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Bases for Sustainable Production

Edited by Ramón Eduardo Rebolledo Ranz

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Published in London, United Kingdom







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http://dx.doi.org/10.5772/intechopen.82888
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First published in London, United Kingdom, 2020 by IntechOpen
IntechOpen is the global imprint of INTECHOPEN LIMITED, registered in England and Wales, registration number: 11086078, 5 Princes Gate Court, London, SW7 2QJ, United Kingdom
Printed in Croatia

British Library Cataloguing-in-Publication Data
A catalogue record for this book is available from the British Library

Additional hard and PDF copies can be obtained from orders@intechopen.com

Modern Beekeeping – Bases for Sustainable Production
Edited by Ramón Eduardo Rebolledo Ranz
p. cm.
Print ISBN 978-1-83880-155-7
Online ISBN 978-1-83880-156-4
eBook (PDF) ISBN 978-1-78985-493-0

Meet the editor

Ramón Eduardo Rebolledo Ranz is a Doctor of agricultural engineering. Since 1986, he has been a professor of agricultural entomology, environmental entomology, and beekeeping at the University of La Frontera Temuco, Chile (both at the undergraduate and graduate level). He has directed more than 80 theses in undergraduate and postgraduate studies. He has participated in 15 research projects and presented more than one hundred works in scientific congresses of entomology, beekeeping and environmental sciences both inside and outside of Chile. He has authored 85 scientific articles in national and international magazines, one book, five book chapters, and has edited two books. He organizes national and international congresses, and is a lecturer at regional, national, and international levels. He is also a scientific advisor to the National Beekeeping Network of Chile. He is a president of different scientific and union organizations in Chile.

Contents

Preface

Beekeeping in recent years has seen a strong increase in both its production and its scientific development in response to an increasing consumption of bee products and pollination requirements to respond to the enormous world demand for safe products.

Despite the aforementioned, beekeeping has faced new challenges that threaten its future and which have undoubtedly become restrictive factors for production.

Such are the problems, that there are scientists who think that this productive activity could become extinct given new diseases, climate change, indiscriminate application of pesticides, and loss of natural spaces where honey and other products of the hive were formerly produced. Habitat loss is one of the serious problems facing global beekeeping.

In addition to the diseases and pests of bees that are currently distributed worldwide, there is the challenge of producing food free of contaminants such as antibiotics and others. This is a great challenge for the world's beekeepers.

Given the great importance that bees have for ecology and man, this book was written in simple and accessible terms for different readers and it includes very relevant topics such as bee diseases, the effect of pesticides on bees and its products, sustainable productive management, analysis of pollen present in honeys, molecular techniques for analyzing products, production of royal jelly, ecological management of bees and their importance in the conservation of plant species, as well as a chapter on meliponiculture.

The chapters have been written by leading world-class researchers and presented in a way that is understandable to the reader. The book is intended for researchers, academics, undergraduate and graduate students, beekeepers, entrepreneurs, as well as the general public, who will find answers in each chapter. The chapters respond to most of the problems that beekeepers face and correspond to the latest research carried out by researchers in their respective subjects.

For this editor, it has been a source of great pride to give shape to the present work and to interact with the different authors with my observations so that the works are written in the best possible way for the reader, be it a researcher, student, professional beekeeper or any person who is interested in starting out in this beautiful profession that brings many benefits both for him and for nature.

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Chapter 1

Effects of Pesticides and Adjuvants on the Honey Bee, *Apis mellifera:* An Updated Bibliographic Review

Raymond A. Cloyd

Abstract

The European or western honey bee, *Apis mellifera*, pollinates approximately 75% of crop species in agricultural and horticultural production systems worldwide at a value of $170–$200 billion per year. While foraging for pollen and nectar in flowering plants, honey bees may be exposed to insecticides; however, they may also be exposed to a multitude of other pesticides and compounds including: fungicides, insect growth regulators, herbicides, and adjuvants. Previous and recent studies show that these pesticides and compounds are directly or indirectly harmful to honey bees, which could negatively impact pollination and colony health. Fungicides can directly and indirectly affect honey bees, and enhance the toxicity (synergize) of certain insecticides, thus increasing their toxic effects to honey bees. Insect growth regulators negatively affect larvae, which impacts brood production in honey bee colonies. Herbicides can indirectly affect honey bee populations by reducing the availability of flowering plants, which decreases pollen and nectar sources during foraging, and consequently reduces colony survival during the winter. Adjuvants, especially surfactants, are a component of pesticide formulations, and are indirectly harmful to honey bees. This book chapter provides a detailed discussion of the effects of fungicides, insect growth regulators, herbicides, and adjuvants on honey bees.

Keywords: fungicides, insect growth regulators, herbicides, adjuvants, surfactants, synergism

1. Introduction

The European or western honey bee, *Apis mellifera* L., is relied upon extensively worldwide for pollinating approximately 75% of crop species in agricultural and horticultural cropping systems at a value of $15–$17 billion per year in the USA and $170–$200 billion per year globally [1–3]. When foraging for pollen and nectar in flowering plants, honey bees can be exposed to a diverse array of pesticides, including: insecticides, fungicides, and herbicides [4–8] that can cause direct or indirect toxic effects to honey bees [9]. Direct toxicity occurs when honey bees are immediately killed when exposed to wet sprays or dried pesticide residues on leaves or flowers [10, 11]. Indirect toxicity is associated with sublethal effects on foraging behavior, development, orientation, reproduction, learning and memory retention, immune system functionality, longevity, and overwintering survival. Indirect

effects may also be related to social interactions resulting from sharing a contaminated food source [11–13]. However, any direct or indirect effects depend on the age of honey bees, because larvae or brood tend to be more susceptible to pesticides than adults [14].

Insecticides are known to be directly or indirectly harmful to honey bees [15–18] with recent research focusing primarily on the direct or indirect effects of neonicotinoid insecticides (imidacloprid, thiamethoxam, dinotefuran, clothianidin, acetamiprid, and thiacloprid) on honey bees, which has resulted in some neonicotinoids, such as: imidacloprid, thiamethoxam, and clothianidin being banned in the European Union and other countries [19–26]. However, although the initial focus has been on insecticides, research demonstrates that other pesticides and compounds can have direct or indirect effects on honey bees, such as; fungicides, insect growth regulators, herbicides, and adjuvants. Therefore, this chapter discusses the issues regarding the effects of fungicides, insect growth regulators, herbicides, and adjuvants on honey bee health.

2. Fungicides

Fungicides are pesticides used to manage fungal plant pathogens of agricultural and horticultural crops [27] and are commonly applied to fruit tree crops during the blooming period when honey bees are most active [9, 18, 28]. Therefore, honey bees are more likely to encounter fungicides than insecticides in agricultural or horticultural settings when foraging for pollen and nectar [9, 29]. Although fungicides are generally considered less toxic to honey bees than insecticides, fungicides may in fact negatively compromise honey bee health [30, 31].

Fungicides are widely detected in honey bee colonies [4], although the effects of fungicide exposure are primarily associated with brood or larvae and not adults [14, 28]. Nonetheless, foraging adults may transport fungicide residues back, along with pollen and nectar, to a hive where the residues are mixed into larval diets, which can result in inhibition of larval and pupal development [28]. In addition, fungicide residues may be present in pollen stores and wax combs, resulting in contamination of food for honey bees [30]. The widely used fungicide, chlorothalonil, which is applied to blooming crops when honey bees are active [14], has been detected at levels up to 300 ppm in bee-collected pollen and wax [4]. Moreover, high concentrations of chlorothalonil were found in bee bread (honey or pollen used as food by bees) samples collected from colonies that died during the beekeeping season [32].

In general, fungicides by themselves, demonstrate minimal direct or indirect effects on honey bee adults [6, 33–36]. However, fungicides are directly or indirectly harmful to honey bee larvae or brood, which can negatively impact colony health [37, 38]. Studies demonstrate that even fungicides alone can negatively affect honey bees, especially larvae. For instance, the fungicide, iprodione, affects the survival of larvae and causes malformations during development, although adults are not affected [28, 37]. Another study demonstrated that honey bee larvae are more sensitive to the fungicide, chlorothalonil, than adults and that dietary exposure to chlorothalonil resulted in a reduction in larval survival by more than 50% [14].

Fungicides may be affiliated with indirect (sublethal) effects on honey bees. The indirect effects of some fungicides can negatively affect honey bees in a way that resembles nutritional deficiencies or weakens honey bees by compromising the immune system, consequently increasing susceptibility to parasites (e.g., varroa mite, *Varroa destructor*) and/or pathogens (e.g., *Nosema ceranae*) [39]. In addition, exposure to the fungicide, myclobutanil, resulted in indirect effects by inhibiting the respiration rate of honey bee workers [31]. The fungicides, boscalid and

pyraclostrobin, were found to negatively impact nutrition and functionality of the immune system in honey bees. Also, a formulated pesticide mixture of pyraclostrobin and boscalid in combination with iprodione was shown to increase adult honey bee worker mortality [40]. Therefore, the indirect effects of fungicides not only can impact adults but can also contribute to colony losses [14, 41].

Another important factor is related to the common practice of tank mixing fungicides with insecticides into a single spray solution or the commercial availability of formulations that blend multiple pesticides into premixtures [7, 42, 43]. Studies report that fungicides can enhance the toxicity of insecticides to honey bees when mixed together [6, 36, 43]. This enhanced toxicity is called synergism or synergistic activity. Synergism is a reaction that occurs when one pesticide in a mixture enhances the toxicity of another pesticide or when the mortality induced by a pesticide combination is greater than the individual pesticides [44–46].

Pesticide mixtures can lead to high levels of toxicity to honey bees and even contribute to a reduction in overall colony health [47]. The ergosterol or sterol biosynthesis inhibiting class of fungicides enhances the toxicity of certain insecticide classes, including: organophosphates, neonicotinoids, and pyrethroids to honey bees [42, 48–51]. The reason for this may be associated with the fact that these fungicides decrease the ability of honey bees to metabolize insecticides [19]. The toxicity of pyrethroid insecticides to honey bees is enhanced—over a thousandfold—when mixed with ergosterol biosynthesis inhibitors [42, 43, 48]. The fungicide, propiconazole, increases the toxicity of the pyrethroid insecticide, lambda-cyhalothrin, to honey bees when the two are mixed together by inhibiting microsomal monooxygenase activity [42]. In addition, propiconazole, when mixed with the insecticide, chlorantraniliprole, resulted in an increase in toxicity to larvae and adult honey bees, which may be associated with propiconazole inhibiting P450 enzymes that are responsible for detoxifying insecticides [9, 50]. Ergosterol biosynthesis inhibitor fungicides also increase the toxicity of thiamethoxam (a neonicotinoid) up to eightfold [51].

However, the synergism of pyrethroid toxicity by certain fungicides is dependent on the proportion or dose of the fungicide in relation to the insecticide in the mixture. The higher the proportion or dose of the fungicide in the mixture compared to the insecticide, the greater the synergistic effects [51, 52]. Furthermore, studies have shown that mixing some neonicotinoid insecticides with certain fungicides can increase honey bee toxicity by as much as a thousandfold. Nevertheless, it is important to differentiate between laboratory and field studies, because synergism associated with honey bee toxicity under laboratory conditions may not predict what occurs under field conditions [43].

3. Insect growth regulators

Insect growth regulators are insecticides that disrupt insect growth and development, eventually leading to death [53]. Insect growth regulators are primarily active on the immature stages (larvae or nymphs) of certain insect pests [53, 54]. There are three categories of insect growth regulators: chitin synthesis inhibitors (diflubenzuron and novaluron), juvenile hormone mimics or analogs (fenoxycarb and pyriproxyfen), and ecdysone receptor agonists/antagonists (azadirachtin, methoxyfenozide, and tebufenozide) [27, 53, 55]. Initially, the effects of insect growth regulators on honey bees were not well known [56]. However, more recent studies show that insect growth regulators are, in fact, directly harmful to honey bees, especially the larvae or brood [12, 13, 57], and there may even be indirect effects on adult behavior [56, 58]. A number of insect growth regulators, associated

with the three categories, and commonly used in agricultural and horticultural cropping systems, directly or indirectly negatively affect honey bees [6].

3.1 Chitin synthesis inhibitors

Chitin synthesis inhibitors disrupt molting of insect larvae by interfering with enzymes responsible for stimulating the synthesis and formation of chitin, an important component of the insect exoskeleton [27, 53, 56, 59, 60]. Studies demonstrate that the chitin synthesis inhibitor, diflubenzuron, negatively affects learning behavior [56], decreases the number of adult honey bees [58], and reduces larval and queen survival [9, 57, 58]. Consequently, this impacts brood production in whole colonies [57, 61–63]. Another chitin synthesis inhibitor, novaluron, is directly toxic to honey bees and negatively affects brood production [64].

3.2 Juvenile hormone mimics

Juvenile hormone mimics (analogs) arrest development and cause insects to remain in an immature stage, which inhibits adult emergence and prevents insects from completing their life cycle [27, 53, 60, 65]. The juvenile hormone mimic, fenoxycarb, affects adult worker honey bees [66], causes adults to age prematurely [67], and, in whole colonies, causes extensive mortality of honey bee larvae, thus reducing the number of brood and size of over-wintering colonies in the subsequent year [58]. In addition, exposure to fenoxycarb affects the ability of colonies to overwinter, which reduces winter survival [58]. The juvenile hormone mimic, pyriproxyfen, affects synthesis and accumulation of vitellogenin (protein in hemolymph from which egg yolk is derived) in young worker bees [68] and negatively affects survival of honey bee foragers [69].

3.3 Ecdysone receptor antagonists/agonists

Ecdysone receptor antagonists/agonists are insect growth regulators that disrupt molting by inhibiting metabolism of the molting hormone, ecdysone, or they bind to ecdysone receptors, resulting in premature molting of larvae or nymphs, and eventually death [54, 60, 70, 71]. Methoxyfenozide does not exhibit any harmful effects on honey bee larvae or adults [9] although Fisher et al. [69] reported that methoxyfenozide negatively affected the survival of honey bee foragers. In general, tebufenozide has been shown to exhibit no direct or indirect harmful effects to honey bee colonies or queen development [58]; however, Abramson et al. [56] found that tebufenozide negatively affected the learning behavior of honey bee adults. Azadirachtin does not indirectly effect brood production, with only minimal harmful effects to honey bee colonies by negatively affecting overwintering survival [58].

4. Herbicides

Herbicides are the most widely used pesticides in agricultural and horticultural cropping systems for control of unwanted vegetation or plant material [27, 35, 72]. Therefore, herbicides should have minimal, if any, direct or indirect effects on honey bees [34, 73, 74]. The post-emergent herbicides, dicamba and picloram, were found not to be directly harmful to adult honey bees or brood [73, 74]. However, the contact, post-emergent herbicide, paraquat, was reported to be directly harmful to honey bees [75]. In addition, laboratory studies found that honey bee colonies

fed two herbicides, 2,4-D and 2,4-trichlorophenoxyacetis acid, resulted in negative effects on brood development, but there were no toxic effects to adult honey bees [33, 73, 74].

Furthermore, the herbicide, glyphosate (sold as Roundup®), which is a broad-spectrum, post-emergent herbicide [76], and is the most widely used pesticide worldwide [77–79], exhibits no direct harmful effects to honey bees [80]. However, research has shown that glyphosate may exhibit indirect effects on honey bees by influencing foraging behavior [79], navigation [81], or beneficial gut microbiota [82]. Nevertheless, it is important to differentiate the effects of laboratory and field studies to assess how glyphosate actually directly or indirectly affects honey bees. There are a host of factors that can influence the direct and indirect effects of herbicides on honey bees including: herbicide used application rate, method and timing of application, and location that honey bees are foraging for pollen, nectar, and water [83].

Herbicides, in general, are more likely to have indirect effects on honey bees by eliminating plants (weeds) that, when in flower, provide pollen and nectar for honey bees during foraging [18, 33, 84]. Consequently, any reduction in floral resource availability (pollen and nectar) could indirectly affect honey bee development, foraging, and survival of managed honey bees [85]. In addition, this could lead to starvation, resulting in a reduction in colony health and winter survival [13].

5. Adjuvants

Honey bees are exposed to a multitude of pesticides while foraging for pollen and nectar in flowering plants, and many formulated pesticides that are applied to control insect and mite pests, or diseases typically contain adjuvants [5, 7, 8]. Therefore, honey bees are likely being directly exposed to adjuvants when foraging [7]. Adjuvants are compounds that are a component of the pesticide formulation (as an "inert ingredient") or are added as a tank-mix additive [86, 87]. Adjuvants are designed to enhance the effectiveness of pesticides, including insecticides and herbicides, by improving or altering deposition, increasing toxicity, improving mixing ability, and/or extending residual activity or persistence [86].

Some of the most widely used adjuvants are surfactants that increase pesticide efficacy by reducing the surface tension of spray droplets, which allows the spray solution to cover more leaf surface area—especially waxy or hairy leaf surfaces of certain plants [5, 7, 88]. In addition, surfactants have been shown to have insecticidal and miticidal properties [89–92]. Initially, surfactants were assumed to be biologically inert with no direct or indirect harmful effects to honey bees [7, 93]. However, studies show that certain surfactants may be toxic to honey bees [88, 94, 95], especially the organosilicone surfactants, which are reported to exhibit direct and indirect harmful effects to honey bees [5, 7, 94, 96]. Nonetheless, the mechanism by which organosilicone surfactants indirectly affect honey bees, such as, impairing learning ability, is not known [5].

6. Conclusion

The European or western honey bee, *Apis mellifera*, is exposed to a diverse array of pesticides when foraging on flowering plants for pollen and nectar. Although insecticides are commonly encountered, honey bees are also exposed to other pesticides (fungicides, insect growth regulators, and herbicides) and compounds (adjuvants) that can result in direct or indirect effects on individual honey bees,

thus affecting colony health. Therefore, it is important to understand the direct and indirect harmful effects of fungicides, insect growth regulators, herbicides, and adjuvants on honey bees, and implement measures that will reduce exposure of honey bees to these pesticides and compounds. These measures include: timing pesticide applications when honey bees are not present, avoid applying pesticides to flowering plants that are attractive to honey bees, select and apply pesticides that are less directly and indirectly harmful to honey bees, and follow specific requirements on pesticide labels regarding honey bee protection.

