the most biologically active of the Sonoran propolis tested, the evaluation of the seasonal effect on the chemical composition and biological activities (antiproliferative, antiparasitic and antioxidant activities) of UP has been done. The collected seasonal samples [spring (sp), summer (s), fall (f) and winter (w)] were analysed by an HPLC–DAD–UV method, wherein from the qualitative point of view, the chemical profile of the seasonal samples was similar; however, the results for antiproliferative effect on M12.C3.F6 cell line [sp (IC50: 11.6 μg/mL) > w (IC50: 26.6 μg/mL) > s (IC50: 49.7 μg/mL) > f (IC50: 54.5 μg/mL)] and antiparasitic activity on *G. lamblia* [s (IC_{50}: 23.8 μg/mL) > w (IC_{50}: 59.2 μg/mL) > sp (IC_{50}: 102.5 μg/mL) > f (IC_{50}: 125.0 μg/mL)] presented significant differences, which suggested that slightly quantitative variations on the bioactive constituents could be implicated in the seasonal effect of biological activities of UP. All propolis samples had weak FRS activity (<25% at 100 μg/mL) [34, 94].

Recently, the immunomodulatory properties of UP were tested (0.2–20.0 μg/mL) in a comparative study with Brazilian (Botucatu, Sao Paulo) and Cuban (Havana) propolis on pro- and anti-inflammatory cytokine production [tumor necrosis factor (TNF)-α and interleukin (IL)-10, respectively] by human monocytes. Brazilian propolis stimulated both TNF-α and IL-10 production by monocytes; meanwhile, Cuban propolis stimulated TNF-α and inhibited IL-10

production. UP exerted the opposite effect, inhibited TNF-α and stimulated IL-10 production. These results are due to qualitative and quantitative differences in the chemical constitution of the three samples, since different constituents that may exert pro- and anti-inflammatory activity depending on concentration. It is reported that the major compounds found in Brazilian, Cuban and Mexican propolis samples used in this study are artepillin C, isoflavonoids and pinocembrin, respectively, [46].

In a recent chemical comparative study performed with the aim to develop and validate a suitable RP–HPLC method to determine and quantify flavonoid markers in Mexican propolis, 11 samples collected at different ecological regions in six states (Estado de Mexico, Puebla, Chiapas, Zacatecas, Tlaxcala and Guanajuato) were analysed. Acacetin, 4′,7-dimethyl naringenin and 4′,7-dimethyl apigenin were used as marker components in this study, and the method was applied to establish some quantitative variations related to seasonal and geographical conditions of the propolis samples. 4′,7-Dimethyl apigenin was selected as an appropriate marker of Mexican propolis, followed by 4′,7-dimethyl naringenin. Both chemical compounds were considered useful for quality control procedures in the geographical origin validation of Mexican propolis [95].

The chemical constitution of a Mexican red-type propolis collected from Champoton at Southern Mexico (Campeche State) was analysed. Three new compounds 1-(3′,4′-dihydroxy-2′-methoxyphenyl)-3-(phenyl)propane, (*Z*)-1-(2′-methoxy-4′,5′-dihydroxyphenyl)-2-(3-phenyl) propene and 3-hydroxy-5,6-dimethoxyflavan were identified [56], in addition to seven known flavanones, isoflavans and pterocarpans that have been described in Cuban and Brazilian propolis. The occurrence of these compounds is related to the chemical profiles of plant exudates from the genus *Dalbergia*, which suggested the botanical relation of red Mexican propolis and *Dalbergia* species [56, 58].

Moreover, in a comparative study about the volatile constituents of propolis from honeybees and stingless bees collected in the Yucatan peninsula, ninety-nine compounds were identified by GC–MS, wherein common compounds were present in both types of propolis. However, styrene, phenylacetaldehyde, trans-sabinene hydrate, nonanal, decanal, 2-undecanone, cyperen, cis-α-bergamotene, massoia lactone, ar-curcumene, cis-calamenene, cardina-1,4-diene, α-cadinene, β-eudesmol, α-bisabolol, neryl linalool, geranyl linalool, manoil oxide, kaur-16-ene, pentacosane and heptacosane were identified only in honeybee propolis [96].

In general, these studies confirm the chemical diversity present in propolis produced by *A. mellifera* in Mexico, suggesting the participation of exudates from different plant species as consequence of the ecological diversity present throughout the country, some of the chemical compounds could be useful as taxonomic markers to differentiate the type of Mexican propolis. In addition, the plethora of biological properties, including antioxidant, antibacterial, antiproliferative, cytotoxicity, antiparasitic and immunomodulatory effects, has demonstrated that Sonoran propolis is a source of bioactive constituents for pharmacology research.

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2.4. Cuba

The isle of Cuba is the largest in the Caribbean Sea, and together with Island of Youth and over 4000 islands comprise the Cuban Archipelago. The moderate tropical climate together with the exposure to different wind currents, topographical variations, diversity in moisture levels and types of soil produce heterogeneous ecological regions, such as the wetlands in the southern coast, tropical desert-like conditions in eastern coast, and pine forest at mountains, which make Cuba an example of almost every ecosystem present throughout the Antilles Islands [97]. Cuban propolis is the most investigated propolis type from North America (including Caribbean and Central America) and several studies have been published in regard to its composition and properties.

The first studies carried out on Cuban propolis were mainly focused on its biological properties, since it is considered as a traditional homemade remedy in this country. However, most of these studies were performed without a certain information on the chemical constitution of Cuban propolis, which makes it difficult to draw a direct correlation between bioactivity reported 20 years ago and the chemical constituents identified at present [58]. The antioxidant effect of ethanolic extracts of Cuban propolis from Baracoa and Pinar del Rio province was analysed by their scavenging action against different species of oxygen radicals (superoxide and alkoxy) using luminol-sensitized chemiluminescence, both propolis preparations showed a high antioxidant activity against superoxide (IC_{50}: 5.0 and 9.5 μg/mL, respectively) and alkoxy (IC_{50}: 0.5 and 0.6 μg/mL, respectively) radicals [98].

Moreover, the hepatoprotective effects of Cuban red propolis (CRP) from Havana region were evaluated in different models of acute liver injury induced by paracetamol (600 mg/kg) [99, 100], and by allyl alcohol (64 mg/kg) [101], both in mice. The intraperitoneal administration of ethanolic extract of propolis (25, 50 and 100 mg/kg) showed to decrease significantly the enzymatic activity of alanine aminotransferase (ALAT), the levels of reduced gluthatione (GSH), and as well showed to reduce liver damage, these protective effects of propolis were produced both, before (30 min.) and after (2 h) paracetamol hepatotoxicity induction [99, 100]. Similar results were obtained with CRP administration before allyl alcohol (30 min.) [101].

Additionally, CRP was evaluated in other two models of acute hepatotoxicity induced by carbon tetrachloride (CCl_4) and by galactosamine (1000 mg/kg) in Sprague–Dawley rats [102–104]. The treatment with CRP (5, 10 and 25 mg/kg) in rats with hepatotoxicity induced by CCl4, showed to reduce ALAT and hepatic malondialdehyde (MDA) levels in blood serum, and as well decreased the triglyceride (TG) levels in liver in comparison with control group [102]. Furthermore, histopathological evaluation revealed rats treated with CRP (25, 50 and 100 mg/kg) exhibited a significant reduction in liver injury, according to the low count of affected cells and the limited extension of steatosis area in comparison with those rats with control treatment [102, 103]. Similar biochemical histopathological results were obtained by CRP against the hepatotoxicity induced by galactosamine [104]. After these experiments, it was suggested that CRP probably exerted its hepatoprotective effects by antioxidant properties (scavenging action against oxygen radicals) [100–104].

The antipsoriatic, anti-inflammatory and analgesic effects of CRP were additionally assessed in another study, wherein CRP induced the formation of granular layer in a mouse tail model, reflecting its antipsoriatic activity; meanwhile, its anti-inflammatory effect was observed using three different test models [Cotton-pellet granuloma assay in rats Sprague–Dawley (dose: 50 mg/kg i.g.), croton oil-induced edema (dose: 25%; 2.5 μL) and the peritoneal capillary permeability test in Swiss albino mice (dose: 10 mg/kg)]. CRP exhibited an analgesic activity using the models of acid-acetic-induced writhing (25 mg/kg i.g.) and hot plate test in Swiss albino mice (40 mg/kg) [105]. Moreover, the sensitizing properties of CRP were assessed, and CRP did not induce erythema, edema, and the study also revealed the absence of dermal and ocular toxicity in guinea pigs and New Zealand rabbits at 24 h. However, a moderate contact allergy potential was identified in CRP treatment by slight induction of erythema [105].

Since Cuban propolis presented several biological activities, subsequent studies came as consequence of the renewed interest in exploring the chemical composition of propolis for drug development [61, 106]. An ethanolic extract of Cuban propolis collected from Nuevitas, Cuba has yielded the isolation and structural determination of propolone A, the first polyisoprenylated benzophenone isolated from tropical propolis [106]. Although the presence of polyisoprenylated benzophenones in propolis from the tropical areas has been previously proposed, the full characterization of individual benzophenone molecules has not happened until this study [107]. In addition, propolone A showed significant antimicrobial and fungicidal activities against several Actinomyces, yeasts and Gram-positive and Gram-negative bacteria [106] that encouraged the study and isolation of other bioactive prenylated benzophenone derivatives present in Cuban propolis.

The presence of polyisoprenylated benzophenones in Cuban propolis suggested that resins from genus *Clusia* could be implied as botanical source [108]. Copey tree (*Clusia rosea*) is widespread distributed throughout Cuba, and nemorosone, a prenylated benzophenone, is one of the major compound present in floral resins of copey tree [108, 109]. In that sense, a phytochemical study of 21 propolis samples collected from different locations along the country was carried out with the aim to track the presence of nemorosone and other benzophenones characteristic of *Clusia* species. Nemorosone was identified as major compound in propolis from the western, eastern and central Cuba. Additionally, a mixture of xanthochymol and guttiferone E, present in resins secreted by fruits but not on floral resins of *C. rosea*, was detected in a lesser proportion in those propolis samples. This study has also provided evidence about the cytotoxic effect of nemorosone on human cancer cell lines HeLa, Hep-2, PC-3 and U251 (IC_{50}:1.9–7.2 μM) and FRS activity (IC_{50}: 44.1 μM). Nemorosone showed to be more biologically active than a mixture of two its methyl derivatives on cytotoxicity (IC_{50}: 29.4–94.5 μM) and antioxidant (IC_{50}: >200) evaluations [109]. Later, comprehensive chemical composition analysis of propolis collected from Guantánamo region led to the structural determination of three new polyprenylated benzophenones derivatives (propolones B-D), in addition to the presence of garcinielliptone I and hyperibone B [110].

By virtue of the differences of endemic plants in tropical and temperate regions and also the ecological diversity of Cuba, further investigations on the chemical composition of propolis from different regions of Cuba were carried out. A scrupulous chemical analysis of a CRP from

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Pinar del río provided a cutting edge input on composition of propolis in general by reporting for the first time the presence and structural determination of isoflavonoids (isoflavones, isoflavans and pterocarpans) in a propolis sample, in addition to gallic acid, isoliquiritigenin, and (-)-liquiritigenin [57]. The occurrence of isoflavonoids is pretty restricted in nature and is characteristic of Leguminosae family, and these findings yield two different Cuban propolis types, suggesting the participation of at least two diverse botanical sources, one that provides prenylated benzophenones and another the isoflavonoids [57].

With the aim to study the chemical similarities and differences on Cuban propolis and to establish a classification system according to the presence of secondary metabolites, 65 propolis samples from different regions of Cuba were chemically analysed by HPLC–PDA, HPLC–ESI/MS and NMR. Cuban propolis types were grouped in three groups, including CRP rich in isoflavonoids; Cuban brown propolis (CBP), characterized by a high amount of polyisoprenylated benzophenones; and Cuban yellow propolis (CYP) that contains aliphatic compounds, which was sub-classified depending on its content on triterpenic alcohols (type A) and polymethoxylated flavonoids (type B) [58, 111–113]. Once CBP was identified as the major type of propolis produced in Cuba, biological assays were performed, especially on its capacity to inhibit *in vitro* cancer cell proliferation, since nemorosone, its main constituent, showed a potent cytotoxic effect on human cancer cells [109, 111]. CBP exhibited anti-metastatic effect in mouse mammary carcinoma Ehrlich's ascites tumour (EAT) cells in NMRI immunocompetent mice (5–23 μg/mL), in addition to cytotoxicity on diverse cancer cell lines, suggesting the potential of Cuban propolis as a source of possible anticancer agents [114].

Other studies showed that BCP induced significant antiproliferative activity on human breast cancer cell lines, preferentially on MCF-7 (estrogen receptor positive; ER α+) rather than MDA-MB-231 (estrogen receptor negative ER α-) in a dose-dependent manner. The antiproliferative effect on MCF-7 was partially related to apoptosis induction after an arrest in G1 phase of cell cycle was detected. Moreover, the co-administration of 17-β-estradiol and an antagonist (ICI 182,780) allowed to hypothesize that BCP possesses an estrogen-like activity, although the effect would not be exclusively considered ER-dependent because the mortality also induced on MDA-MB-231 by BCP [115].

Otherwise, the treatment with nemorosone on MCF-7, MDA-MB-231 and LNCaP cells induced a selective antiproliferative effect on MCF-7 by arresting the cell cycle in G0/G1 phase, in addition to a reduction in the expression of pERK1/2 and PAkt. Nemorosone did not induce antiproliferative effect on MDA-MB-231 nor human prostate cancer cells LNCaP (which express ERβ but not ERα), which suggested that nemorosone is the main responsible for the antiproliferative effect of BCP on ERα+ breast cancer cells, and it could have therapeutic applications in breast cancer treatment since its activity on ERα+ cells [116]. Moreover, nemorosone exhibited cytotoxicity in neuroblastoma cell lines, including their clone selected for resistance to chemotherapeutic compounds. It induced a G0/G1 arrest on cell cycle that yields a reduction in S-phase population, in addition to the detection of an upregulation of $p21^{Cip1}$, presence of apoptotic DNA laddering, the activation of caspase 3 activity, dephosphorylation of ERK1/2 in LAN-1 and the inhibition of Akt/PKB [114]. Due to the reported correlation of nemorosone cytotoxicity on cancer cell lines to direct action on estrogen receptor

(ERs), other studies have been done. By *in vitro* tests [recombinant yeast assay (RYA) and E-screen assay], the antiestrogenic activity of nemorosone was demonstrated, exhibiting this benzophenone a reduction on the cell proliferation induced by 17-β-estradiol (E2). Additionally, the treatment with nemorosone did not induce DNA damage in breast cancer cells MCF-7 BUS or in normal breast cells MCF10A. These results suggested that nemorosone could be a promising adjuvant for ER antagonists [117].

In addition to the presence of nemorosone in CBP, there have been identified mucronulatol and plukenetione A as cytotoxic and antiproliferative compounds that could contribute to the potent inhibitory activity of CBP on cancer cell proliferation. The occurrence of plukenetione A has previously been identified in *Clusia plukenetii*, and in later studies in CBP. Plukenetione A is reported to exert considerably cytotoxicity in a panel of cancer cell lines, including colon, ovarian, prostate carcinomas and neuroblastoma cells (IC_{50}: between 1.7 and 16.3 μg/mL); in addition, it induced in HCT8 cells that the depletion of S phase transitory cells as consequence of a G0/G1 arrest in cell cycle, followed by the presence of apoptotic DNA laddering, changes in gene expression patterns of genes required for cell replication and maintenance, accompanied by the inhibition of the enzymatic activity of both topoisomerase I and DNA polymerase [118]. The isoflavonoid mucronulatol has been described as one of the most cytotoxic constituents for Caribbean propolis. In general mucronulatol showed cytotoxicity MDR1–/MDR3+ cells (2.7–10.2 μg/mL), but not on MDR1+ cells (> to 100 μg/mL), which resulted as consequence of an interruption of cell cycle progression, by blocking at G1, accompanied by an upregulation of $p21^{Cip1}$ and $p27^{Kip1}$ and a downregulation of cyclin E and CDK4, interfering in general with the cell cycle machinery [119]. The presence of mucronatol has also been reported in Brazilian and Mexican red propolis [56].

Other different biological properties of nemorosone have been evaluated, including the mutagenic, antimutagenic and estrogenic effects. The mutagenic and antimutagenic activity of nemorosone were assessed by the Ames test on *Salmonella typhimurium* strains (TA97a, TA98, TA100 and TA102), wherein nemorosone did not induce any mutagenic activity; meanwhile, nemorosome exhibited a moderate to strong protective effect (31 and 53% of inhibition, respectively) in association with mutagens in strains TA100 and TA102. Nemorosone induced estrogenic activity detectable by recombinant yeast assay at various concentrations (EEq of 0.41 ± 0.16 nM), concluding with those results that nemorosone could have a chemotherapeutic application in breast cancer research [120].

Red propolis has also been described in other tropical countries, including Brazil, Mexico and Venezuela. Brazilian red propolis (BRP) is a propolis type produced by honeybees from the resinous exudates of *Dalbergia ecastophyllum* in Northeastern Brazil [121, 122], and its chemical composition is mainly comprised by isoflavonoids, neoflavonoids, flavonoids and polyisoprenylated benzophenones (PPBs) [123, 124], which suggested some similarities to Cuban red propolis (CRP). In order to investigate the chemical composition, the botanical source and to draw a relation between different red propolis from Americas, CRP and BRP were analysed in a comparative study by HPLC–DAD–MS, in addition to *D. ecastophyllum* exudates (DEE) [125]. The presence of flavanones, isoflavones, isoflavans, pterocarpans and a chalconoid was identified in both red-type propolis (BRP and CRP) and DEE. In addition, guttiferone E/

xanthochymol and oblongifolin A were exclusively detected in BRP. The flavans retusapurpurin A and the new retusapurpurin B were found to be the pigments responsible of the red colour in those samples. Indicating these results similarities in phytochemical composition of propolis collected from different tropical zones in Americas, since they apparently share exudates from *Dalbergia* species (probably DEE) as main plant source. However, the presence of PPBs in BRP suggested a complementary botanical origin in this sample. This study provided valuable information to attempt a more appropriate propolis classification [125].

All these studies contribute to understand the chemical diversity present in propolis from tropical zones, wherein *Populus* species are not present and honeybees have to gather bioactive exudates from other plant sources. In addition, these studies provide the pharmacological characterization of prenylated benzophenones and isoflavonoids, chemical constituents that represent a promissory source of therapeutic agents and could be used as chemical markers in future standardization of Cuban propolis. Subsequent studies are necessary in order to understand the mechanism of hepatoprotective effects of CRP, and as well to determine the chemical compounds involved.

2.5. El Salvador

As observed, honeybees had to find other different plant sources of bioactive resins in the tropics to those commonly visited in the temperate zones; thus, the chemical diversity present in tropical propolis, and the pharmacological properties of its constituents has attracted so much interest. In tropical propolis, it has been reported a variety of compounds, including the presence of polyprenylated benzophenones, isoflavonoids, and triterpenes in Cuban propolis [57, 111, 113], the occurrence of isoflavonoids and polyisoprenylated benzophenones in Brazilian propolis and, and 1,3-diarylpropane derivatives, flavonoids, and isoflavonoids from Mexican propolis [56].

With the aim to continue the studies of propolis from tropical Central America, a sample collected in the vicinities of Usulutan, El Salvador, was studied and two new chalcones were isolated and characterized by NMR (2′,3′-dihydroxy-4,4′-dimethoxychalcone and 2′,3′ 4-trihydroxy-4′-methoxychalcone) by first time in propolis. Both compounds presented a good antibacterial activity on *S. aureus* (29 ± 3 and 23 ± 1 mm of inhibition zone, respectively) but not on *E. coli*. In addition, those chalcones inhibited *C. albicans* growth (19.3 ± 0.6 and 29 ± 1 mm, respectively); thus, better antibacterial and antifungal activities than original propolis extract were presented (12 ± 1 and 11 ± 1 mm, respectively). However, none of them exhibited a more effective cytotoxicity in brine shrimp (*A. salina* nauplii) lethality bioassay than propolis extract (LC_{50}: 39 ± 9 mg/mL) [126].

In another study, two diterpene glycosides (*ent*-8(17)-labden-15-*O*-α-$_L$-rhamnopyranoside and *ent*-8(17)-labden-15-*O*-(3′-*O*-acetyl)-α-$_L$-rhamnopyranoside) were isolated from a propolis sample from the Eastern region of El Salvador, and both were reported by the first time in propolis. These new labdenol glycosides exhibited good antibacterial activity against *S. aureus* (21.0 ± 1 and 20.3 ± 0.6 mm at 4 mg/mL, respectively) and showed to be more effective than the propolis ethanolic extract (12 ± 1 mm at 4 mg/mL), but those glycosides did not induce any effect on *E. coli* and *C. albicans*. Additionally, *ent*-8(17)-labden-15-*O*-(3′-*O*-acetyl)-α-$_L$-

rhamnopyranoside exhibited a better cytotoxicity (LC_{50}: 15 ± 7 mg/mL) than the extract (LC_{50}: 39 ± 9 mg/mL) in brine shrimp lethality bioassay [127].

2.6. Honduras

In Central America, there are a high richness of endemic species, and since chalcones and diterpene glycosides were found in Salvadoran propolis [126, 127], and not in other tropical propolis analysed (Cuban, Brazilian and Mexican), the chemical investigation of other sample from Central America was carried out in order to establish differences and similarities in tropical propolis from Americas. To the best of our knowledge, only one study of Honduran propolis has been reported, wherein a sample collected in Marcala was fractionated and led to the isolation of cinnamic ester derivatives, including a new (E,Z)-cinnamyl cinnamate, in addition to flavanones, triterpenes, aromatic acids and one chalcone. Honduran propolis inhibited the ATPase activity of Pdr5p (70% at 100 μg/mL), a protein responsible for a multidrug resistance phenotype in *Saccharomyces cerevisiae*, and four of its most abundant constituents (*E*)-Cinnamyl-(*E*)-cinnamate (IC_{50}: 2.58 μM), (*E*)-Cinnamyl-(*E*)-*p*-coumarate (IC_{50}: 1.54 μM), 6*β*-Hydroxy-3-oxo-lup-20(29)-en-28-oic acid (IC_{50}: 1.03 μM) and sakuranetin (IC_{50}: 1.20 μM) were also potent inhibitors [88].

The presence of these cinnamic ester derivatives has been described in exudates and volatile fractions of *L. styraciflua* (Honduras styrax, Hamamelidaceae). Interestingly, high amount of the triterpene compound 6*β*-hydroxy-3-oxolup-20(29)-en-28-oic acid have been reported in the cones of *L. styraciflua*, which suggested that *L. styraciflua* would be the botanical source of this Honduran propolis sample, in addition to the relative abundance of this plant in the surrounding areas to the beehives [88]. Cinnamic ester derivatives and 6*β*-hydroxy-3-oxolup-20(29)-en-28-oic acid were later described in North Carolina propolis, in addition to the presence of characteristic poplar flavonoids. Therefore, *L. styraciflua* exudates were proposed as secondary botanical source of North Carolina propolis. In that sense, Honduran and North Carolina propolis share a common plant origin, changing in some way the paradigm of poplar-type propolis in temperate areas, such as North Carolina, by additional incorporation of resins gathered from plants present in tropical and temperate zones of North America [87, 88].

3. South American propolis

South America is characterized by possessing the highest plant diversity of any other region in the world, which is due to several aspects, including its continental size and location (latitude and longitude), the presence of the largest extension of tropical forest and finally the Andes Mountains that form the biggest mountain system in the world, representing a linkage between tropical and temperate latitudes across South America [128, 129]. Propolis samples from different regions of South America have been studied. Brazilian propolis is by far the most analysed propolis in South America, followed by Argentinean, Chilean, Uruguayan and Venezuelan. Recently, propolis from Colombia and Bolivia has been studied; meanwhile,

Peruvian propolis has only been included in biological comparative studies. In this section, most relevant and recent advances in South American propolis research topic are summarized.

3.1. Colombia

Colombian vegetation possesses a great biodiversity with a high number of endemic plant species distributed in a variety of tropical forests, steppe and grasslands, representing a source of great variety in phytochemical substances [130]. Propolis is used in Colombia as a folk remedy, and in cosmetic and food industries, however, few studies in Colombian propolis have been done [131]. In a recent study, propolis collected from Medellin region, and subsequently extracted with n-hexane/methanol and fractioned with dichloromethane, exhibited an inhibitory effect on mycelial growth against *Botryodiplodia theobromae* (23.5% at 1 mg/mL) and two different strains of *Colletotrichum gloeosporioides* (38.1 and 47.6% at 1 mg/mL, respectively), which are important postharvest fungi that affect tropical and subtropical fruits. Through antifungal bioassay-guided fractionation of dichlorometane fraction, three labdan-type diterpenes were characterized: isocupressic acid [15-hydroxylabda-8(17),13E-dien-19-oic acid], (+)-agathadiol [labda-8(17),13-diene-15,19-diol] and epi-13-torulosol [8(17),14-1abda-dien-13*S*, 19-diol], chemical compounds that could be responsible of the antifungal effect of Colombian propolis [131].

The presence of isocupressic acid and other labdan diterpenes has been described in Brazilian green propolis, a propolis type that has its plant sources in the resins of *Baccharis* spp. and *Araucaria heterophylla* [23]. In addition, the occurrence of agathadiol, isocupressic acid and torulosol has been reported in Algerian propolis, where its main botanical origin seems to be *Cistus* spp. (Cistaceae) [132]. In temperate regions of America, including Central America, inhabit three genera of Cistaceae (*Crocanthemum* spp., *Hudsonia* spp. and *Lechea* spp.), however, in order to draw a phytochemical origin of those compounds, the botanical origin has to be investigated. Moreover, in another study, a propolis sample collected from La Union (Antioquia), showed a weak to moderate antifungal activity (MIC_{50}: >1 mg/mL) on *C. acutatum*, *C. gloeosporioides*, *Aspegillus* sp. and *Penicillium* sp., in addition to a bacteriostatic (1.0 mg/mL) and bactericidal (10.0 mg/mL) effect against *B. subtilis* (Gram+). By GC–MS were detected fatty acids, and their esters, sesquiterpenes, pentacyclic tripterpenes and bicyclic labdan-type diterpenes, such as isocupressic acid, agathadiol and 13-epi-turolosol [133]. The presence of terpenes in both analysed samples of Colombian propolis suggested a possible common botanical source that has to be investigated in order to understand the origin of those phytochemicals and the biological activities of Colombian propolis.

3.2. Venezuela

As Colombia, Venezuela is a tropical region in South America that represents an enormous source of endemic plants and a great variety of bioactive compounds. One of the first chemical compositional analysis in tropical propolis was performed with 38 samples collected in different tropical areas of Venezuela, including Bolívar, Nueva Esparta, Cojedes, Yaracuy, Monagas, Mérida, Táchira, Portuguesa, Sucre, Baringas, Aragua and Carabobo, with the aim to identify phytochemical evidence for the botanical origin of Venezuelan propolis. Most of

these propolis samples contained similar chromatographic patterns characteristic of phenolic compounds, specifically of prenylated benzophenones, since flavonoids were identified only in few samples, and they were methylated 6-oxygenated flavones. The HPLC profile of resins excreted by the flowers of *Clusia minor* provides a very similar phenolic profile, suggesting that the botanical source of polyprenylated benzophenones present in Venezuelan propolis was the exudates from *C. minor* and other *Clusia* spp. [107].

Moreover, in a later study on Venezuelan propolis from Trujillo State, the presence of the already reported polyisoprenylated benzophenones scrobiculatones A and B was identified, together with their derivatives 18-ethyloxy-17-hydroxy-17,18-dihydroscrobiculatone A and 18-ethyloxy-17-hydroxy-17,18-dihydroscrobiculatone B (first time described in this study). Additionally, a mixture of scrobioculatones A-B exhibited a significant antibacterial activity against *S. aureus* (MIC: 125 μg/mL), a moderate toxicity towards *A. salina* nauplii (LC_{50}: 14 ± 6 μg/mL) and low FRS activity by DPPH assay (10%) [134]. The chemical composition and the antioxidant effect of other three samples (V-1, V-2 and V-3) collected in San Antonio de los Altos, Venezuela, was subsequently analysed. The presence of prenylated benzophenone derivatives, diterpenes and triterpenes was detected by GC–MS, suggesting again the participation of *Clusia* spp. resins in Venezuelan propolis collected from different regions. Additionally, propolis samples (0.04% wt.) increased the oxidation stability of triacylglycerol molecules in comparison with control, and the effect of V-2 and V-3 samples was higher than V-1 (approximately 1.5-fold) [135].

3.3. Peru

Peruvian propolis has not yet been analysed from the chemical compositional point of view; however, there is a comparative report wherein propolis from Peru, Brazil, Netherlands and China was studied to determine the cytotoxic, hepatoprotective and FRS capacity (DPPH method) of the collected samples. Methanol (MeOH ext.) and water (Wt ext.) extracts were prepared for each propolis samples. Peruvian propolis showed the lowest FRS activity among the other propolis samples (ED_{50}: MeOH ext. 82.3 μg/mL and Wt ext. 94.9 μg/mL), although it exhibited moderate cytotoxicity on murine colon 26-L5 carcinoma (51.1 μg/mL) and HT-1080 fibrosarcoma cells (76.4 μg/mL). The hepatoprotective activity of propolis was tested on primary cultures of mouse hepatocytes induced to cell death with D-galactosamine (D-GalN) and tumor necrosis factor-α (TNF-α), wherein the MeOH extract of Peruvian sample showed toxicity at 200 μg/mL; meanwhile, the water extract exhibited significant hepatoprotective activity at 200 μg/mL (approximately 45%) [136].