Acknowledgements

I want to thank Dr. Mary Beth Kirkham from the Department of Agronomy and Dr. Nathan J. Herrick from the Department of Entomology at Kansas State University (Manhattan, KS) for reviewing initial drafts of the book chapter, and providing valuable feedback.

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Chapter 2

Detailed Review on Pesticidal Toxicity to Honey Bees and Its Management

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Abstract

This chapter deals with the effects of different pesticides used in agro-ecosystem on honey bees and other pollinators and probable measures to manage this escalating problem of global decline of managed as well as the wild insect pollinators. This chapter describes different routes from which pollinators, especially honey bees get exposed to the different toxicants, followed by poisoning symptoms in honey bees. Further, this chapter focuses on the classification of different toxicants in different classes as per their nature. Finally, the management of these different toxicants and their toxicity to avoid bee poisoning has been considered in the later portion of the chapter.

Keywords: pesticide, toxicity, honey bees, pollinator, management

1. Introduction

Honey bees contribute more than 90% of the global pollination of more than approximately 85% of the total cross-pollinated plant species in the world, the blessing of being able to hover around and pollinate this much diverse array of floral plant species comes with a curse to these bees, of being in unremitting contact with a wide array of stresses like, parasites, predators, diseases, chemical, etc. present in the environment [1–3]. As being the most successful and commercially exploited pollinators in agro-ecosystems not just for their pollination duties but for commercially valuable by-products such as, honey, wax, propolis, etc. as well, honey bees faces more diverse stresses in nature. Honeybees (*Apis mellifera*) are exposed to an ever changing array of xenobiotics from both natural and synthetic sources. Thousands of older foraging worker honey bees travel as far as 10 km from the hive in the course of collecting the nectar, pollen, water, and propolis needed to sustain a colony of tens of thousands of young adult workers, immature bees, and male reproductive or drones [4]. While foraging over this large area for collecting pollen and nectar to satisfy the carbohydrate needs of the colony, bees forage in various flowering plants with different nature, but these food sources are not always entirely pure every time, having either different plant derived chemicals or the widely used toxic agro-chemicals mixed with them. With the ever increasing use of synthetic chemical pesticides in agriculture honey bees and other wild pollinators have faced a serious threat to their global biodiversity in recent decades [5–7]. Nectar and pollen may contain environmental pollutants or

systemic pesticides drawn from the soil, or they can be contaminated from topical pesticide applications or drift from such applications. Different agro-chemicals like, herbicides, fungicides and most importantly the insecticides, alone and in combination with other factors such as elevated temperature, production of hybrid varieties with lesser quantity of pollen and nectar in their flowers, have caused a devastating effect on honey bees at a global level [2, 8, 9]. Bee foragers may bring such contaminated floral rewards back to the colony for feeding and storing as a resource for future generations [10]. Pesticides which are being used in agriculture crops are highly toxic to bees as they kill the bees through many ways such as, direct killing of foraging workers with their acute toxicity, drifting out from agricultural land to nearby apiaries, thus, making the whole colony more susceptible to different pathogens and to some point reduce their possibility to thrive in the nature by getting accumulated in the pollen inside the colony. Nectars produced by some flowering plant species also contain plant-synthesized chemicals which are toxic to different pollinators [11].

The introduction of chemical pesticides and further increase in its demand in the market is not actually the basic requirements of the farmer; rather it is their mere ignorance. The farmers discriminately use these agro-chemicals with a purpose to manage the pests; instead, they end up with killing their own beneficial insects, i.e., the pollinators. Honey bees and other pollinators get exposed to different toxic agro-chemicals in nature through different paths and these different pesticides affect honey bee colonies to different levels thus; to minimize the losses to pollinators from the adverse effect of pesticide poisoning is a burning topic of interest for human to protect the pollinators.

2. Routes of exposures to different pesticides

The different types of pesticidal formulations travel across the plant through different ways in order to protect the plant or part of it from different factors such as weeds, pathogen, insect pests or rodents, etc. According to the nature of the different pesticides, three principal application methods that are often used to treat crops are: direct spray, which is often used around homes and gardens; soil applications and seed applications, typically used in larger treatment systems. These different methods of application play a crucial role in the exposure of these chemicals to the insect pollinators, visiting a crop (**Figure 1**).

Thus, on the basis of different application methods and the persistence of different pesticides in nature, bees get exposed to different pesticides through these major routes:

1. Direct contact with chemical during foraging on a treated plant

2. Pesticide particles of dust formulations sticking to the foraging bees or to whole colony via drift through wind

3. Pesticidal runoff from the treated fields to nearby water reservoirs

4. Pesticidal drift to non-treated foraging plants growing nearby to the treated crop [12]

5. Pesticidal residues in pollen through seed treatment [12]

These different routes for exposure of various pesticides to honey bees facilitate their entry into a honey bee colony system, but still the mode by which, these

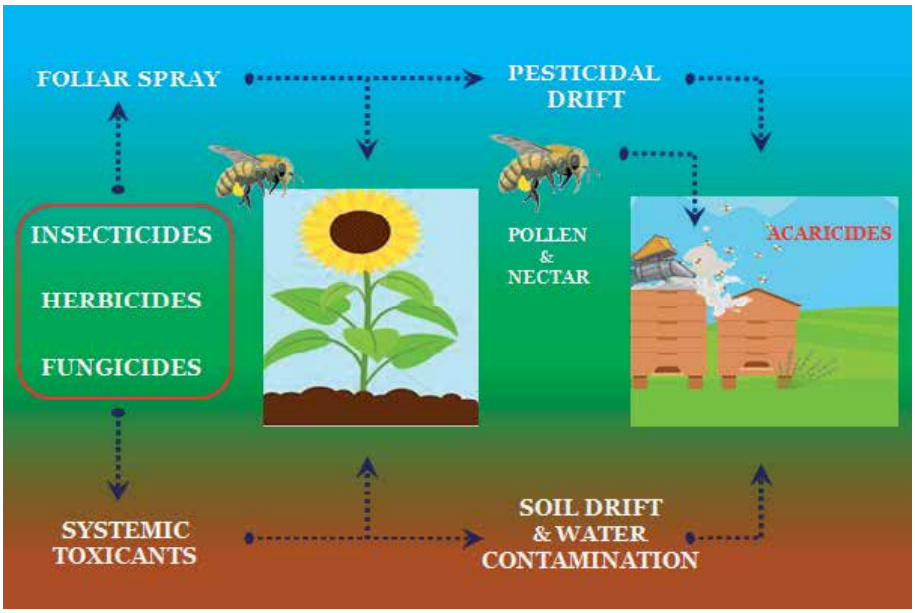

Figure 1.
Different routes of pesticidal exposure to honey bees.

chemicals are pulled out by the forager bees from the fields are quite different being either by oral, respiratory or dermal intake. These different modes of intake of such chemicals are described here.

3. Mode of intake of toxicants

3.1 Oral intake

Oral intake of chemical pesticides from fields is facilitated through the foraging worker bees. Plants treated with different systemic insecticides, produce nectar and pollen containing these insecticides and thus, worker bees collecting this floral resource carries to the colonies to store it into the colony and further use to feed the young developing brood [13]. Several reports of an extremely high concentration of different pesticidal compounds, including insecticides, fungicides, miticides and herbicides have been reported from pollen samples of several crops [12, 14]. These events, from collecting the pollen in the field to feeding to the developing brood results in to a chain of catastrophic events as: foragers are killed during collecting and transporting such contaminated pollen, nurse bees are killed while storing and feeding pollen and the brood are killed by consuming the toxic pollen, thus, leading to a total collapse of the colony.

3.2 Respiratory intake

Respiration of pure oxygen plays a vital role in an organism's growth and development since it ensures proper functioning of various organs in the organism. But, respiration of air admixture with toxicants causes various abnormalities such as abrupt behavioral changes and degradation of learning ability [15]. Pesticide formulations like, dust and fumigants travels through the air called 'drift' can either be carried out onto the body surface of foraging bees or can be absorbed through trachea (respiratory organs) in sufficient concentrations to be toxic to the bees (**Table 1**).

	N_2O	CO_2	Ammonium nitrate	Control
With pollen	0	0	0	15
Without pollen	16	33	25	25

Source: Data extracted from Mackensen [22].

Table 1.
Pollen gathering by bees after anesthetic treatments.

3.3 Dermal intake

This is the major mode of intake of toxicants by the bees since they are exposed to direct contact of pesticides in the fields while foraging on the crops. Honey bee foragers come in direct contact with several pesticides while foraging and such chemicals can be lethal even in small quantities. Dermal toxicity through topical spray has been reported for various insecticides and thorax has widely been regarded as a major route of dermal exposure of pesticides to the honey bees [16, 17]. Other than thorax, insect wings have been reported as a more lethal route of exposure to the bees [18]. Some of the majorly important and frequent ways of dermal intakes are following:

- Majority of bee poisoning is due to application of insecticides to crops during blooming period.
- Bees coming in contact of treated areas.
- Bees coming in contact with insecticides residues on plants and collect insecticide dust with pollen.
- Bees drinking or touching contaminated water or honeydew on the ground or foliage or from nearby water bodies.
- Contamination through treated nectar sources. Dimethaote is the only systemic insecticide known to be excreted in hazardous amount in nectar under field conditions.

4. Symptoms of bee poisoning

- One of the obvious signs of pesticide poisoning is the presence of a large number of dead or dying bees at the hive entrance. These bees are foragers who have been exposed to pesticides sprayed in the fields.
- Another common symptom includes the presence of a moist and sticky mass of dead bees at the hive entrance. This results from poisoning by some fast-acting pesticides, e.g., organophosphorus pesticides. Dying bees extend their tongues through which nectar is regurgitated resulting in sticky and moist dead bees. Bees that have been exposed to a pesticide may regurgitate a thick and dark fluid.
- Swiftly-acting insecticides kill foraging bees in the field itself, while only some of them manage to return to the hive. Sometimes, while spraying is done in the nearby fields to the apiaries, if such chemicals come in direct contact with colony, the whole colony may also die instantly. Stronger colonies suffer greater

losses from pesticidal poisoning than weaker ones, because they have larger numbers of foraging bees.

- Foraging bees often carry residual pesticides in their pollen loads while returning to the hive. As a result, the behaviour of bees in the hive changes abruptly. Honeybees in such colonies become more aggressive or agitated. When a hive containing pesticide-affected bees is opened, the bees fly out of the hive sometimes straight at the face of the beekeeper handling them.

- Other symptoms include stupefaction, paralysis, aggressiveness and abnormal behaviour, jerky, spinning movements. Slowing down of activity and crawling of bees around the hive entrance. They lose their ability to fly and ultimately die 2 or 3 days after poisoning. Poor egg laying patterns or abnormal supercedure of queens.

- Within the hive, a break in the brood cycle (stages of young bees) or a spotty pattern of the brood could also indicate a pesticide problem.

5. Classification of toxicants

Classification of different toxicants to honey bees can be done either on the basis of levels of toxicity or on the basis of sources in the nature. Different toxicants have variable toxicity levels, according to their mode of action on bees and this toxicity level is measured as LD_{50}, which is the dose at which 50% of the bee population dies due to the intoxication. On the basis of their LD_{50} levels, toxicants have been classified into four different categories [19].

- highly toxic (acute LD50 < 2 μg/bee)

- moderately toxic (acute LD50 2–10.99 μg/bee)

- slightly toxic (acute LD50 11–100 μg/bee)

- nontoxic (acute LD50 > 100 μg/bee) to adult bees

Second type of classification is based on the type of toxicants which are originated from different sources, due to human interventions. These toxicants include:

5.1 Inorganic toxicants

5.1.1 Carbon dioxide

The modern time has become an era of the greenhouse gases and global warming. The ever-increasing concentration of various poisonous gasses in the atmosphere has affected a vast majority of the living beings in this world and honey bees are also not any exception with carbon di oxide being the most important of these gasses. Several scientists have worked on the toxicity of carbon dioxide on honey bees and have found some drastic effect of this gas on honey bee at both the levels including individual bees as well as at the colony level, which includes narcosis in foraging honey bees [11], earlier oviposition of queen [20], reduction in life expectancy [21] and reduction in pollen gathering by foraging bees [1].

5.1.1.1 Narcotic effect of CO_2

Bees treated with a mixture of air and CO_2 for 5 min have shown to stop their movement and went motionless but regaining their activity, once it was left for 30 min. The same experiment when repeated with only air for comparative study showed that only air did not anesthetize the bee. Thus, it can be concluded that the CO_2 at even low concentrations is detrimental to honey bees since they have a narcotic effect [1].

5.1.1.2 Effect of CO_2 on pollen gathering ability of the forager

The drastic effect of different chemicals used in the apiaries has been shown to decrease the pollen gathering ability of the forager bees. This decrease in the pollen supply during the blooming seasons can cause a severe threat to the reserves of the colony during winter season, where most of the pollen is consumed for the survival of the colony. This decrease in pollen gathering ability in the worker bees have been observed by different workers and such chemicals like, carbon dioxide, nitrous oxide and ammonium nitrate have been found to be decreasing the pollen gathering capacity of as many as 40 treated worker bees with a threatening outcome of no bee being able to gather pollen after the exposure to abovementioned chemicals, which are widely used in apiaries for different purposes [22].

5.1.2 Metal toxicity

Continued anthropogenic pressure due to the ever-highest human population, which has no signs to slow down in near future have put an alarming metal and metalloid pollutants pressure over the past century because of anthropogenic emissions into the environment. These pollutants may have negatively impacted the pollinators that reside in the soul of machinery responsible for the food production that sustains this human population. Metal pollutants are discharged into the air, water, and soil through different human activities including mining, agriculture, coal burning, hydraulic fracturing to extract gas and oil, and industrial and municipal waste production. Of all the toxic metals collected cadmium, copper and lead have been proved to be the most toxic to bees [23]. These three metals (Cd, Cu and Pb) have also been reported to change the feeding behavior in bees with increased sensitivity towards sucrose.

Once in soil, Cd and Cu is actively absorbed by plant roots, transferred via vascular bundles into the nectar and pollen, and subsequently accumulates in the pollinators and bee products since the pollinators collect the contaminated pollen and nectar. Copper is an essential trace element in plants and is able to accumulate in different plant tissues. Cu co-acts with several essential proteins to enhance growth and development of honey bees but it is toxic when it exceeds the cellular needs.

As lead is not easily trans-located within plants, thus, is also shown to be having a residual effect on forager honey bee. Lead gets trans-located within bees due to transfer through air and dislodgeable residues, resulting from deposition on surface contacted by bees [23].

5.2 Toxins used in bee keeping

A proper maintenance of an apiary depends upon the sanitation aspect of beekeeping but a proper and timely application of different synthetically formulated chemicals is also important for avoidance or management of severe health

problems. Different health problems like infestation of *Varroa destructor*, wax moth (*Galleria mellonella*), tracheal mite and other pathogenic diseases along with the long debated CCD of U.S. apiaries exerts a severe pressure and a serious threat of total loss of millions of honey bee colonies throughout the world and have been successfully managed by the use of various chemical treatments known to be less harmful to the honey bees. These chemicals have been shown to be far more successful than the other treatments, but at the same time, their toxicity towards bees has been highly neglected or has been less explored. One of the best examples for this has been the introduction of formic acid and oxalic acid, for the better management of honey bee parasitic mite, *Varroa destructor*. Medicated strips impregnated with synthetic acaricides such as, fluvalinate-tau and coumaphos have been used for many years for the management of this pest but both the coumaphos and fluvalinate are known to be highly toxic to older bees then young bees [24–26]. Workers that were subjected to less stress appear to be more resistant to fluvalinate and coumaphos poisoning [27, 28]. However, the appearance of resistant mite populations has resulted into a sharp rise in the practice to use formic acid (FA) and oxalic acid (OA). Both of the two organic acids, are varroacides in nature and serve as an attractive natural options for chemicals like coumaphos and fluvalinate as both of them have been reported to be naturally present in *A. mellifera* honey [29, 30]. These pesticides have lower efficacy against the *Varroa* mite but when used in an integrated pest management strategy, they have known to provide an efficient way to control *Varroa* populations. FA is most effective by evaporation of an impregnated substrate with 65% FA inside the hive and OA is most effective when applied in honey bee colonies either by dripping or spraying or through fumigation [9].

Both FA and OA have been proved to be effective to control *Varroa* mite but very less work has been done to establish its negative effect on honey bees. Schneider et al. [31] highlighted the detrimental effects of FA and OA on honey bees which include:

1. Increased mortality

2. Negative effects on brood development

3. Reduced fitness of treated colony

4. Decreased division of labour

5. Reduced hive cleaning and increased self-grooming

5.2.1 Formic acid toxicity

The mode of action of FA against *Varroa* is by inhibition of electron transport into the mitochondria via binding to the last enzyme of electron transport chain, cytochrome c oxidase [32]. Formic acid may produce different toxicity symptoms in honey bees, including reduced longevity of the worker bees [33] and reduced rate of brood survival [34]. Other negative effects of formic acid treatments to honey bee colony mainly includes, increased number of dead bees in front of colonies during the FA treatment period, rejection of queen, worker bees may repel from the colony and a comparatively lower honey yield from the FA treated colony [9].