3.4. Bolivia

Bolivia is located at the centre of South America, and its territory comprises a transition into humid tropical and dry subtropical climatic zones. The geographical characteristics result in a great biodiversity present along the country; however, scarce phytochemical studies of Bolivian flora have been done. Propolis is used in Bolivia as antimicrobial remedy to treat infections and respiratory illnesses [137]. Bolivian propolis has been recently studied, and according to the chemical profile of ten samples collected from the main centres of beekeeping

in Bolivia, two main types of propolis have been identified. The first correspond to propolis from the valley regions (Cochabamba, Chuquisaca and Tarija) which were characterized by the presence of prenylated phenylpropanoids in a high amount, including *p*-coumaric and caffeic acid derivatives, such as drupanin (3-prenyl-p-coumaric acid) and artepillin C (3,5-diprenyl-p-coumaric acid) that were previously reported on Brazilian green propolis resulting from *Baccharis dracunculifolia* exudates [137, 138]. Meanwhile, the second type of Bolivian propolis came from La Paz and Santa Cruz regions, and their chemical composition was mainly comprised by cycloartane and pentacyclic triterpenes, including cycloart-24-en-3β,26-diol, cycloart-24-en-3-one, cycloart-24-en-26-ol-3-one, mangiferonic acid methyl ester and lup-20(29)-en-3-one, which were triterpenes identified for first time in propolis in general. The antioxidant capacity of these ten samples was determined by DPPH, FRAP and ABTS assays, propolis samples rich in phenolic compounds presented moderate to strong antioxidant activity, while propolis rich in triterpenes showed a weak active [137].

Moreover, the antimicrobial properties of those ten Bolivian propolis samples and their main constituents were tested against 11 bacterial pathogenic strains of clinical relevance (*S. aureus* ATCC 25923, methicillin-resistant *S. aureus* ATCC 43300, *Escherichia coli* ATCC 25922, the clinical isolated *E. coli* 121, *E. coli* 122, *E. coli* LM2, *Salmonella enteritidis* MI, *Salmonella* sp. (LM), *Yersinia enterocolitica*-PI, *Pseudomonas* sp. and *Proteus mirabilis* 94-2) using micro-broth dilution method. Propolis samples exhibited different effects, from inactive (MICs > 1000 μg/mL) to low (MICs 250–1000 μg/mL), moderate (62.5–125 μg/mL) and high antibacterial activity (MIC 31.2 μg/mL). The samples that were rich in phenolics showed the high antibacterial activity; in comparison, terpene-rich samples were mostly inactive and some presented low activity. Kaempferol-3-methyl ether and drupanin were the most active constituents of Bolivian propolis. Additionally, the activity of propolis samples towards promastigotes of *Leishmania amazonensis* and *L. braziliensis* was evaluated and the results obtained were similar to those of antibacterial assays. The most active samples against *L. amazonensis* and *L. braziliensis* were from Cochabamba (IC_{50}: 12.1 and 7.8 μg/mL, respectively) and Tarija (IC_{50}: 8.0 and 10.9 μg/mL, respectively) [139]. These studies provided an insight in phytochemical variations of propolis from different regions devoted to beekeeping in Bolivia, suggesting the participation of at least two different botanical resins, Which should be characterized in order to associate the pharmacological activities of those propolis samples to a certain plant origin. These results suggested that Bolivian phenolic-rich propolis could a promissory source of antibacterial and antiparasitic therapeutic agents.

3.5. Brazil

Brazil has an extensive territory that comprises the richest flora in the world, a huge plant biodiversity with over 56,000 species, which represents approximatively 19% of the total flora of the world [140]. Thus, Brazilian propolis possess a particular chemical diversity, involving different plant resins from dissimilar eco-regions, resulting in a variety of propolis types, including those with characteristic chemical composition of both tropical and temperate regions. At present, Brazilian propolis has been by far one of the most investigated propolis types, and a significant number of chemical compounds have been identified in samples

collected throughout the country. Due to the large amount of chemical and pharmacological studies of Brazilian propolis, herein, we present the most significant reports in an attempt to summarize hundreds of studies.

One of the first studies, focused on chemical characterization of bioactive constituents of Brazilian propolis, was carried out with a sample collected in Sao Paulo state, which yielded the isolation of three new compounds: 3,5-diprenyl-4-hydroxycinnamic acid, 3-prenyl-4-dihydrocinnamoloxycinnamic acid and 2,2-dimethyl-6-carboxyethenyl-2H-1-benzopyran [141]. These Brazilian propolis constituents presented antimicrobial activity against *Bacillus cereus* (MIC 15.6, 31.3 and 125 μg/mL, respectively), *Enterobacter aerogenes* (31.3, 62.5 and 125 μg/mL, respectively) and towards *Arthroderma benhamiae* (15.6, >250 and 62.5 μg/mL, respectively). Although the occurrence of cinnamic acid and other phenylpropanoid acid derivatives has been previously reported in propolis from temperate regions, the finding of prenylated derivatives was new for propolis [141].

In other studies, the occurrence of fifteen more cinnamic acid derivatives, in addition to nine *p*-coumaric acid derivatives, was identified from two different samples collected in Minas Gerais, together with other compounds, including six caffeoyl quinic acid derivatives, nine flavonoids, one prenylated phenolic acid, four diterpenoic acids and one lignin [142, 143]. Additionally, benzofuran derivatives (A and B) together with two known isoprenylated compounds were isolated from Brazilian propolis (not specified region). A moderate cytotoxicity was showed by benzofurans A and B against highly liver-metastatic murine colon 26-L5 carcinoma (12.4 and 13.7 μg/mL) and human HT-1080 fibrosarcoma cells (13.9 and 43.2 μg/mL) [144]. At present, hundreds of studies have been performed in order to chemically characterize the different samples of Brazilian propolis, revealing the great variety of compounds in those samples, including more than 145 constituents that have been identified and some of them were additionally tested to characterize their pharmacological activities [28, 58].

In order to establish the basis of Brazilian propolis classification, 500 samples were collected from different regions, including southern (Paraná and Rio Grande do Sul), Southeastern (Sao Paulo, Minas Gerais) and Northeastern (Bahía, Piauí, Ceará and Pernambuco) of Brazil. According to their chemical profile, the samples were classified into 12 groups, wherein five groups were identified in the Southern, one from Southeastern and six from Northeastern Brazil, suggesting a greater plant diversity on Northeastern and Southern than Southeastern region [145, 146]. The antimicrobial activity against *S. aureus* and *S. mutans* was tested, and propolis from group 6 originated from Bahia state (Northeastern Brazil) showed the highest inhibitory effect on both microorganisms (inhibition zone: 6 and 9 mm, respectively) [146]. Subsequently, the botanical origin of one representative group of each region was investigated. The foraging behaviour of honeybees that produced Brazilian brown propolis (BBP) from the South (Paraná, group 3), Brazilian red propolis (BRP) collected in Northeastern (Bahia, group 6) and Brazilian green propolis (BGP) collected from Southeastern (Sao Paulo, group 12) was observed to identify which plant is visited to gather the respective resins. The plant buds or unexpanded leaves were cut off to extract the resinous material, and using reversed-phase high-performance thin-layer chromatography (RPHPTLC), reversed-phase high performance liquid chromatography (RPHPLC), and GC–MS, the plant origin was revealed [145].

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The main compounds identified in propolis from group 3 were flavonoids and organic acids, and the chemical profile of that sample was similar to that of poplar exudates, in particular to *Populus alba*, which were assumed as the main botanical source of this propolis from Paraná state. Interestingly, poplar trees are not native in Southern South America; nevertheless, European immigrants planted poplar trees in the temperate Southern Brazil [71, 147]. In propolis from group 6, the presence of some aromatic compounds, terpenoids and fatty acid esters was recognized and exudates from *Hyptis divaricata* Pohl were suggested to be the main botanical source according to the chromatographic profile [71]. However, since some disparities were observed in the chemical profile of *H. divaricata* and propolis of group 6, in addition to the identification of prenylated benzophenones (hyperibone A) in a later study, both facts suggested the participation of resins from some species of *Clusia* genus as supplementary botanical source [58, 148].

In group 12, the presence of prenylated derivatives of *p*-coumaric acid, the characteristic artepillin C (3,5-diprenyl-4-hydroxycinnamic acid), among other aromatic acids (dihydrocinnamic, *p*-coumaric, ferulic, caffeic and caffeoylquinic acids) and some flavonoids, including kaempferid, 5,6,7-trihydroxy-3,4′-dimethoxyflavone and aromadendrine-4′-methyl ether, and other compounds were found. Based on chemical evidence, *Baccharis dracunculifolia* resins resulted to be the main botanical source of this propolis type, in addition to *Araucaria angustifolia* and *Eucalyptus citriodora* exudates [59, 71]. The antibacterial and antifungal effect of propolis from group 12 against *S. aureus* and *C. albicans* (10.5 and 15 mm of inhibition zone, respectively) allowed to determine that the most bioactive resins came from *B. dracunculifolia* leaf exudates (9 and 16 mm, respectively) [59]. Subsequent studies on BGP and *B. dracunculifolia* exudates (Minas Gerais, group 12) by HPLC–APCI–MS and GC–MS allowed to identify 126 constituents present in those samples, including mainly cinnamic acid and its derivatives, flavonoids, benzoic acid and a few benzoates, non-hydroxylated aromatics, and aliphatic acids and esters, in addition to prenylated compounds, alkanes and terpenoids [138]. Moreover, particular triterpenoids have been identified in BGP, including lupeol, esters of lupeol, α- and β-amyrins, cycloartenol, lanost-7,24-dien-β-ol [58].

With the aim to establish a more precise correlation among Brazilian propolis types, thirty-eight samples were collected, from Minas Gerais, Sao Paulo, Paraná, Mato Grosso do Sul, Bahia and Algoas, and were analysed by ESI-MS and tandem mass spectrometry (ESI-MS/MS). A principal component analysis (PCA) has been applied to determine similarities and differences in the secondary metabolite fingerprint of propolis samples. Brazilian propolis was divided into groups, according to their chemical profile and geographical origins. BRP from Algoas and Bahia was divided into two main groups (R1 and R2), and as well propolis from South and Southeastern Brazil were divided in one BGP group, which contained the greatest number of samples, and two groups of BBP (B1 and B2) from Paraná state. Seven compounds were used as markers and allowed the classification of Brazilian propolis into those five groups according to PCA analysis, wherein the presence of intersection of constituents among samples was detected. Pinocembrin was in BRP R2 and BBP B1, and chsysin in BBP B1, 2,2-dimethyl-6-carboxyethenyl-2H-1-benzopyran in BBP B2, 3-prenyl-4-hydroxycinnamic acid, 3,5-diprenyl-4-hydroxycinnamic acid and dicaffeoylquinic acid were identified in BGP and BBP B2, *p*-

coumaric acid in BGP and BBP (B1 and B2), and 3-methoxy-4-hydroxycinnamaldehyde in BBP (B1 and B2). As observed, there are chemical crossing in the five groups [84].

In regard to the chemical composition of BRP, recent studies have reported the presence of prenylated benzophenones, such as guttiferone E/xanthochymol mixture, the isoflavonoids isosativan and medicarpin, the triterpenoid ketone 20(29)-lupen-3-one, and also a naphthoquinone epoxide (isolated for the first time from a natural source) in a sample collected from the state of Algoas [124]. The presence of these compounds suggested a mixed plant origin, since prenylated benzophenones are described in floral resins of *Clusia* species (Clusiaceae); in particular, the mixture of guttiferone E/xanthochymol and the triterpenoid have been reported in Cuban propolis [58]. Meanwhile, isoflavonoids have been related to *Dalbergia* species (Leguminosae). Additionally, isosativan, medicarpin and the guttiferone E/xanthochymol mixture presented antibacterial activity by inhibiting *S. aureus* (14, 23 and 19 mm), *E. coli* (0, 14 and 12 mm) and *C. albicans* (15, 26 and 0) growth. Moreover, the mixture guttiferone E/xanthochymol exhibited FRS activity by DPPH assay (49% at 48 μM) [124]. It is also reported that the presence of retusapurpurins A and B (C30 isoflavanes) provides the red pigment to those red propolis in different regions of Americas [125].

More studies on chemical composition of BRP collected from Algoas have been done, identifying methyl o-orsellinate, methyl abietate, medicarpin, 2,4,6-trimethylphenol, and the isoflavonoids, homopterocarpin, 4′,7-dimethoxy-2′-isoflavonol and 7,4′-dihydroxyisoflavone. Biological tests have been carried out for the BRP extract, including antimicrobial against *S. aureus* ATCC 25923 and *S. mutans* UA159 (MIC: 25–50 μg/mL), antioxidant (57% at 90 μg/mL) and cytotoxic activity against HeLa cell line (IC_{50}: 7.45 μg/mL) [149]. In another study of BRP, the cytotoxicity of a sample collected from the South coast of Paraiba State was evaluated on Human pancreatic cancer cells (PANC-1), and since Paraiba BRP exhibited a 100% cytotoxicity at 10 μg/mL, a subsequent phytochemical analysis was carried out and led to the isolation of 43 compounds, mainly flavonoids, including pterocarpanes, flavanonols, isoflavanones, chalcones, isoflavans, isoflavones, flavanones, lignans, a flavonol, a isoflavanonol and a neoflavonoid. Three novel compounds: (6aS,11aS)-6a-ethoxymedicarpan, 2-(2′,4′-dihydroxyphenyl)-3-methyl-6-methoxybenzofuran and 2,6-dihydroxy-2- [(4-hydroxyphenyl)methyl]-3-benzofuranone, in addition to (6aR,11aR)-3,8-dihydroxy-9-methoxy-9-methoxypterocarpan, exhibited the most potent effect (100% cytotoxicity at 12.5 μM) on PANC-1 cells [123].

The chemical composition of an essential oil extract obtained from Brazilian propolis collected in Rio de Janeiro was characterized, and 26 constituents were identified, wherein β-caryophyllene (12.7%), acetophenone (12.3%) and β-farnasene (9.2%) were the major constituents, in addition to the new compounds found linalool (6.47%), -elemene (6.25%), methyl hydrocinnamate, ethyl hydrocinnamate, α-ylangene and valencene. This essential oil extract presented antimicrobial activity against *S. aureus* (14 mm, inhibition zone), *S. epidermidis* 25/04 (10 mm), *S. epidermidis* 194/02 (10 mm), *S. pyogenes* 93007 (14 mm), *S. pyogenes* 75194 (18 mm) and *E. coli* (17 mm) by agar diffusion method [150].

Since Brazilian propolis is continuously produced all over the year, it was important to determine the seasonal effect on propolis composition and evaluate the influence of season on its biological activities. By GC and GC–MS, the seasonal variations in chemical composition of

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BGP collected from Sao Paulo state were investigated. All the seasonal samples contained phenolic compounds, mainly cinnamic acid derivatives as major constituents; however, the presence of diterpenes appeared in summer sample and reached a predominate percentage in the autumn sample, but being absent during the other seasons, which suggested the participation of at least two plants as botanical source [59]. These chemical variations could be important for the practical application of propolis, and in order to evaluate the effect of these slight changes on Brazilian propolis composition, antibacterial assays on bacterial strains isolated from human infections were carried out. The growth of Gram-positive bacteria, *S. aureus*, was inhibited at low concentrations of seasonal propolis samples (0.4% v/v), whereas Gram-negative, *E. coli*, bacteria were less susceptible to propolis treatment. It was concluded that there was no significant difference on the inhibitory action of propolis samples, discarding a seasonal effect on antibacterial activity of propolis from Sao Paulo, Brazil [30].

Propolis possesses immunomodulatory activities, and it has been reported that its immunostimulant effect is produced via macrophage activation, which enhances macrophage phagocytic capacity [28, 151, 152]. The effect of BGP on activation by reactive oxygen (H_2O_2) and nitrogen (NO) species was determined on peritoneal macrophages obtained from male BALB/c mice. Both *in vivo* and *in vitro* experiments were carried out, and propolis stimulation resulted to induce a slight augmentation in H_2O_2 releasing and a moderate inhibition of NO generation in a dose-dependent manner, suggesting that propolis acts on host non-specific immunity by macrophage activation [153]. Moreover, the effects of BGP on fungicidal activity of macrophages against *Paracoccidioides brasiliensis* were evaluated. Considering that cell-mediated immunity plays a significant role in host defence against this pathogen, peritoneal macrophages from BALB/c mice were stimulated with BGP and subsequently challenged with *P. brasiliensis*. This study suggested an increase in fungicidal activity of macrophages after propolis stimulation [151].

The role of toll-like receptors (TLR) in microbial pattern recognition and as well as the action of pro-inflammatory cytokines are important to trigger the initial events of immune response. Thus, the immunomodulatory action of BGP has been evaluated in regard to those mechanisms of innate immunity, and BGP treatment resulted to upregulate TLR-2 and TLR-4 expression, together with the production of pro-inflammatory cytokines (IL-1β and IL-6) in peritoneal macrophages and spleen cells of Male BALB/c mice treated with BGP of Sao Paulo State (200 mg/kg) for 3 days, suggesting the favourable action of BGP by enhancing immune responses in macrophages and spleen cells of treated mice [154]. Moreover, the action of BGP on antibody production was studied in rats immunized with bovine serum albumin, wherein BGP (10%), independently of the season of the year, stimulated antibody production, which was concluded to be a consequence of synergic effects, since isolated compounds and *B. dracunculifolia* exudates did not induced the antibody production [155].

Other studies in regard to the effects of BGP on immune response of acutely and chronically challenged stressed mice have been also investigated. After 3 days treatment under acutely stress conditions, BGP (200 mg/kg) restored TLR-2 and TLR-4 expression and increased IL-4 production in mice, in comparison with control [156, 157]. Meanwhile, BGP (200 mg/kg) during 7 days treatment in stressed mice increased the production of H_2O_2 in macrophages and

reduced the alterations found in spleen [158]. Moreover, the BGP treatment (200 mg/kg) for 14 days in mice submitted to chronic stress exerted similar effects, demonstrating these experiments the immunomodulatory activity of BGP under *in vivo* experimental stress conditions [28, 152, 159, 160].

Once the effect of BGP on the initial steps of the immune response was evaluated in murine models, the immunomodulatory effect of BGP was evaluated on receptors expression, cytokine production and fungicidal activity of human monocytes. The BGP treatment (5, 10, 25, 50 and 100 μg/mL) resulted to upregulate TLR-4 and CD80 expression and decreased the production of TNF-α and IL-10 as concentration treatment increased. The fungicidal activity of human monocytes after incubation with BGP and challenged with *C. albicans* was increased in a dose-dependent manner. Moreover, cytokine production was reduced by blocking TLR-4, whereas the fungicidal activity was affected by blocking TLR-2, suggesting the involvement of these receptors in the mechanism of action of BGP [161].

Later, in an attempt to investigate the BGP constituents involved in the immunomodulatory effects on the innate immunity, caffeic and cinnamic acids (5, 10, 25, 50 and 100 μg/mL) have been assessed on human monocytes. Caffeic acid downregulated TLR-2 and HLA-DR expression. Otherwise, cinnamic acid downregulated TLR-2, HLA-DR and CD80; meanwhile, it upregulated TLR-4 expression depending on concentration. Both phenolic acids inhibited TNF-α and IL-10 production, whereas they increased the fungicidal activity of monocytes against *C. albicans*, without affecting cell viability. These data suggested that caffeic and cinnamic acid are partially involved in BGP effect on cell receptors expression and cytokine production, although the fungicidal activity of monocytes treated with those phenolic acids could be due to different mechanisms, possibly involving reactive oxygen and nitrogen species [162, 163].

In another study, the antioxidant and anti-inflammatory action of BGP and caffeic acid were determined. By DPPH assay, FRS activity of caffeic acid (EC_{50}: 2.5 μg/mL) resulted to be more effective than that of BGP (EC_{50}: 18.51 μg/mL), and the treatment with BGP and caffeic acid (5, 10, 25, 50 and 100 μg/mL) exerted anti-inflammatory action, by inhibiting nitric oxide (NO) production in RAW 264.7 cells. Moreover, both treatments suppressed LPS-induced signalling pathways, namely p38 MAPK, JNK1/2 and NF-κB, and did not induce hepatotoxicity at the tested concentrations, suggesting that caffeic acid may be involved in the antioxidant and anti-inflammatory effects of BGP [164]. BGP and its main botanical source, *B. dracunculifolia* exudates have been demonstrated to exert cytotoxic effect on several human cancer cell lines, including HEp-2, CaCo2, HCT116, HT-29 and SW480, and moreover on canine osteosarcome cells [165–168].

All these results obtained for Brazilian propolis reflect the enormous plant biodiversity of the country, obtaining different phytochemical patterns in samples collected from different geographical origins. Therefore, it is necessary to further investigate the chemical composition of samples produced in different zones in order to understand the plant origin of the bioactive compounds present in propolis from entire Brazil. The broad pharmacological activities tested for Brazilian green, red and brown propolis are a great example of biological interest in this beehive product, which makes it one of the most characterized propolis in the world. It is

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important to continue the studies on Brazilian propolis to provide a more precisely insight about the possibilities of tropical, temperate and a mixture of both types of propolis from this biodiverse country.

3.6. Uruguay

Uruguay is covered by the temperate sub-humid grasslands, a biome extended through the vast plains of Southern South America, including part of Northeast and Central Argentina and Southern Brazil [169]. Uruguayan propolis has been investigated, and one of the first studies reported the identification of 22 different phenolic acids and flavonoids in six samples collected from different regions of the country. The presence of these constituents was detected by HPLC–PDA–UV, and resins from *Eucalyptus globulus*, *Populus* sp., *Betula* sp. and *Salix* sp. were suggested to be the botanical source of Uruguayan propolis. Those six propolis samples showed antibacterial activity against *B. subtilis* and *S. aureus* (MIC: 80–130 μg/mL); otherwise, the inhibitory effect of Uruguayan propolis against *E. coli* (MIC: 800–1000 μg/mL) was less efficient. Alkylperoxyl radical (ROO●) scavenging potential activity was additionally exhibited by those propolis samples (3.4–4.1 μg/mL) [170].

In another study, the chemical constitution of propolis collected from Montevideo was further investigated using HPLC–MS and NMR techniques and led to the isolation of eighteen flavonoids, four aromatic carboxylic acids and eleven phenolic acid esters, in addition to 3 new compounds elucidated: pinobanksin-3-*O*-isobutyrate and pinobanksin-3-*O*-(2-methyl)butyrate and 2-methyl-2-butenyl ferulate. The major constituents in that sample were pinobanksin-3-*O*-propanoate, pinobanksin-3-*O*-acetate, pinobanksin, pinostrobin, pinocembrin, techtochrysin, chrysin, galangin and cinnamyl *p*-coumarate, which suggested similarities to Southern Brazil, North American and European propolis [171]. Moreover, by RP–HPTLC, GC–MS and RP–HPLC analyses, it was determined that the phytochemical profile of propolis from Southern Brazil, Argentina and Uruguay was similar and correlated with that of *P. alba* resins; therefore, it was concluded that resins of poplar trees are the main plant origin of Uruguayan propolis [147].

Subsequently, a propolis sample from Montevideo was included in a comparative study with different geographical samples, wherein Uruguayan propolis was qualitatively similar to those from the United States, New Zealand, Hungary, China and other temperate samples included. Additionally, Uruguayan propolis presented a moderate antioxidant activity by a FRS method (DPPH assay: ≥30% at 20.0 μg/mL) and by β-carotene–linoleic acid system (≥45% at 10.0 μg/mL) [82]. The antioxidant properties of ten propolis samples collected in Southern Uruguay were further evaluated by *in vitro* (FRS by ORAC and inhibition of lipid and protein oxidation) and by cellular assays. Uruguayan propolis showed a high FRS activity by ORAC assay (800 μmol trolox equiv/g propolis). Uruguayan propolis inhibited LDL lipoperoxidation and protein nitration, and it was effective at cellular level by increasing endothelial nitric oxide synthase (eNOS) expression and inhibited endothelial NADPH oxidase, indicating a potential benefit by increasing nitric oxide bioavailability in the endothelium [172].

The volatile compounds of three samples from Central-southern Uruguay were analysed by static headspace technique coupled with a GC–MS, and compared with volatile content of

Brazilian, Estonian and Chinese propolis. Monoterpenes (α- and β-pinenes) were predominant, and the volatile profile of Uruguayan and Brazilian propolis was composed mainly by α-pinene and β-pinene (64.6–77.6%). Brazilian propolis was distinguished by a high amount of β-methyl crotonaldehyde (10.1%), and one of Uruguayan samples displayed the presence of limonene (15.6%). By principal component analysis of the volatile profile found in samples showed high differences, falling into separate clusters. In this study, the geographical origin of Uruguayan and Brazilian propolis is not specified; however, by the results obtained, it could be suggested that Brazilian propolis is from the southern region, since the similarities in volatile composition to Uruguayan propolis [173]. The results obtained in these studies for Uruguayan propolis confirm the chemical characteristics of propolis from temperate zones.

3.7. Argentina

Argentina has a vast territory and a great diversity of ecological regions, including subtropical rain forests and temperate sub-humid grasslands or "Pampas." However, two thirds of Argentinean mainland is comprised by extensive arid and semi-arid region between the Andes and cold subantartic zones. Natural arid and semi-arid plain regions include western Chaco, Monte and Patagonian steppe, and these are ecologically similar to those semi-arid regions of North America, present in Sonora, Sinaloa and Great Basin, respectively [174, 175]. Several studies have differentiated propolis into two main groups, one from temperate zones and the other from tropical regions; however, few studies have been done in regard to propolis from arid and semi-arid areas [89]. Propolis from different regions of Argentina has been investigated, twenty-five samples collected in temperate, arid and semi-arid lands in Northern Argentina (Santiago del Estero, Tucumán, Chaco, Salta, Catamarca, Jujuy and Misiones) were chemically and biologically analysed. By absorption spectra, RP–HPTLC and RP–HPLC analysis, it was found that 16 of the 25 samples presented a similar phenolic profile to that exhibited by samples from Southern Brazil and Uruguay. The main phenolic constituents identified in those 16 samples were pinobanksin, pinocembrin, chrysin, galangin, tectochrysin and 1,1-dimethylallylcaffeic acid [176]. Propolis samples from Catamarca and Tucumán presented the highest phenolic content, exhibiting greater amounts of pinocembrin, 1,1-dimethylallylcaffeic acid, ferulic acid and cinnamic acid in comparison with the other 20 samples analyzed by Isla et al. [176]. Additionally, these two propolis presented the higher antibacterial activity against *S. aureus ATCC* 25923 As well (inhibition zone: 4 and 5 mm, respectively) and presented the high FRS activity by DPPH assay (>40%) [176].

Moreover, other biological properties of propolis from Tucumán have been tested, including cytoxicity with the lethality test of *A. salina* (LD_{50}: 100 μg/mL), toxicity and mutagenicity on both *S. typhimurium* TA98 and TA100 (did not exhibit toxicity at 300 μg/plate), genotoxicity on *Allium cepa* (not presented) and antimutagenicity, inhibiting the effect of isoquinoline (IQ) and 4-nitro-*o*-phenylenediamine (NPD) (ID_{50}: 40 and 20 μg/plate, respectively). Furthermore, the chemical constituent 2′,4′-dihydroxychalcone of Tucumán propolis showed cytotoxic activity (LC_{50}: 0.5 μg/mL) and was able to inhibit the mutagenicity of IQ (ID_{50}: 1 μg/plate), whereas genotoxic or mutagenic effects were not observed [177]. Additionally, the antimycotic activity of Tucumán propolis and its compounds, pinocembrin and galangin, has been tested against

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Trichoderma spp., *Penicillium notatum*, *Aspergillus niger*, *Fusarium* sp., *Phomopsis* spp., *Saccharomyces carlsbergensis*, *Rhodotorula* spp. by bioautography, hyphal radial growth, hyphal extent and microdilution in liquid medium. Wherein propolis inhibited filamentous fungal growth and exhibited an antifungal moderate activity (MIC: 77–349 μg/mL). Pinocembrin, and galangin presented higher antifungal activity (MIC: 77 349 μg/mL), and their assumed as partially responsible for the fungitoxic activity of Tucumán propolis [178].

Similar results have been obtained from Tucumán propolis against human opportunistic and pathogenic fungi, specifically on dermatophytes and yeasts (*C. albicans* ATCC 10231, *C. tropicalis* C 131, *S. cerevisiae* ATCC 9763, *C. neoformans* ATCC 32264, *A. flavus* ATCC 9170, *A. fumigatus* ATTC26934, *A. niger* ATCC 9029, *T. rubrum* C 110, *T. mentagrophytes* ATCC 9972, and *M. gypseum* C 115). All the dermatophytes and yeasts were inhibited by three different Tucumán propolis samples (MIC: 16–125 μg/mL). The most susceptible fungi were *M. gypseum*, *T. mentagrophytes* and *T. rubrum*. A bioassay-guided isolation of Tucumán propolis and chemical characterization by NMR and HPLC–ESI-MS/MS yield two bioactive chalcones: 2′,4′-dihydroxy-3′-methoxychalcone and 2′,4′dihydroxychalcone that displayed strong activity against clinical isolates of *T. rubrum* and *T. mentagrophytes* (MIC: 1.9–2.9 μg/mL). In addition, pinocembrin, galangin, 7-hydroxy-8-methoxyflavanone presented a moderate antifungal activity. By the presence of those compounds was identified in exudates of *Zuccagnia punctata* Cav. (Caesalpinieae), which was assigned as the botanical origin from Tucumán propolis samples [179].