5.2.2 Oxalic acid (OA) toxicity

As OA is generally provided to the honey bee colony in sugar syrup, in order to increase its efficacy against the *Varroa* mite by increasing its stickiness on to the

mite body, thus, its repeated application to a colony can be proved as lethal to the honey bees as well. A high queen mortality and reduced number of sealed brood have been reported in the colonies treated with OA [35]. The OA treatment has also been reported to be associated with an increased apoptosis in bee midgut [36]. Worker bees have been reported to be showing an abnormal age-related patterns problem while treated with oxalic acid during their early life stages. A dose of 3.5% oxalic acid dehydrates at after 24 h of emergence have been reported to have a disturbance in the normal age-related patterns of worker honey bees. All the age-related patterns of the workers appear in the natural chronology: they shows first events of behavioral patterns for nursing, followed by, honey or pollen manipulation, wax manipulation and patrolling at the same time but in different intensity. The bees start showing all age-related patterns somewhat earlier than the normal. Treated bees show an increased self-grooming, a superior tendency to inactivity and decreased nursing behavior. For all other behavioral patterns, including trophallactic interactions, house-cleaning, honey manipulation and patrolling, bees show no significant changes than the normal chronology [27].

5.3 Agrochemicals

5.3.1 Insecticides

Chemical control for insect pest management contributes as the major part of insect pest management strategies used all over the world [37]. Insecticides have been used since early 1940s for the effective pest management and have been a successful tool for the pest management as saving serious crop losses through insect pest infestations [38]. But, at the same time, the negative effects of these synthetic chemicals have created havoc throughout the world by suppressing the overwhelming populations of several non-target insect species, mainly including the biological control agents and the pollinators. Honey bees are susceptible to many insecticides and different harmful effects of these insecticides are believed to be the prime most reason for the decline in global honey bee populations [9, 39, 40]. The different insecticides have been highly criticized for their possible role in widely discussed and seriously concerning worldwide losses of honey bee colonies [41, 42]. Since the first detailed report and description of the term 'colony collapse disorder' (CCD) in 2006 [43] in America and followed by Europe, had again initiated the long term agitation of banning the use of insecticides, posing a serious threat to the billion dollar industry. Since the CCD, possible role of insecticidal residues in weakening the honey bee colonies for an increased susceptibility towards different environmental and pathogenic pressure on different colony levels has widely been discussed in scientific community [7, 44–50].

Lethality of any pesticide to honey bee is measured during toxicological tests of lethality by observing the mortality of bees after the application of pesticides either by oral administration or by topical application. The bee is usually considered dead when it exhibits "no movements after prodding". Investigation on lethality of any insecticide includes the use of correlation metrics to link the lethality and dose of a toxic chemical or substance to the bees [51, 52]. List of lethality of different class of insecticides was compiled from supporting information from Hardstone and Scott [53], and for the same information regarding fungicides and herbicides was compiled through ECOTOX database [54].

The different class of chemical insecticides poses variable threat to the individual honey bee and a colony level health, thus, the toxic effects and toxicity symptoms of different insecticides can be discussed under one umbrella of major classes of insecticide causing toxicity to the honey bees which is described here.

5.3.1.1 Acetyl cholinesterase Inhibitors

The two widely used groups of insecticides, organophosphates and carbamates acts on insects in a similar way as acetyl cholinesterase (AChE) inhibitors which in normal conditions, inhibits the activity of neurotransmitter acetylcholine in the insect nervous system [53]. These two groups of insecticides have deeply investigated for their toxic effects on honeybees and have been reported to have high larval as well as chronic toxicity to the adult bees causing toxicity symptoms like memory loss and behavioral agitations [55–60]. These two classes of insecticides have a variable amount of topical toxicity to the bees with LD_{50} ranging between 0.018 and 31.2 μg/bee [61, 62], with some of the widely used insecticides enlisted in **Tables 2** and **3**.

Insecticide (organophosphate)	**LD50 (μg/bee)**		**Risk ranking**
	Mean	**Range**	
Chlorpyrifos	0.01	—	High
Coumaphos	31.2	—	Low
Diazinon	0.2	—	High
Dicrotophos	1.62	0.410–3.05	High
Dicholorvos	2.73	0.290–5.01	High
Fenitrothion	1.66	0.180–3.83	High
Malathion	0.2	—	High
Methidathion	0.236	—	High
Methyl parathion	1.66	0.610–3.24	High
Paraoxon	0.600	—	High
Parathion	1.36	0.100–3.50	High
Phorate	2.45	0.910–3.20	High
Phosmet	1.06	—	High
Phosphamidon	4.89	0.020–14.5	High
TEPP	0.410	0.010–1.20	High

Source: Data compiled in Hardstone and Scott [53].

Table 2.
List of organophosphate insecticides with respective toxicity to the bees.

Insecticide (carbamates)	**LD50 (μg/bee)**		**Risk ranking**
	Mean	**Range**	
Oxamyl	0.094	—	High
Methomyl	0.16	—	High
Carbaryl	1.1	—	High
Carbofuran	1.55	1.49–1.60	High
Aldicarb	2.36	1.52–2.85	High
Bendiocarb	2.64	1.00–4.28	High
Aminocarb	4.40	0.85–11.2	High

Source: Data compiled in Hardstone and Scott [53].

Table 3.
List of carbamates insecticides with respective toxicity to the bees.

5.3.1.1.1 Toxic symptoms of organophosphates

- Regurgitation of ingested food
- Disoriented movements
- Distended abdomens
- Erratic movement of the bees
- Wings hooked together, held away from body
- Extended tongues
- Death of the bee

5.3.1.1.2 Toxic symptoms of carbamates

- Erratic movement of the bees
- Stupefaction (numb)
- Paralysis
- Break in brood cycle
- Queen ceases egg laying
- Development of supersedure queen bees
- Most bees die at colony

5.3.1.2 Nicotinic acetylcholine receptor agonists

Leaves of *Nicotiana tabacum*, the plant producing the nicotine which mimics the neurotransmitter acetylcholine, activates the nicotinic acetylcholine receptor (nAChR), and promotes the generation of action potentials in postsynaptic nerve cells, contain up to 90,000 ppm of the nicotine, its pollen may contain up to 23 ppm and nectar 0.1–5 ppm alkaloid content [21, 46]. Adult bees have been proven to be successfully detoxifying nicotine in nectar with a median lethal concentration of 2000 ppm for nicotine [21], whereas the larva are sensitive to nicotine and usually die at the third or fourth larval instar at 5 ppm [46].

The neonicotinoids which are synthetic analogs of nicotine insecticides have a greater affinity to nAChR in the insect nervous system, including bees as well. In recent years, several studies and workers have portrayed these insecticides as the most serious cause of well discussed CCD [63–66]. However, these studies have been criticized for using unrealistic doses and duration of exposure [67]. The nitro guanidine neonicotinoids, including imidacloprid, clothianidin and thiamethoxam have been reported to be highly toxic to bees [68], with toxicity levels ranging from 0.004 to 0.075 μg/bee [69, 70] (**Table 4**). The insecticides like, thiacloprid and acetamiprid which are the member chemicals of cyanoguanidine neonicotinoid group, were much less toxic to the bees with topical or contact LD50 in a range of

Insecticide (neonicotinoids)	LD50 (μg/bee)		Risk ranking
	Mean	Range	
Acetamiprid	8.1	—	Moderate
Imidacloprid	0.0039	—	High
Thiacloprid	17.32		Low
Thiamethoxam	0.0005	—	High
Clothianidin	0.00368	—	High
Dinotefuran	0.0023	—	High

Source: Data compiled in Hardstone and Scott [53].

Table 4.
List of neonicotinoid insecticides with respective toxicity to the bees.

7.1–14.6 μg/bee [70]. This relatively lesser toxicity of cyanoguanidines to the bees is probably due to rapid cytochrome P450 detoxification.

The nitroguanidine insecticides also show their toxic effect through impairing the ability of foraging honey bees to return to the hive [28, 71, 72].

5.3.1.3 Voltage-gated Na^+ channel agonists

Pyrethrin insecticides, produced by pyrethrum flowers (*Chrysanthemum cinerariaefolium*) are again a widely used group of insecticidal compounds. Even though, the pyrethrin has a natural origin, still these chemicals are known to be highly toxic to the bees (LD_{50} = 0.05–0.21 μg/bee) [73] (**Table 5**).

Other than pyrethrins, the pyrethroids, and organochlorine insecticides, show their action on the voltage-gated Na^+ channel in the axons of nerve cells, by delaying the closing of the Na^+ channel and prolonging the recovery period of the nerve cells, following the transmission of an action potential [74]. Bees show more tolerance towards some of the pyrethroids because of their rapid detoxification by cytochrome P450s. Being a pyrethroid, tau-fluvalinate a widely used miticide also

Insecticide (organophosphate)	LD50 (μg/bee)		Risk ranking
	Mean	Range	
Bifenthrin	0.0146	—	High
Cyfluthrin	0.037	—	High
Esfenvalerate	0.017	—	High
Fenpropathrin	0.05	—	High
Gamma-Cyhalothrin	0.0061	—	High
Lambda-cyhalothrin	0.038	—	High
Permethrin	0.024	—	High
Pyrethrin + PBO	0.002	—	High
Pyrethrum	0.022	—	High
Zeta-cypermethrin	0.181	—	High

Source: Data compiled in Hardstone and Scott [53].

Table 5.
List of pyrethroid insecticides with respective toxicity to the bees.

appears to be less toxic or safer to the honey bees but in higher concentrations this chemical has been reported to affect the health of different castes of honey bee colony. Colonies exposed to high doses of tau-fluvalinate had smaller queen bees [75]. Drones exposed to tau-fluvalinate during development were also reported to be affected with lesser chances of attaining sexual maturity [14].

5.3.1.3.1 Toxic symptoms of synthetic pyrethroids

- Regurgitation of ingested food
- Erratic movement of the bees
- Paralysis
- Many bees die between foraging area and colony

5.3.2 Fungicides

"A fungicide is a specific type of pesticide that controls fungal disease by specifically inhibiting or killing the fungus causing the disease."

It is believed to be nontoxic to bees by farmers and hence it is mostly applied during the flowering of plant coinciding with maximum bee activity. Thus, fungicides often account for most of the pesticide content of pollen [9]. An alarming concentration of fungicide chlorothalonil (99 ppm) has been reported from the honey bee pollen [76]. Other than chlorothalonil, in other studies, fungicides like vinclozolin (32 ppm) and iprodione (5.5 ppm) captan (contact) and difenoconazole [77] have also been reported from beebread. While fungicides are considered to be fairly safe for use around adult honey bees, beekeepers have reported losses of brood in larval and pupal stages coinciding with fungicide use during bloom. Fungicide applications also have been determined to trigger hypothermia in adult honey bees [78]. Fungicide was causing toxic effects to honey bee brood based on finding malformed, and frequently wingless, pupae and recently emerged adult bees. The affected bees accumulated on the bottom boards and at the entrance so hives about 2 week after applications. The toxicity levels for different fungicides lies in the range of LD_{50} > 200 to as small as 0.2 μg/bee (**Table 6**).

Active ingredient	Trade name	LD50 (μg/bee)
Dicloran	Botran	0.2
Captan	Captan	10
Dodine	Syllit FL	12.5
Propiconazole	Bumper	25
Ziram	Ziram	46.6
Thiram	Thiram	74
Sulfur	Disperss	>100
Mancozeb	Dithane	178.9
Trifloxystrobin	Flint	>200

Source: Data compiled from ECOTOX database [54].

Table 6.
List of fungicides toxic to bees.

Herbicide	LD50 (μg/bee)
2,4-DB acid	14.5
2,4-DP-P, dimethylamines	25
Trifloxysulfuron-sodium	25
Pendimethalin	49.5
Triclopyr, butoxyethyl ester	62.5
Alachlor	68.1
Simazine	96.7
Atrazine	97
Picloram, potassium salt	100
Glyphosate, isopropylamine	100
2,4-D, 2-ethylhexyl ester	100

Source: Data compiled from ECOTOX database [54].

Table 7.
List of herbicide toxic to bees.

5.3.3 Herbicides

Even though the main purpose of using different herbicides is to control the unwanted weed populations in the fields and there is no such objective to kill insects through them. The toxicity level of herbicides is known to be very less to most of the insects and due to this these pesticides are applied without any restrictions regarding insects. Bees usually come across these chemicals in higher concentrations [79] and toxic effects of these have also been reported on honey bees. Toxicity levels in LD_{50} values differ from one chemical to another with a range of 14.5–100 μg/bee (**Table 7**). A widely used herbicide, paraquat has been reported to be toxic to the bees in laboratory conditions, causing median life of worker ten times reduced than the normal, on injecting at the rate of 15 μg per worker and death within a span of 3 days' time, when sprayed at the rate of 4.5 kg AI/ha [79]. These pesticides may harm the bees in other way around as well as they reduce the number of plants offering floral resources to the bees.

6. Management of pesticidal toxicity to the honey bees

- **Use pesticides only when needed**: insect pests, pathogen or any environmental factor infest or infect the particular crops during specific growth stages of the plant and pesticide application should be done only after surveying the crop fields for the presence of weeds, pest population or disease incidence for threshold levels. This helps in safeguarding the population of insect pollinators, beneficial insects.

- **Do not apply pesticides while crops are in bloom**: use of different pesticides should only be performed only when the crop concerned is not in flowering stages.

- **Apply pesticide when bees are not flying**: the most pollinators are active during 8 a.m. to 5 p.m. and in such favorable conditions pesticides should not be sprayed to help in protecting the forager bees from coming in the direct contact

with pesticide applied. To avoid such condition of direct contact of the pollinators with the pesticides, the application can be mostly in the early evening hours. This late application of the pesticides allows time for these chemicals to partially or totally decompose during the night.

- **Do not contaminate water**: contamination of nearby standing water through pesticides run off should be avoided to prevent the bee losses, as the bees collect water from these water sources to cool down the temperature of the colony during the summer season.

- **Use less toxic compounds**: if the situation allows, then the compounds which are less toxic to the bees should be given preference over the highly toxic chemicals. The pesticide labels should notify the possible hazards to honey bees. If no other alternate option remains then the variations in dosages can be applied.

- **Use less toxic formulations**: many pesticides work equally, when prepared in different formulations.

- **Microencapsulated insecticides** are found to be more toxic to honey bees than any other formulation. As the size of these capsules is similar to that of pollen, thus, it facilitates their transport directly into the colony, where these compounds remain poisonous for long time and can also be fed to the developing brood. Use of this formulation should strictly be prohibited if; there is any chance of collection of pollen by a foraging bee from the treated crop.

- Dusts are more hazardous than the liquid formulations as these chemicals can reach and enter a honey bee colony through drifting along with the air current. Ultra-low-volume (ULV) formulations are also more hazardous than the other liquid formulations as they can enter or reach a colony in the same manner as well.

- Emulsifiable concentrates are less hazardous than wettable powders.

- Granular formulation is also safer for the bees as these chemicals are provided to the lower parts of the plant canopy, which minimizes their direct contact with any flower visiting pollinator.

- **Identify attractive blooms**: attractive blooms in and around the field to be sprayed should be check before the application as most of the times such blooms of weed flora attracts the foraging bees and the pesticidal drift to such blooms can be hazardous to the visiting pollinators. In order to avoid such incidents the blooms of weed plants can be removed before the application.

- **Notify beekeepers**: beekeepers should be notified well before the application, as this time period will allow them to move their colonies to a distance where, pesticidal drift is minimal. Colonies can also be covered with the cloth to confine bees into the box itself to avoid any foraging for 1 or 2 days.

7. Conclusion

Pollinators in general, either insects or the handful of other animal species are of utmost importance for their continuous support to most of the cross pollinated plant species for their reproduction. The honey bees, which is considered as the

most important among all the pollinators is responsible for achieving of global food production demand every year. With ever increasing population, human have constantly been searching for a way to maintain this demand of global food production and in order to achieve this goal, the conventional agriculture has evolved over the centuries. In this sequence, for the proper management of insect, plant pathogens and weed plants in agro-ecosystem various chemical pesticides were discovered in the nineteenth century. Ever since the introduction of these chemical pesticides, the serious debate on their effects on non-target insects and other organisms have also started. Thus, this chapter focuses on the different routes, modes and effects of interaction between various pesticidal applications and their toxic effects on honey bees, at both individual and colony level. Agrochemicals used in fields focusing mainly on minimize the crop losses are harmful for non-target organisms and hundreds of pollinator species, including honey bees are also no exception to this. Being the worker caste of the colony honey bee foragers visit various fields and gather pollen and nectar from different plant sources, which makes them in a phase of constant exposure to various chemicals, either natural or synthetic in nature. These foraging workers collect provisions from floral resources from chemically treated plants and carry them to their colony and thus, unknowingly with each visit they carry with them, a serious threat to their own life as well as to their colony as well. The different kinds of agrochemicals may be a fungicide residue, remaining in a plant after the seed treatment; a herbicide molecule, sprayed directly over the weed plants; an insecticide residue either coming through a direct spray or reaching the colony via air current (drift). Other than these agrochemicals, a serious threat for honey bee colonies has also been imposed by the various synthetic chemicals applied to the bees in apiaries itself for the proper management of honey bee health. Several such chemicals, used for the management of honey bee pests have also been reported to be toxic to the bees.

Although, several studies have been put forward regarding pesticidal toxicity to honey bees, but still a proper management strategy in order to minimize the honey bees exposure is still lacking. However, all pesticidal applications should be done in a way to minimize their exposure to honey bees, so as to prevent the further decline of honey bee population throughout the world. Furthermore, there exists a need of an extension program, for the farmers and beekeepers to spread the awareness regarding the hazardous effects of different agrochemicals to the honey bees, in order to make the existing management strategies more effective in future.

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Chapter 3

Commercial Pollination of Apple Orchards: Val di Non Case Study

Luciano Pilati, Paolo Fontana and Gino Angeli

Abstract

This chapter presents the results of a survey conducted in spring 2019 within the beekeepers who rent their colonies for the pollination of apple orchards in Val di Non, an alpine area in North Italy. The commercial pollination of apple orchards in this area is managed in an associated form by their cooperatives. The survey, carried out in collaboration with the local farmer cooperatives, submitted to the beekeepers a questionnaire containing questions on the economic and apidological aspects of their migratory beekeeping. The answers, referring to 43 questionnaires, show that beekeepers mostly: plan the migration itinerary at the beginning of the year; proceed to balance the colonies of honey bees before the pollination of the apple orchards; believe that the strength of the colonies must affect the pollination fee paid by the farmers and that the concentration of the colonies for the pollination of crops is not a relevant factor in the spread of bee diseases. The winter losses of honey bee colonies suffered by the responding beekeepers are on average 11.9%. The average cost of feeding the honey bee colony amounts to 19.1 €/colony. Finally, there is a wide interest in beekeepers to ensure the honey bee colonies.

Keywords: commercial pollination service, apple orchards, Val di Non, cooperative management of pollination service, survey, economic and apidological results, sustainability

1. Introduction

The use of managed western honey bee (*Apis mellifera* Linnaeus, 1758) colonies for commercial pollination of crops is redesigning modern beekeeping. Farmers must use managed honey bee colonies for generalized loss or reduction of not managed pollinator biodiversity in intensively cultivated areas [1, 2] and to support crop yield and/or to improve crop quality [3, 4]. Conversely, beekeepers are interested in hiring their bee colonies to farmers to cash colony rental fees and in addition, if the pollinated crop produces valuable nectar, to obtain honey productions. The matching between the demand of farmers and the supply of beekeepers has given rise to markets in the world of commercial pollination services [5–9] with very heterogeneous economic and apidological characteristics. The possibility of sequentially integrating the production of honey and commercial pollination services during the year has led beekeepers to undertake the migration management of honey bee colonies. The movements of the honey bee colonies follow privileged itineraries [10–13], and according to some authors [14], these itineraries are designed by the beekeepers before the start of the migration itinerary. The commercial pollination of crops is posing some new economic issues. Traditionally, the pollination fee per hive

was contracted by the beekeeper without taking into account the robustness of the honey bee colony [15]. However, some research has shown that robustness significantly affects the productivity of the bees [16–19]. Consequently, farmers should choose the stocking density on the basis of their robustness to obtain the optimal pollination. In reality, the inhomogeneity of colony-to-colony robustness reflects the effects on the pollination of the crop. For this reason, balancing of honey bee colonies before the start of pollination service is to be counted among the good beekeeping practices [20]. The migratory management of modern beekeeping offers advantages but also involves risks: the concentration of numerous honey bee colonies coming from the most disparate places in a restricted area to be pollinated can facilitate the spread of honey bee diseases [21, 22]; displacement of honey bee colonies over long distances can affect their health [23, 24] and also the percentage of winter losses [25]. Moreover, when the supply of the commercial pollination service involves the movement of bees of a subspecies of not managed *Apis mellifera* within the natural range of another subspecies, or when honey bees belonging to the so-called commercial hybrids are handled, damage can be created both to the not managed populations of *Apis mellifera* of the other local subspecies and to honey bees managed by local beekeepers [26].

Even the intensive cultivation of the apple trees shows some problems from the point of view of pollination [27]. The intensification of cultivation has made the survival of local not managed pollinators difficult, especially where the cultivations reach great extents, while at the edge of the cultivated area, near the natural vegetation (woods or grasslands), not managed pollinators can easily satisfy the residual pollination needs. To support the pollination provided by wild pollinators in areas where these do not constitute stable and conspicuous populations, apple producers use the pollination service provided in general by the managed honey bee colonies. We used honey bee colony and beehive as synonyms even if this latter is formed by the colony of honey bees and the hive (box) that contains it.

Commercial pollination is a consolidated practice in Val di Non (North Italy), an alpine area specialized in intensive apple cultivation. In this area the pollination contract with the beekeepers is stipulated by the cooperatives to which the farmers confer apples for storage, processing, and marketing. The combined management of the pollination service allows to overcome the technical-economic limit deriving from the typical pulverization of the land structure of the local farms. In order to gather information about beekeepers who support the pollination of the apple orchards in Val di Non, a survey was conducted through a questionnaire. The survey was filled anonymously. Participants were asked questions about some technical-economic and apidological questions concerning mainly migratory beekeeping. The objective of the survey is twofold: on the one hand to verify if there are differences between the answers provided by small- and large-scale beekeeping operations on quantitative aspects, such as the level of bee colony losses in winter and the number of kilometers traveled annually, but also on qualitative aspects such as balancing of bee colonies and the propensity to ensure bee colonies, and on the other hand, to compare the results obtained with those of other surveys on beekeeping. Unfortunately, this comparison will remain confined to a few aspects because many questions we submit to beekeepers are lacking terms of comparison.

The structure of this chapter is the following. After the presentation in Section 2 of the materials and methods of investigation, in Section 3 five economic and five apidological aspects considered worthy of attention will be briefly discussed, not all those considered in the questionnaire, to avoid that the analysis becomes too dispersive. The results obtained will be shown and briefly discussed in Section 4. Section 5 will present some proposals for the future of migratory beekeeping in Val di Non (but not only).

2. Materials and methods

The exploratory survey on beekeepers supporting apple pollination in Val di Non was conducted during the spring 2019 using a special questionnaire. Submitting questionnaires to the beekeepers to collect business information is a fairly common practice. The winter losses of bee colonies [28, 29], the pollination fees [30, 31], and the movement of bee colonies during the year [32, 33] have been surveyed with this instrument.

The associated management of the pollination service in Val di Non facilitated the investigation; in fact, the cooperative managers contacted beekeepers to interview, distribute, and collect questionnaires. Beekeepers filled in a questionnaire with 20 questions: in some cases, the interviewee was asked to provide a dichotomous answer (yes/no) as shown in **Table 1**, Section 1; in other cases, the questions

	Number of honey bee colonies					
	1–80		**>80**		**All**	
Section 1						
	Yes (%)	No (%)	Yes (%)	No (%)	Yes (%)	No (%)
Plans honey bee colony movements?	93.1	6.9	100. 0	0.0	95.2	4.8
Interest in ensuring honey bee colonies?	63.3	36. 7	92.3	7.7	72.1	27.9
Balance honey bee colonies?	93.3	6.7	100.0	0.0	95.3	4.7
Section 2						
Disease spread risk			%			
Irrelevant	34.5		46.2		38.1	
Low	41.4		46.2		42.9	
Significant	24.1		7.7		19.0	
Honey bee colony losses in summer			%			
<5%	92.8		74.6		90.3	
5–10%	3.6		15.4		7.3	
>10%	3.6		0.0		2.4	
Honey bee robustness vs pollination fee			%			
No	10.0		8.3		9.5	
Low	30.0		25.0		28.6	
High	60.0		66.7		61.9	
Commercial hybrids are an opportunity?			%			
No	43.3		38.5		41.9	
Yes	10.0		30.7		16.3	
Do not know	47.7		30.8		41.8	
Section 3						
Cost of feeding honey bees (€/colony)	21.1		14.7		19.1	
Kilometers traveled per year	3538		13,592		6650	
Winter honey bee colony losses (%)	12.5		10.3		11.9	

Table 1.
Beekeepers replies to the questionnaire.

were multiple choice and the interviewee could choose between several pre-coded answers (**Table 1**, Section 2); in other cases, the answer was in an open form where the interviewee entered numeric data (**Table 1**, Section 3).

A total of 43 completed questionnaires were returned by the beekeepers. However, the number of valid answers varies from question to question. As a whole, the respondent beekeepers, given the stocking density average locally applied to apple orchards, provide almost half of the commercial pollination needs in the area under investigation.

The commercial pollination of the apple orchards in Val di Non is always practiced at the beginning of the migration itinerary of the beekeepers of Northern Italy. In this period of the year, beekeepers are not able to provide data on the loss of honey bee colonies in summer and winter, on the average feeding cost of the honey bee colony. For these aspects, the questionnaire asked the beekeeper to refer to the situation of the previous year (2018) or the last two years or to indicate the expected value under normal conditions. The averages of the continuous variables and the percentages of the answers to the pre-coded questions were calculated. The statistical analyzes of the data collected with the questionnaire are placed, for the peculiarity of the questions set out and for the lack of information on the distribution of the variables, in the context of non-parametric statistics. To process the collected data, the R program, an open source programming language designed specifically for statistical analysis [34], was used. In order to ascertain the effect of the company size, beekeepers were divided into two groups based on the number of bee colonies they managed: a) up to 80 honey bee colonies; and b) more than 80 honey bee colonies.

3. Technical-economic and apidological aspects of apple pollination

3.1 Technical-economic aspects

Most apple cultivars require cross-pollination with a compatible pollinizer to increase apple tree yield and fruit quality. Some exceptions to this are the diploid varieties as Golden Delicious, the more cultivated in Val di Non. Although this variety is partially self-fruitful, it will produce better apples with cross-pollination by means of honey bees. This has been observed in Val di Non where, despite being close to vast areas of meadow and wood, the pollination action carried out by wild pollinators is sometimes limited due to the low temperatures of the flowering period. To get the maximum quality of apple production, it is necessary that pollination takes place at the first flowering phase, when 2–4% of the flowers of apple are open (first king flowers). The commercial pollination of the apple tree does not occur in Val di Non simultaneously because the local flowering periods of the apple orchards depend on the altitude and exposure and on the different varieties cultivated. Depending on the altitudinal level, at the same variety, the flowering window is about 14–18 days, while it's between 5 and 8 days the period of beginning of flowering among the varieties grown on the same altitudinal plane. The honey bee colonies are distributed in orchards to be pollinated in small batches according to precise stocking density, which in the case of apple intensive cultivation is placed in the range 8 ± 4 honey bee colonies per batch. The optimal stocking density varies according to some parameters, in particular the varieties of apples locally grown (few varieties or multi-varieties). Even if one colony per hectare could generally be enough, it is advisable to distribute honey bee colonies at a rate of 1.5 per hectare. The permanence of honey bee colonies for pollination of apple orchards has a theoretical duration of 18 ± 2 days, generally included between the first week of April and May; operatively this period is greatly influenced by the weather pattern [35].

If the first phase of full flowering takes place with very favorable weather for the flight of honey bees, the hives can be transferred to the next site already after 7–10 days.

The problem of the use of pesticides assumes in the case of the Val di Non and, more generally in the alpine territory, a particular connotation due to the blossoming of apple orchards that is strictly correlated to the variation of the elevation. In fact, it may happen that active honey bees on a site where apple orchards are in bloom and therefore pesticide treatments are not yet performed can reach apple orchards located at a lower elevation where the bloom is already over (or is considered such by growers), and therefore the treatments with pesticides have already started. The problem is aggravated by the presence of new apple plants which, especially in the first year of plantation, usually bloom 3–4 weeks later than the others. These young apple trees are often nearby or mixed (in rows) with productive ones and can after that receive pesticides while blooming.

The apple growers of the Val di Non confer (with few exceptions) their production for the subsequent conservation, processing, and marketing to 16 cooperatives associated in turn in a Consortium. The area cultivated with apple trees from the 4000 producers associated with the cooperatives of the Val di Non extends over 6400 ha located at an altitude between 450 and 900 m above sea level (https://www.melinda.it/il-consorzio/il-consorzio.html). In the Val di Non, the average size of the apple orchards owned by single farmers is only 1.6 ha, and the land parcels are so small that they evoke the image of "patches of land." The management of the pollination service at the farm level would be almost impossible, given that the flight of the honey bee exceeds 1 km and does not respect the boundaries of the land parcels. In these conditions, some farmers may behave as free riders, i.e., wait for others to implement their apple orchard pollination service and then benefit from it free of charge. Acting in this way would get the service without paying the fee; lastly, however, they would compromise, due to the reaction of those who paid for the service, the commercial pollination on the whole area (or almost) reaching a socially inefficient situation. The problem of the free rider found a solution in Val di Non thanks to the coordination function carried out by the farmer cooperatives. The latter, acting as territorial authority, organize the pollination service on behalf of their members: they decide the stocking density to be applied to the apple orchards, settle the payments to beekeepers who have provided honey bee colonies for hire, and divide the cost of the service among the producers in proportion to the pollinated surface. In some areas of the Val di Non, the commercial pollination service is managed by the land improvement consortium to replace the cooperative.

The associated management of the pollination service generates a further economic advantage as it allows to contain the transaction costs [36]. The pollination contract is in fact stipulated by the cooperative without the direct involvement of thousands of farmers.

The most significant economic aspects among those submitted to beekeepers with the questionnaire are the following:

a. If they plan the movements of honey bee colonies before the start of the migration route. This hypothesis, however plausible it may be, remains to this day unproven.

b. If they consider the robustness of the honey bee colonies to be relevant in determining the pollination fee of the apple orchards. The effectiveness of the pollination service depends critically to know if the beekeepers who support the pollination of the apple orchards in Val di Non are aware of the need to consider this factor in the calculation of the pollination fee of the commercial pollination service.

c. What is the average cost borne by the beekeeper for feeding the honey bee colony in order to obtain strong colonies early in the season. The data on the costs of big beekeeping operations for the year 2018 reported by Sumner and Champetier [37] show that the cost of the food purchased suffered a very strong increase compared to 1976.

d. How many kilometers the beekeeper travels to manage the movements of his bee colonies; this is a significant aspect for measuring the migratory footprint of the beekeeper.

e. Whether the beekeeper is interested in securing his honey bee colonies. This is an economic aspect of primary importance. In the last few decades, the role of insurance has grown enormously in many productive sectors of alpine agriculture, especially in those afflicted by adverse climatic conditions or plant diseases. Surprisingly, in apiculture the insurance instrument has not found wide applications (at least in Italy) although the effects of climatic adversities have become increasingly evident in the last few years.

3.2 Apidological aspects

The intensification of apple cultivation, even in the Val di Non, has made the survival of local not managed pollinators difficult due to the drastic reduction of the flora capable of supplying pollen and nectar, for the reduction of nesting and overwintering sites for some bees and even more for the use of agrochemicals. The presence of not managed Apoidea is generally very scarce in intensively cultivated areas. Stable populations of Apoidea and other wild pollinators are present only in areas adjacent to meadows, pastures, and forests [38]. However, these populations provide a limited contribution because they have normally reduced mobility, not exceeding 80–100 m of home range. Since the cultivation of the apple tree in Val di Non follows linearly the path of the Noce river forming on the two sides of the river strips of variable width often fragmented by areas of natural vegetation, the presence of not managed pollinators in apple orchards is closely related to their ability to reach them from adjacent areas that depends on the width of these strips. The immediate implication of the peculiar apple orchard cultivation configuration in Val di Non, in relation to the management of the commercial pollination service, is that the stocking density to be applied is not uniform but varies according to the need for integration of "natural" pollination. The choice of stocking density to be applied in order to optimize the pollination of the apple orchards must carefully consider the level of robustness of the honey bee colonies. In fact, the pollination potential of the honey bee colonies depends both on the number of foraging bees as well as on their health status. Each bee is able to make 3–10 daily flights, during which it can visit up to 3000 flowers [39]. Since the health status is difficult to estimate quickly and economically, the pollination contract is limited to prescribing, if provided, the minimum strength (population) of the colonies quantified on the basis of the number of "inhabited" frames/combs.

At the beginning of the spring season, i.e., at the end of the wintering period, honey bee colonies have a poor robustness, and beekeepers must reinforce them with artificial preventive nutrition or enter the market to buy colonies or brood combs. Among the apidological aspects submitted to beekeepers in the questionnaire, the following are the most relevant:

a. If they proceed to balance the colonies of honey bees before the pollination of the apple orchards.

b. What is the percentage of losses of bee colonies found in the winter rest period 2017/2018 and 2018/2019.

c. What is the percentage of losses of bee colonies found in the summer period 2017/2018 and 2018/2019, classifying it as follows: less than 5%, between 5 and 10%, and over 10%.

d. If they consider that the concentration of bee colonies in the area to be pollinated facilitates the spread of diseases and parasites among bees.

e. If the use of commercial hybrids (i.e., the so-called Buckfast bees) constitutes an opportunity. Commercial hybrids offer opportunities for their great vigor, for the production of abundant broods, and for the consequent formation of populous honey bee colonies, but they carry with them the risk of not reaching the robustness of the colonies themselves at the beginning of the productive season. Commercial hybrids, strongly selected for the production of honey, find hard to develop their colonies in the early stages of the production season when they are used for pollination of crops.

4. Results

The responding beekeepers manage on average 93.3 honey bee colonies. Thirty beekeepers have less than 80 honey bee colonies with an average of 27.9. The remaining 13 beekeepers with more than 80 honey bee colonies have an average of 244.2. All beekeepers with less than 80 honey bee colonies have their headquarters in the local area, specifically in the Trentino-Alto Adige region, while the remaining 13 come from other Italian regions. It follows that the size classes and the classes of origin coincide. Overall, the responding beekeepers have in total 4011 honey bee colonies; those from outside the region have only 3175 colonies that are almost 80% of the total number. The "local" beekeepers therefore prevail numerically over the others, but they are minority in terms of colonies of honey bees possessed.

With regard to the economic aspects (see **Table 1**, Section 1), a clear majority percentage (95.2%) of the responding beekeepers declares that they plan the migration itinerary ex ante. The percentage reaches 100% for the beekeeper with over 80 honey colonies. This result supports the basic assumption of the model of the migratory beekeeper on the planning of the movement sequence at the beginning of the year.

Most beekeepers (61.9%) believe that the strength of the honey bee colony should affect highly the pollination fee paid for pollination of the apple tree (see **Table 1**, Section 2). This percentage is low compared to the importance attributed to balancing the honey bee colonies; it could be distorted due to the convenience of beekeepers with less robust honey bee colonies to declare the parameter irrelevant.

The distance traveled by beekeepers takes into account not only the movements of bee colonies but also the visits to apiaries located at various sites during the season. Responding beekeepers traveled (see **Table 1**, Section 3) an average of 6650 km during 2018 (both for pollination services and honey production). The distance traveled by beekeepers with less than 80 honey bee colonies is much lower, being on average 3538 km. Beekeeper with more than 80 colonies traveled an average of 13,592 km during 2018. The greater distance traveled by beekeepers belonging to the latter size class finds an easy explanation in their origin from outside the region and in their farm size. The reduced number of kilometers traveled by beekeepers with

less than 80 honey bee colonies shows that these beekeepers have a weak migration footprint.