Other studies focused on the characterization of propolis from Catamarca Province in Northwestern Argentina have been done. Propolis collected from El rincón presented FRS activity by ABTS assay (SC_{50}: 6.9 μg/mL) and by β-carotene–linoleic acid antioxidant assay (SC_{50}: 2.0 μg/mL), additionally exhibited antibacterial effect on methicillin resistant *S. aureus* (MRSA) by the microdilution method and bioautographic assays (MIC: 65 μg/mL). This propolis led to the isolation and characterization by NMR of twelve compounds, wherein the most bioactives were 2′,4′-dihydroxy-3′-methoxychalcone, 2′,4′-dihydroxychalcone, 2′,4′,4-trihydroxy-6′-methoxychalcone, 5-hydroxy-4′,7′-dimethoxyflavone, 4′,5′-dihydroxy-3,7,8-trimethoxyflavone and 7-hydroxy-5,8-dimethoxyflavone [180].

FRS activity by DPPH assays of Argentinean propolis collected in the regions of Mendoza, Rio Negro, La Pampa, and Entre Rios was evaluated. Almost all of the propolis samples presented FRS activity (40-60% at 20 μg/mL), in exception to the samples from La Pampa and Entre Rios. Greater amounts of caffeic acid, ferulic acid, caffeic acid phenetyl ester were found by HPLC–PDA–UV analysis in propolis samples with the stronger FRS activity. In general, the presence of flavonoids, such pinocembrin, chrysin, pinobanksin, pinobanksin-3-*O*-acetate and galangin, was found in almost all samples [181]. Moreover, three samples collected from two different pythogeographical regions (Prepuna and Monte) exhibited FRS activity by DPPH assays (IC_{50}: 28–43 μg/mL) and by β-carotene–linoleic acid antioxidant assay (IC_{50}: 2.0–8.4 μg/mL, respectively). The samples from Monte region showed the highest inhibitory effect on different strains of *S. aureus* and *E. faecalis* (MIC_{100}: 50 and 100 μg/mL, respectively). Nine compounds were identified by HPLC–DAD–UV, and two of them were only identified in samples from Monte region 2′4′-dihydroxychalcone and 2′,4′-dihydroxy 3′-methoxychalcone. These two

bioactive chalcones, present in propolis from El Rincon and Monte region, have been detected in propolis from Tucumán and in *Zuccagnia punctata*, a perennial shrub in Argentinean arid regions, which is identified as botanical source of this propolis from Argentinean arid lands [182].

The chemical composition of propolis collected from the Andean locality of Bauchaceta in San Juan province was characterized by HPLC–ESI-MS/MS and GC–MS techniques and revealed a lignan and volatile organic acid profile that matched with the exudates of *Larrea nitida*, which was suggested as its main botanical source. Andean propolis presented antifungal activity against Dermatophytes and yeasts (MIC: 31.3–125 μg/mL). A bioassay-guided isolation of the most active compounds yield two lignans characterized as 3′-methyl-nordihydroguaiaretic acid (NDGA) and nordihydroguaiaretic acid (NDGA) that showed high inhibitory activity against *T. mentagrophytes*, *T. rubrum*, *M. gypseum* (15.6–31.25 μg/mL) and clinical isolates of *Candida* spp., *Cryptococcus* spp., *T. rubrum* and *T. mentagrophytes* (MIC: 31.3 62.5 μg/mL). In addition, 4-[4-(4-hydroxy-phenyl)-2,3-dimethyl-butyl]-benzene-1,2-diol, 4′-methyl-nordinydroguaiaretic acid and two epoxylignans meso-(rel 7S,8S,7′R,8′R)-3,4,3′,4′-tetrahydroxy-7,7′-epoxylignan and (7S,8S,7′S,8′S)-3,3′,4′-trihydroxy-4-methoxy-7,7′-epoxylignan) were isolated and identified by their spectroscopic data in NMR experiments. These six compounds were isolated from propolis for the first time [183]. Similar antifungal activity results were obtained from other eleven samples collected in San Juan province, and the identification and isolation of MNDGA and NDGA. The flavonoids chrysin, pinocembrin and galangin were the most bioactive compounds [184].

A reverse phase LC–DAD–MS method has been developed to quantify phenolic acids and flavonoids present in propolis and found that the most abundant constituents from Argentinean, European and Chinese propolis were chrysin (2–4%), pinocembrin (2–4%), pinobanksin-acetate (1.6–3%) and galangin (1–2%) [185]. Furthermore, the typical fingerprint of propolis from Argentina, Italy and Spain was determined by on-line HPLC–ESI/MS analysis, and those propolis samples showed the same total ion chromatogram (TIC) profile, in addition to a similar amount of pinocembrin (39-49% of the total identified flavonoids), suggesting a poplar-type origin for Argentinean propolis [186]. Interestingly, the subtropical montane forest of "El Siambón" in Tucumán region is characterized by the presence of native vegetation including members of Lauraceae, Myrtaceae, Fabaceae, Juglandaceae, Salicaceae and Nyctaginaceae families, as well as the introduced poplar, eucalyptus and pinus trees, which are described as the most visited trees by honeybees for resins in the Tucuman region [178].

However, in another study, propolis samples from 30 different regions of Santiago del Estero province were analysed and classified into three groups according to the main arboreal species in the region. Nineteen chemical constituents were identified in these 30 samples, pinocembrin, quercetin, kaempferol, chrysin, salicylic, 4-hydroxybenzoic, benzoic, ferulic and gallic acids were observed in most of the analysed samples. Some quantitative differences of each component were found in the three groups, and interestingly, no *Populus* species were found in apiary environments, where the most abundant plant species were *Geoffroea decorticans*, *Prosopis alba*, *Prosopis nigra*, *Schinopsis lorentzii*, *Acacia aroma*, *Cercidium praecox*, *Schinus fasciculatus*, and *Larrea divaricata*. These studies suggest that flavonoids characteristic of

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temperate zones, which are present in Argentinean propolis, could be gathered from other plant species than poplar [187].

Furthermore, the antibacterial, antiradical and antioxidant activities of those 30 propolis samples from Santiago del Estero province were analysed, and variability in bioactivity was found. All samples presented a substantially similar inhibitory effect on *S. aureus* ATCC 25923 (8.5–11.4 mm), and about 77% of the samples showed an inhibitory zone with diameter longer than 9 mm. Additionally, there was observed a moderate correlation between antibacterial activity and total polyphenol and flavonoid contents. However, antimicrobial activity correlated better with pinocembrin content than with total polyphenol content. Results for the antioxidant activity by the β-carotene–linoleic acid assay presented high variability among samples (16.2–84.7%) as consequence of quantitative differences in chemical composition. Similar results were obtained by FRS activity on DPPH assay (20.4–89.9%) [188].

All these studies carried out to characterize the chemical composition and biological properties of Argentinean propolis have demonstrated that samples collected from different ecological regions throughout the country exhibited different chemical profiles; however, flavonoids are present in the most of the analysed samples, and chemical compounds such as lignans and chalcones could be used as markers to suggest the participation of a particular botanical source; thus, the phytochemical diversity found in Argentinean propolis has its botanical origin on several plants, including *Populus alba*, *Larrea nitida*, *Larrea divaricata* and *Zuccagnia punctata*.

3.8. Chile

Chile extends along the Southwestern coast of South America, between the geographical barriers of the Andes on the East and Pacific Ocean on the West, resulting in a unique flora developed as consequence of the environmental isolation that consists in many endemic plant species. Chile owns the Atacama Desert on the North, Chilean Mediterranean-type region in North-central, temperate forest in South-central region and subpolar forests at south [189, 190].

Some studies have been carried out in Chilean propolis collected from different regions. A propolis sample from Quebrada Yaquil in Santa Cruz (Region VI) in the Mediterranean semi-arid region of central Chile have led to the isolation and structural determination by NMR of five lignans, including 3 novel compounds: 1-(4-hydroxy-3-methoxyphenyl)-1,2-bis{4-[(E)-3-acetoxypropen-1-yl]-2-methoxyphenoxy}propan-3-ol acetate, and two different optical isomers ($[\alpha]^{25}D$ + 8.8° and $[\alpha]^{25}D$–15.6°, respectively) of 1-(4-hydroxy-3-methoxyphenyl)-2-{4-[(E)-3-acetoxypropen-1-yl]-2-methoxyphenoxy}propan-1,3-diol 3-acetate, in addition to the already reported 3-acetoxymethyl-5-[(E)-2-formylethen-1-yl]-2-(4-hydroxy-3-methoxyphenyl)-7-methoxy-2,3-dihydrobenzofuran, and 3-acetoxymethyl-5-[(E)-3-acetoxypropen-1-yl)]-2-(4-hydroxy-3-methoxyphenyl)-7-methoxy-2,3-dihydrobenzofuran. The hives were located in a region with the dominant species sclerophyllous shrubs and herbaceous species, such as *Lithrea caustica*, *Quillaja saponaria*, *Cryptocarya alba*, *Kageneckia oblonga*, *Colliguaja odorifera*, *Trevoa trinervis*, *Baccharis linearis*, *Peumus boldus*, *Madia sativa*, *Helenium aromaticum* and *Pasithea caerulea*, which were considered as possible botanical sources of resins [190].

In another study, propolis was collected from hives located at Cuncumen (also Mediterranean-type climate), and fractionated to yield viscidone, vanillin, 3′,4′-(methylendioxy)acetophenone, 3-ethoxy-4-methoxybenzaldehyde, cinnamic acid and 3-methoxy-4-hydroxymethyl ester. These chemical compounds were already described; however, this was the first report on propolis composition of an arid, and a Mediterranean-type climate area. Additionally, the frequency of pollen grains observed in this Cuncumen propolis by optical and scanning electronic microscopy (SEM) allowed to suggest that resins of *Eucalyptus* spp. and *Salix humboldtiana* are the main botanical source of *A. mellifera* in central Chile. Pollen grains of *B. linearis*, *Q. saponaria* and *L. caustica* were also present in propolis sample in lower amounts [191]. Therefore, in order to understand more about the chemical composition and botanical sources available for honeybees in Central Chile, the same research group studied other sample from sclerophyllous shrubland coast (Colliguay), and by chromatographic procedures led to the isolation and characterization by NMR of seven phenolic compounds, including pinocembrin, acacetin, galangin, izalpin, kaempferide, prenyletin and diarytheptane. The botanical origin of Colliguay propolis was investigated by palynological analysis in optic microscopy, and the most abundant pollen grains and leaf fragments found in the sample were related to the plants *Escallonia pulverulenta*, *S. humboldtiana* and *Eucalyptus globulus*, which suggested their participation as botanical sources of this Chilean propolis. Probably honeybees obtain resins rich in pinocembrin from *E. pulverulenta*, since this flavanone was formerly reported from this source [192].

Other studies have been carried out in propolis from Central Chile. Six samples collected from the region of Santiago (Curacaví, Lo Cañas, Buin, Caleu, Cajón del Maipo and pireque) were analyzed by HPLC–ESI-MS/MS, and 30 chemical phenolic compounds were identified, including pinocembrin, pinobanksin, pinobanksin-3-*O*-alkanoates, caffeic acid phenethyl ester (CAPE), chrysin, galangin, kaempferol, kaempferide, ferulic acid, quercetin and quercetin methyl ether derivatives. The antioxidant properties of those samples were analyzed by FRAP, ORAC-FL, ORAC-PGR and DPPH radical methods. All samples exhibited a different FRS activity. The samples that presented CAPE (Curacaví, Buin and Cajón del Maipo) and quercetin (Buin and Lo Cañas) exerted the best antioxidant activity. Pinobanksin was found in all the samples, a compound that would be a suitable candidate for the standardization of propolis from the region of Santiago [193]. In a subsequent study, the anti-inflammatory effect of propolis from Caleu and Buin was tested through mice ear edema model, in addition to the *in vitro* assessment of nitric oxide (NO) production by RAW 264.7 cells stimulated with lipopolysaccharide (LPS). Buin propolis presented anti-inflammatory effect in the murine model (64%), and moreover, significantly decreased the NO release in RAW 264.7 cells in a dose-dependent manner [194].

In addition, a propolis sample collected from San Vicente de Tagua-Tagua (VI Region) was biologically assessed, and showed FRS activity by DPPH (100% at 80 μg/mL) and scavenger effect on superoxide anion (100% at 0.78 μg/mL), as well this Chilean propolis presented antiproliferative activity (at 80 μg/mL for 72 h incubation) by MTT assays on human tumour cell lines KB, Caco-2 and DU-145 (9, 45 and 23% cell viability, respectively). Galangin, caffeic acid, p-coumaric acid, ferulic acid and CAPE were identified by HPLC analysis in San Vicente

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sample, and since the most abundant plant species in this region were *Peumus boldus*, *Q. saponaria*, *P. alba* and *Pinus radiata*, the botanical origin of this propolis according to the presence of those compounds would be the resins of *P. alba* [195].

Moreover, the chemical constitution, the botanical origin and antibacterial activity of twenty samples collected from Central and Southern regions of Chile (Valparaíso, Metropolitana, Libertador Bernardo O'Higgins and La Araucanía Regions) were investigated. Quercetin, myricetin, kaempferol, pinocembrin, coumaric acid, caffeic acid and CAPE were identified in propolis samples by HPLC–MS. All Chilean propolis samples presented a growth inhibitory effect on *S. mutans* and *S. sobrinus* (MIC: 0.9–8.2 μg/mL). By palynological analysis, the plant structures of native species, such as *Trevoa quinquenervia*, *Aristotelia chilensis*, *L. caustica*, *Retanilla trinervia*, *Q. saponaria*, and species of the genus *Escallonia* were found in propolis samples from central regions, whereas *Lotus uliginosus*, *Aextoxicon punctatum*, *B. linearis* and *Eucryphia cordifolia* were identified in samples from Southern Chile, additionally no structures of the genus *Populus* were detected in all the samples. These results suggested that honeybees could obtain CAPE and those flavonoids from other species rather than poplars [196].

With the aim to determine whether the bioactivity against *S. mutans* of Chilean propolis from "La araucanía" region was influenced by the year of collection, three different samples from spring of 2008, 2010 and 2011 were studied. The chemical composition of the annual samples presented qualitative differences by LC-MS analysis. Apigenin, genistein, kaempferol, myricetin, pinocembrin, quercetin, CAPE, caffeic acid, *p*-coumaric acid and ferulic acid were found in all the samples. Otherwise, the presence of daidzein, rutine, and chlorogenic and gallic acid was not constant. The antimicrobial activity of the annual samples did not presented significant variations (MIC: 0.91, 0.22 and 0.39 μg/mL); however, the biofilm formation in *S. mutans* cultures treated with Chilean propolis showed to be influenced by the year of collection [197].

These reports provide important information about chemical composition of Chilean propolis. At present, two main types of Chilean propolis are described, those with lignans as main compounds, and the others with flavonoids and phenolic acids related to propolis collected from temperate regions; however, the palynological evidence obtained indicated that native plants, such as *S. humboldtiana*, *E. globulus*, *E. pulverulenta*, *L. uliginosus*, *B. linearis*, *Q. saponaria* and *L. caustica* are related to the botanical origin of Central and Southern Chile. Moreover, the antibacterial, cytotoxic, antiproliferative and antioxidant activities of Chilean propolis make it a bioactive product for further investigations.

4. Conclusions

Propolis produced by honeybees in Americas represents an important source of diverse bioactive compounds, such as nemorosone and other prenylated benzophenones, artepillin C, CAPE, terpenes labdane type, chalcones, flavonoids, lignans, among others. These secondary metabolites are biosynthesized by different plants present in diverse ecological regions throughout the continent. Thus, propolis from Americas possess constitutional particularities,

and some samples share both temperate and tropical chemical constitution, and even characteristic compositional mixtures are present in some propolis from North and South America, resulting in a huge propolis diversity. In addition, propolis from temperate zones is not restricted to poplar, birch, pines and horse chestnut exudates as main and exclusive botanical source, since recent findings on propolis from United States and Honduras tracked liquidambar species as botanical source, providing a peculiar and transcontinental alternative source of resinous material.

At present, the chemical constitution of North American and South American propolis is partially characterized; however, more studies are needed to understand and identify the bioactive compounds found in those samples and to determine the mechanism of action through which they exert their pharmacological activities. Additionally, more studies should be done in order to understand the plant resin bee foraging behaviour, the role of propolis inside the hive and the benefits of botanical chemistry available along the ecological regions present in America, which imply interdisciplinary work to draw a relationship between bee health and the ecosystem implied. Finally, in accordance to the broad spectrum of biological activities, the high variability and complexity of North American and South American propolis, it becomes necessary to develop a more precise classification of this propolis diversity that combine the qualitative and quantitative plant origin fingerprint, and as well as the biological properties of this beehive product.

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Chapter 6

Fruit Tree Pollination Technology and Industrialization in China

Guo Yuan, Ma Weihua, Wu Wenqing and Song Huailei

Additional information is available at the end of the chapter

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Abstract

This work investigates the bee pollination of fruit trees, especially apples and pears in the field. We first introduce research carried out into bee pollination of crops in China, and then our own pollination experiments with managed bees such as *Apis mellifera* in the field. We monitor the efficiency of bee pollination of fruit trees by regulating hive bees and tree arrangement. In addition, we develop some methods to attract bees to visit fruit trees. Our research shows that the number of beehives and the arrangement of trees greatly influence bee pollination. The results provide a comprehensive tutorial on the best practices of bee pollination of fruit trees.

Keywords: Bees, pollination, fruit tree, apples, pears, pollination efficiency

1. Introduction

About 75% of all crops require pollination by bees. Some pollination is done by domesticated honeybees, but the pollination of most crops is done by wild insects, including wild bees. The decline of wild bee diversity in China has forced farmers to depend on managed bees such as *Apis mellifera*. The most prominent example can be found in southwest China where the cultivated area devoted to apple and pear trees is being expanded year by year, but at the cost of decrease in the number of wild pollinators because of environmental degradation, air pollution, pesticide usage, and so on. This means that crops cannot get sufficient pollination. Those places where there is a serious shortage of pollinators even make use of manpow-

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er to replace insect pollination, which increases the cost of production dramatically. To reduce the cost of pollination and improve the efficiency of pollination, this study looks at how best to get bees to pollinate apple and pear trees, including such factors as how to attract bees to pollinate, selection of optimum bee species, and optimum bee configuration. The work provides a basis for the application of bee pollination technology for crops.

2. Pollination problem facing development of the fruit industry in China

2.1. Development of the fruit industry

China is a large country with many natural habitats and rich fruit tree resources. The cultivation of fruit trees involves more than 50 families of fruit trees, more than 300 species, and more than 10,000 varieties. Principal among these are apple, pear, peach, plum, apricot, plum, grapes, cherry, walnuts, citrus fruit, lychee, longan, loquat, olives, kiwi fruit, fig, pomegranate, *Phyllanthus emblica* (Indian gooseberry), banana, pineapple, durian, mangosteen, breadfruit, cocoa, and betel nuts, the majority of which are widely distributed throughout China. The area devoted to fruit cultivation and fruit yield are among the highest in the world.

Apple trees have a wide distribution in China, can be evergreen or deciduous, and are widely grown across the Yunnan–Guizhou plateau. The deciduous varieties can be broken down into temperate deciduous, deciduous with dry temperature, dry cold deciduous, and hardy deciduous (**figure 1**)[1]. In 2011 the cultivated area devoted to apples in China reached 2.177 million hm^2, accounted for about 42.0% of the world's total area devoted to the cultivation of apple, total output reached 35.985 million t, represented about 54.2% of total world yield, and had an output value of 160.52 billion yuan[2].

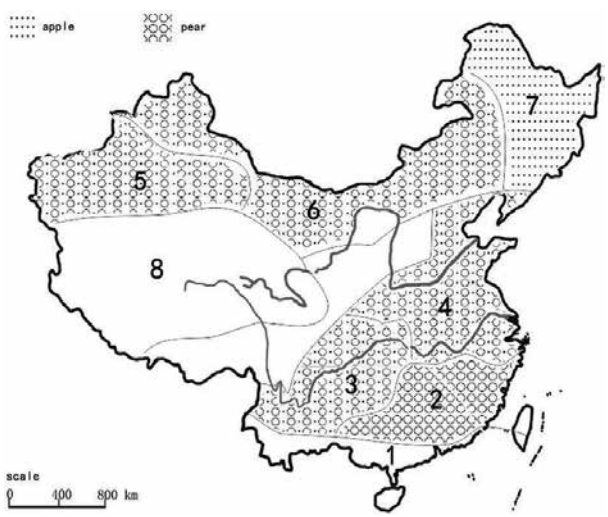

Figure 1. Distribution of fruit trees in China.

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Pear trees are also important; they are grown across five fruit zones. They too can be deciduous or evergreen. Subtropical evergreen trees are grown in the south, whereas cold and dry deciduous fruit trees are grown in the north[1] (**figure 1**). By the end of 2012, according to statistical data released by the United Nations Food and Agriculture Organization[3], China's harvest pear covers an area of 1.138 million hm^2, production is 1.721 million t, and area and yield are among the highest in the world.

Peach trees grow in temperate areas of China. They are mainly distributed in the Yunnan–Guizhou Plateau. They too can be evergreen or deciduous. They can be broken down into evergreen and deciduous mixed, temperate deciduous, dry-temperature deciduous (**figure 1**) [1]. According to statistical data published by the United Nations Food and Agriculture Organization[3] by the end of 2010 the area devoted to peach cultivation in China was 732,000 hm^2 and yield reached 10.828 million t, both of which were the highest in the world.

Fruit production has made great progress in China in recent years. This mainly applies to producing larger varieties, breeding, developing good fruit quality, and marketing of popular market varieties. The biological characteristics of tree species, their adaptability to the environment, improvement of plant varieties, and growing stock in the most appropriate biome possible, all help to optimize efficiency[4, 5] and improve fruit market competitiveness and economic benefits.

2.2. Serious shortage of fruit tree pollinators

Most of the apple, pear, plum, apricot, and chestnut varieties as well as almost all the sweet cherry varieties need be cross-pollinated for the production of seed[6]. Because the pollen grains of nuts and fruits are big, heavy, sticky, and have an outer wall with a pattern of bumps, the wind cannot spread them easily. These fruits rely mainly on insect pollination, especially bees[7,8].

According to a survey of the literature, in the major apple-producing areas in Shanxi Province a total of 23 species of insects visit apple flowers. They belong to 4 orders and 14 families, mainly comprising hymenopteran (Hymenoptera) bees (Apidae), an anthophorid bee (Anthophoridae), leafcutting bees (Megachilidae), an andrenid bee (Andrenidae), and a dipteran (Diptera, Syrphidae). Among them, the Italian bee (*Apis mellifera ligustica*), which accounts for 61.5 to 99.4% of pollinators, is the apple's main pollinator. The foraging peaks of the Italian bee and *Anthophora plumipes* (Pallas, 1772), another local pollinator known as the hairy-footed flower bee, stagger, reducing competition between each other[9]. Lu Yanguo et al. visited insect-pollinated apple blossom in central and southern loess plateau regions. The results show that the bee is the main pollinator in Tianshui and Liangdi, where it accounted for 92.6 and 60%, respectively, of all insect pollination[10]. Yang and Wu surveyed the number and species of insects pollinating kiwi fruit flowers. They identified 16 species of pollinator, including bees (11), food aphid flies (4), and a dung beetle (1). Statistical analysis of the pollination behavior and pollination frequency showed that the bee *Apis cerana cerana* and the Italian bee are the best pollinators, with other insects much less active, carrying less pollen, and having much less of an impact[11]. Zhang Yunyi et al. investigated species of pollinators and the quantity of large cherry trees in Shanxi, and found that hymenopterans accounted for 64.83 to 74.81% of

the pollinators. Hymenopteran pollinators are the most important. Interestingly, pollinators in mountainous area are richer in species than those in the plain[12].

In recent years the number of bees and other pollinators has fallen sharply, which has drawn wide attention across the world. It is likely due to the use of pesticides resulting in a significant decline in the number of wild pollinators[13, 14]. In addition, large-scale clearing of land for farming in the 15th and 16th centuries led to serious restriction of the habitat of wild insects. Climate change may cause inconsistency in plant phenophases and pollinator development periods, resulting in inadequate pollination[17]. In short, fruit tree pollination is the most pressing problem, with artificial pollination having to be adopted in some areas (**figure 2**); for example, about 30% of China's pear trees are artificially pollinated[18].

Figure 2. Artificial pollination.

3. Present situation of fruit industry pollination technology in China

3.1. Chinese institutions engaged in research on fruit tree pollination technology

Chinese literature from 1980 to 2013 reveals that there are 161 institutions engaged in fruit tree pollination research, principal among which are the Chinese Academy of Agricultural Sciences' Institute of Bees, the Horticulture Institute of the Shanxi Academy of Agricultural Sciences, and the Beijing Academy of Agriculture and Forestry. Between 1980 and 2011, there were 324 research papers on bees pollinating trees (**figure 3**), including 66 articles written between 1980 and 1992 (an average of 5 articles per year), a relatively stable number of articles

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between 1993 and 2001, and 204 articles from 2001 to 2011. Research content covered a wide range of factors from bee pollination methods, bee species selection to effects on cultivation and pollination evaluation. There were a few papers looking into how bee pollination increases production, bee pollination and ecology, crop breeding, and pollination colony management (**figure 4**).

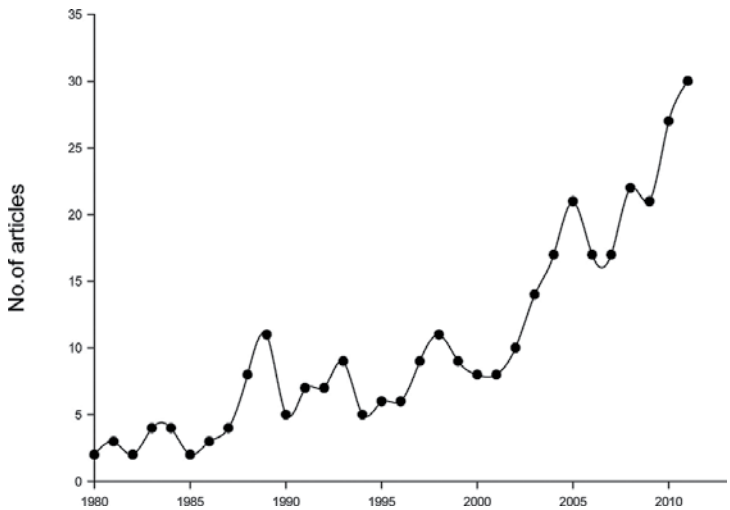

Figure 3. Literature about bee pollination from 1980 to 2011.

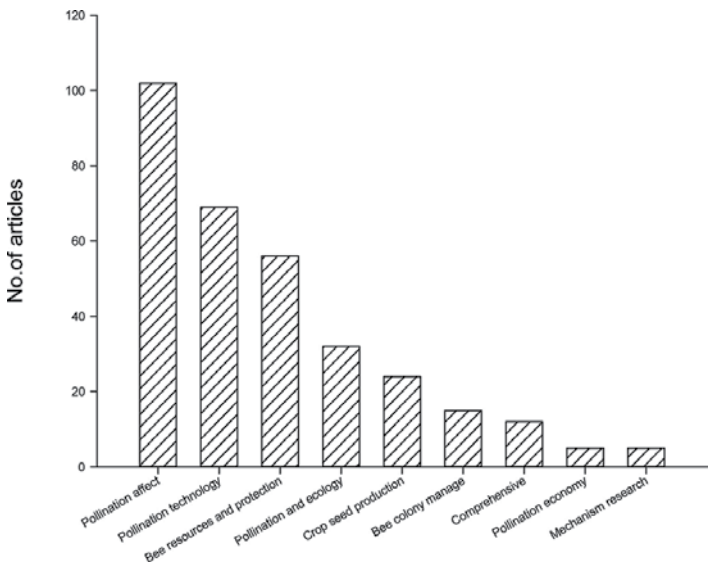

Figure 4. Bee pollination literature by research content.

Between 2001 and 2011, nine books were published on insect pollination technology research and application (**table 1**).

Monograph title	Author	Publishing date	Press	Page extent
Technology of Bee Breeding and Pollination	Zeng Zhijiang	2001.07	Shanghai Popular Science Press	274
Bee Pollination	Shao Youquan	2001.09	Shanxi Science and Technology Press	113
New Technology of Osmia Pollination for Fruit Tree	Zhou Weiru	2002.01	Jindun Press	143
Pollination Insect and Technology of Pollination	Wu Jie	2004.01	Chinese Agric ulture Press	204
Bee Pollination Handbook	Zhang Zhongyin, An Jiandong, Luo Shudong et al.	2008.10	Chinese Agri culture Press	93
Apiculture and Ecology	Wang Yong	2009.05	Chinese Agriculture Science and Technology Press	127
Yield Increasing Techniques of Insect Pollination for Fruit and Vegetable	Shao Youquan, Qi Haiping	2010.05	Jindun Press	202
Bumblebee Artificial Propagation and Pollination	Liu Xinyu, Gao Chongdong	2011.05	Northwest Agric ulture and Forestry University Press	133
Technique of Crop Yield Increasing: Bee Pollination	Wu Jie, Shao Youquan	2011.07	Chinese agriculture press	173

Table 1. Monographs on bee pollination in China.

According to the State Intellectual Property Office patent database, from 2000 to 2013 there were 54 classes of bee pollination patents: 43 invention patents and 11 utility model patents. They covered a wide range of factors from bee pollination application technology, pollinating bee breeding technology, pollinating bee management methods, to the induction of bee pollination methods, design and transformation of pollination hives, and bee pollination control devices. Patentees came from a number of provinces and cities, with Beijing, Zhejiang, and Shandong ranking in the top three. Between 2000 and 2007 a total of 15 patents were

granted, and between 2008 and 2013 the total was 39. Patents related to bee pollination are clearly on the increase.

From the National Network of Scientific and Technological Achievements we retrieved 41 classes of bee pollination, principal among which were 17 classes on bee species breeding and selection accounting for 41.5% of total results. In addition, there were classes covering bee application and technology research (14), beehive design (2), and pollinating bee species resources (3). The results show that of the pollinating bees—bumblebees, osmia bees, and leafcutters—bumblebee research was the most impotant. From the point of view of achievements, institutes in Beijing, Jilin, and Shanxi were in the top three. Regarding the number of achievements the Beijing Forestry Academy of Sciences, the Chinese Academy of Agricultural Sciences' Institute of Bees, and the Horticulture Institute of the Shanxi Academy of Agricultural Sciences were in the top three. The Beijing Academy of Agriculture and Forestry made great progress in providing facilities for crop pollination, bee species breeding, utilization, demonstration, and pollination hive development. The Chinese Academy of Agricultural Sciences' Institute of Bees made a breakthrough in bumblebee breeding, utilization, and application. The Horticulture Institution of the Shanxi Academy of Agricultural Sciences had a lot of success as a result of providing improved facilities for vegetable production technology research and application.

3.2. Effectiveness evaluation of bee pollination for fruit trees

After the bee *Osmia cornifrons* (Ra doszkowski) was imported by the Biological Control Research Institute of the Chinese Academy of Agricultural Sciences from Japan in 1987 the pollination effectiveness of the fruit-setting rate and fruit quality of apricot, cherry, peach, pear, and apple in Hebei and Shandong were remarkable.

After using *Apis mellifera ligustica* for apple pollination, Zhang Guiqian et al. found that, compared with natural pollination, bee pollination increased the "Red Fuji" apple fruit yield by 46.8%, reduced the misshapen fruit rate by 22.4%, and increased yield to 14,124 kg/hm^2 [20]. He Weizhi and Zhou Weiru researched the use of the concave-lipped bee *Osmia excavata* Alfken, the Italian bee, and artificial pollination for "Red Fuji" apple pollination. The results showed the apple fruit yield of the six kinds of pollination was significantly higher than that of natural pollination; the pollination effect of osmia bees combined with Italian bees was best with a high inflorescence fruit rate of 99.6% [21]. Lou Delong et al. found that the "Red Fuji" apple fruit yield, production, and coloring index of bee pollination were higher than those of natural pollination by 15, 36.26, and 17.07%, respectively[22]. Using bee pollination for apple and pear, Zhao Zhonghua et al. found that fruit yield was more than 20% higher than artificial pollination and the average production of each acre was 224.4 kg, 335.3 kg with the increase rate of 8.7% and 11.3% [23]. Yuan Feng et al. used osmia bees and honeybees to pollinate "Red Fuji" apple trees and found that fruit yield increased by 14.68 and 10.95%, respectively, over the natural pollination yield and the fruit abscission rate reduced by 32.9 and 20.27%, respectively[24].

In addition, the effect of using a variety of bees for pear and peach pollination was clear. Liu Jinli et al. increased the fruit yield of crown pears, emerald pears, south fruit pears, and gold

pears by using concave-lipped osmia for pollination by 11 to 18.4% compared with that of artificial pollination[25]. Guo Yuan et al. researched different pollination methods for pear and found that the fruit yield of bee pollination was 32.9%, artificial pollination 13.05%, and natural pollination only 2.83%[26]. Dong Jie et al. used Italian bees and *Bombus hypocrita* to pollinate peach trees; the results showed that any difference in peach fruit yield and fruit nutritional quality of the two kinds of bee pollination was not significant and that both were significantly better than that of artificial pollination[27]. Mu Hongjie used bumblebees and bees to pollinate fruit trees; the results showed that the fruit yield of bumblebee pollination was higher than that of bee pollination, with an increase of 25.5% in the nectarine yield[28]. Means within a column followed by the different letters are extremely significant different at P 0.01 level.

From 2008 to 2015, researchers from the Horticultural Institute of the Shanxi Academy of Agricultural Sciences carried out research into bee pollination for the "Red Fuji" apple; the results show that bees can significantly improve the fruit-setting rate (**table 2**).

Variety	Pollination pattern	Flower number	Fruit number	Fruit-setting rate (%)
"Red Fuji"	Bee	1004	240	23.9
	Nature	1044	96	9.2
"Starkrimson"	Bee	1258	438	34.8
	Nature	1098	93	8.5

Table 2. Fruit-setting rate by bee and natural pollination.

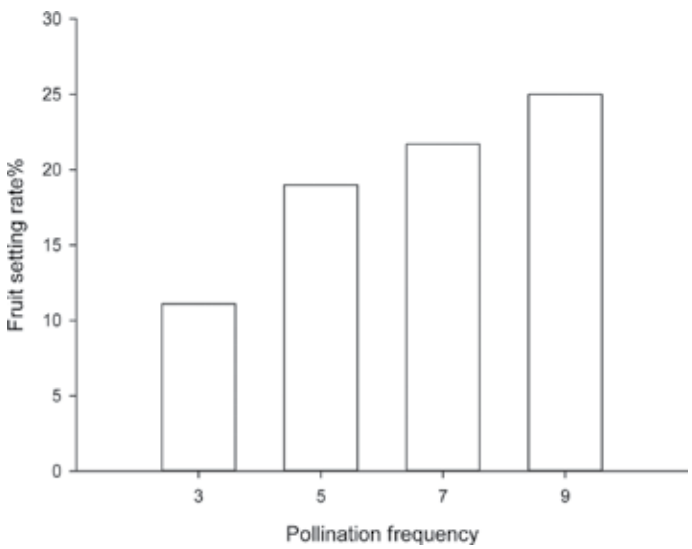

Figure 5. Fruit-setting rate by number of pollination visits.

In addition, three, five, seven, and nine lots of bee pollination resulted in increased fruit yield to the tune of 11.1, 19, 21.7, and 25%, respectively, as shown in **figure 5**. So the greater the number of visits made by bees to pollinate the higher the fruit yield.

Bee pollination stimulates the growth of young fruit. The average yield of each tree after bee pollination was 69.8 kg compared with 31.9 kg of natural pollination. The fruit shape index of bee pollination and natural pollination were similar, but the coloring index of bee pollination was significantly better than the natural pollination group; the results are shown in **table 3**.

Pollination pattern	Fruit shape index	Color index (%)	Chemical quality		
			Solid content (%)	Acidity (%)	TSS:acid ratio
Bee	0.8282	67.33	13.53±1.63A	0.34±0.05B	40.98 A
Nature	0.8516	47.33	13.93±1.34A	0.43±0.07A	32.71 B

Notes: Fruit shape index: The ratio of longitudinal diameter to transverse diameter. Color index=Σ(Fruit number of each class×Extreme value)/(Total fruit number×The highest series)×100%. TSS, total soluble solids.

Table 3. Different pollination patterns.

3.3. Key technology underlying bee pollination of apples and pears

3.3.1. Configuration of pollination trees

About 70% of trees under production in orchards in Shanxi receive insufficient or no pollination. We have researched the ratio between pollination partners and the cultivation of fruit trees. In some pear gardens, pollination branch grafting guarantees bee pollination. Research shows that self-incompatibility occurs when pear trees have the same S-type genotypes, hence cultivation of at least one S genotype of different varieties as pollination partners should be undertaken. **Table 4** outlines the main culture of some varieties and their appropriate pollination partners.

Main varieties	Pollination partners
Pyris pyrifolia Nak. *cuiguang*	*P. pyrifolia* Nak. *qingxiang, huanghua* *Pyris bretschneideri* Rehd. *huangguang, xinya*
P. pyrifolia Nak. *xizilv*	*P. bretschneideri* Rehd. *zaosu, hangqing* *P. bretschneideri* Rehd. *huangguang* *P. bretschneideri* Rehd. *zhongliyihao*
P. bretschneideri Rehd. *huan gguang*	*jimi, P. bretschneideri* Rehd. *zhongliyihao, fengshui*
yuanhuang	*xianhuang, fengshui, huanghua, xueqing*
fengshui	*huanghua, xinshui, P. bretschneideri* Rehd. *dangshansu, P. bretschneideri* Rehd. *huangguang*

Main varieties	Pollination partners
Pyris ussriensis Max. *nanguo*	*P. bretschneideri* Rehd. *pingguo,* Bartlett Williams, *P. bretschneideri* Rehd. *ren*, *P. bretschneideri* Rehd. *ya*
xingao	P. *bretschneideri* Rehd. *ya, P. ussriensis* Max. *jingbai*, *P. bretschneideri* Rehd. *dangshansu*, *fengshui*
P. bretschneideri Rehd. *dang shansu*	*P. bretschneideri* Rehd. *ren*, *P. bretschneideri* Rehd. *ya*, *matihuang*, *P. bretschneideri* Rehd. *zhongliyihao*, *P. bretschneideri* Rehd. *huangguang*
P. bretschneideri Rehd. *xuehua*	*P. bretschneideri* Rehd. *ya*, *P. bretschneideri* Rehd. *zaosu*, *jimi*, *P. bretschneideri* Rehd. *huangguang*
P. bretschneideri Rehd. *ya*	*P. bretschneideri* Rehd. *dangshansu*, *P. ussriensis* Max., *jingbai*, *jinhua*
P. bretschneideri Rehd. *korla*	*P. bretschneideri* Rehd. *ya*, *P. bretschneideri* Rehd. *xuehua, P. bretschneideri* Rehd. *dangshansu*, *P. bretschneideri* Rehd. *pingguo*
P. bretschneideri Rehd. *hong xiangsu*	*P. bretschneideri* Rehd. *dangshansu*, *P. bretschneideri* Rehd. *xuehua, P. bretschneideri* Rehd. *ya*, *fengshui*

Table 4. Configuration of main pear variety and appropriate pollination partners in Shanxi.

The ratio between pollination partners and the main variety can be 1:4–1:8; 1:6 has been found to be optimal. To ensure full pollination and prevent flowering inconsistency the main variety should be paired with two pollination partners; namely, six main varieties and one pollination variety.

In addition, we can increase the supply of pollen by means of grafting pollination branches (**figure 6**).

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Figure 6. Central branch grafting of pear pollination partners.

3.3.2. Selection of pollinating bee species

Apis mellifera cv. "Kaqian Black Ring Bee", *Apis mellifera* cv. "Mr. Northeast Black Bee", *Apis mellifera caucasica*, *Apis mellifera* cv. "Honey-proplis 1 Bee", *Apis mellifera* cv. "Carpathian Bee", *Apis mellifera* cv. "Heimeiyi", *Apis mellifera carnica*, the Italian bee, and *Apis cerana cerana*

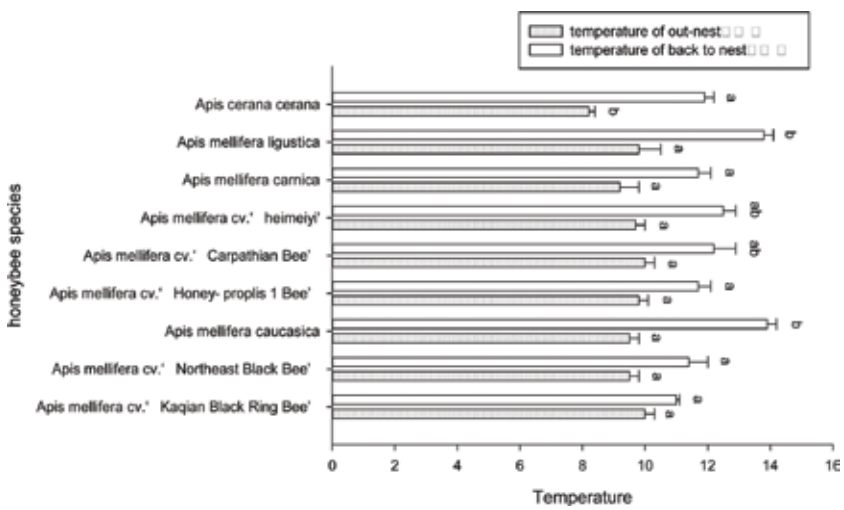

Figure 7. Temperature of outflying and backflying bee varieties.

Fabricius were usually used for pollination. However, the nine varieties differ in their life habits (**figure 7**), their ability to carry pollen (**figure 8**), and their collection of pear flower powder proportion (**figure 9**). Therefore, in the process of pollination we need to choose appropriate bees for pollination according to different fruit trees.

Figure 7 shows that the outflying temperature of *Apis cerana cerana* Fabricius was 8.2°C, which was significantly lower than western bees (9.2– 10°C). *Apis mellifera carnica* can fly out of the nest at 9.2°C; there were no significant differences among western bees. The average backflying temperature of all bee species was 12.1°C and the average pollen-carrying temperature of *Apis mellifera* cv. "Kaqian Black Ring Bee", *Apis mellifera* cv. "Northeast Black Bee", *Apis mellifera* cv. "Honey-proplis 1 Bee", *Apis mellifera carnica*, and *Apis cerana cerana* Fabricius was significantly lower than *Apis mellifera caucasica* and the Italian bee.

The weight of total pollen and pear pollen collected by bees in one hour was compared and analyzed (**figure 8**). *Apis mellifera carnica* collected more pollen than the others, and *Apis cerana cerana* Fabricius collected the least. *Apis mellifera* cv. "Kaqian Black Ring Bee" collected the most pear pollen in one hour and *Apis mellifera* cv. "Honey-proplis 1 Bee" and *Apis cerana cerana* Fabricius collected the least pear pollen. *Apis mellifera* cv. "Kaqian Black Ring Bee" and *Apis mellifera carnica* were much the same but they both collected more pear pollen than the others. They can collect five times more pear pollen than *Apis cerana cerana* Fabricius.

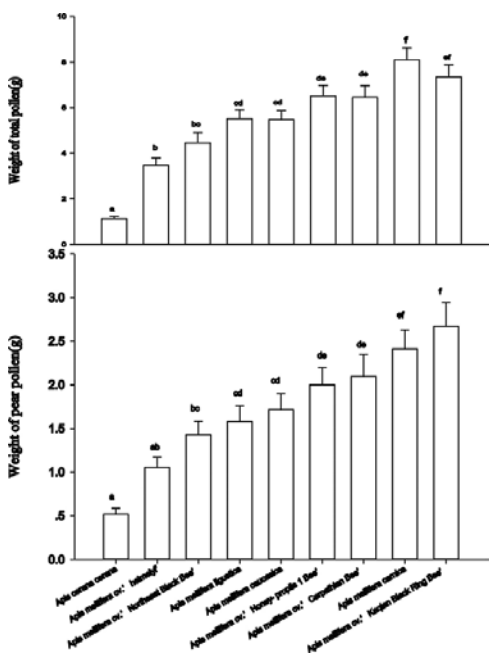

Figure 8. Total pollen and pear pollen collected by different bee varieties in one hour.

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The proportion of pear pollen collected by *Apis mellifera carnica* was highest (45.2%) and *Apis mellifera* cv. "Kaqian Black Ring Bee" was the second highest (42.4%). Nevertheless, there was no significant difference among the species (**figure 9**).

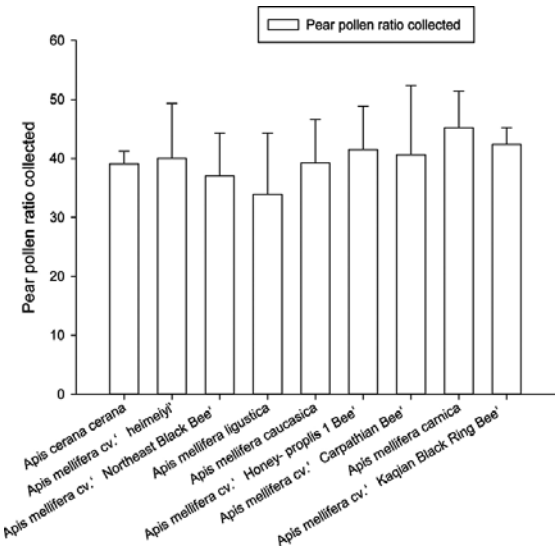

Figure 9. Pear pollen collected by different bee varieties.

Since pear trees bloom throughout China at different times, there is a need for pollinating bees that are not only good at pollen collection but adapt well to the environment. Pear trees flower early when the temperature is low, so bees have little choice but to pollinate pear trees in low temperatures. Of the nine species of bees selected, *Apis mellifera* cv. "Kaqian Black Ring Bee" and *Apis mellifera carnica* adapted best to the environment and were best at collecting pollen, especially pear pollen. So they can be recommended to pollinate pear trees.

3.3.3. Control of pollination bees

Insect pollination can be used to improve the fruit-bearing rate and yield, but it does not follow that the higher the fruit-bearing rate the better the yield. If the fruit-bearing rate is too high, nutrients will be depleted resulting in small-sized fruit and poor yield. Therefore, the key technical problem is to adjust the number of bees to control the fruit-setting rate.

Table 5 shows that the greater the varieties of bees the greater the subsidence on stigma pollen, the higher the fruit-setting rate, and the greater the yield. The fruit-setting rate is low with one to four varieties of bees; however, when the varieties of bees are increased to six or eight there could be an increase to 9.6 or 17.9%, respectively. In addition, using different varieties of pollination bees can also affect the quality of apples (**table 6**). When six varieties of pollination bees were employed the fruit not only met the appropriate requirements but tasted good too. All in all, when the ratio between pollination partners and main varieties is 1:4, each tree can meet production requirements with six bees.

Number of bee varieties used	Pollen amount on stigma	Fruit-setting rate (%)	Average yield/each tree (kg)
1	678.52	2.87	**65.75**
2	832.08	3.07	**65.5**
4	1,112.9	3.31	**66.5**
6	1,145.8	9.63	**79.25**
8	**1,360.4**	**17.87**	**119.25**

Notes: Experiments were conducted in net houses. The ratio between pollination partners and main varieties is 1:4.

Table 5. Stigma pollen count, fruit-setting rate, and yield employing different numbers of varieties of pollination bees.

Number of bee varieties used	Soluble solids (%)	Acid (%)	Hardiness in the sun (N/cm^2)	Hardiness in the shade (N/cm^2)	Fruit shape index	TSS-acid ratio
1	15.0527bc	0.302d	9.154b	9.24b	86.44	49.79
2	14.8423c	0.318cd	10.809a	10.72a	87.81	46.7
4	15.9480ab	0.3906b	10.509a	10.23a	86.96	40.82
6	16.6893a	0.502a	10.46a	10.31a	88.49	33.25
8	15.6733bc	0.375bc	9.485b	8.73b	85.65	41.78

Note: TSS, total soluble solids. Means within a column followed by the different letters are significantly different at P 0.05 level.

Table 6. Fruit quality employing different numbers of varieties of pollination bees.

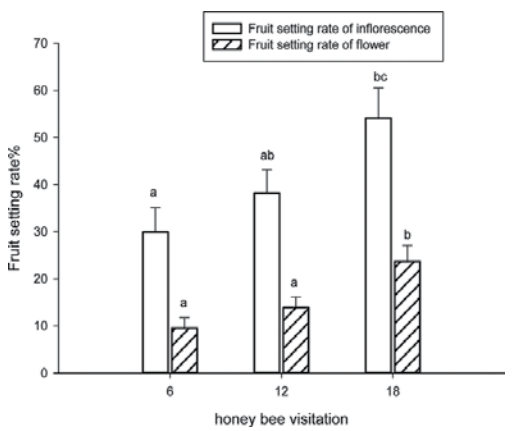

Figure 10. Number of varieties of pollination bees employed and fruit-setting rate.

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When the ratio between the main variety of "Red Fuji" apples and pollination partners of "Qinguan" is 20:1, the fruit yield, fruit shape index, seed number, and deformity fruit rate of each tree employing 6, 12, and 18 varieties of bees are, respectively, shown in **figure 10**, **table 7**, and **table 8**. Notes: Means within a column followed by the same letter are not significantly different.

Number of bee varieties used (each tree)	Average longitudinal diameter (mm)	Average diameter (mm)	Fruit shape index
6	66.41	75.26	0.9111±0.0103a
12	68.2	75.21	0.9076±0.0094a
18	67.65	74.28	0.8821±0.0121a

Table 7. Relation between number of varieties of pollination bees employed and fruit shape index.

Number of bee varieties used (each tree)	Total number of seeds	Plump seed	Plumpness (%)	Irregular fruit rate (%)
6	140	134	95.7	56a
12	247	243	98.38	26b
18	290	288	99.31	18c

Table 8. Relation between number of varieties of pollination bees and fruit seed number and irregular fruit rate.

In the absence of pollination partners the number of bee varieties used for pollination and the fruit-setting rate are shown in **figure 11**.

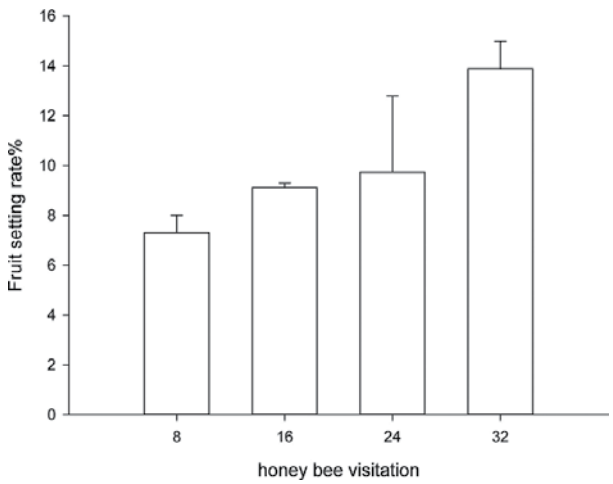

Figure 11. Fruit-setting rate of "Red Fuji" apple trees by bee pollination in the absence of pollination partners.

The configuration of pollination partners is a major factor affecting the number of bee varieties to be used for pollination. A good configuration will allow employment of six varieties of pollination bees, enough to guarantee production requirements. If the configuration leads to insufficient pollination partners, it will be necessary to increase the number of varieties of bees to at least 12 for pollination purposes. In the absence of pollination partners, 16 varieties of bees will be needed to achieve a fruit-setting rate of 9.12%.

3.3.4. Scale of the pollination apiary

The distance between buzzers and fruit trees had a significant effect on pollination. Foraging bees and fruiting percentage at different distances using 20 colonies are shown in **tables 9** and **10**.

Distance (m)	Number of foraging bees	Flower number	Fruit number	Fruiting percentage
0	354	715	138	19.30
50	246	610	94	15.41
100	227	764	97	12.70
150	194	935	112	11.98
200	86	1,825	126	6.90

Notes: 20-colony treatment involved setting up 5 survey spots, 1 every 50 m from the colony out to 300 m. At every spot one apple tree of consistent variety, tree potential, on-year yield (high), and off-year yield (low) was selected. At every spot foraging bees were counted for 45 minutes and the fruiting percentage after 15 days was calculated.

Table 9. Number of foraging bees and fruiting percentage at different distances using 20 colonies in 2009.

Distance (m)	Flower number	Fruit number	Fruiting percentage	Pollen amount on stigma
0	1,723	286	16.6	6,050
50	1,843	304	16.49	5,450
100	3,090	417	13.5	6,889
150	1,738	228	13.12	5,850
200	1,790	202	11.28	6,050

Table 10. Fruiting percentage at different distances using 20 colonies in 2010.

With increase of the distance between fruit trees and bee colonies the number of foraging bees gradually reduced. Bee pollination at a distance of 150 m between bee colonies and fruit trees was found to give the ideal fruiting percentage; therefore, the bee pollination effective radius was 150 m when 20 colonies are employed.

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With increase of the distance in the 0 to 200-m range using 20 colonies the fruiting percentage decreased from 16.60 to 11.28%; however, neither the trend nor change in the pollen count on the stigma were obvious. Despite there being more foraging bees and the fruiting percentage increasing with decrease in the distance from the colony within the 0 to 200-m range, 2010 was an off-year with low apple tree yield, reduced flower total quantity, and enlarged bee gather distance.

The number of foraging bees and fruiting percentage at different distances using 50 colonies are shown in **tables 11** and **12**.

Distance (m)	Number of foraging bees	Flower number	Fruit number	Fruiting percentage
0	907	1,070	339	31.68
50	593	1,775	236	13.30
100	600	1,250	140	11.20
150	342	1,865	208	11.15
200	262	1,400	148	10.57
250	241	1,255	70	5.58
300	189	1,580	97	6.14

Notes: 50-colony treatment involved setting up 7 survey spots, 1 every 50 m from the colony out to 300 m. At every spot one apple tree of consistent variety, tree potential, and on-year and off-year yields was selected. At every spot foraging bees were counted for 45 minutes and the fruiting percentage after 15 days was calculated.

Table 11. Foraging bees and fruiting percentage at different distances using 50 colonies in 2009.

Distance (m)	Flower number	Fruit number	Fruiting percentage	Pollen amount
0	1,561	309	19.8	5,400
50	592	93	15.71	5,050
100	1,200	135	11.25	5,167
200	1,425	203	14.25	4,800
250	1,097	215	19.60	4,000
300	1,373	202	14.71	4,000

Note: There were no 150-m data because tree vigor was poorer.

Table 12. Fruiting percentage at different distances using 50 colonies in 2010.

With increase of the distance in the 0 to 300-m range using 50 colonies, in 2009 fruiting percentage decreased from 31.7 to 6.1%, fruiting percentage at 200 m was 10.2%, and fruiting

percentage at 250 m was 5.6%; therefore, the bee pollination effective radius was 200 m when 50 colonies are employed. In 2010, apple pollen counts on stigmas overall declined with increasing distance. This shows that foraging bees were fewer with increasing distance, yet fruiting percentage showed no significant change in trend.

The bee pollination effective radius was 150 m with 20 colonies and 200 m with 50 colonies. When apple trees gave on-year yields, production practice chose 50 colonies for bee pollination. When apple trees gave off-year yields the bee pollination effective radius was larger than apple trees in on-year yields and production practice chose 20 colonies for bee pollination.

3.3.5. Technology behind getting bees to visit fruit trees

The attraction of pollinators to some fruit trees is poor; one such is pear. When there are other plants such as rape, dandelion, and paulownia flowering near the target trees at the same time, foraging insects rarely alight on pear trees[29]. Artificially inducing bees to pollinate fruit trees when more desirable plants are available is a problem that must be solved. Many fruit trees bloom early in the year and flowering time is short; for example, apple trees flower for between 10 and 15 days and pear trees flower over a shorter period (about 7–10 days). Another technical difficulty is activating the foraging enthusiasm of the swarm.

In an effort to improve the foraging enthusiasm of bees, our team studied foraging behavior after using attractants. *Apis mellifera* 30 hives with six combs every hive had a consistent colony structure. The test involved 10 treatments and 3 colonies. Three treatments, respectively, used attractant I, II, and III, which our team prepared. Nine treatments involved feeding six compounds to bees: 1-mM methionine, 1-mM lysine, 1-mM arginine, 1-mM gallic acid, 500-μM 8-Br-cGMP, and pure syrup as a control treatment. Attractants were start-fed to *Apis mellifera* once every evening before the pear blossom appeared and then every 2 days until the end of the flowering. Pear flower load after sorting from total pollen was weighed, number of foraging bees on pear flowers were recorded, and percentage pear flower load and foraging bee number on pear flowers were calculated.

The weight of pear pollen load collected in a day is shown in **figure 12**. All treatments enhanced the foraging ability of bees for pear pollen. The foraging effect of hanging attractant I in hives (77.56 + 1.59 g/group) was significantly higher than other treatments. Those treatments that involved feeding the bees Arg (62.05 +/– 2.01 g), Lys (62.2 + 2.3 g), 8-Br-cGMP (64.45 + 4.55 g) and hanging attractant II in hives (64.20 + 2.72 g) were better than the control treatment (49.11 + 1.03); other treatments showed no significant difference from the control treatment.

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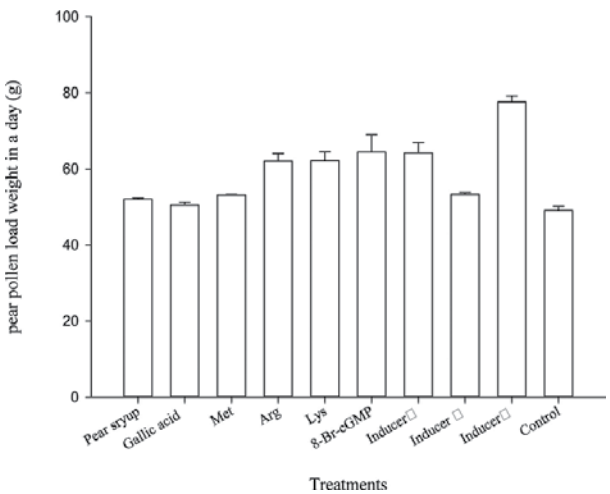

Figure 12. Weight of pear pollen collected in a day.