The average annual cost for the honey bee colony feeding stands at 19.1 €/colony, being higher for beekeepers with less than 80 colonies (21.1 €/colony) and minor (14.7 €/colony) for those with a higher number. It is surprising that the big beekeeping operations have a lower average cost for feeding the colony than the small ones. The first possible explanation of the difference is the amount of food provided to honey bees, and the second one is that beekeepers with less than 80 colony pay a higher price because they buy smaller quantities.

In total 72.1% of beekeepers declare an interest in securing honey bee colonies being the 92.3% within the beekeepers belonging to the class with more than 80 colonies. This high percentage is a good starting point to implement an insurance strategy suitable on the needs of beekeepers.

Regarding the apidolological aspects, almost all (95.3%) the responding beekeepers balance their honey bee colonies before the apple pollination service. The percentage reaches 100% for beekeepers with more than 80 colonies.

The losses of honey bee colonies suffered in the winter period in the 2-year period 2017/2018 and 2017/2019 amounted to the average of 11.9% (see **Table 1**, Section 3). This is a lower percentage than that documented by the Coloss survey [29] for European beekeepers, which reached an average of 16.4% during winter 2017/2018 with variations from 2.0 to 32.8% between countries. Beekeepers with a number of colonies greater than 80 undergo winter losses that are lower in percentage (10.3%) than in those with a lower number of colonies (12.5%). This result is aligned with that of the Coloss survey.

The losses of honey bee colonies suffered by beekeepers during the productive, summertime period, with few exceptions, are less than 5% in the area under investigation (see **Table 1**, Section 2). The overall average loss of honey bee colonies during the 2-year period considered therefore remains less than 16.9%.

Only 19% of interviewed beekeepers consider the risk of spread of honey bee diseases as significant due to the movement and the subsequent concentration of honey bee colonies. This risk is considered irrelevant or low by 92.4% of beekeepers with more than 80 honey bee colonies.

To the question of whether commercial hybrids represent an opportunity, the answers "do not" and "don't know" are on a par with 41.9 and 41.8%. Only 16.3% of beekeepers believe that commercial hybrids are an opportunity. However, the answers to this question are very different in the two size classes. 30.7% of beekeepers with more than 80 honey bee colonies compared to a meager 10.3% of beekeepers of the other size class believe that commercial hybrids are an opportunity. 47.7% of beekeepers with less than 80 bee colonies and 30.8% of the other size class do not know how to answer this question, demonstrating their lack of knowledge of the subject.

4.1 Discussion

The survey carried out using the questionnaire highlighted some interesting differences between migratory beekeeping implemented in the Val di Non for pollination of apple orchards and the pollination service conducted in other beekeeping contexts such as the USA. The major differences concern the average annual cost for the honey bee colony feeding, the risk of spread of honey bee diseases, and the losses of honey bee colonies suffered in the winter period. These differences derive both from the specific morphological and climatic conditions of the Val di Non and from the main address and the size of the beekeeping operations involved in the pollination service. The Val di Non is a typical alpine valley where the apple

orchards are of small extension and the altitudinal variability generates a straightness of blooms. It is therefore necessary to move the honey bee colonies from one site to the next one in rapid succession, and this obviously favors small- and medium-sized, more flexible, and dynamic beekeeping operations.

The average annual cost for the honey bee colony feeding stands in Val di Non at 19.1 €/hive, a value much lower than that reported by Sumner and Champetier [37], equal to 50.21 $/hive, referred, however, to large-scale commercial migratory beekeepers (1000 hive operation) from California. The differences between the two contexts make the data for the cost for the honey bee colony feeding difficult to compare even if they refer to the same year (2018). The small size of beekeeping operations that support the commercial pollination of apple orchards in Val di Non probably favors greater efficiency in the management of honey bee colonies, and, on the other hand, the main production address (honey production) does not allow the use of large artificial feeds before the production season, in order not to contaminate the honey product with noncompliant sugars and not to stimulate swarming.

Almost all the beekeepers that support the commercial pollination of the apple orchards in Val di Non balance their honey bee colonies before the apple pollination. Beekeeping in North America, in particular that aimed at the large-scale pollination service, does not provide for colony balancing, but on the contrary, these, kept in hives of different conception, are divided before the migration. The most common hives used by North American beekeepers are multi-nest body Langstroth hives, while in Italy the most common ones are the Dadant-Blatt hives with single nest body. With these hives the balancing and moderate feeding of the honey bee colonies before their displacement in cultivated agricultural areas are normal in Italy in order to anticipate their demographic development and after that to better perform pollination and honey production.

The risk of spread of honey bee diseases due to the concentration of honey bee colonies is considered irrelevant by beekeepers interviewed above all by those who have more than 80 honey bee colonies. It is a result in contrast with the resonance that this problem has aroused in the USA and Australia. Most likely, the concentration of a few thousand bee colonies in an area of about 6000 ha that develops linearly along the two sides of the Val di Non is not perceived by the responding beekeepers as a real risk of spread of honey bee diseases.

The losses of honey bee colonies suffered in the winter period in the 2-year period 2017/2018 and 2017/2019 assumes a lower average value than that documented by the Coloss survey [33] for European beekeepers. Winter losses are lower in percentage for the large-scale beekeeping operations than in those with a higher number of colonies. The greater distance traveled annually by large beekeeping operations for the transfer of honey bee colonies between forage sites therefore does not exercise the feared negative effect in the opinion of beekeepers who support pollination of the apple orchards in Val di Non.

5. Conclusions

The pollination of the apple orchards in Val di Non has peculiar characteristics that make it a case of study: the orography and landscape structure could favor the integration between "natural" pollination and "managed pollination"; pollination of apple orchards is managed in a cooperative manner and not by each farmer; and the pollination of the apple tree is also supported by the honey bee colonies managed by small local beekeepers not contractually involved in the pollination service.

Among the economic and apidological aspects covered by the survey on beekeepers who operate the apple pollination service in Val di Non, two deserve to be highlighted for their immediate operational implications:

- The robustness of the honey bee colonies is considered, by the majority of interviewed beekeepers, a parameter that must have a large impact on pollination fee, and therefore apple tree pollination service contracts should include clauses that subordinate the level of pollination fee to the robustness of honey bee colonies.

- The high propensity of the responding beekeepers to ensure their honey bee colonies are the prerequisite for pushing insurance companies to place on the market "cutoff" policies on the needs of beekeepers, in particular for the coverage of risks of honey bee colony losses and of honey production reduction.

Regarding the future of apple orchard pollination in Val di Non (and not only), it is to be hoped that the value of unmanaged pollinators will be enhanced by means of an integrated crop pollination strategy [40] that includes the protection of plant biodiversity also in agroecosystems and the protection of sensitive sites in view of the reproduction of wild pollinators.

Conflict of interest

No conflict of interest.

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Chapter 4

Melissopalynological Analysis of Honeys from Paderu Forest Division of Visakhapatnam District in Andhra Pradesh, India

Ravula Devender, Hari Ramakrisha and Sonte Niranjan

Abstract

Palynological examination of 17 honey samples procured from 8 localities in Paderu forest division in Visakhapatnam district, Andhra Pradesh, India, produced assemblage of pollen in terms of quantity and diversity. According to melissopalynological assessment of the honey samples, 6 were unifloral, i.e., 3 from *Ageratum conyzoides* and 1 each from *Schleichera oleosa*, *Psidium guajava*, and *Mimosa pudica*, and 11 were multifloral. The dominant taxa include *Mimosa pudica*, *Syzygium cumini*, and *Centipeda minima*. The taxa such as *Terminalia arjuna*, *Dendrophthoe falcata*, *Lamiaceae*, *Asteraceae*, and *Phyllanthus emblica* were minor sources of nectar and bee forage, as indicated by low frequencies of their pollen. The numerous pollen types and their diversity show that bees travel considerable distance to collect the nectar for honey production.

Keywords: pollen analysis, honey, Paderu forest division, unifloral, multifloral

1. Introduction

Melissopalynology is a branch of plant sciences that studies pollen found in honey. Precision in interpreting pollen data recovered from the honey has always been a primary goal of those who study pollen and honey. We are using pollen count to determine the nectar source of a honey sample and recognize the types and percentage of recovered pollen in the honey. This study is the fact that honey bees utilize certain natural raw materials which are identifiable in honey. These natural raw materials include pollen and nectar [1]. The growth and development of honey bees depend on nectar as the source of carbohydrates and pollen as the source of proteins [2, 3]. Palynological analyses of honey and pollen loads are used to know about honey bee foraging ecology, habitat and vegetation [4–11]. Pollen of honey samples provides reliable information on floral resources of honeys along with the relative preferences of bees among the diverse assemblages of plant species flowering synchronously [12, 13].

The significant melissopalynological works have been reported from different sectors of this state, dealing with pollen analysis of honey [14–16]. Similar melissopalynological research work has been worked out in Karnataka [17–19],

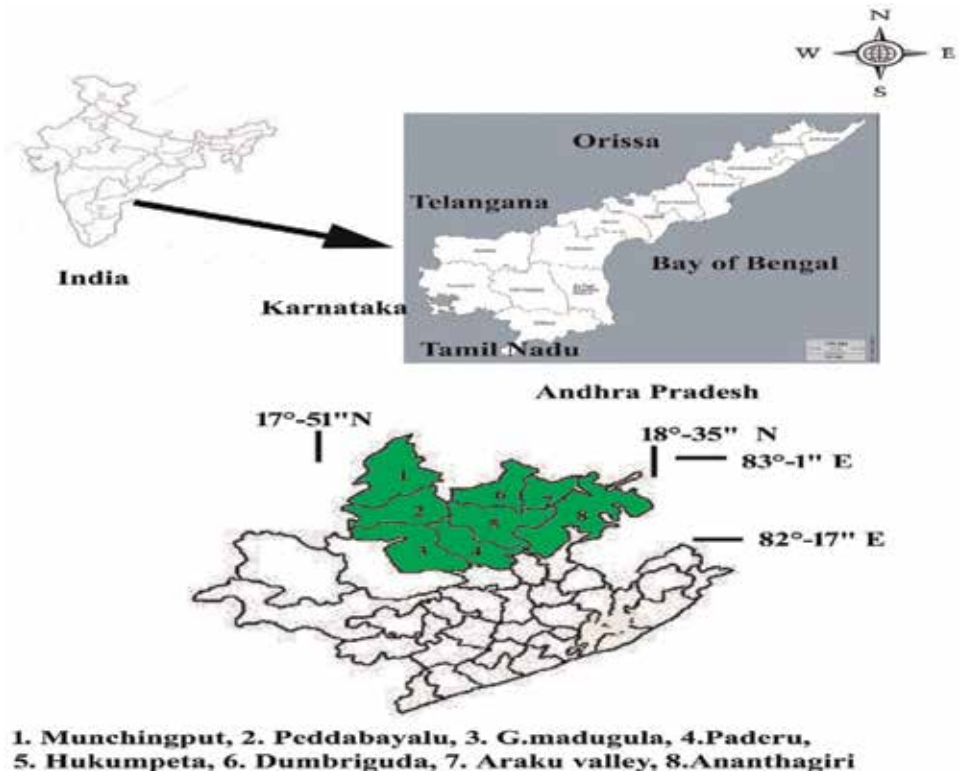

Figure 1.
Study area of Paderu forest division in Visakhapatnam district, Andhra Pradesh.

Bihar [20], Madhya Pradesh [21], Maharashtra [22], Uttarakhand [23–26], Uttar Pradesh [27, 28], and West Bengal [29–32], but the information is still rather sketchy. Qualitative and quantitative melissopalynological analyses in the east coast regions of India demonstrate that these regions are rich in bee plants with potential for producing adequate unifloral honeys, have an extended honey flow period, and thus can be utilized commercially for a moderate- to large-scale apiculture enterprises [33].

The study area includes the Paderu forest division of Visakhapatnam district, Andhra Pradesh. This division is the higher altitude zone in the hilly tracts of Eastern Ghats of Andhra Pradesh. It has the second highest tribal population in Andhra Pradesh. Paderu forest division (**Figure 1**) lies in between latitudes of 17°-51″ and 18°-35″ north and longitude of 82°-17″ and 83°-1″ east with a total geographical area of 3,24,965 ha, out of which the forest area under the control of the division is 104811.91 ha. The division comprises of a series of hills with an average annual rainfall of 2800 mm and a rich diversity of plant wealth.

2. Materials and methods

The materials for the present study are 17 honey samples (50 ml each) that were procured from 8 different blocks of Paderu forest division during 2011–2013, i.e., 3 samples from Ananthagiri (Af-13, Af-34, and Af-57), 4 from Paderu (Af-10, Af-40,

Af-44, and Af-64), 2 from Munchingiputtu (Af-14 and Af-54), 2 from G.Madugula (Af-3 and Af-48), 3 from Peddabayalu (Af-11, Af-53, and Af-65), and one each from Araku Valley (Af-37), Dumbriguda (Af-9), and Hukumpeta (Af-51). For palynological assessment, the honey samples were chemically processed using the acetolysis method, i.e., 1 ml of honey sample was dissolved in 10 ml of distilled water and centrifuged. The supernatant liquid was drawn out. The resultant sediment was treated with 5 ml of glacial acetic acid and centrifuged. After decanting acetic acid, the sediment was treated with acetolysis mixture (5 ml). It was prepared by nine parts of acetic anhydride and one part of con. sulfuric acid and then heated under water bath until the liquid turned chestnut-brown color. After cooling it was again centrifuged and the supernatant liquid was decanted. The sediment then treated with glacial acetic acid, later centrifuged, and the supernatant liquid was decanted off. And the sediment was washed with distilled water, and 50% aqueous glycerin (5 ml) was added and centrifuged for 10 min. The supernatant liquid was decanted off, and the tubes were inverted upside down on a filter paper for a few minutes [34].

The pollen sediment was taken on a pellet of glycerin jelly and transferred to the center of the slide. After being warmed slightly, the melted jelly with pollen sediment was covered by cover slip. Cover glass was later sealed with paraffin wax and labeled with their respective codes. Three slides were prepared for each sample and studied critically for their pollen contents.

Identification of pollen recovered from honey samples were carried out through the consultation of reference pollen slides available in Paleobotany and Palynology lab, UCS, Saifabad, OU. Quantitative pollen analysis was based on the method recommended by the International Commission for Bee Botany [6]. Pollen contents were taken at random, covering the maximum mounted area to avoid repletion. Once identified and counted, the pollen grains were placed into one of following pollen frequency classes-predominant pollen types (>45%), secondary pollen types (16–45%), important minor pollen types (3–15%), and minor pollen types (<3%). Honey samples containing more than 45% of a single type of pollen were considered as unifloral honey. The pollen types were placed into arboreal and non-arboreal taxa for making honey pollen spectra (**Figure 2**). A detailed list which included sample number, locality, nature and type of honey, collection season, and frequency of pollen types recovered is given in **Table 1**.

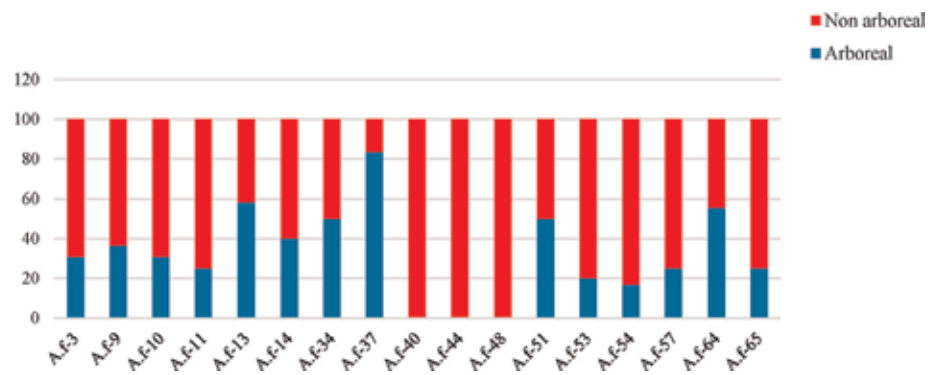

Figure 2.
Pollen spectra showing frequency of non-arboreal and arboreal taxa from different honeys of Paderu forest division.