Pear pollen was sorted from total pollen. The percentage of pear pollen in total pollen load collected in a day is shown in **figure 13**. The results show that treatment groups fed pear syrup and methionine showed no significant difference from the control group; the other seven groups were higher than the control group. Feeding lysine (76.3%), hanging attractant I in the hives (79.3%), and hanging attractant II in the hives (80.2%) were all significantly higher than other treatment groups ($P < 0.05$). Hanging attractant II in the hives was the most effective and had the highest percentage of pear pollen; hanging attractant I in hives coupled with the lysine groups was the next best.

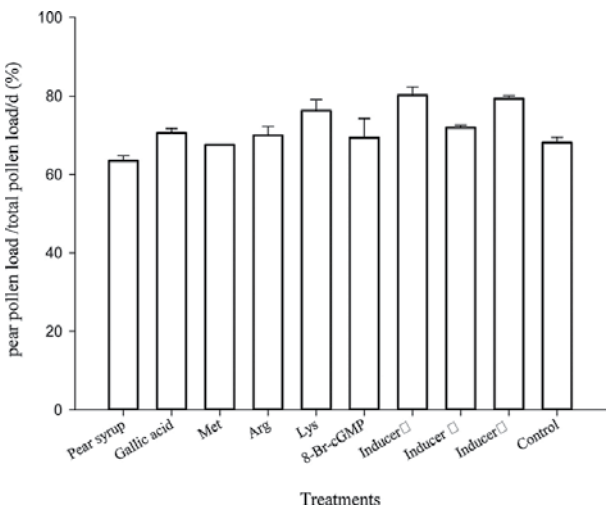

Figure 13. Percentage of pear pollen in total pollen.

Cameras were installed at the entrance to the hive, worker bees returning to the hive were recorded for 5 minutes every hour. The percentage of foraging bees on pear flowers out of total foraging bees is shown in **figure 14**. The results showed that treatment groups fed pear syrup, gallic acid, and methionine were significantly lower than the control group, whereas the group fed arginine and 8-Br-cGMP showed no significant difference from the control group. The groups fed lysine (85.81%), hanging attractant I (86.74%), attractant II (87.27%), and attractant III (85.67%) were significantly higher than the control group. The percentage of foraging bees improved by 3 to 4.5% after application of attractants I, II, and III.

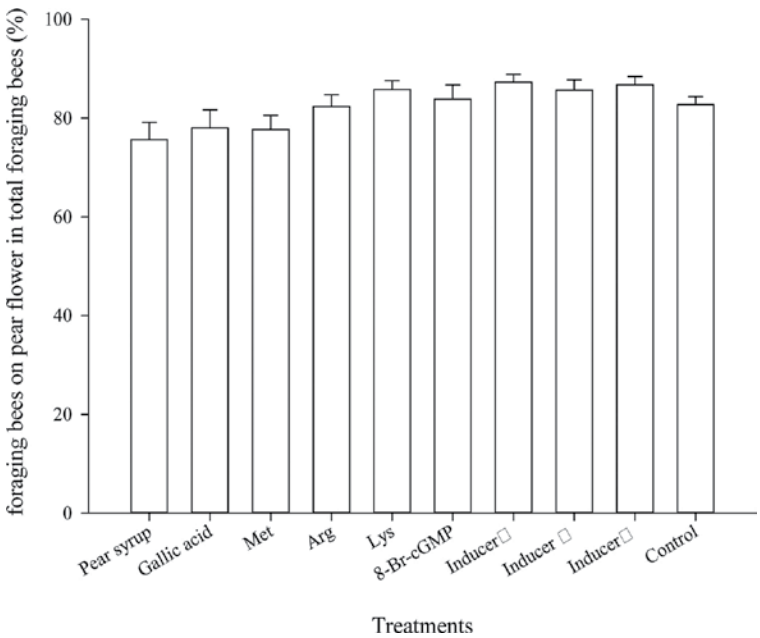

Figure 14. Percentage of foraging bees on pear flowers out of total foraging bees.

The above results show that feeding bees lysine brings about the best effect of all the feeding treatments, increasing both the percentage of pear pollen load and the number of foraging bees on pear flowers. The effect of hanging attractant in the hives is better than feeding treatments. Hanging attractant II in the hives resulted in the highest percentage of pear pollen load, whereas hanging attractant I in the hives resulted in the highest weight of pear pollen. Both treatments can effectively increase foraging behavior.

3.3.6. Technology underlying bees carrying pollinizer pollen

Since some pear orchards have no pollinizers whatsoever, we developed a kind of bee-carrying powder device (**figure 15**). This device is installed at the entrance to the hive, fresh pollen is put in the upper part of the device, and pollen will leak out from the bottom when bees leave the hive. Bees that carry pollinizer pollen will pollinate leading cultivars.

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Figure 15. Device for bees to carry pollen.

4. Industrialization of fruit tree pollination

4.1. Professional bee breeding for pollination

Apis mellifera and *Apis cerana cerana* are the foremost pollination bees, primarily employed for the production of bee products (like honey), although they are sometimes used to pollinate fruit trees. However, they are not ideal pollination bees[30]. In the 1990s, researchers in China made a breakthrough in the artificial breeding of wild bumblebees, mastered the key technology to breed bumblebees indoors, domesticated six kinds of bumblebees, and established several production bases that could be gradually applied to facilities for orchard pollination[31]. In an effort to fill the gap in agricultural practical development needs, researchers have bred and domesticated osmia bees, stingless bees, and andrenids in recent years. Our hope is that these technologies might play an important role in fruit tree pollination in years to come.

4.2. Induced bee pollination technology: The need for further research

Researchers have cultivated a special colony used exclusively for fruit tree pollination, developed a pollination technology that does not depend on a queen bee, and solved the bee-butting-greenhouse problem in facilities crop . Although many advances have been made in bee pollination, There are remain many technical problems that must be solved such as how to get bees to visit fruit trees they do not favor or how to get the bees to improve pollination? Answers to these questions involve the study of the correlations between plants and bees, as

well as the relationship between the spatial layout of fruit trees and the spatial distribution of foraging bees.

4.3. Pollination professional development is slow

At present, some areas in China have established bee pollination intermediary service agencies, bee industry cooperatives, pollination professional companies, and a few corresponding pollination intermediary services. However, these organizations have failed to provide the necessary market supply-and-demand information and technical training, or to set up a bee pollination service and relevant policies. There are a number of reasons for this: The scale of the industry in China is small, specialist companies are few in number, the degree of organization is low, and it is difficult to form a pollination network.

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Chapter 7

Beekeeping in Jalisco, México

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Additional information is available at the end of the chapter

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Abstract

The purpose of this study was to analyze the socioeconomic factors that influence the beekeeping process and describe the current situation in beekeeping technology development in the south and southeast regions of Jalisco. The study was conducted by reviewing secondary sources of documentary information and the primary information was obtained by means of a survey, analyzing demographic, social, technological, and economic variables. From January to April 2011, a stratified sampling was conducted of six strata of beekeepers, with a final sampling of 183 beekeepers. We applied a frequency analysis, ANOVA (Waller-Duncan), and contingency tables (χ^2). The average age observed for the beekeepers was 47 years, with fewer women participating in the activity, and an above national average level of education. The majority keep their apiaries in rented premises, a high percentage outside the municipality where they live. The honey obtained is multiflora and the main harvest is in the autumn, with a honey yield per hive below the national average. A number of problems affect the production sector including environmental factors, production costs, and varroa. We observed little diversification; in addition to honey only beeswax is recovered, and only a minority keep a record of production costs. There is wide participation in beekeeping associations and in training provided by different public and private bodies. There is a willingness to adopt new technologies and equipment for honey production with good practice standards.

Keywords: beekeepers, management, innovation, technology, socioeconomic aspects

1. Introduction

Apiculture is a production area that has been carried out under a broad mosaic of systems and vertical and horizontal integration of the production process. It is an important activity in Mexico

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within the food, economic, social, and ecological areas and has developed in different parts of the country, through small and medium producers with an important share of the international market and local consumption of 190 grams per capita during the nineties, increasing to 320 grams in 2010. This increase is because of its use as a raw material in the preparation of foods such as yoghurt, cereals, confectionery, baked goods, and cosmetic products.[1] In 2010, Mexico was the sixth largest honey producer in the world with 1.8 million hives producing 56,883 tons a year, and the third largest exporter, exporting 25,000 tons that same year, mainly to the European market,[2] positioning apiculture among the top three sources of foreign currency in the national livestock field.[3] More than 2,400 tons of beeswax and close to 8 tons of royal jelly are produced each year.[4] Apiculture directly benefits 400,000 people who form part of the beekeeping production chain by constructing beekeeping equipment and packaging and marketing honey and other bee products. In addition to benefiting agricultural crops through pollination, with an estimated value of 2 billion dollars a year, beekeeping also helps to maintain the ecological balance in various ecosystems, through the pollination of wild plants.[5]

The state of Jalisco is one of the main honey producers in Mexico, with a census of 157,827 hives producing an average of 5,698 tons of honey per year between 2005 and 2009 and a 10% market share positioning it in third place nationally behind only Yucatán with close to 10,000 tons (15%) and Campeche with 7,500 tons (12.9%). There are almost 1,000 beekeepers in the state, of whom 50% are in the south and southeast, the main regions in this productive environment; the activity is mainly a sideline to agriculture and livestock.[1,6,7]

In recent decades, the beekeeping sector has faced substantial changes, the result of urbanization, globalization, and population growth, thus developing a new environment in itself.[8, 9] Actions have been taken to improve production, increase diversity in the end product, and try new schemes of organization, giving rise to new commercial dynamics and methods of insertion into the world market.[1] Government actions have focused on promoting productive restructuring, diversification of traditional crops, technological assessment, and the generation of infrastructure and technology innovation.[9,10]

Several studies have drawn attention to the fact that national apiculture is affected by a wide range of issues, including Africanized bees, global climate change (encompassing factors such as erratic rainy seasons, drought and extreme heat, and freezing temperatures), in addition to the lack of training and organization of beekeepers, and not least diseases such as varroa and foulbrood. Middlemen and competition on the international market have also contributed to a worrying instability.[3,6,11]

Honey was already shaping up with major annual sales projections until 2007.[12] This positive forecast has a growing international honey market as current production does not satisfy total demand.[13] However, marketing is another of the core problems within this production sector. In terms of product development, there need to be changes in the collection process, presentation, and business dynamic for it to be considered a primary activity and not just an additional source of income. In general, honey in Mexico is considered a by-product and few producers and companies have invested in research and development, conservation, and quality improvement, as well as differentiated forms of sale and marketing strategies and channels.[14]

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Within Mexican apiculture, more than 75% of beekeepers are low-income farmers who see apiculture as a means of boosting their income; they have on average fewer than 100 hives,[6] numbers that are declining because of the problems already mentioned. The way these small producers carry out the activity does not follow business logistics, making it difficult to obtain reliable data regarding the income they perceive; they keep no records of production, spending, or income.

Given the economic and social importance of apiculture in the south and southeast regions of Jalisco as already described, and a scenario of constant change, it is important to characterize beekeepers and agents of innovation taking into account socioeconomic, technological, and productive variables. Thus the aim of this work was to identify the influential socioeconomic factors within the beekeeping process and describe the current situation in the technological development of beekeepers in the south and southeast regions of Jalisco, to have an updated, objective view of the situation of the apiculture sector that allows the development of a frame of reference, a fundamental decision-making tool within government support programs for the benefit of beekeepers.

2. Materials and methods

The documentary information was obtained from secondary sources to get a frame of reference about aspects of production and commercial statistical behavior of the apiculture production chain, as well as the methodological framework.

The study design is exploratory and quantitative. Exploratory investigation is used to define the study problem and its context through the analysis of secondary data. The quantitative investigation was descriptive and cross-sectional, applying a person-to-person nominal scale survey and a single sampling. The primary information was obtained by means of a survey using a structured questionnaire (See Appendix 1).[15,16] We analyzed demographic, social, technological, and economic variables, which included questions such as gender, age, how often hives were inspected, treatments for varroa, price of honey per kilo, apiculture products, type of extraction equipment, labeling, and marketing the honey, main diseases and their treatments, type of feed and frequency, extraction equipment and production costs, among others.[17]

A pilot survey was applied beforehand to 30 people to make adjustments to the final questionnaire. A stratified sampling of beekeepers was made in six strata: 1 to 25 hives, 25 to 50 hives, 50 to 100 hives, 100 to 500 hives, 500 to 1,000 hives, and over 1,000 hives. From a population of 1,000 beekeepers in Jalisco, 50% live in the study zones, resulting in a population of 500 beekeepers. The final sampling was of 183 beekeepers surveyed, with a 95% confidence level, 3% accuracy, 5% participation, and sample size adjusted to 15% losses. For the data analysis we used a descriptive and quantitative method to identify, understand, correlate, and prove the hypothesis of the study. To analyze the information collected, it was processed using SPSS version 19® statistics software. We applied frequency analysis, ANOVA (Waller-

Duncan), and contingency tables (χ^2) to find whether or not there is an association between the social, economic, and technological profile variables in the apiculture production chain.

3. Results and discussion

3.1. Demographic aspects

The majority (59%) of beekeepers in the study region manage fewer than 100 hives, although some were observed to have more than 1,000 hives ([**Figure 1**). The average age of the beekeepers in the study zone was 47 years for men and 45 years for women. Only 3% were younger than 20 years, and the beekeepers with most hives were also the oldest, evidence of the lack of interest in beekeeping among young people. In the peninsula of Yucatán, the average age is 47 years,18] unlike Michoacán where a 2004 study mentioned 43 years.[19,20] In contrast, on the island of Tenerife, Spain, the average age is reported as 59 years.[21] Age is an important factor to consider in terms of the present and future management skills of beekeepers; older beekeepers are less willing to change their traditional production methods and learn new production or management techniques. Likewise, working on projects with young beekeepers under 25–30 years increases instability because of temporary or definitive migration because of a lack of sources of work in the field or perhaps for reasons of study.[22]

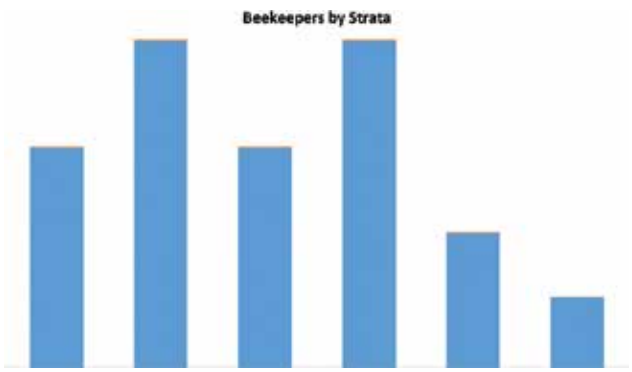

Figure 1. Distribution of beekeepers by strata. (Livestock Research Laboratory, Department of Agriculture Production, CUCSUR, University of Guadalajara).

3.2. Location of apiaries

As far as access to land for beekeeping, there is a significant difference ($p = 0.046$, χ^2) where 61.2% of beekeepers rent the premises where they have their apiaries and the rest use small-holdings and, to a lesser extent, *ejido* or common ground. Beekeepers with 101 to 500 hives are more likely to rent, a similar situation to that reported in communities in Michoacán, where the majority of beekeepers do not own the premises where they set up their apiaries.[23] This situation limits apiculture development as producers must pay, either in cash or kind, for the lease of the lands, and also limits the assurance of the availability of the space to maintain the

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apiaries; this, in addition to competition for better spaces not only among beekeepers but also farmers and other branches of livestock. In Turkey, 90.59% of beekeepers have their apiaries installed on private property.[20]

Some 60% of beekeepers installed their apiaries in the municipalities where they were born and the rest look elsewhere for suitable flowering spaces, showing a significant difference ($p = 0.000$, χ^2) where beekeepers with more than 100 hives have greater mobility in search of better yields. They also mention a wide saturation of hives in their municipalities, this being another of the main issues raised within the beekeeping production system. This is related to the average distance of 25 km they have to travel to inspect the apiaries (in a range from 1 to 200 km), where a significant difference ($p = 0.000$, χ^2) was observed, beekeepers with less than 50 hives traveling less than 10 kilometers to install their apiaries, while those with over 500 hives travel distances in excess of 60 kilometers. Such a situation is unique to this region; a study in Chile describes a high concentration of apiaries in certain zones,[22] a fact that goes against the environmental management requirements for good farming practices. A different situation exists in Yucatán, where close to 50% of beekeepers travel more than 10 kilometers to reach the apiary, while 22.2% travel less than 2 kilometers, which leads to strong competition among the bees to obtain food, since 88.9% have apiaries at a distance of less than the recommended 3 kilometers.[24]

3.3. Months of honey production

About 100% of the beekeepers refer to honey harvested is multiflora origin, since flowering of the area is varied in the area, and production depends on environmental conditions and the availability of floral resources producing pollen and nectar. The main honey harvest occurs in autumn; 30.1% of the beekeepers in the study area harvest in October, 74.5% harvest in November, the strongest month, and 48.1% in December. The secondary harvest, with less production, is done in the spring starting in March with 12%, rising to 27.9% in April, with a significant reduction in the activity in May with 11.5%.

The seasonality of honey production is marked at two different times of the year in most of the country, in the southeast and coastal regions it is obtained from March to May (spring-summer), generating 40% of production. The second harvest is obtained in the Altiplano and north of the country between September and November (autumn-winter), obtaining the remaining 60% of production. Honey production in the Yucatán Peninsula occurs during winter and spring from December to June and comes from toothleaf goldeneye (*Viguiera dentata*), tzitzilché (*Gymnopodium floribundum*), and some vines (1, 25). In Michoacán, most beekeeping activity takes place in spring and summer (August and September), with the most significant peak during June. This variation in seasonality by zone around the country allows honey to be available throughout the year.

3.4. Problems in honey production

There is a significant difference ($p = .000$, χ^2) among the complaints of beekeepers where those with more than 500 hives express the lack of available spaces for placing the apiaries; in recent

years, new beekeepers have emerged who establish their apiaries less than 2 kilometers away, thus invading flowering spaces, which decreases production. Meanwhile, beekeepers with less than 500 hives give priority to environmental factors, mentioning situations of high deforestation and fires, reduced and erratic seasons, the indiscriminate use of pesticides with the resulting damage to bee populations; followed by production costs, which have increased because of the high cost of sugar, one of the basic inputs, the purchase and exchange of queens because of Africanization, as well as the cost of the treatment of diseases, lack of support for the purchase of extraction equipment and hive management, and roads and tracks in poor condition, which affects the beekeeper going to inspect the hives, and the fact that where support does exist it is insufficient (**Figure 2**).

Figure 2. Problems facing beekeepers for honey production (Livestock Research Laboratory, Department of Agriculture Production, CUCSUR, University of Guadalajara).

As far as disease, attention is focused on the varroa mite to control the health problem. A similar situation is found in Yucatán, where beekeepers also complain of lack of training and unfavorable market conditions, not unlike the situation in the study zone. This is in contrast to the problems described in Nigeria, where beekeepers are affected by theft of the hives, fires, abandonment of hives, lack of better technology, lack of technical assistance, and the aggressiveness of the bees.[25]

3.5. Bee products

In the productive field, all (100%) beekeepers obtain conventional honey; in first place as an alternative product is beeswax, produced by 58.6%. This is followed in second place by nucleus colonies and propolis, and to a lesser extent, royal jelly, queens, and pollen, with little or no participation in the pollination process (**Table 1**). Although pollination is not a product but a service provided by apiculture, in many parts of the country it is an alternative source of income. In fact, in the states of Sinaloa, Chihuahua, and Coahuila, it is the main purpose of bee

exploitation, honey production being a secondary activity,1] and in Michoacán pollination generates important economic income for 32.4% of beekeepers. In contrast, only 29.1% of beekeepers in Michoacán recover beeswax, whereas in the study zone this figure is more than double (58.5%).[19]

Alternative product	Frequency	Percentage
Beeswax	107	58.5
Nucleus	52	28.4
Propolis	33	18
Queens	25	13.7
Royal jelly	23	12.6
Pollen	18	9.8

Table 1. Products other than honey obtained by beekeepers in the south and southeast regions of Jalisco (Livestock Research Laboratory, Department of Agriculture Production, CUCSUR, University of Guadalajara).

Although obtaining organic honey generates higher economic profits, it implies additional costs both for equipment and the necessary certification processes as well as the application of different production protocols to guarantee a product free of chemical substances. Organic bee farming also presents strategic technical challenges in training to obtain quality products and resource management for the acquisition of processing equipment and physicochemical product analyses which, when done professionally, make the activity more competitive. Organic honey is an area of opportunity for beekeepers in the study zone; the best price for organic honey may be 30% more than the price of conventional honey.[1,19] The obtaining of other products and pollination could improve the producers' income; however, these activities require the investment of more time and as this is not the main economic activity of more than 50% of the beekeepers in the study regions, further diversification is stifled and they remain within traditional exploitation with the production of primarily honey, beeswax, and bee nucleus colonies, which is contrary to the so-called integral exploitation.

3.6. Economic aspects

No differentiation is made in the management of the honey whether sold by the bottle or by the bucket as only 14% of beekeepers sell their products with a label. However, a 2012 study mentions that in Jalisco, sales of private label bottled honey are less than 1% of the production sold by those producers, an action that represents an important step toward the end consumer and the added value of the product.[26]

This form of commercialization has facilitated the sale of adulterated honey and even high-fructose corn syrup as if it were honey, thus deceiving many people who purchase it believing it to be real honey at a very low price.[1] Limited classification of the product by color and/or flowering, bulk sale, and the lack of technology to enable value-added export position the honey industry as a commodity.[27]

In terms of the sale price per kilo of honey, a significant difference is observed in the analysis of variance Waller-Duncan of 0.000; beekeepers in strata 1 with up to 25 hives receive an average of 52.71 pesos while those in strata 5 with 501 to 1,000 hives receive 37.42 to 40 pesos. The trend observed is that the fewer the hives, the higher the sales price, which is because of the sale being made directly to the consumer while big producers sell their product wholesale and often receive a price close to 40 pesos per kilo (**Table 2**). In 2008, however, Jalisco was considered the best paid state, in that year receiving a price of 30.57 pesos per kilo, above the national average of 24.52 pesos. It should be noted that the price quadrupled in the decades from the nineties to 2008 going from 5.86 to 24.54 pesos nationally, which is attributed to the issues this production sector faces.19]

Variable		*N*	Average	Sig.
Sale price of honey in 2011 (kg)	1–25	31	52.71	0.000
	26–50	46	48.00	
	51–100	30	43.50	
	101–500	46	40.26	
	501–1,000	19	37.42	
	1,001 o more	10	40.10	
	Total	182	44.57	

Table 2. Sales price of honey per strata in the south and southeast regions of Jalisco (Livestock Research Laboratory, Department of Agriculture Production, CUCSUR, University of Guadalajara).

As far as the export of honey, only 6.55% of beekeepers mention exporting honey to Germany. The beekeepers in strata 6 with more than 1,000 hives are the ones who export the most, there being a significant difference ($p < 0.001$ using χ^2) compared with strata 2 with 26 to 50 hives. One of the problems observed in the states in the study is that the production is bought by intermediaries who often pay for the harvest in advance and are responsible for positioning the product on the European market. This is a situation that prevails in countries such as Argentina, where it is reported that more than 95% of honey production is for exportation, and which is handled by only a few actors (three or four companies); the crucial points applied to exportation, such as quality control, storage, transport, and retail outlets form part of the marketing and supply strategies of the exporting companies in the area.[27]

Only around 30% of beekeepers know the quality standards required on the international market. Beekeepers in strata 4, 5, and 6 (more than 100 hives) are better trained in these aspects ($p < 0.66$, χ^2) compared with beekeepers with fewer hives, who also show little interest in the export process considering it to involve too much bureaucracy. Producers need to know the quality standards required by the international market, as well as packaging, packing, and prices be competitive.[28]

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3.7. Honey marketing problems

Close to 40% of beekeepers interviewed expressed problems in marketing the honey, and among the problems they face are low prices, mentioning that sometimes they recover only the production costs. Likewise, street vendors (carts) of adulterated honey at low prices have become unfair competition for beekeepers, as consumers have no knowledge of the quality and purity of the honey. A similar problem occurs in Argentina, where adulterated honey is rife on the local market. In addition, the abundance of red tape for exporting and the need for intermediaries demotivates producers from exploring the international market. In strata 1, 2, and 3 beekeepers express their concern about the low per capita consumption of honey, which only reaches 320 grams per year.[27]

Within the production process, 43.7% of those interviewed keep a record of production costs. It is mostly the beekeepers in strata 4, 5, and 6 (more than 100 hives) who carry out this activity to a significant extent ($p < .028$, χ^2). Similarly, Torres[22] observed in Chile that 50% of those interviewed said they did not maintain written accounts or sales records.

Of those beekeepers who do maintain records, not all were able to provide complete information, hence only 38.25% (70) of those interviewed were considered in the calculation of production costs, which include containers, treatments, gas, electricity, equipment repair and maintenance, vehicle maintenance, queen bees, beeswax, labor, feed (sugar, and others), protection equipment, and hive management equipment. In this area, there were significant differences ($p < 0.43$, Waller-Duncan) between the strata, observing that beekeepers with more than 500 hives (strata 5 and 6) have lower production costs (16.43 and 19.62 pesos, respectively), while beekeepers with less than 50 hives (strata 1 and 2) have higher production costs at 46.87 and 34.47 pesos, respectively. Lower production costs in strata with more hives may be directly related to the high volumes of inputs purchased to carry out the beekeeping activity and to group purchases to obtain better prices by buying wholesale.

The exploitation of bees that are more defensive, swarming, and evasive leads beekeepers to make changes in the way they are managed, such as relocating apiaries to more distant locations, thereby increasing the costs of transportation and labor (each worker manages fewer hives per day than when working with European bees), and also the protective equipment required against more defensive bees (coveralls and gloves),[29] and the purchase of queens, which before Africanization was minimal. In addition to this is the cost of bee feed, which in recent years has become one of the major costs, given the excessive increase in the price per kilo of sugar. It is estimated that production costs in managed colonies have increased around 30% in comparison with European bees[4] and because of treatments, particularly for the varroa mite. In spite of this, 66.6% of beekeepers believe bee farming is profitable. However, profit margins vary widely in a range from 5% to 200% as a consequence of such great differences between beekeepers.

3.8. Social aspects

About 90% of beekeepers are members of a beekeepers association; 52% belong to 4 of the 11 associations registered in the study regions (Table 3).

Local livestock association of beekeepers (municipality)	Frequency	Percentage
Sayula	26	14.2
Apiteca	17	9.3
Asociación de Tamazula	22	12.0
Gómez Farías	9	4.9
Atoyac	4	2.2
Asociación San Gabriel	19	10.4
Asociación de Zapotiltic	23	12.6
Asociación de Tapalpa	6	3.3
Zapotlán	24	13.1
Zacoalco	9	4.9
Atemajac de Brizuela	6	3.3
Does not belong to any association	18	9.8
Total	183	100.0

Table 3. Participation in beekeeper associations in the south and southeast regions of Jalisco (Livestock Research Laboratory, Department of Agriculture Production, CUCSUR, University of Guadalajara).

A strategic challenge in the technology field is for small beekeepers to communicate clearly with research bodies, to generate a greater degree of professionalism and scientific rigor to meet the competitive challenges emerging in the industry; such communication is more feasible with producers who participate in organizational bodies. This is an important factor to push the competitive development of apiculture production units toward higher levels of social engagement for economic and productive purposes. It is also important to carry out coordinated actions to achieve a common goal, through the identification and planning of collective actions, and confront the control exerted by intermediaries, which would allow better prices for bee products and lead to the activity no longer being considered as merely for subsistence.[19,30]

Associated beekeepers in the study zones indicate that the support they have received from the association to which they belong consists of guidance for obtaining technical resources and training. Through the producers' alliance, they have been able to obtain government economic resources for the construction and equipping of extraction rooms based on the safety requirements within the honey production process. Beekeepers have opted to associate in various ways to deal with their lack of resources and knowledge; however, the way in which they have become associated has often been linked to obtaining government support, as in the Yucatán

Peninsula, beekeeping organizations and cooperatives facilitate the adoption of technology, equipment acquisition, storage, and sale of better quality honey, and are promoted by public institutions and civil organizations.[24]

Associative schemes, whether for productive or commercial purposes, are a valuable tool for beekeepers to achieve their objectives; however, these alone are no guarantee of success; any tool has advantages and disadvantages and being aware of these and analyzing them will avoid any false expectations.

Among the associated beekeepers, 26% think it is unnecessary to make any changes within the operation of the association; however, others mention that changes are required, such as better organization and integration among the members of the associations, referring to greater responsible participation of the assemblies. They also express the need for more resource management and technical support. Nevertheless, they recognize that they have obtained an important benefit by participating in the organization, namely training, and they believe that the honey they produce is recognized for its excellent quality because of their training in best practices in apiary management.

Regarding participation in programs or institutions for support management, close to 80% of beekeepers mention having obtained support from the Secretariat of Agriculture, Livestock, Rural Development, Fisheries and Food (*Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación*, SAGARPA), followed by the Secretariat of Rural Development (*Secretaría de Desarrollo Rural,* SEDER), with 13.1%. In addition, 6.6% have received support from the Livestock Productivity Incentive Program (*Programa de Estímulos a la Productividad Ganadera*, PROGAN), which provided economic resources, support for hive identification and payment for technical assistance and training, as well as direct support of 75 pesos per hive to beekeepers of 10 to 175 hives and 75 pesos per hive from 175 to 1,500 hives. This contrasts to the participation in the Secretariat of Social Development (*Secretaría de desarrollo Social*, SEDESOL) program, with 2.7%, which unlike the others promotes social and micro business development. Of the 26% who have not received support from any institution, the majority have less than 50 hives (Table 4).

Institution	Frequency (beekeepers)	Percentage
SAGARPA	146	79.8
SEDER	24	13.1
PROGAN	12	6.6
FIRA	6	3.3
SEDESOL	5	2.7
DIF	3	1.6
None	26	14.20

Table 4. Institutions that have provided support to beekeepers in the south and southeast regions of Jalisco (Livestock Research Laboratory, Department of Agriculture Production, CUCSUR, University of Guadalajara).

Similar participation in support programs is observed in Michoacán and Yucatán, which beekeepers consider is mainly because of there being no requirement for guarantees.[19,24]

3.9. Technological aspects

Of the beekeepers interviewed, 84% have access to training including support by the SEDER through PSP technicians. Of these, 80% say they have put into practice the knowledge obtained both in congresses and during training with technicians, mainly in disease control, feeding, and honey production with good practices. This is interesting as the percentage of beekeepers with technical or higher education is very low. This coincides with what happens in the Alhué commune in Chile, where 70% are interested in training on beekeeping topics, as indicated by Torres,[22] who reports that 80% of beekeepers mention having attended training courses and the rest consider themselves self-taught.[31] This is in contrast to what happens in Santa Catarina in Brazil, where beekeepers do not put into practice knowledge obtained in different forums because the majority engage in beekeeping as a secondary activity, in addition to financial difficulties.[32] It should be mentioned that in the study zones, 47% engage in beekeeping as a primary activity, which is perhaps why they are more likely to put innovation into practice.

Of those interviewed, 94.5% carry out pest and disease control in January, February, June, and August ([Table 5). Varroa is the main problem affecting 91% of their bees, with foulbrood to a lesser extent at 24%, and chalkbrood at 16%. Yucatán presents similar figures for Varroa but with chalkbrood at 44.4%.30] In India, treatment is provided to 86.7% of hives, mainly against varroa and moths.[33] Similarly, in Canada it has been reported that varroa is the main cause of death for bee colonies during winter, being associated with 85% of cases of mortality.[34] Furthermore, in the United States, Europe, and Japan bee colony deaths have also often reported (*Apis mellifera* L.). The *Varroa destructor* mite and the combination of some viruses have been implicated in recent disappearances of bee colonies, making it a particularly serious threat to the health of bees.[35]

MONTHS	Bee feeding		Disease treatment	
	Frequency	% Beekeepers	Frequency	%Beekeepers
January	25	13.7	94	51.4
February	27	14.8	39	31.3
March	21	11.5	25	13.7
April	31	16.9	21	11.5
May	74	40.4	33	18
June	139	76	80	43.7
July	145	79.2	49	26.8
August	139	78	54	29.5
September	89	48.6	25	13.7

MONTHS	Bee feeding		Disease treatment	
	Frequency	% Beekeepers	Frequency	%Beekeepers
October	19	10.4	4	2.2
November	1	0.5	4	2.2
December	6	3.3	18	9.8

Table 5. Feeding and disease control by month in the south and southeast regions of Jalisco (Livestock Research Laboratory, Dept. of Agriculture Production, CUCSUR, University of Guadalajara).

Given that varroa is the main pathological problem in the study zones, 45% of producers have focused on controlling mite infestation in bee colonies using mainly Bayvarol®, 28.4% use natural products, and 16% use Apivar®. Only 7.7% did not apply any treatment. There are few studies in Mexico that show the detrimental effect of varroa on honey production; however, in Valle de Bravo in Mexico State, colonies treated with an acaricide against *V. destructor* were observed to produce significantly more honey than untreated colonies,[36] but it should be noted that environmental conditions and the type of bee may influence the effect of varroa on the productivity of the bees.[37]

3.10. Feeding frequency and feed type

Of the beekeepers interviewed, 96.7% provide maintenance feed to their bees; 81% of these provide energy feed mainly in syrup, and only 33% provide protein feed, unlike Brazil, where 63.6% provide maintenance feed and only 9% protein feed.[38] The use of fructose and confectionery waste is an uncommon practice. In Yucatán, 77.8% feed their bees with a sugar syrup, while only 14.8% feed them with honey, the rest use granulated sugar.[28] In California, the use of honey and sugar syrup is described,[39] and in Chile feeding with honey is also practiced;[31] however, this practice endangers the health of the colonies if the honey does not come from safe sources.

The frequency of feeding is from 7 to 10 days (36.1%), 11 to 15 days (45.4%), and 16 to 30 days (17.5%). Various types of feeders are used to feed, the most popular being a plastic bag (close to 27%), followed by a 1-liter tub (25.7%), the Doolittle feeder (18.6%), and less frequently the Miller feeder (14.2%) and plastic soda bottle (13.7%). Feeding and feeding frequency is a management practice that guarantees vigorous colonies when the nectar is flowing, which translates into higher production levels. Feeding is one of the main production costs and beekeepers indicate that in recent years bees need to be fed for longer periods because of changes in rainfall cycles and lack of flowering. In this respect, the practice of migratory beekeeping, which is negligible in these regions, could reduce feeding costs, however, the costs of moving the hives and the wide competition for spaces to place apiaries would have to be considered.

The majority of the beekeepers in the study manage modern jumbo or Langstroth hives. In contrast, beekeepers in Ethiopia use predominantly rustic hives even though they mention having adopted technological innovation (86%) and notice production increases; nevertheless the modern hive has not gained popularity because of its high cost and lack of awareness.[40]

In the north of Ethiopia, an average of 33 and 16 kg of honey per colony was observed in modern and traditional hives, respectively; production is more than doubled with just the transition to a modern hive.[41]

3.11. Quality control

As far as the implementation of a quality system, 66% carry out some practice for this purpose, mainly maintaining hygiene in the equipment, harvest and post-harvest, and avoiding the use of pollutant fuels and to a lesser extent using vegetable oil instead of paint to protect the hives.

Within the honey harvesting process, 82% of beekeepers interviewed use a smoker to remove the bees from the racks, either alone or in combination with shaking or brushing. The fuel they use is wood chips and corn cobs. This is consistent with the authorized physical means to repel the bees from combs for harvesting (air, shaking, brushing, and smoke through the use of clean fuels). Only a few (4.9%) use chemical repellents (carbolic acid, propionic anhydride, and benzaldehyde), which are restricted because of their residual action on honey and because they are considered carcinogenic. It is also inadvisable to use hydrocarbons and their derivatives (diesel or liquid gas) or materials impregnated with chemicals, paints, resins, or organic waste such as manure as fuels.[9] These are important aspects to consider in the honey production process to preserve and even improve Mexico's privileged position on the international market.

Among honey processing equipment, 60% of beekeepers said they had an extraction room, and the rest mention having a prepared space or resorting to the rental or loan of a room to carry out the extraction: 66% of the beekeepers say the rooms in which they work are equipped with running water. Close to 70.5% of the beekeepers in the study have stainless steel equipment (extractor and settling tank), an indispensable requirement within the good practices of honey production. In addition, 16.4% of beekeepers claim to have galvanized metal extractors and 11% mention other types of materials, among which some are made by the beekeepers themselves; 64.5% of the beekeepers use drip trays in the honey harvesting process in the field, with which they protect the supers from possible field contamination; and 66. 7% of beekeepers in Michoacán have an extractor and only 39.5% a settling tank, but the kind of materials these are made from are not described, and although they have incorporated technology, they have not updated it in accordance with current demands for safe food products.[19]

About 63.4% of beekeepers sieve or strain the honey as part of the process once it is extracted, while the rest mention only letting it settle for a period of 48 hours in the tank, and bottling it from there. On the other hand, it was observed that 87% of Michoacán producers filter the honey.[19] The technological level is a competitive factor intended to speed up the production process.[38] This leads to an increased volume of honey and reduces costs by improving equipment and tools with the innovation of the beekeeping production system.

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4. Conclusions

In the south and southeast regions of Jalisco, beekeeping is practiced by older people, with little appeal to the young and few women participants. Small and large producers with over 1,000 hives participate in the activity, although the majority are small producers who do not have enough hives to justify a full-time commitment. This is reflected in a considerable reduction in honey production that is below the national average, due mainly to environmental factors, high production costs and health problems in which varroa is the producers' major challenge. There is little diversification and differentiation between bee products, so it is necessary to work on strategies to differentiate the quality of honey to maintain their position as global exporters. The beekeepers in general are unaware of the destination of the production, and only have general references of those who buy large quantities, the destination being simply exportation. Similarly, there is little knowledge of the quality standards demanded by export markets. The level of education of producers has encouraged them to attend various training and technical assistance forums, as well as the assimilation of technological innovation both in hive management and harvest and post-harvest of honey and derivatives, through the incorporation of stainless steel equipment in the extraction rooms.

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Appendices

Appendix 1

Survey

Beekeepers of the South and Southeast Regions of Jalisco, Mexico.

Universidad de Guadalajara, Universidad Popular Autónoma del Estado de Puebla

Date:_____Interviewer_____

Name of respondent_____

E-mail_____ Telephone_____

Address_____

Age: **_____Sex _____ Education _____**

Type of Tenure

☐ Common land ☐ Smallholding ☐ Co-ownership ☐ Rented

Town and municipality _____

Length of time as a beekeeper _____

Primary economic activity _____

a. GENERAL INFORMATION ABOUT THE ACTIVITY

1. How many hives do you have?

a) 1 to 25 b) 26 to 50 c) 51 to 100 d) 101 to 500 e) 501 to 1000

2. What is the average distance from your apiary to your home?

_____

3. Geographical location of the apiaries (Municipality)

_____

4. During which months is honey produced?

_____

5. What is the average yield per hive? (Indicate unit of measurement)

_____

6. What are the problems you face in producing honey? (In order of importance)

_____

7. Indicate the bee products you currently produce:

Pollen ☐ Royal jelly ☐ Beeswax ☐ Propolis ☐ Queens ☐ Nucleus ☐ Others (specify)

_____

c) ECONOMIC ASPECTS

8. Where do you sell honey?

☐ Local market (neighbors, friends, nearby communities)

☐ National market: Intermediary ☐ Bottler ☐ Other ☐

☐ Industry Which?

_____

9. Do you sell the honey? ☐ **Bottled** ☐ **In bulk** ☐ **Both**

10. At what price have you sold honey in the last 5 years?

_____

11. Do you label your products?

Yes ☐ No ☐

12. ***Do you export honey?***

Yes ☐ *No* ☐ ***To which countries***? _____

13. **What quality standards does the international market demand?**

_____

_

14. Mention the problems you face to market honey**?**

_____

15. Do you keep a record of production costs? Yes ☐ No ☐
Why? _____

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Inputs	Unit cost	Amount	Total Value
Containers:			
Drum			
Tub			
Jar			
Treatment per hive for			
Nosemosis			
Foulbrood,			
Varroa			
others			
Gas (kg)			
Electricity (Kw.)			
Repair and maintenance of extraction and field equipment (nails, vegetable oil, etc.)			
Gas, vehicle repair and maintenance. (Km)			
Queen replacement			
Stamped beeswax (kg)			
Paid labor ($ / working day)			
Feed (kg sugar per hive per year) and other feeds			
Protective equipment (veil, overall and gloves)			
Hive management equipment (hive tool, smoker, brush)			
Others			

16. Indicate under each heading your expenses for producing honey (per year)

_____

17. Has the activity been profitable in the last 5 years?

Yes ☐ No ☐ What is your profit margin? _____%

b. SOCIAL ASPECTS

18. Do you belong to a beekeepers association?

Yes ☐ Which? _____

No ☐ Why not? _____

19. What type of support do you receive from the beekeepers association?

_____

20. What changes do you think are necessary to improve the functioning of beekeepers associations?

_____

21. Which organizations or government programs have given you support? a) SAGARPA b) Local livestock associations c) FIRA d) Other (Specify)

_____

C. TECHNOLOGICAL ASPECTS

22. Do you have access to beekeeping training courses? Yes ☐ No ☐

23. What institutions offer training courses?

_____

24. How often do you attend beekeeping courses or conferences?

a) Once a year b) Twice a year c) 3 or more times a year d) Never

25. What training events do you attend regularly?

_____

26. Have you implemented any of the knowledge you obtained in the training courses in your apiaries?

Yes ☐ No ☐ Which?

_____

27. Do you carry out any bee disease control?

Yes ☐ No ☐ Which? _____

28. In which months do you carry out disease control?

_____

29. Which medicines do you use for disease control?

_____

30. How do you administer the medicines?

_____

31 Do you feed the bee colonies? Yes ☐ No ☐

32. In which months do you feed the bees?

_____

33. How often do you feed the bees?

a) Every 7 to 10 days b) Every 11 to 15 days c) Every 16 to 30 days

34 What kind of feed do you give the bees?

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a) Energy b) Protein c) Both

35. What kind of energy feed do you give the bees?

a) Sugar b) Fructose c) Confectionery waste d) Other

36. What kind of feeder do you use?

_____

37. What variety of honey do you produce?

_____

38. Do you use any quality control system in the honey production?

Yes ☐ No ☐ Which?_____

39. What technique do you use to remove the bees from the honeycombs?

a) Smoke b) Air c) Strong blow d) Repellents e) Brush f) Other

40. Do you have an extraction room?

Yes ☐ No ☐

41. Do you use drip trays to transfer the harvested supers?

Yes ☐ No ☐ Other

42. Do you have running water in the extraction room?

Yes ☐ No ☐

43. What kind of material are your extractor and settling tank made of?

_____

44. Do you sieve or filter the honey?

Yes ☐ No ☐

Chapter 8

From Extraction to Meliponiculture: A Case Study of the Management of Stingless Bees in the West-Central Region of Mexico

Alejandro Reyes-González,
Andrés Camou-Guerrero and
Salvador Gómez-Arreola

Additional information is available at the end of the chapter

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Abstract

Currently, stingless bees' populations are declining due to environmental degradation. In this context, the authors have developed a research project in the central-western region of Mexico with the goal to generate strategies for conservation and sustainable management of stingless bees. The chapter aims to present the process of this investigation and its main results in terms of a) local knowledge and management strategies of stingless bees, and b) the social process of technological appropriation of meliponiculture by beekeepers. We recognized specific knowledge on the biology and ecology of stingless bees that result in a system for identifying species and management strategies of wild populations of these bees based on the extraction of nests. The implementation of an innovative productive activity based on the principles of meliponiculture and current techniques has been well received by producers, which has led to the formation of the Meliponicultores Michoacanos del Balsas Association, which grows five species of stingless bees. The research suggests that conservation associated with the use of bees (integral meliponiculture) can be enhanced in the region. Faced with the loss of biodiversity and environmental crisis, it is essential to maintain and enhance local knowledge of stingless bees and management practices. This represents an alternative to develop management schemes that allow the raising and breeding of these bees, while its products are obtained.

Keywords: stingless bees, meliponiculture, Balsas River Basin, Michoacán, México

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1. Introduction

Bees represent one of the most important functional pollinator groups for terrestrial ecosystems [1, 2]. It is estimated that nearly 73% of cultivated vegetation species and more than 75% of the world's total vegetation is pollinized by bees [1, 3, 4]. However, in spite of the importance of this group of organisms, there is clear evidence of their population decline, putting at grave risk the pollination services they provide [5] as well as ecosystem and agrecosystem maintenance [6, 7]. This has profound ecological and economic implications. Some of the more relevant causes of this pollination crisis are forest loss and fragmentation, the use of agrochemicals, bee pathogens, invasive species, and climate change, among others [1, 5, 8–18]. In the particular case of stingless bees, the extraction of wild nests and habitat alteration have been cited as primary causes of population deterioration for this group [19].

There are approximately 20,000 species of bees [20] of which *Apis mellifera* has received greater attention due to their ecological and productive qualities having been introduced all over the world [21]. However, in the tropics, one of the Apidae of greatest ecological and sociocultural significance are the stingless bees (*Meliponini*) [2, 16, 20, 22, 23]. The authors of this chapter have developed a research project in the west-central region of Mexico with the purpose of generating strategies for the conservation and sustainable management of stingless bees. The objective of this chapter is to present the investigation process and the results in term of a) diversity, knowledge, and strategies of local managers of stingless bees; and b) the social process of technological appropriation of integrated meliponiculture by the managers in the study area. The chapter is divided into three sections. The first section discusses the diversity of stingless bees in Mexico. The second portion analyzes the management of stingless bees, and in the third and last section, we present a case study of technological appropriation of integrated meliponiculture in the Alto Balsas region of Michoacán, Mexico.

2. Stingless bee diversity in Mexico

Stingless bees form part of the order *Hymenoptera*, family *Apidae,* tribe *Meliponini*. Worldwide there are 24 genus, 18 subgenus, and between 400 and 500 species [20]. The American continent is considered the center of diversity for this group and in the case of Mexico there have been a total of 16 genus and 46 species present (**Table 1**), of which 26.% (12 species) are endemic [24]. After a review of the specialized literature of the total species described for Mexico, 43.5% (20 species) are under some form of human management (**Table 1**).

Species[1]	Local name	Management	Region of managed species	Reference (for useful species)

1 **Cephalotrigona oaxacana*

(Schwarz, 1948)

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Species[1]	Local name	Management	Region of managed species	Reference (for useful species)
2 *Cephalotrigona zexmeniae* (Ayala, 1999)				
3 *Cephalotrigona*zexmeniae (Cockerell, 1912)	E'hol, Tajbak	(*e*)	Peninsula de Yucatán	[25]
4 **Frieseomelitta nigra* (Cresson, 1878)	Sak-Xic'/ Abeja zopilota	(*m*,*e*)	Península de Yucatán, Sierra de Manantlán Jalisco, Cuenca del Balsas Michoacán	[19, 25, 26]
5 **Geotrigona acapulconis* (Strand, 1919)	Colmena de tierra	(*e*)	Cuenca del Balsas Michoacán	[19]
6 **Lestrimelitta chamelensis* (Ayala, 1999)	Abeja limoncilla	(*e*)	Cuenca del Balsas Michoacán	[19]
7 *Lestrimelitta niitkib* (Ayala, 1999)	Niitkib, Limón kab Limoncillo		Península de Yucatán, Soconusco Chiapas	[25, 27]
8 *Melipona beecheii* (Bennett, 1831)	Xunaan-Kab / Abeja real/Ajau-chab	(*m*)	Península de Yucatán, Soconusco Chiapas, Tabasco, Veracruz	[19, 25, 27, 28, 29, 30]
9 *Melipona colimana* (Ayala, 1999)	Colmena real	(*e*)	Volcán Colima	[31]
10 **Melipona fasciata* (Latreille, 1811)	Colmena real	(*m*,*e*)	Cuenca Balsas Michoacán, Sierra Atoyac Guerrero	[19, 32, 33]
11 **Melipona lupitae* (Ayala, 1999)		(*e*)	Cuenca Balsas Michoacán	[31]
12 *Melipona solani* (Cockerell, 1912)	Abeja real roja	(*m*)	Soconusco Chiapas	[27]
13 *Melipona yucatanica* (Camargo, Moure,	Tsets.	(*m*)	Península de Yucatán	[25]

Species[1]	Local name	Management	Region of managed species	Reference (for useful species)
Roubik, 1988)				
14 **Nannotrigona perilampoides* (Cresson, 1878)	Mehenbol / Doncellita prieta / Mumu/ Abeja trompetera	(*m*,*e*)	Península de Yucatán, Soconusco Chiapas, Sonora y Sinaloa, Cuenca Balsas Michoacán	[19, 25, 27, 28]
15 *Oxytrigona mediorufa* (Cockerell, 1913)	Pringadora		Soconusco Chiapas	[27]
16 *Paratrigona guatemalensis* (Schwarz, 1938)				
17 *Paratrigona opaca* (Cockerell, 1917)				
18 **Partamona bilineata* (Say, 1837)	Esculcona/ Mordelona	(*e*)	Cuenca Balsas Michoacán	[19]
19 *Partamona orizabaensis* (Strand, 1919)				
20 *Plebeia cora* (Ayala, 1999)				
21 **Plebeia frontalis* (Friese, 1911)	Us-Kaab/Yaaxich/ mosquito	(*m*,*e*)	Península de Yucatán, Tehuacán Puebla	[25]
22 **Plebeia fulvopilosa* (Ayala, 1999)	Abeja sapito	(*m*,*e*)	Cuenca Balsas Michoacán	[19]
23 *Plebeia jatiformis* (Cockerell, 1912)				
24 *Plebeia llorentei* (Ayala, 1999)				
25 *Plebeia manantlensis* (Ayala, 1999)		(*e*)	Colima, Jalisco	[31]
26 *Plebeia melanica* (Ayala, 1999)				
27 **Plebeia mexica* (Ayala, 1999)				

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Species[1]	Local name	Management	Region of managed species	Reference (for useful species)
28 **Plebeia moureana* (Ayala, 1999)				
29 *Plebeia parkeri* (Ayala, 1999)				
30 *Plebeia pulchra* (Ayala, 1999)				
31 *Scaptotrigona mexicana* (Guérin-Méneville, 1844)	Pisil-nekmej/ Abeja congo	(*m*)	Sierra Norte Puebla, HuastecaPotosina-Veracruz, Soconusco	[27, 30, 34]
32 *Scaptotrigona pectoralis* (Dalla Torre, 1896)	Kantsak/Abeja congoalazana	(*m*)	Chiapas. Península Yucatán, Soconusco Chiapas	[25, 27]
33 **Scaptotrigona hellwegeri* (Friese, 1900)	Abeja Bermeja	(*m*,*e*)	Cuenca Balsas Michoacán y Guerrero	[19, 32, 33]
34 *Scaura argyrea* (Cockerell, 1912)				
35 *Tetragona mayarum* (Cockerell, 1912)				
36 *Tetragonisca angustula* (Latreille, 1811)	Doncellita/sayulita	(*m*)	Soconusco Chiapas	[27]
37 *Trigona corvina* (Cockerell, 1913)	KurisKab		Península Yucatán.	[25]
38 **Trigona fulviventris* (Guérin-Méneville, 1844)	MuulKab, Culo de buey		Península Yucatán, Soconusco Chiapas	[25, 27]
39 *Trigona fuscipennis* (Friese, 1900)	Kuris-kab, Tamagaza, Basurera		Península Yucatán, Soconusco Chiapas	[25, 27]
40 *Trigona nigerrima* (Cresson, 1878)	Tamagaza, Basurera		Soconusco Chiapas	[25]

Species[1]	Local name	Management	Region of managed species	Reference (for useful species)
41 *Trigona silvestriana* (Vachal, 1908)				
42 *Trigonisca azteca* Ayala, 1999				
43 *Trigonisca maya* (Ayala, 1999)	Puup, Chachem			
44 *Trigonisca mixteca* (Ayala, 1999)				
45 **Trigonisca pipioli* (Ayala, 1999)	Puup, Chachem, Cepimilla	(*e*)	Península Yucatán, Cuenca Balsas Michoacán	[19, 25]
46 *Trigonisca schulthessi* (Friese, 1900)				

[1]Based on [58]. *Species reported for Michoacán. (*e*): Extraction. (*m*): Meliponiculture.

Table 1. Diversity of meliponini in Mexico and useful species.

As we mentioned, stingless bees are distributed in tropical and subtropical regions around the world. In Mexico, the distribution is in Neotropical areas [35, 36], intimately associated with dry tropical and evergreen forests, though some species have been found in mountain ecosystems and mesophillic forests as well as temperate mixed pine-oak forests [24]. In the west-central region of Mexico, there is considerable *Meliponini* diversity particularly in two specific regions: 1) the Pacific coast and 2) the Balsas River Basin (an area of relevance in terms of endemism for this group) [19, 24, 31].

The bees from the *Meliponini* tribe are anatomically distinct from those with a functioning stinger. Moreover, they present a notable reduction in the venation of the anterior wings, simple, non-bifurcated spurs and a line of thick comb-like hairs along the internal distal margin of the posterior tibia, called *penicillium* [24]. At the same time, this group of bees shows diverse behavior patterns (for example, there are species that show cleptobiosis or thievery) and different nesting habits (species that build their nest in tree cavities, underground or exposed similar to termites) [37].

To demonstrate the ecological importance of stingless bees, it is estimated that they pollinate from 30 to 50% of all plant species in the lowlands in tropical America [22]. In Mexico, it is estimated that more than 80% of cultivates for human consumption depend in various degrees on these pollinators for efficient production [38].

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3. Management strategies of stingless bees

3.1. Traditional management

From a sociocultural perspective, stingless bees are of great significance in the social, economic, and religious aspects of diverse areas in which have been developed various systems of managing and breeding of these insects. In tropical America, from México to Brazil, this activity goes back to the Pre-Hispanic era [30, 33, 39, 40, 41]. The traditional knowledge and management practices associated with the stingless bees still exist in the indigenous communities in Mexico and Latin America that coexist with them.

In Mexico, there are four areas where stingless bees have been traditionally and contiguously managed: 1) the Yucatán peninsula, 2) the Gulf coast of Mexico, 3) the Pacific coast between and Sinaloa y Jalisco, and 4) the Balsas River Basin in Guerrero and Michoacán [28, 33, 42, 43]. In each of these areas exist important management strategies and practices from the extraction of derivative products and breeding to a process called "meliponiculture."

Typically, Mesoamerican meliponiculture has been developed with the goal of harvesting the goods produced by stingless bee, which represent a significant nutritional and medicinal dietary component. The honey is used mainly as a medicinal supplement and treatment for such things as ocular infection, fractures, muscle pain, sprains, cutaneous wounds, as well as gastrointestinal and respiratory illness [19, 44]. Likewise, the pollen (which they call "*pasacuareta"* in the Balsas region [19] is consumed either by itself or mixed with the honey for respiratory infections and "weakness" or fatigue. Another important product is the wax (called "Campeche wax"), which acted as a valuable trade resource during colonial times.

Today, these traditional practices associated with Mesoamerican meliponiculture are only conserved in few specific areas in the Mexican tropics. These practices are particularly significant in the Yucatán peninsula (Mayas) [45, 46], the Sierra Norte of Puebla by the Nahuas and Totonacos [29], in the south of Veracruz by the Popolucas [34], and in the Itzmo de Tehuantepec by the Zapotecos, Mixes, Zoques, Popolucas and Nahuas [47].

Apart from the traditional meliponiculture, extraction of stingless bee products has been documented in other regions in Mexico. Bennett (1964) mentions the presence of stingless bees in areas not considered part of their normal range as in the Sierra Tarahumara and northern Sinaloa, where there is previously documented knowledge of the meliponini, specificaly *Nannotrigona perilampoides* [48].

As previously mentioned, other regions of importance with regard to meliponiculture are the western and southwestern portions of Mexico; From Nayarit, southern Jalisco to the Balsas River Basin, found in the States of Guerrero, Michoacán y Morelos [33, 42, 43]. In these regions, the extraction of honey used to be principally from the species *Scaptotrigona hellwegeri* [49]. Historically, this was an activity of economic and socio-political importance as it was used as an offering to the Valle de México, which was the seat of the Mexica Empire [43]. Hendrichs [42] mentions that the Balsas region was known for its honey production (*"mieleros"),* by groups that would form expeditions to seek out honey and beeswax during the dry season

(November–December). It is notable that, according to the author the extraction methods in the Balsas region was an activity that systematically sought specific nests and was considered a specialized trade but that resulted in the destruction of the nest. More recently Reyes-González and collaborators [19] reported product extraction in the same region but in the State of Michoacán.

3.2. Technical advancements in management

Today, the practice of meliponiculture persists in spite of long periods of inattention and substitution for other production activities, including apiculture. However, there has been resurgence in interest for this particular activity, which has been the impetus for strategies to rescue traditional meliponiculture making it more efficient with the goal of meliponini conservation and alternative productive projects. This resurgence is directly linked with the growing demand for natural, organic, and homeopathic products, which include honey, pollen, propolis, and beeswax. New techniques and production methods have been developed (largely through academic institutions) that has been termed "integrative meliponiculture." This has allowed for more efficient and sustainable management of these insects where they are present. We see examples of this modern management in the States of Chiapas, Yucatán, Campeche, Guerrero, and Veracruz [27, 32, 46].

Integrative meliponiculture is a practice that takes into account not only the production factor but also the conservation and maintenance of viable colonies of stingless bees as well as the integration of the families of those interested in participating in this activity as well as the implication for community benefit. In the production sense, the goal of integrative meliponiculture is to obtain goods like honey, pollen, and propolis from the same nests while encouraging specialized ecosystem benefits like pollination in greenhouses and agricultural fields as well as the landscape in general. Environmental education is also an important aspect of this activity, where possible. Integrative meliponiculture limits the destruction of wild nests and favors their maintenance, rescue, and propagation. This activity also helps to limit the traffic and introduction of non-native species in the areas of the stingless bee's natural distribution. These principles are the main challenge for meliponculture currently developed in various regions of the planet.