S. no	Sample code	Locality	Nature of honey	Type of honey	Season collected	Predominant pollen type (>45%)	Secondary pollen types (16–45%)	Important minor pollen types (3–15%)	Minor pollen types (1–3%)	Pollen present (0.5–<1%)
1	V-P-GM-Bp-AF-3	G.Madugula	Multifloral	Squeezed	Spring	—	*Carum copticum* (21.74%)	Urticaceae type (15.02%), *Eucalyptus globulus* (13.22%), *Syzygium cumini* (12.78%), *Psidium guajava* (9.40%), *Tridax procumbens* (6.05%), *Blumea oxyodonta* (4.70%), *Bombax ceiba* (4.03%), *Brassica nigra* (3.81%), *Vernonia cinerea* (3.65%)	*Spilanthes calva* (2.69%), *Hakea laurina* (1.79%), *Saccharum officinarum* (1.12%)	—
2	V-P-DG-Vb-AF-9	Dumbriguda	Multifloral	Squeezed	Spring	—	*Mimosa pudica* (28.62%)	*Crotalaria juncea* (13.01%), *Tridax procumbens* (8.57%), *Psidium guajava* (8.55%), *Andrographis echioides* (8.17%), *Terminalia arjuna* (7.94%), *Schleichera oleosa* (7.06%), *Commelina suffruticosa* (5.57%), *Ageratum conyzoides* (5.20%)	*Dendrophthoe falcata* (2.60%), *Acacia chundra* (2.48%), *Pedalium murex* (2.23%)	—
3	V-P-P-Sa-AF-10	Paderu	Unifloral	Squeezed	Spring	*Ageratum conyzoides* (68.25%)	—	*Schleichera oleosa* (7.93%), *Parkinsonia aculeata* (6.03%), *Citrullus lanatus* (3.17%)	*Peltophorum pterocarpum* (2.22%), *Crotalaria juncea* (2.22%), *Ocimum basilicum* (2.53%), *Tridax procumbens* (1.90%), *Mimosa pudica* (1.58%), *Terminalia arjuna* (1.58%)	*Cyperus rotundus* (0.95%), *Acacia chundra* (0.95%), *Pedalium murex* (0.69%)
4	V-P-PB-PB-AF-11	Peddabayalu	Multifloral	Squeezed	Autumn	—	—	*Coriandrum sativum* (15.90%), *Mimosa pudica* (15.19%), *Syzygium cumini* (12.36%), *Ageratum conyzoides* (11.30%),	*Vicoa indica* (2.47%), *Amaranthus spinosus* (2.47%), *Pedalium murex* (2.47%),	—

S. no	Sample code	Locality	Nature of honey	Type of honey	Season collected	Predominant pollen type (>45%)	Secondary pollen types (16–45%)	Important minor pollen types (3–15%)	Minor pollen types (1–3%)	Pollen present (0.5–<1%)
								Ocimum sanctum (6.00%), *Schleichera oleosa* (5.30%), *Eucalyptus globulus* (4.94%), *Polygonum barbatum* (3.53%), *Blumea oxyodonta* (3.53%), *Casuarina equisetifolia* (3.18%)	*Vernonia cinerea* (2.12%), *Cajanus cajan* (2.12%), *Solanum nigrum* (1.76%), *Celosia argentea* (1.41%), *Lagerstroemia parviflora* (1.06%), *Commelina suffruticosa* (1.41%), *Xanthium strumarium* (1.48%)	
5	V-P-AG-Ch-AF-13	Ananthagiri	Unifloral	Squeezed	Autumn	*Schleichera oleosa* (55.00%)	—	*Mimosa pudica* (13.07%), *Eucalyptus globulus* (8.65%), *Phyllanthus emblica* (6.92%), *Dendrophthoe falcata* (3.07%), *Leucas aspera* (3.69%)	*Manilkara zapota* (2.88%), *Psidium guajava* (1.73%), *Careya arborea* (1.69%), *Vicoa indica* (1.50%), *Dillenia pentagyna* (1.34%)	*Clerodendrum inerme* (0.46%)
6	V-P-MUN-L-AF-14	Munchingiputtu	Multifloral	Squeezed	Autumn	—	—	*Eucalyptus globulus* (13.46%), *Tridax procumbens* (8.77%), *Centipeda minima* (8.32%), *Ageratum conyzoides* (7.41%), *Terminalia arjuna* (6.80%), *Psidium guajava* (6.80%), *Crotalaria juncea* (6.95%), *Syzygium cumini* (6.95%), *Commelina suffruticosa* (6.95%), *Sapindus emarginatus* (4.84%), *Erythrina variegata* (3.02%), *Leucas aspera* (3.78%)	*Datura stramonium* (2.26%), *Cassia occidentalis* (2.42%), *Vernonia cinerea* (2.11%), *Citrullus lanatus* (2.11%), *Albizia lebbeck* (1.96%), *Conyza stricta* (1.96%), *Lannea coromandelica* (1.66%), *Lantana camara* (1.47%)	—

S. no	Sample code	Locality	Nature of honey	Type of honey	Season collected	Predominant pollen type (>45%)	Secondary pollen types (16–45%)	Important minor pollen types (3–15%)	Minor pollen types (1–3%)	Pollen present (0.5–<1%)
7	V-P-AG-Gb-AF-34	Ananthagiri	Unifloral	Squeezed	Spring	*Ageratum conyzoides* (81.03%)	—	*Phyllanthus emblica* (12.06%), *Madhuca indica* (5.86%)	*Saccharum officinarum* (1.05%)	—
8	V-P-AR-Me-AF-37	Araku Valley	Unifloral	Squeezed	Spring	*Psidium guajava* (55.03%)	*Mimosa pudica* (24.59%)	*Terminalia arjuna* (10.53%), *Delonix regia* (5.85%)	*Acacia chundra* (2.34%), *Gardenia lucida* (1.66%)	—
9	V-P-P-Sp-AF-40	Paderu	Unifloral	Squeezed	Spring	*Ageratum conyzoides* (92.83%)	—	*Dendrophthoe falcata* (4.98%)	*Tridax procumbens* (2.19%)	—
10	V-P-P-Vm-AF-44	Paderu	Unifloral	Squeezed	Spring	*Mimosa pudica* (69.57%)	—	*Hyptis suaveolens* (14.56%), *Conyza stricta* (13.59%)	*Tridax procumbens* (1.26%), *Cardiospermum halicacabum* (1.02%)	—
11	V-P-GM-Ak-AF-48	G.Madugula	Multifloral	Squeezed	Spring	—	*Mimosa pudica* (32.91%), *Conyza stricta* (28.30%), *Tridax procumbens* (17.81%).	*Pedalium murex* (5.24%), *Hyptis suaveolens* (3.56%), *Saccharum officinarum* (3.18%), *Sida acuta* (3.14%), *Ageratum conyzoides* (3.14%)	*Tribulus terrestris* (2.72%)	—
12	V-P-HP-R-AF-51	Hukumpeta	Multifloral	Squeezed	Spring	—	*Eucalyptus globulus* (35.48%), *Mimosa pudica* (17.41%).	*Hygrophila auriculata* (14.83%), *Cocos nucifera* (11.61%), *Borassus flabellifer* (10.96%), *Cucumis sativus* (9.71%).	—	—
13	V-P-PB-L-AF-53	Peddabayalu	Multifloral	Squeezed	Spring	—	*Syzygium cumini* (41.82%), *Centipeda minima* (18.87%)	*Mimosa pudica* (14.59%), *Vernonia cinerea* (11.08%), Urticaceae type (6.80%)	*Caesalpinia bonduc* (1.36%), *Bombax ceiba* (2.33%), *Dendrophthoe falcata* (1.36%)	*Sida acuta* (0.97%), *S. cordata* (0.82%)

S. no	Sample code	Locality	Nature of honey	Type of honey	Season collected	Predominant pollen type (>45%)	Secondary pollen types (16–45%)	Important minor pollen types (3–15%)	Minor pollen types (1–3%)	Pollen present (0.5–<1%)
14	V-P-MUN-Sk-AF-54	Munchingiputtu	Multifloral	Squeezed	Autumn	—	*Syzygium cumini* (37.63%), *Centipeda minima* (18.70%), *Mimosa pudica* (18.27%)	Urticaceae type (11.82%), *Vernonia cinerea* (7.74%), *Tridax procumbens* (5.84%)	—	—
15	V-P-AG-Mt-AF-57	Ananthagiri	Multifloral	Squeezed	Autumn	—	*Syzygium cumini* (26.97%)	*Spilanthes calva* (13.40%), *Mimosa pudica* (13.24%), *Centipeda minima* (12.43%), *Cyathocline purpurea* (12.76%), *Leucaena leucocephala* (10.10%), *Tridax procumbens* (5.71%), *Vernonia cinerea* (5.39%)	—	—
16	V-P-P-Rb-AF-64	Paderu	Multifloral	Squeezed	Autumn	—	*Tridax procumbens* (27.95%), *Schleichera oleosa* (19.88%), *Erythrina variegata* (16.42%)	*Eucalyptus globulus* (12.96%), *Leucaena leucocephala* (6.34%), Urticaceae type (5.47%), *Cocos nucifera* (4.32%), *Amaranthus spinosus* (3.45%), *Dendrophthoe falcata* (3.21%)	—	—
17	V-P-PB-L-AF-65	Peddabayalu	Multifloral	Squeezed	Autumn	—	*Mimosa pudica* (41.0%), *Schleichera oleosa* (26.61%), *Dendrophthoe falcata* (19.42%)	*Hakea laurina* (12.97%)	—	—

Table 1.
Pollen content in honey samples of Paderu forest division in Visakhapatnam district.

3. Palynological results

The qualitative and quantitative analyses of 17 squeezed honey samples procured from different blocks of Paderu forest division in Visakhapatnam district of Andhra Pradesh were conducted. The pollen results provide new insights into the pollen composition of these honey samples. A total of 69 pollen morphotypes (**Figures 3**–**5**) in 35 families were identified, including 65 entomophilous pollen

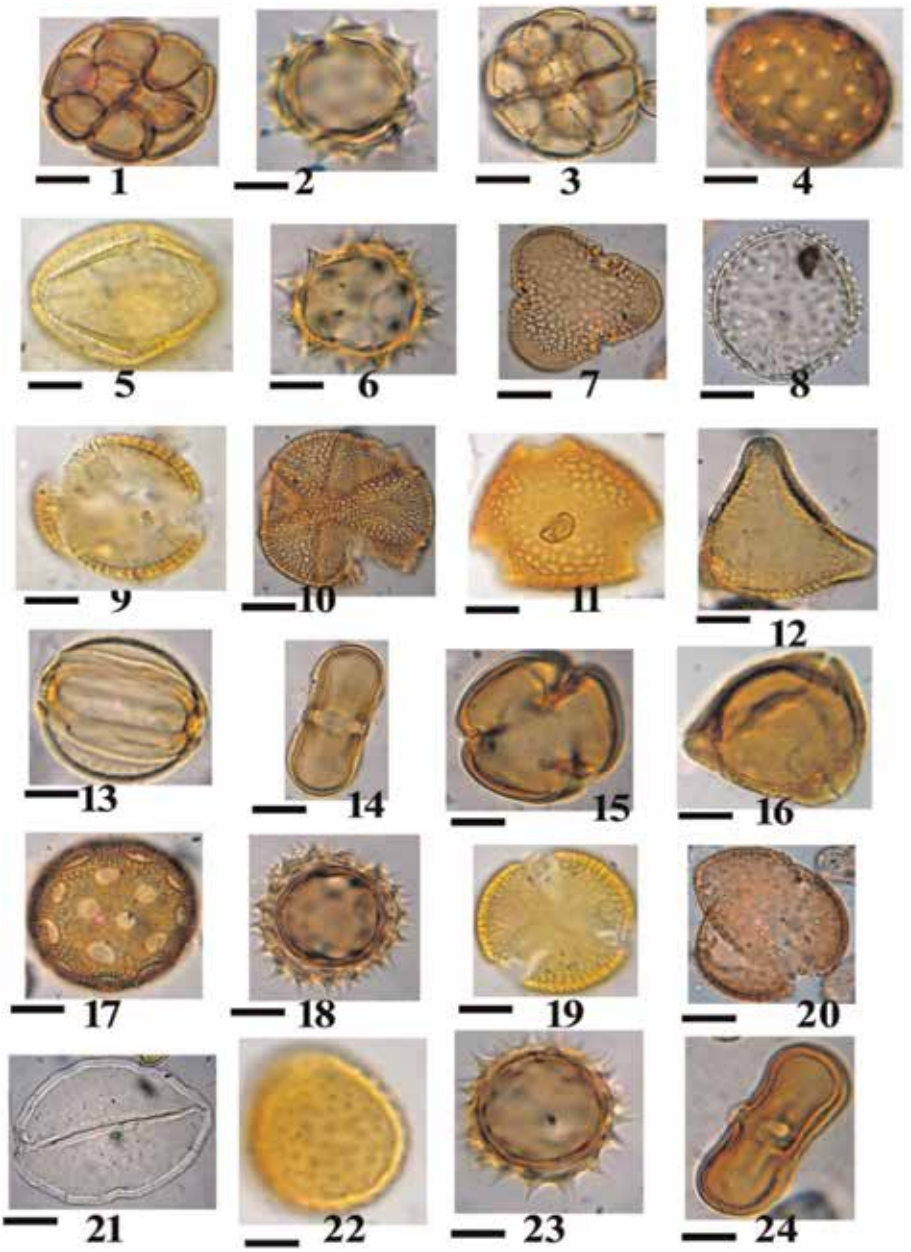

Figure 3.
(1) Acacia chundra, (2) Ageratum conyzoides, (3) Albizia lebbeck, (4) Amaranthus spinosus, (5) Andrographis echioides, (6) Blumea oxyodonta, (7) Bombax ceiba, (8) Borassus flabellifer, (9) Brassica nigra, (10) Caesalpinia bonduc, (11) Cajanus cajan, (12) Cardiospermum halicacabum, (13) Careya arborea, (14) Carum copticum, (15) Cassia occidentalis, (16) Casuarina equisetifolia, (17) Celosia argentea, (18) Centipeda minima, (19) Citrullus lanatus, (20) Clerodendrum inerme, (21) Cocos nucifera, (22) Commelina suffruticosa, (23) Conyza stricta, and (24) Coriandrum sativum. Scale bar: 10 *µm.*

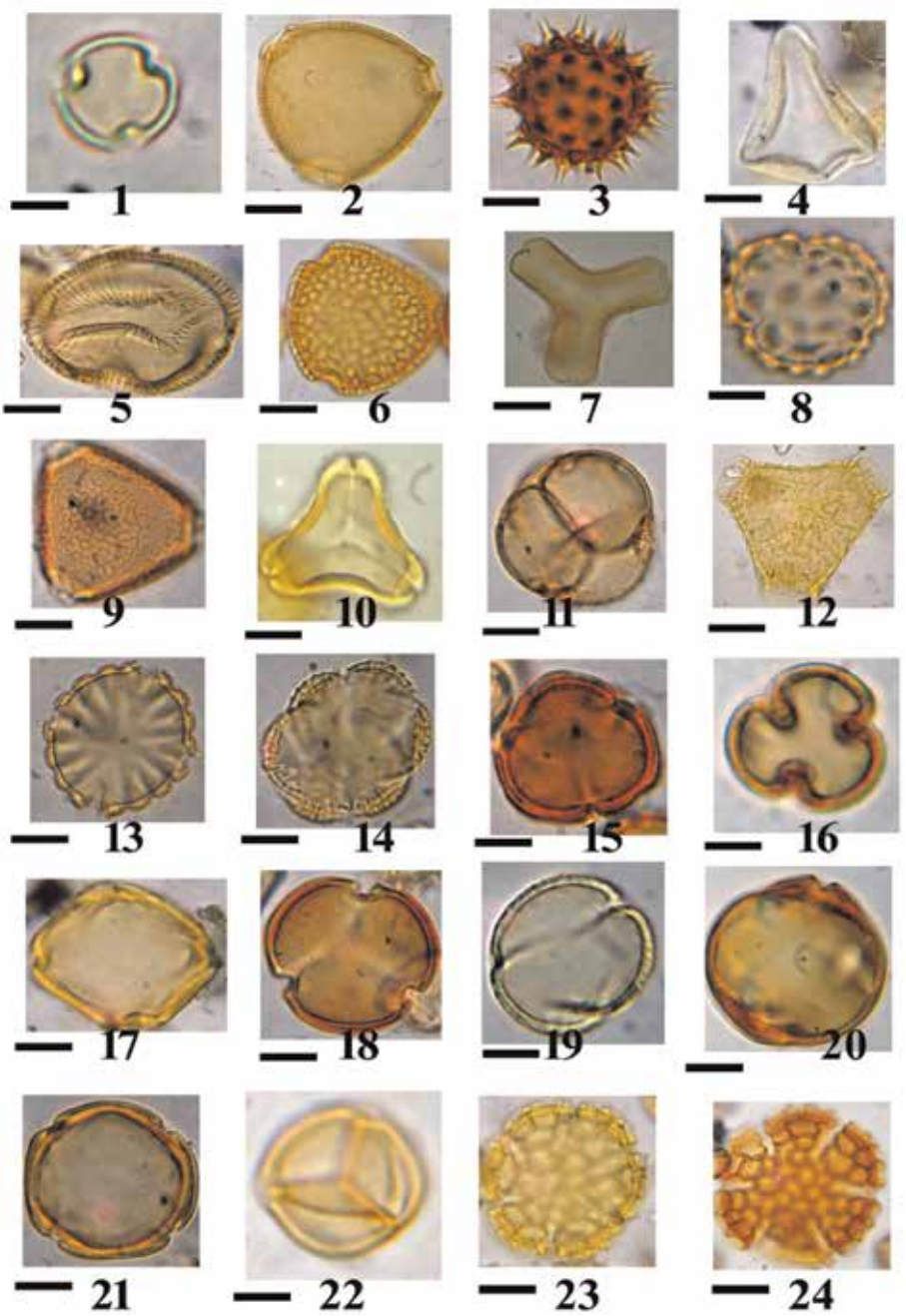

Figure 4.
(1) Crotalaria juncea, (2) Cucumis sativus, (3) Cyathocline purpurea, (4) Cyperus rotundus, (5) Datura stramonium, (6) Delonix regia, (7) Dendrophthoe falcata, (8) Dillenia pentagyna, (9) Erythrina variegata, (10) Eucalyptus globulus, (11) Gardenia lucida, (12) Hakea laurina, (13) Hygrophila auriculata, (14) Hyptis suaveolens, (15) Lagerstroemia parviflora, (16) Lannea coromandelica, (17) Lantana camara, (18) Leucaena leucocephala, (19) Leucas aspera, (20) Madhuca indica, (21) Manilkara zapota, (22) Mimosa pudica, (23) Ocimum basilicum, and (24) Ocimum sanctum. Scale bar: 10 μm.

types (i.e., *Acacia chundra*, *Ageratum conyzoides*, *Albizia lebbeck*, *Andrographis echioides*, *Blumea oxyodonta*, *Bombax ceiba*, *Borassus flabellifer*, *Brassica nigra*, *Caesalpinia bonduc*, *Cajanus cajan*, *Cardiospermum halicacabum*, *Careya arborea*, *Carum copticum*, *Cassia occidentalis*, *Casuarina equisetifolia*, *Centipeda minima*,