4. Case study

4.1. Study area

The Balsas River Basin makes up 6% of Mexico's continental mass and covers various economically important regions of the west-central Pacific coast and south-central area between 17°00′-20°00′ N and 97°30′ y 103°15′ E. This traverses eight States in the Mexican Republic: Morelos (100%) and portions of the States of Tlaxcala (75%), Puebla (55%), México (36%), Oaxaca (9%), Guerrero (63%), Michoacán (62%), and Jalisco (4%) (**Figure 1**). For the state of Michoacán, this basin can be divided into three subregions, taking an altitudinal criteria:

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Alto Balsas, Medio Balsas, and Bajo Balsas (Tepalcatepec). The specific area of study falls inside the sub-region of Alto y Medio Balsas, and includes the municipalities of Charo, Madero, Carácuaro, Nocupétaro, Tacámbaro y Tzitzio (**Figure 1**).

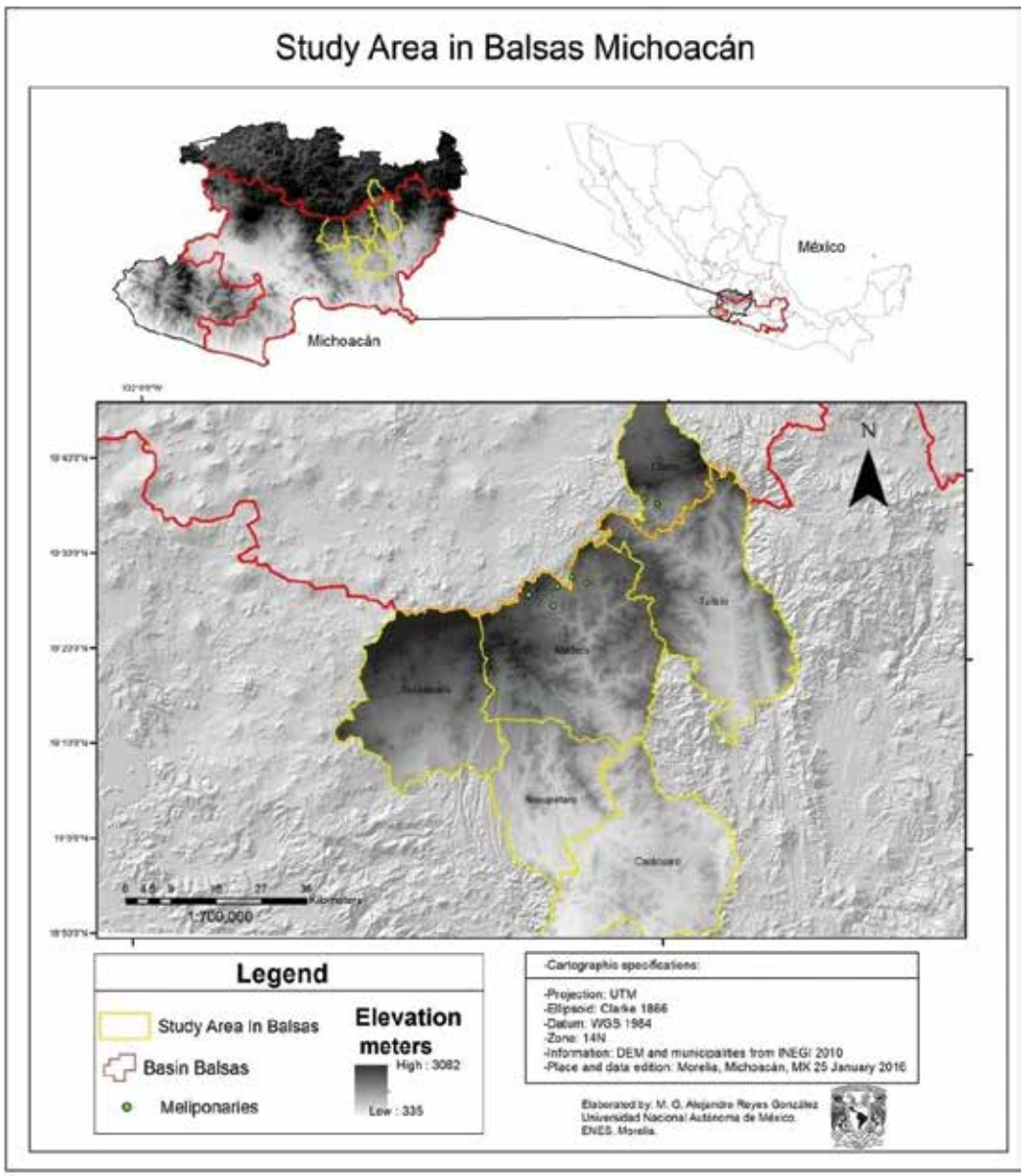

Figure 1. Location map of the study area in the Alto and Medio Balsas, Michoacán, México.

The study area falls in the foothills of the transmexican volcanic belt, flowing from the perennial streams that descend from an altitude of 3000m from the Turicato, Taretio, and El Perdido hills located approximately 40 km to the southeast of Morelia, Michoacán [50]. These subregions of the Balsas in the state of Michoacán has an altitudinal gradient that extends from 3000m to sea level with various climate regions associated with the altitudinal changes. These climate variations principally include temperate, tropical, warm sub-humid, temperate sub-humid, and semiarid [51]. According to Rzedowszki [52], the Balsas basin is considered one of the most biologically diverse regions in the world with a wide range of vegetation types where the principle ecosystems are the tropical dry forest and mixed pine and oak forest [52]. These climatic and vegetative conditions allow for bee species richness where, according to [24], the Balsas zone between Guerrero and Michoacán demonstrates notably high endemism for stingless bees.

Studies show that temperate zones with mixed pine-oak forest have extensive land use change from forest to agriculture and horticulture where avocado is predominant [53, 54],which has serious implications in the study area. Likewise, in the tropical forest areas, land use change is prevalent for livestock that converts natural cover to induced grasslands [55]. Such perturbations and transformations of the natural or mildly transformed landscapes results in strong repercussions in the stingless bee presence since populations of these important pollinators diminish as important vegetative sources disappear or are degraded eliminating sources of pollen, nectar and resins as well as niches for nests [56, 57].

4.2. The process of technological appropriation

The process that has been designed to orient sustainable management proposals for the stingless bees in the Alto Balsas region of Michoacán, has 4 main stages. The first stage consists of an inventory of the stingless bee species in the proposed area. The second consists of the documentation of local management practices of the species present there. The third is the selection of the working group that determines the selection of species, transference of hives, and outreach and communication of the work. The fourth and final stage is the maintenance and monitoring of the managed hives.

4.3. Local knowledge and management of the stingless bees in the study area

Through exhaustive fieldwork which involved extensive collection in the different climatic zones in the study area, workshops with the local apiculturists and "*colmeneros*" (experts in extracting products from wild nests of stingless bees) and thorough interviews, it was possible to document the stingless bee species of that zone as well as local knowledge and management practices. Of the species listed for Mexico, 15 species (32% of the total) were reported to be present in the State of Michoacán (**Table 1**). It is relevant to reiterate that there is a high species richness of stingless bees in the study area where 9 species (69% of the total species reported for Michoacán) were encountered (**Table 2**). The altitudinal distribution range of stingless bees showed a gradient that extends from 300 to 2000 m, covering the dry tropical forest in the

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warmest section and mixed pine-oak forest in the temperate extreme of the range. The species limited to the warm zone (300–1600 m) are*Scaptotrigona hellwegeri, Trigonisca pipioli, Frieseomelitta nigra.* The species limited to the temperate zone (1700–2000m) are*Partamona bilineata, Plebeia fulvopilosa, Nannotrigona perilampiodes, Melipona fasciata.* Lastly, there are two species of broad distribution (300–2000 m) that are *Geotrigona acapulconis* and *Lestrimelitta chamelensis.*

Local name	**Scientific name**	**Behavior (local knowledge)**	**Morphology (local knowledge)**	**Nesting**	**Distribution**
1) Abeja Bermeja	*Scaptotrigona hellwegeri*	Defensive (gets tangled in the hair and bites).	Intense red dish median bee.	In hollow trunks.	300 –1,600 m Tropical dry forest.
2) Abeja Cepimilla	*Trigonisca pipioli*	Bee type that likes people sweat.	Very small bee.	In hollow trunks, very small nests.	300–1,600 m Tropical dry forest.
3) Abeja Esculcona mordelona	*Partamona bilineata*	Defensive (gets tangled in the hair and bites).	Black middle bee	Aerial and exposed nest as termite mound.	1700–2000 m Oak and pine forest.
4) Abeja Limoncilla	*Lestrimelitta chamelensis*	Docile and attack other bees.	Small dark bee with strong lemon scent.	In hollow trunks.	300–2000 m wide distributions
5) Abeja Sapita	*Plebeia fulvopilosa*	Very docile and timid.	Small dark bee	In hollow trunks and between the trunk and the ground	1700–2000 m Oak and pine forest.
6) Abeja Trompetera	*Nannotrigona perilampiodes*	Very docile and timid.	Small bee.	In hollow trunks. The nest entrance is shaped trumpet (made of beeswax).	1700–2000 m Oak and pine forest.
7) Abeja Zopilota	*Frieseomelitta nigra*	Docile	Median dark bee, very bright with white wing tips.	In hollow trunks.	300–1,600 m Tropical dry forest.
8) Colmena real	*Melipona fasciata*	Defensive (gets tangled in the hair and bites).	Similar to *Apis mellifera* in size, color more reddish abdomen that *Apis*.	In hollow trunks	1700–2000 m Oak and pine forest.
9) Colmena de Tierra or Prieta de tierra	*Geotrigona acapulconis*	Very docile and timid.	Medium bee completely dark.	Buried in the ground.	300–2000 m Wide distribution

Local name	Scientific name	Behavior (local knowledge)	Morphology (local knowledge)	Nesting	Distribution
10) Abeja pintilla	–	–	More small than *Apis mellifera* in size and color similar.	In hollow trunks.	300–1600 m Topical dry forest.
11) Abeja Prieta esculcona	–	–	Black middle bee	Buried in the ground and cavities between the trunk and the ground."	300–1600 m Wide distribution

Table 2. Local knowledge and distribution on stingless bees at the Balsas Region, Michoacan. Based on [19].

There is a high level of local knowledge in the study area with regard to the bative stingless bees which are called *colmenas* or *colmenas de palo*. It is important to mention that apart from the 9 species encountered, the apiculturists and *colmeneros* mention 2 other types of stingless bees that have not yet been collected: *abeja pintilla* and the *abeja prieta esculcona* (**Table 2**). This implies that the species richness could be higher than recorded in the study area. As shown in **Table 2**, the local apiculturists and *colmeneros* are aware of the morphological characteristics, nesting habits, foraging habits, and defense tactics.

As previously described [19], the regional management in Balsas in the State of Michoacán relies on the direct extraction from wild hives with simple tools (axes and machetes). This was an important activity until the 1980s when the *colmeneros* relied on seasonal periods to extract wax and honey. The beeswax was used in candle making and for sale in other regions as material for fruit tree grafting. Honey is still a popular product for medicinal purposes associated with ocular infections, wounds, bruising, as well as an effective sweetener. However, in spite of the importance of these bee products there is much knowledge that has been forgotten with regard to management practices due to cultural changes and the diminished bee populations as a result of anthropomorphic land change [19]. Apiculture has been increasing in popularity as a common and generalized activity in rural families in the Alto Balsas, Michoacán region. For decades, in almost all households in the area the inhabitants had rustic hives using the *abeja de castilla* (*Apis mellifera*). These rustic hives were installed near the keepers' houses or sites very close to their homes for easier access and individual household consumption or local sale. However, this activity has been largely compromised with the introduction of the Africanized bee in Balsas during the period 1988 to 1989, in which the families not only stopped having rustic hives but also stopped using the European bee. In spite of the complications that came as a result of the introduction of the Africanized bee, the value placed on the natural bee products and in the face of the need to diversify livelihood activities and subsistence in rural areas is providing impulse to apply alternative projects in which integrative meliponiculture have been developed in the Alto Balsas in Michoacán.

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4.4. Forming the working groups

As previously discussed, the Balsas region in Michoacán has significant diversity of stingless bees and their actual diversity and distribution is not fully known. However, with the investigation efforts made in this study resulted in a useful pilot project with regards to integrative meliponiculture in the municipalities of Nocupétaro and Madero. Due to the influence and direction of the researchers in this study, the group "Meliponicultores Michoacanos del Balsas" has become an organization dedicated to the management and conservation of stingless bees. Most importantly, it must be noted that the initiative came out of a genuine interest by the apiculturists whose objective is the wider recognition of the importance of these bees along with the conservation and proliferation of these species as well as alternative livelihood potential that the bees offer.

4.5. Species selection and hive relocation

We started with collecting and documenting the bees and the location of wild hives in the study area (**Figure 1**). As a result, we observed that in the transitional ecotones between temperate forest and dry tropical forest, the most frequently encountered species were those of *Nannotrigona perilampoides* and *Plebeia fulvopilosa*. In the warmer zones, *Frieseomelitta nigra and Scaptotrigona hellwegeri* was more common. The relocation of hives was initiated with these three species, where we placed the entire hive into the customized bee boxes. Later, we sought out *Melipona fasciata* and *Scaptotrigona hellwegeri*, which are considered the most apt for production according to past experience [32] and by preference of the local managers.

Initially, we adopted techniques and management processes that had been employed in other regions of the country for the same genus found in this area [27, 32], but with undesired results since the percentage of adaptation and retention of nests (*Scaptotrigona hellwegeri* and *Melipona fasciata*) was 30%. Also other species as *Nannotrigona perilampoides* and *Plebeia fulvopilosa* did not develop their nests. The exercise did serve as a means to better understand biological particulars in the development of each genus that we worked with.

Through this process, we learned that it was of fundamental importance to use hives at risk of destruction or disappearance either by extraction or habitat destruction, which was causing significant impact on the bee populations. To ensure this means of selection, we developed a series of communication strategies to find out the location of hives and in particular those hives that were at risk.

Through direct dialogue with the inhabitants of the study area and formal presentations in municipal meetings pertaining to rural development organized by the local governing agencies (which were attended by all heads and representatives of the local ejidos, landholders, and communities), we presented the project and activities of the Meliponicultores Michoacanos del Balsas. This allowed us to establish rescue strategies for the nests that were at greatest risk (**Figures 2** and **3**).

Figure 2. Transfer of wild nest of *Plebeia fulvopilosa* who was in a oak tree (*Quercus* sp) at El Herrero, Madero, Michoacán.

Figure 3. Rescue of pillaged nest. One portion of the nest is removed, and recapped for maintenance. *Melipona fasciata* nest in a pine tree at Pie de la Mesa, Tzitzio, Michoacán.

We also employed a collection technique in which only part of the hive was extracted with only a fragment of the hive resources (honey combs and pollen) that were transported immediately to the new locations where the bee keeping boxes were already prepared for their arrival. This meant very careful extraction from the wild hives to avoid damaging the preexisting internal structure and covering the nest after extraction was completed. If the nest was located in a tree trunk or crevice, the cutting was made with power saw, which allowed for lower disturbance so that the remainder nest was not moved or altered from the original spot and could continue developing in its original location as we have seen in about 80% of nests worked.

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We worked with the following five species of meliponini in the study area: *Frieseomelitta nigra,Melipona fasciata*, *Nannotrigona perilampoides*, *Plebeia fulvopilosa* and *Scaptotrigona hellwegeri* (**Figure 4**). For each species, we have made every effort to adapt the management strategies and box design to their particular development needs. The smaller boxes have been used with *Frieseomelitta*, *Nannotrigona*, and *Plebeia* genera. By contrast, *Melipona fasciata* requires much larger boxes because the size of their brood combs, pots of storage and bee population (**Figure 4**).

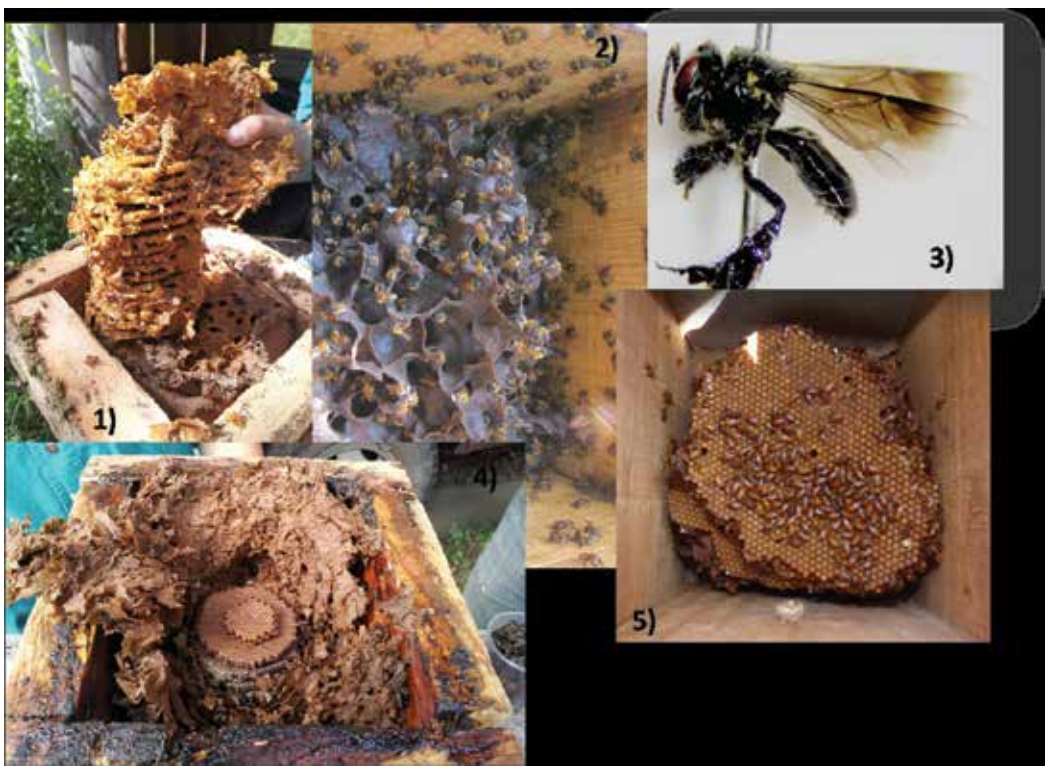

Figure 4. Managed species by the meliponicultores Michoacanos del Balsas: 1) *Nannotrigona perilampoides,* 2) *Melipona fasciata,* 3) *Frieseomelitta nigra,* 4) *Plebeia fulvopilosa,* 5) *Scaptotrigona hellwegeri*.

4.6. Hive maintenance and product commercialization

Once the hives have become established in the modern nesting boxes, it was important to monitor them for parasitic fly infestations by *Pseudohyphocera kerteszi*. In the cases where infestations were detected, apple-cider vinegar traps were used along with directed elimination of the flies. We also ensured that the nests had a stable resource stock for the population (pollen and honey) and if reserves were low we supplemented with honey from *Apis mellifera* nests having abundant resources.

In the hives where development was unhampered by external influences or resource limitations, we were able to obtain (in the fall—Oct-Nov) the following quantities of honey (per hive): *Melipona fasciata* 1500–2000 ml; *Scaptotrigona hellwegeri* 800-1,000 ml; *Nannotrigona perilampoides* 100–250 ml; *Frieseomelitta nigra* 100–250 ml and *Plebeia fulvopilosa* 40–80 ml (**Figure 5**).

Figure 5. Honey harvest of Melipona fasciata by decanting method. Piumo, Michoacán.

Figure 6. Selling products at fairs and festivals.

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In the experience of the working group, though there is a period of flowering in the spring and honey production as a result, products were not extracted from the hives. Instead that was the time when hives were divided to augment the bee and product inventory. As a result of this strategy to allow the hives to take advantage of natural production and leave their nutritional reserves, the monitoring of the nests was not as frequent over the rest of the year when harvesting or dividing were not occurring.

The division of the nests was carried out by taking half of the relocated nests (after they were well established) and dividing the storage vessels. In this regard, *Nannotrigona perilampoides* showed the greatest productivity and growth followed by *Plebeia fulvopilosa*. The species that responded less favorably in terms of growth and production after division was *Melipona fasciata* with an estimated 40% success rate after each division.

At the moment, Meliponiculture en el Balsas is being developed on a small scale, it is an innovative activity and has been well received by a group of apiculturists concerned about the rescue and conservation of stingless bees. The products obtained from these bees have a niche in the local and regional market and are sold directly from the producer to the consumer (**Figure 5**). The honey made into a suspension and is sold as a treatment for ocular infections at a price of around de $50 MN for 25 ml ($3 US dollars). This provides an earning of approximately $120 US dollars per liter of honey made into suspension. The pure honey is sold locally with a cost per liter of approximately $1000 MN ($65 US dollars). In the regional market, the working group has participated in various commercial events like the State fair and gastronomic events, among others (Image 6). The pollen is also sold, mixed with the honey at a proportion of 100 g of bee pollen per 1000 ml de honey of the species *Apis mellifera*. This product is marketed as a nutritional supplement with high protein and energy potential at a price of $150 MN ($9 US dollars) per 250 ml of the mix.

4.7. Challenges and prospects for meliponiculture in Alto Balsas, Michoacán

During this experience, it was necessary to adapt to various ecological and biological contexts, in particular the specific needs of each of the species we worked with. Environmental conditions like flowering periods, seasonality (resulting in limited resources during part of the year), temperature fluctuations, presence and abundance of wild nests, among others, influenced the management decisions and resulting interventions. All of this speaks to adaptive management.

Without a doubt, the most influential factor was the distribution and abundance of wild nests for the species we worked with. For example, *Melipona fasciata* is found exclusively in mountainous zones in areas where mixed pine-oak forest is well conserved. This has been a significant complication for the meliponiculture in the region due to the limited presence of species with the highest production potential. In particular, Melipona fasciata, "la colmena real" is quite scarce and has low resilience to disturbance in the area around nesting sites as it is only found in areas where the vegetation is well conserved and in the hollowed trunks of oaks (Quercus sp).

This was the only species that required special management attention. Initially, we tried to apply the same relocation strategies as for the other species of *Meliponini*; however, we realized

that they were not adapting to the relocation sites where they would either abandon them or showed high susceptibility to predation by *Pseudohyphocera kerteszi*. As a result, the managers experimented with extraction by moving them to larger rustic nest boxes located in the same sites where the wild nest was located. As such, it was observed that these bees were able to adapt and continue developing and producing leading to the conclusion that this species is "hermit" and prefers to be far from human settlements. This resulted in the experimentation with the continued use of rustic nests versus the modernized ones (creating hollows in oak trunks) for use as relocation sites for complete or divided wild nests already being managed. This is comparable to the Mayan technique of "jobones" which is the hollowing of oak trunks as described [46].

5. Conclusions

The interaction of the human populations with bees has been of great importance among diverse cultures in America. The relationship between the Mesoamericans and stingless bees was always been of great significance and continues to persist in some tropical areas in Mexico. Various management schemes exist to take advantage of the products of these insects. Today stingless bee populations are in decline as a result of environmental degradation primarily in the form of land use change, deforestation, and degradation [19]. Likewise, the traditional knowledge and related management practices are also at risk of disappearance as a consequence of cultural changes, economic pressures and environmental change.

In the face of these risks, it is of fundamental importance to maximize local knowledge about stingless bees and management strategies in accordance with the realities of the local context. This research program presents a development alternative to develop alternate strategies in the breeding and reproduction of these bees to aid in their conservation, while at the same time taking advantage of the products they provide. This is a viable means to promote the conservation of stingless bees and the environmental services they provide, strengthen local knowledge and encourage production activities that offer sustainable alternative to the rural communities that manage these insects.

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Chapter 9

Rearing Bumble Bees for Research and Profit: Practical and Ethical Considerations

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Additional information is available at the end of the chapter

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Abstract

The commercial production of bumble bee colonies is a multi-million dollar business worldwide. The pollination of greenhouse tomatoes is largely dependent on this industry. However, microparasites are prevalent in many of these colonies and can spread to wild populations of bumble bees. Academic researchers now commonly purchase colonies for their work. I believe that this raises some questions: (a) What is the danger of exacerbating the problem of spread of parasites and pathogens to wild population of bumble bees from field studies using purchased colonies? (b) How representative studies are done on only a few species, for example, *B. terrestris, B. impatiens*? (c) Does the purchase and use of these colonies give tacit approval to the industry, which may be having a detrimental effect on the native populations of bumble bees? This is an ethical issue. (d) Loss of "feeling for the organism" by researchers and particularly graduate students. These issues were discussed, and the classical method of bumble bee rearing which avoids these problems was described.

Keywords: *Bombus*, rearing methods, parasites, bumble bees, pathogens

1. Introduction

The mass rearing of bumble bee colonies for commercial purposes started in the mid-1980s and since then has expanded into a worldwide industry worth millions of euros [1]. In the mid-1980s bumble bees (*Bombus* spp.) were found to be particularly effective and economical for the pollination of greenhouse tomatoes and have replaced labour intensive mechanical methods of pollination or hormonal treatments [1]. There are now over a million colonies produced per year and exported to and from countries in Europe, North and South America, and Asia [1]. In addition

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to the tomato, there are 19 other commercially important crops pollinated by bumble bees and the use of bumble bees for pollination on this scale has been an enormous benefit for the production of essential food crops giving higher yields, and better fruit quality with lower costs [1].

There are more than 30 commercial producers worldwide; however, the market is dominated by three companies: Koppert Biological Systems and Bunting Brinkman Bees (BBB) both headquartered in The Netherlands and Biobest in Belgium [1]. The production of large numbers of colonies requires the development of rearing methods that can be carried out on an industrial scale. These methods involve manipulating aspects of the bumble bee life cycle to be able to produce colonies at any time of the year and in numbers as needed. The exact methods used by each company are proprietary secrets, although the general methods have been published [1, 2].

In spite of the benefits this domestication of bumble bees has brought, it also has unwittingly engendered detrimental effects on wild populations of bumble bees, two of which have been well documented, to wit: the escape of non-native bees from greenhouses and their establishment in the wild, and associated with this, the spread of parasites from these bees to the wild populations of native bees.

However, I also believe that there have been some insidious effects on the way in which academic research on bumble bees is being conducted. In this paper I will discuss some concerns I have regarding the use of commercially reared colonies of *Bombus* species for fundamental (i.e., "pure") academic research. It has become more and more common for academics at universities to purchase colonies from the various commercial bumble bee rearing companies for use in their research and for their graduate students' research. This is particularly the case in North America and in Europe. The issues fall into two categories, the first two being tangible and the second pair being intangible.

a. What is the danger of exacerbating the problem of spread of parasites and pathogens to wild population of bumble bees from field studies using purchased colonies?

b. How representative are studies done on only a few species, for example *B. terrestris, B. impatiens*?

c. Does the purchase and use of these colonies give tacit approval to the industry, which may be having a detrimental effect on the native populations of bumble bees? This is an ethical issue.

d. Loss of "feeling for the organism" by researchers and particularly graduate students.

I believe that these are some important issues which should be discussed by the entomological and conservation community. In this paper, I will first review the biology and the life cycle of bumble bees, then briefly discuss some aspects of the commercial colony rearing industry, and review the potential for the spread of parasites from infected colonies to bees in greenhouses and in the wild. Next, I will discuss the use of commercially reared colonies for 'pure' research and the issues surrounding this practice. Finally, I will summarize the 'classical' methods for rearing bumble bee colonies and discuss the advantages of using these for research work.

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2. Bumble bee biology and life cycle

Bumble bees all belong to one genus, *Bombus*, and there are only about 250 species worldwide [3]. The species are generally confined to the northern temperate regions, but also occur naturally in South America. A few species have been introduced to New Zealand and Tasmania where they are non-native.

Bumble bees generally have an annual life cycle. Reproductive individuals, young queens and males, are produced by colonies towards the end of summer, and mate after they leave their natal nests [4]. The new queens then enter hibernation with the sperm from the males stored in a sac, called the spermatheca. In most species, queens only mate once [5]. The queens emerge from hibernation in the spring, and spend their time foraging for nectar and searching for a suitable nest site. Bumble bees nest, where there is some pre-existing nesting material, for instance underground in an abandoned rodent (e.g., mouse) nest, or on the surface in a ball of dried grass, or around human habitation (e.g., in the insulation of houses) [4]. Once a queen has chosen her nest site, she then forages for pollen to eat to develop her ovaries, and she also collects pollen and deposits it on the floor of her nesting cavity. On this she makes cells out of wax extruded from glands between the segmented plates on her abdomen. She then lays an initial brood of 6–10 eggs. The queen also builds a honey pot in which she stores honey. The first 3–4 weeks comprises the solitary phase of the life-cycle, in which the queen alone forages and tends to her first brood.

Bumble bees like all Hymenoptera have a haplodiploid genetic system, in which fertilized eggs develop into females, while unfertilized eggs develop into (haploid) males. This allows a queen to control the sex of her offspring, either by releasing or by withholding sperm from the spermatheca. For the first part of the season, the eggs laid by the queen will be fertilized and will develop into workers. Once the first brood of workers emerge, the queen remains in the nest and the workers take over the tasks of foraging, defence, and colony maintenance [4]. The colony grows rapidly for 2–3 months, and when the worker force is large enough to accumulate sufficient resources, young queens are produced [4]. A successful colony can produce large numbers (>50) queens [6]. The foundress queen also switches to laying unfertilized eggs to produce males. At the end of the summer, the old queen, the workers, and the males die, leaving the inseminated new queens to repeat the cycle the following spring.

Bumble bee (*Bombus*) species, with their varying tongue-lengths, ability to forage at lower temperatures, and capacity to buzz pollinate, are one of the most effective pollinators of wild plants and crops [4].