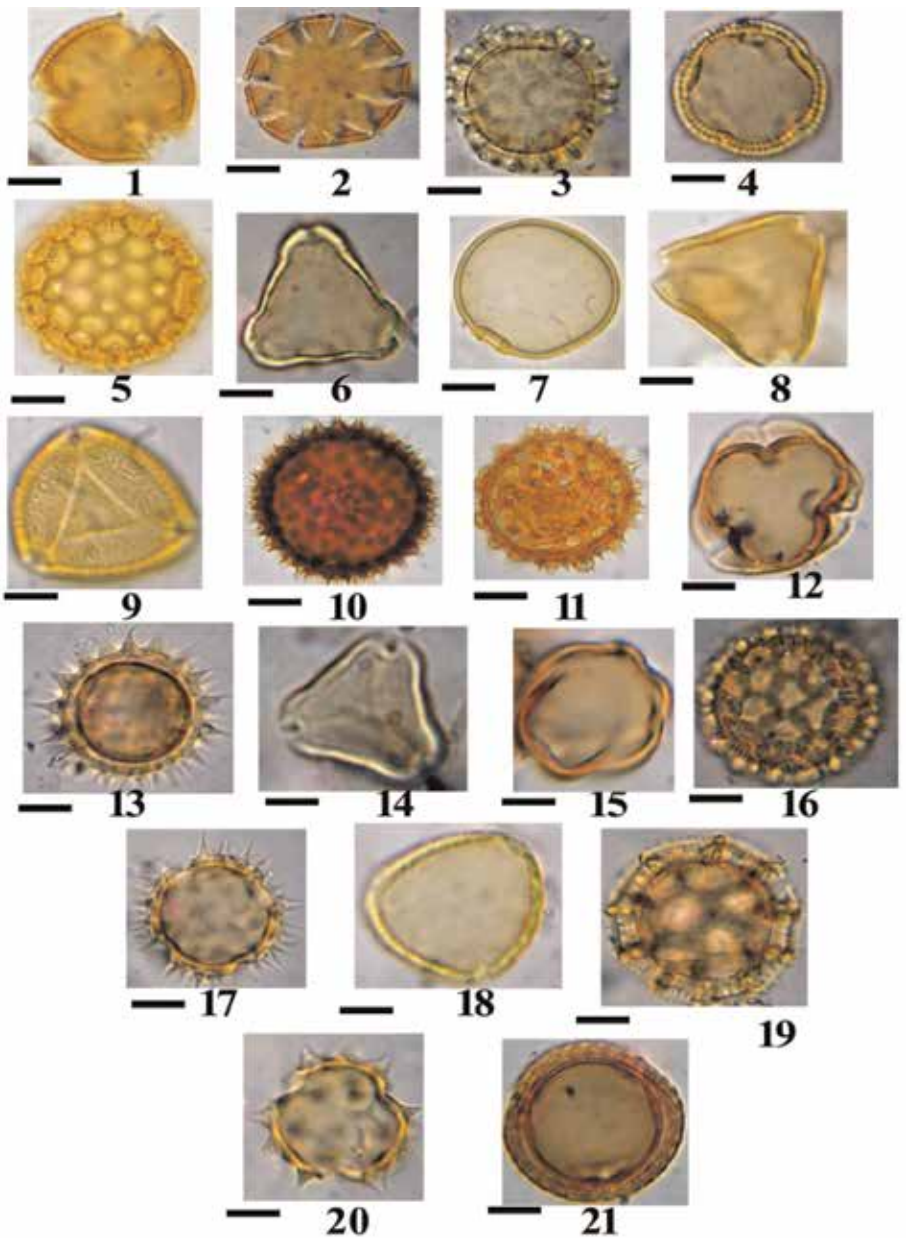

Figure 5.
(1) Parkinsonia aculeata, (2) Pedalium murex, (3) Peltophorum pterocarpum, (4) Phyllanthus emblica, (5) Polygonum barbatum, (6) Psidium guajava, (7) Saccharum officinarum, (8) Sapindus emarginatus, (9) Schleichera oleosa, (10) Sida acuta, (11) S. cordata, (12) Solanum nigrum, (13) Spilanthes calva, (14) Syzygium cumini, (15) Terminalia arjuna, (16) Tribulus terrestris, (17) Tridax procumbens, (18) Urticaceae type, (19) Vernonia cinerea, (20) Vicoa indica, and (21) Xanthium strumarium. Scale bar: 10 *µm.*

Citrullus lanatus, *Clerodendrum inerme*, *Cocos nucifera*, *Commelina suffruticosa*, *Conyza stricta*, *Coriandrum sativum*, *Crotalaria juncea*, *Cucumis sativus*, *Cyathocline purpurea*, *Datura stramonium*, *Delonix regia*, *Dendrophthoe falcata*, *Dillenia pentagyna*, *Erythrina variegata*, *Eucalyptus globulus*, *Gardenia lucida*, *Hakea laurina*, *Hygrophila auriculata*, *Hyptis suaveolens*, *Lagerstroemia parviflora*, *Lannea coromandelica*, *Lantana camara*, *Leucaena leucocephala*, *Leucas aspera*, *Madhuca indica*, *Manilkara zapota*, *Mimosa pudica*, *Ocimum basilicum*, *O. sanctum*,

Parkinsonia aculeata, *Pedalium murex*, *Peltophorum pterocarpum*, *Phyllanthus emblica*, *Polygonum barbatum*, *Psidium guajava*, *Sapindus emarginatus*, *Schleichera oleosa*, *Sida acuta*, *S. cordata*, *Solanum nigrum*, *Spilanthes calva*, *Syzygium cumini*, *Terminalia arjuna*, *Tribulus terrestris*, *Tridax procumbens*, Urticaceae type, *Vernonia cinerea*, *Vicoa indica*, *Xanthium strumarium*) and 4 anemophilous pollen types (i.e., *Amaranthus spinosus*, *Celosia argentea*, *Cyperus rotundus*, and *Saccharum officinarum*). Pollen analysis data of each sample is discussed below according to provenance of the samples.

G.Madugula (V-P-GM-Bp-AF-3, spring collection): the sample proved to be multifloral with secondary pollen taxa including *Carum copticum* (21.74%), followed by important minor pollen types, Urticaceae type (15.02%), *Eucalyptus globulus* (13.22%), *Syzygium cumini* (12.78%), *Psidium guajava* (9.40%), *Tridax procumbens* (6.05%), *Blumea oxyodonta* (4.70%), *Bombax ceiba* (4.03%), *Brassica nigra* (3.81%), and *Vernonia cinerea* (3.65%).

Dumbriguda (V-P-DG-Vb-AF-9, spring collection): the sample procured is productive and proved to be multifloral with secondary pollen taxa *Mimosa pudica* (28.62%). *Crotalaria juncea* (13.01%), *Tridax procumbens* (8.57%), *Psidium guajava* (8.55%), *Andrographis echioides* (8.17%), *Terminalia arjuna* (7.94%), *Schleichera oleosa* (7.06%), *Commelina suffruticosa* (5.57%), and *Ageratum conyzoides* (5.20%) are recorded as important minor pollen types.

Paderu (V-P-P-Sa-AF-10, spring collection): the sample proved as unifloral with one predominant pollen taxon, *Ageratum conyzoides* (68.25%), followed by *Schleichera oleosa* (7.93%), *Parkinsonia aculeata* (6.03%), and *Citrullus lanatus* (3.17%) as important minor pollen taxa.

Peddabayalu (V-P-PB-PB-AF-11, autumn collection): the sample procured is multifloral with pollen of *Coriandrum sativum* (15.90%), *Mimosa pudica* (15.19%), *Syzygium cumini* (12.36%), *Ageratum conyzoides* (11.30%), *Ocimum sanctum* (6.00%), *Schleichera oleosa* (5.30%), *Eucalyptus globulus* (4.94%), *Polygonum barbatum* (3.53%), *Blumea oxyodonta* (3.53%), and *Casuarina equisetifolia* (3.18%) as important minor pollen taxa.

Ananthagiri (V-P-AG-Ch-AF-13, autumn collection): the sample procured is productive and proved as unifloral as evidenced by the predominant pollen taxon *Schleichera oleosa* (55.00%), followed by the recovery of *Mimosa pudica* (13.07%), *Eucalyptus globulus* (8.65%), *Phyllanthus emblica* (6.92%), *Dendrophthoe falcata* (3.07%), and *Leucas aspera* (3.69%) as important minor pollen types.

Munchingiputtu (V-P-MUN-L-AF-14, autumn collection): the sample proved as multifloral with pollen of *Eucalyptus globulus* (13.46%), *Tridax procumbens* (8.77%), *Centipeda minima* (8.32%), *Ageratum conyzoides* (7.41%), *Terminalia arjuna* (6.80%), *Psidium guajava* (6.80%), *Crotalaria juncea* (6.95%), *Syzygium cumini* (6.95%), *Commelina suffruticosa* (6.95%), *Sapindus emarginatus* (4.84%), *Erythrina variegata* (3.02%), and *Leucas aspera* (3.78%) as important minor pollen types.

Ananthagiri (V-P-AG-Gb-AF-34, spring collection): the sample procured is unifloral as palynologically evidenced by a single predominant pollen taxon, *Ageratum conyzoides* (81.03%), followed by *Phyllanthus emblica* (12.06%) and *Madhuca indica* (5.86%) as important minor pollen types.

Araku Valley (V-P-AR-Me-AF-37, spring collection): the sample procured is productive and proved to be unifloral with predominant pollen taxon *Psidium guajava* (55.03%), followed by secondary pollen taxon *Mimosa pudica* (24.59%), *Terminalia arjuna* (10.53%), and *Delonix regia* (5.85%) pollen as important minor pollen types.

Paderu (V-P-P-Sp-AF-40, spring collection): the sample procured is productive like the AF-34 samples which is unifloral with predominant pollen taxon, *Ageratum conyzoides* (92.83%) followed by important minor pollen taxon, *Dendrophthoe falcata* (4.98%).

Paderu (V-P-P-Vm-AF-44, spring collection): the samples is palynologically productive and proved to be unifloral with single predominant pollen taxon, *Mimosa pudica* (69.57%), followed by the important minor pollen types like *Hyptis suaveolens* (14.56%) and *Conyza stricta* (13.59%).

G. madugula (V-P-GM-Ak-AF-48, spring collection): the samples proved as multifloral as evidenced by secondary pollen types like *Mimosa pudica* (32.91%), *Conyza stricta* (28.30%), and *Tridax procumbens* (17.81%). The remaining taxa are represented as important minor pollen types like *Pedalium murex* (5.24%), *Hyptis suaveolens* (3.56%), *Saccharum officinarum* (3.18%), *Sida acuta* (3.14%), and *Ageratum conyzoides* (3.14%).

Hukumpeta (V-P-HP-R-AF-51, spring collection): the sample is palynologically proved to be multifloral as evidenced by secondary pollen types, *Eucalyptus globulus* (35.48%) and *Mimosa pudica* (17.41%), followed by important minor pollen types, *Hygrophila auriculata* (14.83%), *Cocos nucifera* (11.61%), *Borassus flabellifer* (10.96%), and *Cucumis sativus* (9.71%).

Peddabayalu (V-P-PB-L-AF-53, spring collection): the sample procured is productive and proved as multifloral with pollen of *Syzygium cumini* (41.82%), *Centipeda minima* (18.87%) recorded as secondary pollen types, followed by the pollen of *Mimosa pudica* (14.59%), *Vernonia cinerea* (11.08%), and Urticaceae type (6.80%) as important minor pollen types.

Munchingiputtu (V-P-MUN-Sk-AF-54, autumn collection): the sample proved as multifloral with evidenced of pollen of *Syzygium cumini* (37.63%), *Centipeda minima* (18.70%), and *Mimosa pudica* (18.27%) represented as secondary pollen types. The remaining taxa are represented as important minor pollen types like Urticaceae type (11.82%), *Vernonia cinerea* (7.74%), and *Tridax procumbens* (5.84%).

Ananthagiri (V-P-AG-Mt-AF-57, autumn collection): the sample procured is productive and proved as multifloral with pollen of *Syzygium cumini* (26.97%) represented as secondary pollen taxon, followed by the pollen of *Spilanthes calva* (13.40%), *Mimosa pudica* (13.24%), *Centipeda minima* (12.43%), *Cyathocline purpurea* (12.76%), *Leucaena leucocephala* (10.10%), *Tridax procumbens* (5.71%), and *Vernonia cinerea* (5.39%) as important minor pollen types.

Paderu (V-P-P-Rb-AF-64, autumn collection): the sample proved to be multifloral with evidenced by secondary pollen types like *Tridax procumbens* (27.95%), *Schleichera oleosa* (19.88%), and *Erythrina variegata* (16.42%). The remaining taxa are *Eucalyptus globules* (12.96%), *Leucaena leucocephala* (6.34%), Urticaceae type (5.47%), *Cocos nucifera* (4.32%), *Amaranthus spinosus* (3.45%), and *Dendrophthoe falcata* (3.21%) recollected as important minor pollen types.

Peddabayalu (V-P-PB-L-AF-65, autumn collection): the sample procured is productive and proved as multifloral with pollen of *Mimosa pudica* (41.0%), *Schleichera oleosa* (26.61%), and *Dendrophthoe falcata* (19.42%) identified as secondary pollen types, followed by single-pollen taxon *Hakea laurina* (12.97%) as important minor pollen taxon.

4. Discussion

The present Melissopalynological study provides new insights into the pollen composition of honey samples from Paderu forest division in Visakhapatnam district of Andhra Pradesh. A total of 69 pollen morphotypes from 17 honeys produced by *Apis florea* were identified. Six honeys were considered unifloral honeys because they contained a predominant pollen type (>45%). The dominant of unifloral honeys, without any toxic pollen grains and with scarce fungal elements, suggests that most of the honeys are of good quality and suitable for human consumption.

The results coincide with the melissopalynological investigation in the peninsular part of India where unifloral honeys are dominant [35]. The diverse flora of India is due to varied climatic conditions in different parts of India. The multifloral source of honeys may be generated by the absence of major ingredients of forest and invasion of secondary forest elements [36]. The Palynological analysis of Paderu forest division honeys reflects that the native flora may be used as a source of good quality honey. In our studied honey-pollen exploration, it is easy to perceive that the honey bee preferred mainly non-arboreal in spring honeys, with the exception of Af-37 as arboreal dominant; *Ageratum conyzoides* is a predominant pollen taxon in three spring samples, *Mimosa pudica*, *Tridax procumbens*, and *Conyza stricta* as secondary pollen taxa. In autumn season also, bees preferred mainly non-arboreal exception of Af-13 and -64 samples, in Af-13 sample as unifloral with *Schleichera oleosa* as predominant pollen taxa.

Based on the above study, bees are preferred mainly on non-arboreal to collect nectar and convert to honey due to the flowering time of the melliferous species; climatic condition and human activities (e.g., farming, reforestation, and forest fires) may be other factors to consider in understanding the presence or absence of some taxa in the pollen spectra of honeys. In recent years, many rural communities have taken up beekeeping as an alternative source of their livelihood strategies. Even the younger generation is showing interest because beekeeping is so easy and simple that anybody can take it as an enterprise. Thus, our melissopalynological investigation may contribute to and favor the possibilities of using rich flora of the studied area in order to develop beekeeping enterprises on a commercial basis, in which self-employment opportunities may be created for many rural communities and develop their livelihood strategies in this area.

5. Conclusions

The analysis of the pollen content of Paderu forest division in Visakhapatnam district of Andhra Pradesh honey samples indicates that the local flora may be used as a source of good quality honey. The overall preponderance of non-arboreal in most of the honeys reflects that the honey bees prefer to visits to collect nectar. The scarce appearance of pollen from nectar-less plants such as *Amaranthaceae*, *Cyperaceae*, and *Poaceae* indicates that they were trapped in the hive incidentally by wind or were inadvertently transported by honey bees. And apiculture may enhance honey production in floristically rich province of Paderu forest division in Visakhapatnam district of Andhra Pradesh and adjoining areas, when job opportunities may be created for many developing rural communities of this state.

Acknowledgements

The authors are thankful to the Principal, University College of Science, Saifabad, OU, and Head of the Department of Botany, Osmania University, for providing laboratory facilities and acknowledge to local beekeepers for sample collection.

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Chapter 5

Application of Environmental DNA: Honey Bee behavior and Ecosystems for Sustainable Beekeeping

Tomonori Matsuzawa, Ryo Kohsaka and Yuta Uchiyama

Abstract

Honey prices can vary widely depending on the production areas and/or the nectar plants, and quality control, therefore, is of great significance. Also, the identification of the nectar plants is one of the major concerns regardless of the purposes of beekeeping, namely, commercial, recreational, or for environmental education. In recent years, the scope for the application of eDNA technology has been expanding. We conducted an eDNA analysis of the 14 types of honey sold in supermarkets. The result showed that all of the honey samples contained DNA of several plants and revealed that there was no monofloral honey. In addition, there were cases where there was a discrepancy between the plants listed on the labels and the species whose DNA was the most prominent in the sample. DNA analysis of honey is considered to have the potential to enhance exponentially the understanding of the plant species that honeybees used as nectar plants and their proportions.

Keywords: environmental DNA, ecosystem services, sustainable beekeeping, multifloral honey, monofloral honey, honey-source plant, urban beekeeping, beekeeping in Japan

1. Introduction

Honey is a relatively expensive food product, but the prices vary largely. The differences in popularity and prices of honey are largely determined by factors such as country of origin and nectar plants, as well as the stories associated with the honey – who made it, when they made it, and where they made it. Hence, it is important to understand these stories behind each honey product.

There are various types of honey in the world, but information that we can obtain is limited. So, in this chapter, we start with a brief explanation of the status of beekeeping in the world, followed by an introduction to the history of Japanese beekeeping that is rarely told in western literatures written in English. We then share an overview of urban beekeeping that has gained popularity in recent years and discuss the risks and regulations surrounding the movement. In the last chapter, we show the results of our eDNA analysis of honey and indicate the validity of these results.