3. The commercial rearing industry

3.1. Economics of the industry

In 2006 when Velthuis and van Doorn [1] reviewed the state of the industry, the growth in commercial sales of bumble bee colonies had reached around one million in 2004 from its beginnings in 1988. It has certainly continued to expand since then, and the supply of bumble

bee colonies is essential for global tomato production. In 2004, 99,000 acres of greenhouse tomato production were pollinated worldwide by bumble bees, with an estimated value of ~ $15 billion [1]. Exact revenues from bumble bee colony sales are hard to estimate because the companies are private. However, since, for example costs of bumble bee colonies sold by Green Methods.com (https://greenmethods.com/) run from US $109.95–$252.95. The industry as a whole must be worth hundreds of millions of dollars.

3.2. Species used

Until recently only species of two subgenera, *Bombus sensu stricto* and *Pyrobombus* [3], have been used for commercial rearing [1]. These are listed in **Table 1**. These are all pollen-storing species, which store pollen in wax cylinders near to the brood clumps, as opposed to the pocket-making species which pack pollen into pockets next to the developing larvae which feed directly. Workers of pollen-storing species feed larvae with a regurgitated mixture of pollen and honey. Pollen-storing species can be fed additional pollen which aids in their domestication. Of the species which have been reared commercially, two species are used predominately: *B. terrestris* in Europe and *B. impatiens* in North America (**Table 1**). Biobest has just started to supply colonies of *B. atratus* for use in South America. This is a pocket-maker, which has large colonies and is a vigorous and aggressive species. *B. occidentalis* is no longer produced commercially as the cultures were severely infected by *Nosema bombi* in 1996, which probably came from wild-caught queens [1].

Species	Subspecies	Origin	Used in	Source (Company[1])
Subgenus *Bombus*				
B. terrestris L.				
	B. t. audax (Harris 1780)	Belgium	U.K. only	Biobest
	B. t. canariensis Pérez	Canary Islands	Canary Islands	Biobest
	B. t. dalmatinus Dalla Torre	south-eastern Europe, Turkey	Europe	Koppert
	B. t. sassaricus Tournier	Sardinia		Koppert
	B. t. terrestris L.	Europe, Turkey, North Africa, China	Europe, North Africa, Asia, Australasia and Chile	Biobest, Koppert

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Species	Subspecies	Origin	Used in	Source (Company[1])
B. lucorum L.		Europe, Asia	East Asia	
B. ignatius Smith		Belgium	Japan only	Biobest
B. occidentalis Greene[2]		Western North America	Western North America	
Subgenus *Pyrobombus*				
B. impatiens Cresson		Canada, Mexico	North, Central and South America	Biobest, Koppert, Green Methods
Subgenus *Fervidobombus*				
B. atratus Franklin		Argentina	South America	Biobest

[1] Example company.
[2] No longer in production.

Table 1. Species and subspecies of bumble bees (*Bombus*) which have been or are currently used for commercial rearing of colonies.

4. Dangers from commercially reared colonies

The use of commercial reared colonies for the greenhouse tomato industry alone has become essential and a reduction in the pollination services provided would have serious economic consequences for the growers. However, the widespread use of these colonies has introduced some dangers for populations of wild bumble bees, which should not be underestimated.

4.1. Escape of non-native species

Species of bumble bees have been intentionally introduced to countries where bumble bees are non-native. For example four species were introduced to New Zealand in 1885 and 1906 for the pollination of red clover [7]. One of these, *B. ruderatus*, was later introduced to Chile in 1982, where there is one species of native bumble bee, *B. dahlbomi* [8]. However, there have also been instances where species have possibly been introduced accidentally. In 1992 *B. t. audax*, most likely from New Zealand, arrived in Tasmania where it has spread at a mean rate of 25 km/year [9]. In Chile, *B. terrestris* colonies were imported in 1998 from Israel and Belgium for use in greenhouses and later used for the pollination of field crops [8]. It is undoubtedly spreading and is likely to become established in the wild. In 2001, I collected a number of *B. terrestris* males at high elevations south of Santiago. In Japan, *B. terrestris* has been imported since 1992 and colonies have established in the wild [1]. More worrying is that hybridization has been recorded between *B. terrestris* and *B. ignitus* in the wild [10]. It is worth noting also that mating between some subspecies of *B. terrestris* in captivity occurs quite readily [11]. In North America, the eastern species *B. impatiens* is being used in unsecured greenhouses in

Alberta and British Columbia. For example, Ratti and Colla [12] collected a queen and five workers in pan traps in fields, a minimum of two km from the nearest greenhouse. Also, *B. impatiens* workers have been collected while foraging on trees next to a commercial greenhouse in Sylvan Lake, Alberta (Beaudin and Owen, unpublished).

4.2. Common parasitic diseases

The escape and establishment of species in areas where they are non-native is a real danger as it is simply not possible to ensure that bees will not escape from greenhouses. Moreover, even in areas where greenhouse bees are the same species as the native bees, escaped bees can carry and spread diseases to native populations of bees. Although commercial operations strive to keep their colonies free of microparasites, a large proportion of colonies are probably infected [13]. Graystock *et al*. [13] assessed levels of nine parasites in colonies produced in 2011 and 2012 from the three main producers of colonies. Using molecular methods, they screened for the three main bumble bee microparasites, all of which are faecal-orally transmitted parasites of adult bees: (1) the trypanosome *Crithidia bombi*, (2) the microsporidian *Nosema bombi*, and (3) the neogregarine *Apicystis bombi*. They also screened for six widespread honeybee parasites: (4) *Nosema apis*, (5) *N. ceranae*, (6) the orally infecting foulbrood bacteria *Melissococcus plutonius*, (7) *Paenibacillus* larvae of bee larvae, (8) deformed wing virus (DWV), which is a common parasite in honeybees and bumblebees, and (9) the orally infecting fungal parasite *Ascosphaera* of bee larvae. They also screened the pollen provided to feed the colonies for the same pathogens. They examined 48 colonies of *B. terrestris* purchased from the three main suppliers in Europe, all of which were imported into the United Kingdom on the producers claim that they were disease-free; however, 37 of the 48 colonies (77%) were infected, and in these 5 parasites were present in 13–56% (depending on parasite) of the colonies [13]. Similarly 24 of 25 pollen samples were contaminated with parasites [13]. Also, when bumble bee workers were fed infected pollen or faeces from the commercially produced colonies they would then become infected, and this reduced their survival. The parasites tested were *Crithidia bombi, Apicystis bombi, Nosema bombi*, and *N. ceranae* [13]. It is necessary to test for honeybee diseases as there has been a spread of some of these from honeybees to wild populations of bumble bees [14]. For example Graystock *et al*. [13] found deformed wing virus (DWV) in about 15% of the *B. terrestris* colonies and in 10% of the pollen samples. The recent spread of DWV has been well documented [14, 15]. DWV is endemic in the European honeybee, *Apis mellifera* [15]; however, it is currently remerging as a global epidemic of honeybees. This resulted from the spread of its vector, the mite *Varroa destructor*, from the Asian honeybee *Apis cerana*, which is its normal host [15]. This leap occurred in the middle of the twentieth century and now *V. destructor* is distributed worldwide. The mites are particularly infective because they bear a heavy load of the virus, as it may replicate within the mite or accumulate in the gut; moreover they also inject the virus directly into the hemolymph of the bee [15]. Both the mite and DWV have been implicated as one possible cause of Colony Collapse Disorder in honeybees [15]. Global movement of honeybee colonies brought the Asian and European honeybees into contact and allowed the spread of the host and the virus [15], and the introduction of infected bees into Hawaii, previously free of *Varroa*, led to an increase in virulence of DWV [15]. Where infected honeybees and bumble bees are sympatric, the latter have higher prevalence of DWV

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than in other locations, and they have lower survival rates than uninfected bees [16]. Thus, potential danger of spillover of pathogens from domesticated bees must be taken seriously.

4.3. Spillover of diseases to natural populations: models and data

One factor implicated in the decline of wild bumble bees is the possible spread or 'spillover' of pathogens from greenhouse populations to the wild bees [17–19]. This has been modelled by Otterstatter and Thomson [19]. An initial question is, if a single infected colony is introduced into a greenhouse along with other non-infected colonies, how will the infection spread through this closed population? Here we are ignoring the loss of bees to the outside. This can be analysed with a deterministic model of pathogen spread. Here we will consider *C. bombi* which is an intestinal protozoan which spreads both within and between colonies. Once ingested, the parasites attach to wall of the gut using their flagellum. Here they multiply and in a few days, infective cells are shed in the host's faeces. There is no direct transmission from bee to bee, but the infection spreads within a colony when a new host comes into contact with cells on substrates in the nest [20]. In the field, *C. bombi* spreads when bumble bee workers pick up infective cells deposited on flowers by infected bees [17, 19]. The cells are either shed from the body surface of the bee or deposited when the bee defecates [19]. Infection by *C. bombi* can have multiple effects including severely reducing the colony-founding success of queens, the growth rate of established colonies, and worker survival and foraging efficiency [19, and references therein].

4.3.1. Spread in a greenhouse population

If we consider a closed population of bees in a greenhouse, then a basic SIR epidemiological model can easily be constructed. *S, I,* and *P* are the densities of susceptible bees, infected bees, and infective pathogen particles in the environment respectively. Let a = the birth rate of the susceptible population, β = the transmission rate of pathogen particles, α = the mortality rate of infected bees, λ = the rate at which infected bees produce and deposit pathogen particles in the environment, and μ is the rate at which pathogen particles breakdown in the environment and are no longer infective (see **Table 2**). It is assumed that (1) the parameters (a, b, α, λ, μ, γ) are constant, (2) there is no vertical transmission (i.e., no within colony transmission), (3) no bumble bee may be infected more than once, (4) the disease does not spread directly from bumble bee-to-bumble bee. Additionally the duration of the epidemic is set to be roughly 90 days (during June to August) while colony growth is occurring and involves only workers.

Parameter	Symbol	Value
Birth rate of the susceptible population	a	0.220 d^{-1}
Natural (non-disease) mortality rate	b	0.183 d^{-1}
Disease-induced mortality	α	0.102 d^{-1}
Pathogen production rate	λ	4.23×10^4 d^{-1}
Pathogen decay rate	μ	12.98 d^{-1}

Parameter	Symbol	Value
Transmission rate	β	1.08×10^{-4} m^2 d^{-1}
Initial host population density	S_0	0.08 m^{-1}
Diffusion coefficient	D	800 m^2 d^{-1}

From: Otterstatter MC and Thomson JD (2008) Does pathogen spillover from commercially reared bumble bees threaten wild pollinators? *PLoS ONE* 3(7): e2771. doi:10.1371/journal.pone.0002771

Table 2. Parameter estimates used by Otterstatter and Thomson (2008) for their model of *Crithidia bombi* spillover to wild bumble bees near greenhouses.

Therefore,

$$\frac{dS}{dt} = (a-b)S + aI - \beta SP$$

$$\frac{dI}{dt} = \beta SP - (\alpha + b)I$$

$$\frac{dP}{dt} = \lambda I - \mu P$$

Thus $S(t)$ = Number (or density) of *susceptible bumblebee*s at time t, $I(t)$ = Number (or density) of *infective bumblebees* at time t, and $P(t)$ = Number (or density) of pathogens present in the environment at time t. As an example, if we start with a population of 100 bees with five of these infected, that is, the non-negative initial conditions are $S(0) = S_0 = 100$, $I(0) = I_0 = 5$, and $P(0) \geq 0$. The parameter estimates are those used by Otterstatter and Thomson [19] and are

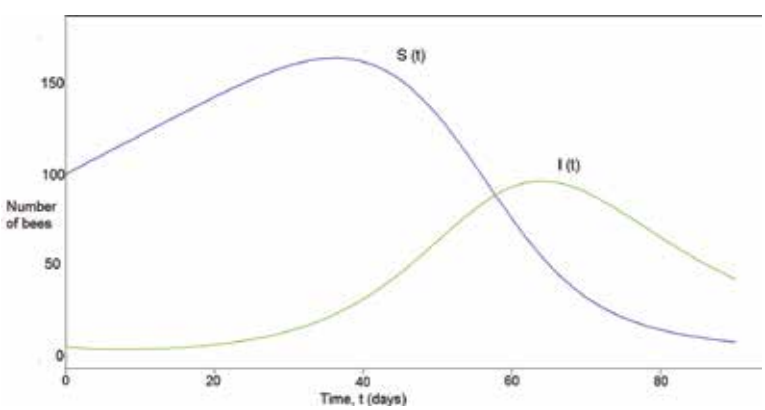

Figure 1. Theoretical course of an infection of *Crithidia bombi* in a closed greenhouse.

given in **Table 2**. As shown in **Figure 1** the infection sweeps through the population in about 80 days leaving the majority of the bees infected. Clearly in closed populations infections are likely to spread easily.

4.3.2. Spatial effects: spillover to wild populations

In the field, horizontal transmission between workers occurs indirectly when infected and susceptible bumble bees share flowers. *C. bombi*, and other pathogens, can spread to wild populations of bees when infected commercially reared bees that escaped from a greenhouse and deposit short-lived pathogen particles on flowers near the greenhouse [19]. Susceptible wild bees foraging near the greenhouse then acquire infection from these particles when foraging for nectar or pollen, subsequently, they become infectious themselves and can introduce this pathogen into their natal colony and also deposit them on more flowers.

Otterstatter and Thomson [19] modelled this by modifying the equations given above to track spread of pathogens not only in time (t), but also in space (x, displacement from starting point).

$$\frac{\delta S}{\delta t} = (a-b)S - \beta SP + D\frac{\delta^2 S}{\delta x^2}$$

$$\frac{\delta I}{\delta t} = \beta SP - (\alpha + b)I + D\frac{\delta^2 I}{\delta x^2}$$

$$\frac{\delta P}{\delta t} = \lambda I - \mu P + D\frac{\delta^2 P}{\delta x^2}$$

Here, the parameters are as defined earlier (**Table 2**) with the addition of D, the dispersal rate of hosts and pathogen particles [19]. Otterstatter and Thomson [19] assumed that wild bees and pathogen particles, which can be picked up and carried on bees' bodies, move about the environment *via* simple diffusion. The model did not include vertical transmission within colonies. Prevalence curves were generated through numerical simulation of the diffusion model using the parameter estimates given in **Table 2**, to predict the long-term dynamics of *C. bombi* spillover [19]. Initially (t= 0–13 wks), pathogen spillover into wild populations is localized around the source; the prevalence of *C. bombi* is about 20% next to greenhouses, and declines to 0% at a distance of roughly 2 km. However in the weeks following, a large wave of infection develops and by 15 weeks, peak prevalence of *C. bombi* near greenhouses has increased to ~75%. By 18 weeks, peak prevalence has reached 100%. The wave spreads through the wild bumble bee population at a rate of 2 km/wk [19].

Otterstatter and Thomson [19] tested the predictions of their model by sampling bumble bee workers from wild populations adjacent to greenhouse populations at two locations in southern Ontario, Canada. Given the parameter estimates used, the model gave a good fit to the pathogen prevalence observed in the field. The model predicted the sharp decline in

pathogen prevalence observed near greenhouses and matched well with the prevalence observed over several kilometres [19].

5. Using bumbles bees for research

Bumble bees have become important and widely used organisms for research. Their study illuminates many areas of biology not only of practical importance, such as crop pollination, but also of theoretical interest, such as the evolution of eusociality and optimal foraging. Since the widespread availability of commercially produced colonies, many research workers have simply purchased colonies for their graduate students' research. The advantages are obvious; no time or effort is required to rear colonies and colonies are available at any time of year. This is particularly useful since research can be carried out in the winter months in the temperate regions. However, I believe that there are potential and actual detrimental aspects to this approach.

Commercially produced colonies have been used for research in the following areas: colony development, worker behaviour, foraging behaviour, estimating mortality rates of workers, and transplantation experiments to assess the pollination efficiency of different subspecies (of *B. terrestris*).

I will now discuss the issues I listed earlier in more detail. The issues fall into two categories, the first two being intangible and the second pair being tangible:

a. What is the danger of exacerbating the problem of spread of parasites and pathogens to wild population of bumble bees from field studies using purchased colonies?

As discussed earlier, the major microparasites of bumble bees are very infectious and liable to spread very rapidly among greenhouse and wild bees. There is a risk of spreading infections from commercially reared colonies to wild populations of bumble bees and this should be avoided.

b. How representative are studies done on only a few species, for example *B. terrestris*, *B. impatiens*?

There is considerable variation among bumble bee species and subgenera in morphology, behaviour, etc. Concentration on only one or two species for detailed study would seem to be inadvisable.

c. Does the purchase and use of these colonies give tacit approval to the industry, which may be having a detrimental effect on the native populations of bumble bees?

The same scientists may also be decrying the spreading of parasites, etc., and supporting petitions to limit the importation and movement of bumble bee colonies while they are using purchased colonies for their research.

d. Loss of 'a feeling for the organism' [21] by researchers and particularly graduate students.

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The phrase 'a feeling for the organism' was how the late Nobel-prize winning geneticist Barbara McClintock described the almost intuitive understanding that a biologist can develop after deep study of a particular species [21]. It is often based on years of observation and work on this organism, and can lead to insights that superficial study cannot provide [21]. I believe that rearing bumble bee colonies goes a long way in giving one a feeling for the organism.

6. The art and science of rearing bumble bee colonies

One advantage of working with bumble bees is that it is possible and very easy to get to know the organism as a whole. The best way to do this is to rear bumble bee colonies using the methods developed years ago, which essentially provide the conditions under which queens will initiate colonies reasonably and naturally in captivity. It is labour intensive and involves work every day of the spring and summer, typically from mid-April/early May until mid-August in northern latitudes. This involves collecting queens and installing them daily, daily inspection of the nest boxes, feeding the queens, and later on feeding and monitoring the developing colonies. If done with sufficient numbers of bees and species, as in **Table 3**, then one gains invaluable knowledge of the nuances of each species and the 'individuality' of each queen.

The method described here is based on the one developed by Plowright and Jay [22] as modified by Owen [23]. It is designed to be of use to students and researchers who want to rear a fair number (up to100 or so in a season) of colonies, without undue effort and under reasonably natural conditions. The idea is to provide a queen with a simulation of what she would encounter in nature, after emerging from hibernation in the spring, that is, a nesting site with pre-existing nesting material and a supply of pollen and nectar.

Species	Year	Dates collected	Median	*N*	Days to B1E	Mean	*N*	Success rate
B. nevadensis	1985	May 7–June 7	14 May	23	5 to 17	9.70	12	12/23 = 52%
	1986	May 2–June 14	19 May	23	5 to 24	12.70	11	11/17 = 65%
B. occidentalis	1985	April 30–May 16	08 May	56	3 to 11	6.50	30	30/55 = 54%
	1986	April 19–May 28	12 May	70	3 to 19	7.40	47	47/67 = 70%
B. terricola	1985	May 2–May 16	07 May	27	4 to 12	7.14	14	14/25 = 56%
	1986	May 2–May 28	09 May	30	3 to 13	7.25	16	16/21 = 76%
B. californicus	1985	June 5–June 18	11 Jun	5	4 to 11	7.50	2	2/5 = 40%
B. bifarius	1985	May 2–June 7	07 May	24	3 to 10	6.10	15	15/24 = 62%
	1986	May 2–May 28	09 May	12	4 to 12	7.70	7	7/10 = 70%
B. frigidus	1985	May 2–May 16	06 May	42	2 to 8	4.20	21	21/42 = 50%
	1986	April 19–May 28	22 May	30	2 to 13	4.40	16	16/20 = 80%

Species	Year	Dates collected	Median	*N*	Days to B1E	Mean	*N*	Success rate
B. huntii	1985	May 2–June 7	13 May	25	3 to 11	6.80	10	10/13 = 77%
	1986	May 2–May 28	12 May	22	3 to 9	5.80	11	11/18 = 61%
B. perplexus	1985	May 3–May 15	08 May	20	3 to 17	7.10	14	14/19 = 74%
	1986	May 3–May 20	09 May	14	3 to 6	4.80	10	10/14 = 71%
B. ternarius	1985	May 2–June 4	07 May	31	3 to 10	6.50	6	6/27 = 22%
Total				454			242	242/400 = 60%

Fifty four of the queens died after installation (12% mortality) therefore the success rate is calculated for the surviving queens. N, the total number of queens collected for each species; n, the number of queens successfully initiating colonies.

Table 3. Dates that queens of nine *Bombus* species were collected in the vicinity of Calgary, Alberta in 1985 and 1986, and the number of days until brood one eggs (B1E) lay.

6.1. Queen collection

It is best to collect queens which have been newly emerged from hibernation; the exact timing depends on the phenology of the species (**Table 3**). These queens are in prime condition and using them gives optimal starting rates and more vigorous colony development. Queens which are gathering pollen have already started their nests and should not be collected. In the early spring the queens forage on pussy-willow (*Salix* spp.) and are easy to find and catch. The bees should be put into 5 dram vials with air holes punched in the lid. They need to be kept cool on a freezer pack or on bags of ice (covered with a 'J-cloth') in a small cooler for a maximum of 4–5 hours before they are transported to the laboratory. Ideally the queens should be installed immediately upon return to the laboratory; however, they can be kept in their vials (with no food) in the fridge (~ 4°C) overnight if necessary. At this stage, bees can be wet-weighed [24], and a data sheet started for each queen. Queens should be inspected for mites; some queens are heavily infested with the mites completely covering their thorax, and these bees should not be installed. However, if only a few mites are present then they can be picked off using forceps. It is best to always transfer queens by using a vial, and it is rarely (if ever) necessary to anaesthetize them. If bees must be picked up, then use broad-tipped forceps and grab the bee by one of its middle legs.

Table 3 gives the dates that queens of nine *Bombus* species were collected in 1985 and 1986. These are two of the years in the 1980s when I was doing intensive collecting of all bumble bee species in Calgary and nearby in southern Alberta. Valuable comparative data can be collected this way, such as the dates of emergence and the number of days until brood one eggs (B1E) laid. Interestingly these earlier records later revealed some important trends in the declining abundance of *B. occidentalis* [25]. The starting or success rate under the laboratory conditions for the different species is also given in **Table 3**. If one assumes that all species establish their colonies equally well in the wild, then clearly some species do better in the lab than others. However, the average starting rate can be counted upon to be about 60%. A queen that does not start a colony within two weeks is very unlikely to do so and can be preserved (frozen or

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in ethanol) for genetic studies, and her wings can be removed for morphometrics [26, 27]. Likewise, queens heading colonies can also be preserved until the end of their life.

6.2. Nest boxes and queen installation

The rearing system is very simple and consists of two wooden boxes: a larger box for foraging and defecating (the 'front' box) and a smaller nesting box (the 'back' box). Only the dimensions of the nesting box are critical, and if constructed from half-inch plywood, its exterior dimensions should be 4"x4"x2". It can be lined with upholsterer's cotton for nesting material (see **Figure 2**). The boxes should be placed on, but not attached to, a ½" plywood board and be provided with glass lids. Bees can be kept at room temperature (~20°C) and ambient humidity, although moist filter paper or a piece of paper towel can be placed in the nesting box if so desired. Light condition or dark/light cycle does not seem to matter. I have found that this works very well in Alberta where the humidity is generally low. However, where the ambient humidity in the spring and summer is higher, as in eastern North America, the nesting boxes can be kept in a room with high heat (~30°C) and humidity as was done in Chris Plowright's lab. However, I am not convinced that this is always necessary given my experience with rearing colonies in Alberta. Honey solution (1:1) or sugar solution (60:40 water: sugar v/v) is supplied in the foraging box. Plexiglass bars with 1 cm deep holes are ideal. A pollen lump is provided in the nesting box and this should be ~1 cm in diameter and ~0.5 cm in height. Pollen lumps of uniform size can be made by using a cork borer and a scalpel. The pollen dough is made by grinding up fresh, clean pollen (which can be stored frozen), with honey or sugar solution, in a mortar. The resulting paste must be of just the 'right' consistency, that is, neither too sticky nor too dry. Pollen can be obtained from honeybee colonies and can be purchased

Figure 2. A queen with her first brood clump and a well constructed honey pot. Note also the honey squirted by the queen on the cotton.

from honeybee breeders. Given the findings of Graystock *et al*. [13] discussed earlier, it is of crucial importance to ensure a disease-free source of pollen, which may prove difficult today.

6.3. Inspection and feeding

Bees should be inspected on alternate days before they have started laying eggs. The plexiglass feeding should also be replaced at this time, and dirty bars should be thoroughly washed in hot water. On the intervening days, the bar can just be topped-up using a squeeze bottle of sugar solution. If no eggs have been laid, the pollen lump is replaced; however, it is often also necessary to rearrange the upholsterer's cotton. If eggs have been laid, or are about to be laid, then small sausage-shaped lumps of the pollen dough are placed next to the incipient brood mass. Once a queen has 'started' then she must be inspected and fed every day, as she will continue to eat pollen herself and, of course, feed it to the developing larvae. If the queen is in the back box incubating her brood, then a gentle tap on this will usually bring the now agitated bee into the front box. The back box can then be move over a cm or two to block her return. Alternatively, the glass lid on the front box can be moved back a fraction followed by blowing on the entrance hole to bring the queen out. Pollen should be provided in a number of small lumps placed around the brood, and the total amount given should be equivalent to about one quarter the size of the brood clump. This is a rule of thumb that works quite well and avoids over-feeding. Any old, dried-up pieces of pollen are removed. If there are any wax pockets in the brood clump, as will be the case with the pocket-making species such as *B. atratus*, then pollen should be pushed into each pocket. This is essential if the species is a pocket-maker, because if this is not done, then the bees will not feed their larvae. With aggressive bees such as *B. atratus*, the feeding room can be kept in the dark and a red light used to feed and manipulate the bees as they cannot see this end of the light spectrum. Interestingly even some pollen-storer species will sometimes make pockets in their first-brood larval clumps, and so pollen should be provided in these pockets if present.

There are a number of signs that a queen may be about to lay eggs, or is starting to develop her ovaries; two important ones are that the pollen lump has been nibbled and that there is pollen in faeces. The latter can clearly be seen on the floor of the front box. Additionally a 'cavity' is often formed in the cotton in the back box, and this should not be disturbed if at all possible. Sometimes the pollen lump is covered with cotton, and then it should be left and a new one added rather than disturb the cavity. Sometimes wax will be deposited on the pollen lump, and also egg cell cups are also formed. In this case, the pollen lump should NOT be replaced, and only pollen sausages should be added around the wax. Construction of a honey pot will usually be started at the same times as or shortly after the queen has laid eggs. Some species, for example, *B. nevadensis*, start their honey pots *before* they actually lay any eggs. In nature, the storage of sufficient honey is crucial for the survival of the queen if there are a few cool, wet days in a row, so in addition to the honey pot, she will sometimes squirt honey on the cotton (**Figure 2**). This is often done at, or just before, egg laying. One drawback of upholsterer's cotton is that it tends to become very matted and sticky, and when this occurs, these patches should be removed. Finally the queen may remove the pollen lump and make

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egg cells directly on the cotton. In this case just add pollen bits around the cells and they will be incorporated into the growing brood clump by the queen.

6.4. Abnormal development

Sometimes a broody queen, or even one that has already started, will show abnormal behaviour. One particularly bothersome one is front box incubating (FBI) when she will incubate on the floor of the foraging box. If this is caught early on, it can be cured by putting wire screen (window screen with a fine mesh) down over the floor. This will usually induce the queen to move to the nesting box and lay her first brood eggs, or will resume their incubation if they are already laid. On the other hand, the condition can continue to worsen and the brood and colony is lost. Occasionally, a queen will deposit wax directly on the floor of the front box and lay her eggs there. In this case it is best to put some cotton around the brood clump and let it develop *in situ*, rather than try to move it into the nesting box. After this, perfectly normal development usually results.

6.5. Colony transfer

About three weeks after the first eggs have been laid, the first brood workers enclose, and the comb must soon thereafter be transferred to a larger nesting box for the remainder of the colony development. It is best to wait a few days until most, or all of the first brood workers have enclosed before moving the comb. Almost any type of larger nesting box will do, for instance a front box can be used. The comb is placed in the middle, and the rest of the space filled with upholsterer's cotton. This is best for colonies that are to be put outside and allowed to free-forage, but is not so convenient for colonies that are to be kept in the laboratory for observation and manipulation. In this case it is better to move the colony to a Porous Concrete Hive (a 'perlite' hive) as described by Pomeroy and Plowright [28]. The advantage of this type of hive is that no nesting material is required, and so these hives are ideal for laboratory based observation and manipulation. The hive can be lined with a cone of cardboard placed on the floor of the hive. This makes final removal of the comb and cleaning of the hive easier.

7. Conclusions

The commercial rearing and use of bumble bee colonies is essential for the production of many food crops. However, there are some serious drawbacks with their use which should be acknowledged.

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Beekeeping and Bee Conservation - Advances in Research presents current issues in the field of bees in multiple contexts and ties together experiments conducted by some of the world's most renowned researchers. The authors' point-of-view and own research results are described in a clear and objective way, which is very useful for beginners in the study of the subject and is likewise valuable for the more experienced on the subject, who may find new hypotheses to be tested and broaden their future prospects in the field. The book is wide in scope, focusing largely on Apis mellifera. Topics range from genetics, to pollination studies, to the conservation of bees. It includes a chapter dedicated to stingless bees and another for bumble bees.

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