The history of beekeeping is showing that beekeeping is a part of culture of human society [1]. The beekeeping culture is historically included in regional food

culture [2]. Urban beekeeping became one of the main ways of beekeeping in different regions of the world [3]. Urban environment can contribute to maintenance of diversity of honeybee species including native species [4]. Alternatively, the issues of pesticides are becoming serious in some countries [5], although the honeybee colony declines can be caused by complex factors including pesticides and global honey trade [6]. Trends of beekeeping and honey production in Japan and South Korea are analyzed in the previous study [7]. The transmission of the knowledge of beekeeping is the one of the research topics in the field of environmental studies [8].

As the IPBES assessment report suggested, management pollination services are an urgent global task. In this circumstance, urban beekeeping is gaining attention in terms of various aspects including ecosystem diversity, genetic diversity of honeybee, educational practices, and so on. The size of urban areas is relatively small as compared with farmland and other habitats of honeybee. However, the roles of urban areas to maintain the genetic diversity of organisms and to enhance the environmental awareness of citizens are suggested by the existing studies [4, 7], and urban beekeeping is expected as a main way of beekeeping. In the promotion of urban beekeeping, rack of scientific evidence of behavior of urban honeybee is a serious issue. To provide the scientific evidence, environmental DNA analysis can be utilized to detect the details of nectar sources. In this regard, this chapter reviews the status and trend of urban beekeeping and discusses the results of the application of environmental DNA analysis. The methods provided in this chapter can be applied for other cases and contribute to accumulating the scientific evidence for making relevant policies of urban beekeeping.

2. Materials and methods

This paper consists of two analytical frameworks. First, we reviewed the historical documents, articles, and relevant policy documents in order to capture status and trends of issues of honey production from bees.

As second phase of analysis, we conducted e-DNA analysis to identify the nectar source of honey produced by the authors and compared them with those products sold in the supermarket.

We extracted the DNA according to the method of Hawkins et al. [9] and amplified the DNA using the ITS-p3/ITS-u4 primer pair [10]. Then, the DNA metabarcoding of the plant contained in honey was implemented using the next-generation sequencer MiSeq.

3. Results and discussion

3.1 The status of beekeeping

3.1.1 The status of beekeeping around the world

Honeybees are important not only as honey producers but also as pollinators for agricultural crops [11]. Their importance has been raised as an easily comprehended example of the ecosystem service brought by nature in the context of conservation of biodiversity. In the engagement of the report of The Economics of Ecosystem and Biodiversity (TEEB), attempts have been made to quantify (and if possible, monetize) various services of the ecosystem, and as such, the importance of the pollinating function is described as "five times value of the production of honey" in the report [12]. Also, at the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services (IPBES), the phrase "Nature's Contribution to People

(NCP) has been advocated instead of the services of the ecosystem. In a report published by IPBES, too, the economic value of the pollinating service of honeybees is estimated to be up to 577 billion dollars, highlighting its importance [13].

The decline of the pollination function has been a major concern socially and economically, as well as scientifically. In the report, which points out the decrease of pollinators and their related pollinating services, some examples of research into the decline of pollinators and related vegetation are showcased as a global concern [11].

In the case of honeybees, examples of Colony Collapse Disorder (CCD) have been observed involving a serious shortage of honeybees in the United States and Europe. In the United States, roughly one third of the honeybees kept for pollination was lost from 2007 to 2008, causing a major concern [14]. In Europe, too, similar phenomena were reported in Germany, Belgium, France, Holland, Poland, Spain, and so on. Further, similar cases were reported in other countries elsewhere including Brazil, India, Taiwan, and Japan.

While a number of theories have been put forward to address the causes of CCD, including agrichemicals, infections, malnutrition, electromagnetic waves, and genetically modified crops, the mechanism of CCD has not been fully understood. However, neonicotinoid agrochemicals are one of the possible candidates as a cause of the disorder [15], and they are thus restricted in EU countries [16].

For beekeeping around the world, *Apis mellifera* is predominantly used. There are said to be approximately 10 species of honeybees, and *Apis mellifera* is considered superior to others in terms of amount of honey production and the ease of keeping. Other species have hardly been domesticated.

3.1.2 Beekeeping in Japan

In Japan, the two species, namely, *Apis mellifera* and *Apis cerana*, are used for beekeeping. Until *Apis mellifera* was introduced in the nineteenth century, only *Apis cerana* was used. In some of the old literature, sketches of beekeeping using *Apis cerana* can be found [17, 18].

In "the Chronicles of Japan (*Nihon Shoki*)", there is a description that in 643, some Koreans attempted beekeeping on Mt. Miwa using four sheets of honeycomb but failed [19].

There is a record from 739 of honey being listed as one of the offerings from Korea, along with other products such as those made from panthers and ginseng, which implies that honey was treated as a precious imported item [18].

Entering the 900s, a record was found that honey and comb honey were presented to the Imperial Court from various countries. Considering the amount of the honey presented from each prefecture was around 2–4 L, it is thought that it was an extremely valuable commodity [18]. "*The Tale of Genji (Genji monogatari),*" the oldest novel in the world written in 1008, describes how honey was used as one of the ingredients to make incense.

Arriving at the Edo-era (after 1600), with the advance in the research into honey production, educational books explaining the beekeeping technology accompanied by illustrations began to be published. As the amount of honey production increased, it came to be used mainly as medicine by the general public (**Figure 1**).

The person who succeeded in the most sophisticated beekeeping in Japan, before the time any modern beekeeping technology was introduced there, was Ichiemon Sada in Wakayama prefecture. He standardized the hive boxes and kept several hundreds of bee colonies. The honey yielded from one of these colonies was 4.7 kg, which is a considerable amount for beekeeping using *Apis cerana* [17–19].

The illustration above is a part of "The Honey Catalogue (*Hachimitsu Ichiran*)," compiled to be exhibited at the World Expo held in 1873 in Austria. It is evident

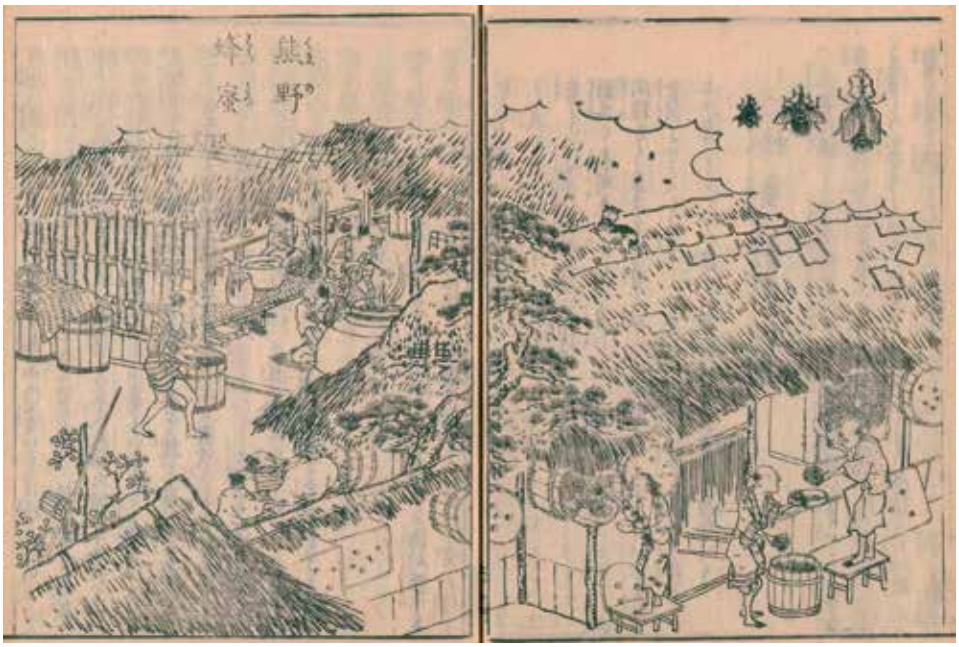

Figure 1.
*Beekeeping in the Edo period using Japanese honeybees (*Apis cerana japonica*). From "Noted Products from Land and Sea of Japan in Pictures (*Nihon sankai meisan zue*)," 1799.*

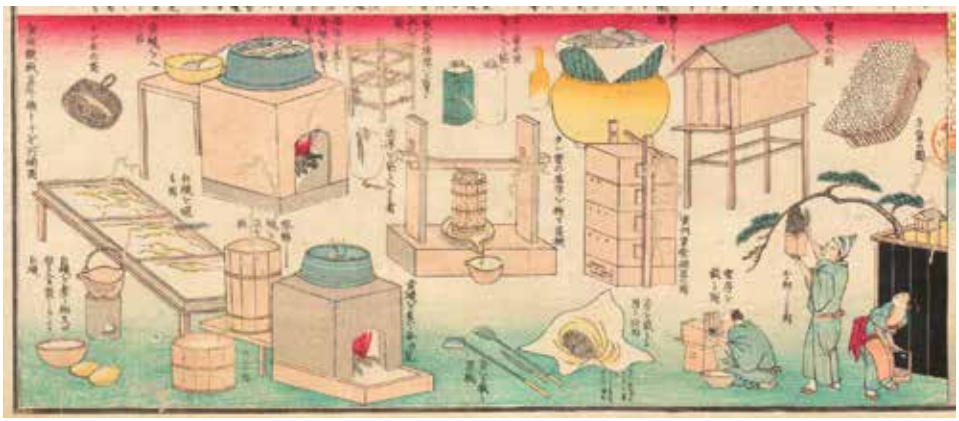

Figure 2.
Illustrations of beekeeping before introduction of Apis mellifera*. This figure shows the process of beekeeping and production of beehoney using native Japanese species in Edo period. The words in the figures are written in ancient languages describing the names and process of production of the individual tools. From "The Honey Catalogue (*Hachimitsu Ichiran*)," 1873.*

from this that a kind of technology very similar to the level of the modern beekeeping was already established (**Figure 2**).

In 1877, *Apis mellifera* was imported from the United States for the first time. From around 1905, the number of domestic beekeepers started to increase rapidly and so did the domestic production of honey. In Japan, honey production reached its peak around 1965 [20, 21].

Advancement of Japanese industrialization accelerated the development of agricultural land and the accompanying reduction of land suitable for beekeeping. The area planted with astragalus, which was a major source plant for honey, decreased to

roughly 11% compared with the level of around 1970, while for rapeseed, it dropped to about 5% over the same period. Meanwhile, relatively the ratio of cheap imported honey increased in the Japanese market, and the aging of domestic beekeepers was accelerated. Furthermore, the number of domestic beekeepers became approximately half compared with before that period [18]. Currently, domestic honey production has been hovering around 2800 tons with the domestic self-sufficiency ratio of approximately 5–7% [22]. Of all the imported honey, 70% comes from China and 10% from Argentina [22]. Domestic honey production was on a declining trend up to around 2010 but has been moving sideways since then.

In Japan, beekeeping using domestic species has been gaining popularity, and some organizations have been set up to share beekeeping know-how [23]. Japanese honeybees (*Apis cerana japonica*) have a mild temperament and are less dangerous but produce less honey, so they are relatively more suited for hobby beekeeping [24]. A common method of beekeeping is to guide wild honeybees into a tree hollow or some artificial hive box with a cavity.

In Japanese honey bees (*Apis cerana japonica*), some aggregation pheromone [a mixture of 3-hydroxyoctanoic acid (3-HOAA) and 10-hydroxy-(E)-2-decenoic acid (10-HAD)], which is found in orchidaceous plants, has been identified [25]. Therefore, beekeepers use a type of orchid *Cymbidium floribundum*, which secretes this aggregation pheromone or the composite thereof in order to induce honeybees into their artificial hive boxes. Also, some of the other beekeepers have been attempting to establish other methods using Langstroth hives or flow hive boxes [26–28].

3.1.3 Urban beekeeping

There are records of beekeeping in urban areas dating from ancient time, but it is only recently when it gained prominence. Since 2005, urban beekeeping began to expand in various European countries, before spreading into North America, Asia, Latin America, and Africa.

In Palais Garnier of the Paris Opera, beekeeping has been going on for the last 30 years, and now, it is seen in various landmark locations in the city such as Orsay Museum and Grand Palais. In the United Kingdom, it has increased by 200% between 1999 and 2006, while in New York, the number of beehives kept has gone up to 10 times since 2010. In Paris, over 700 bee colonies are in existence [29].

In South Korea, there are business entities specializing in urban beekeeping, while in Japan, one of the beekeeping traders with the longest history is engaged in commercial beekeeping in the surrounding areas of the Imperial Palace.

In addition to honey production, urban beekeeping is thought to be contributing to the conservation of biodiversity by compensating for the function of the indigenous pollinators such as *Apis cerana* that had decreased due to the development of the natural environment over the years. In fact, in the surrounding areas of the Imperial Palace in Tokyo, known for the cherry blossoms, more cherry fruits have been observed after the blossoms. This suggests that the increase of urban beekeeping near the Imperial Palace may be a factor.

In the United Kingdom, community groups play an important role in the development of urban beekeeping, but there is also a support from local government to promote it [30].

It is generally understood that urban beekeeping has a greater role in improving quality of life as it provides a form of hobby and a communication tool, in addition to the function of honey production and pollinating. In fact, commercial beekeeping is rare in urban areas, and for the most part, the number of bee colonies is usually only up to a few per area. In Japan, where NGOs, private companies, as well as local government are involved in urban beekeeping, the primary objective there is

to revitalize civic activities through encouraging collaboration among the residents and enhance their understanding of the environment and ecosystem services [31].

Beekeeping at the Paris Opera and the White House is widely known, and the main purpose of much of these examples of urban beekeeping is awareness of the environmental issues and improvement of quality of life. In the Kyodo district in the Setagaya Ward in Tokyo, local residents began to keep the swarming of honeybees in the green area on the rooftop of an apartment block. As a result of this, this area, which was hardly in use before, became a focal point for the local residents where they would gather collectively mainly on weekends for potluck parties using the honey collected. This led to strengthening of the bond between the residents as well as voluntary planting and cleanup activities in the area. In Tokyo, where greenery is scares in residential areas, the value of properties with well-managed green spaces tends to be high, and beekeeping can be a contributing factor for raising property values.

Ginza on the eastside of the Tokyo railway station is a commercial district known for its land prices that are the highest in Japan. An NGO called "Ginza Honeybee Project" began urban beekeeping in 2006 using the rooftop of a building in Ginza [32]. It was initially started as a means for environmental and dietary education, but through the years, its achievements such as greening of the urban areas, the large amount of honey collected (producing around one ton per year), and successful sale of other agricultural and processed products began to be publicized nationwide as best practice examples of community revitalization. Today, similar activities have grown and expanded into over 30 cities throughout Japan [31, 33].

3.1.4 Governance issues around urban beekeeping

On the other hand, beekeeping in urban areas also caused negative results – some of them were predictable by urban dwellers, but others were not. The problem most easily predicted would be stings. Improper access to beehives could lead to stings. However, in reality, there are not so many cases of damage caused by stings in urban areas with a concentrated population. As anybody involved in beekeeping would know, it is very rare for a person to be stung by honeybees away from beehives. Nonetheless, sight and buzzing of a large number of honeybees kept on a balcony of an apartment would probably be enough to scare the neighbors, and they may find it dangerous for their children to play freely outside.

Further, damage caused by the feces of honeybees often poses as important issues of concern. Honeybees flying out into the first rays of the spring sun after enduring the long winter make a large amount of feces. Unfortunately, they love white and yellow colors and pure white sheets, and your favorite yellow T-shirt would appear to them as the mark of "enticing toilets." The stains and smell from the honeybee feces dropped on laundry, and cars are a practical threat to city dwellers.

Although not being given too much attention until recently, urban beekeeping can be a cause for ecological competition. In other words, alien species that were not present in the unique biodiversity of a particular region could bring about adverse impact on the indigenous species [34].

Apis mellifera is an indigenous species in Europe, Africa, and the Middle East but not in the most parts of the Americas, Australia, and Asia. However, as the modern beekeeping with *Apis mellifera* spread to various parts of the world, these bees began to be kept in the fields worldwide [35].

As honeybees are herbivorous, they would not cause such direct adverse impact like attacking other creatures for consumption, but it is conceivable that other indirect damage may result. There are essentially two possibilities of negative impact: one is the competition with other species over limited honey sources [36] and the other, the spread of diseases and parasites [34, 37, 38].

Generally speaking, in the natural environment, the amounts of flowers available as the sources of nectar change dramatically. In the times when there are flowers in abundance, there can be nectar in excess of what the users can consume, but at other times, competition over limited nectar sources usually prevails. The potential competitors for honeybees include other types of honeybees, bumble bees, insects such as butterflies, bird species such as hummingbirds, and mammals like bats [39–41].

These competitors may not regard the honeybees brought into urban areas by humans as their desirable neighbors. It is quite possible that the nectar reserve available to them may have been reduced because of these urban honeybees. Such examples have rarely been verified at the level of scientific research, but there is a case study reported from some countries [42, 43].

Also, urban honeybees may bring with them more serious, undesirable guests. At the moment, one of the most worrying factors troubling beekeepers worldwide is honeybee mites. The two particularly well-known species, Varroa mite/Varroa destructor and honeybee tracheal mite/*Acarapis woodi*, can often cause fatal damage to honeybees.

In addition, there are infectious diseases such as Nosema and Sacbrood, which are caused by parasites, microbes, and viruses. They have been traveling with the honeybees by beekeepers and have now spread to most parts of the world. In their new habitats, these diseases can pose as new threats of infectious diseases to the indigenous species.

There has been a report of a rare case of "sugar theft" by honeybees. This problem came into light when mysteriously colored red and green honey was produced in the apiary adjacent to a candy factory in Manhattan (the colors were the same as those of the syrups used for candy production) [44].

Due to the large number of benefits that honeybees bring to humans, these negative impacts are rarely discussed where they should be sufficiently. Especially in urban areas, where the number of species making up the biodiversity is usually small, a loss of a species with specific functions could lead to serious consequences [45].

Therefore, there should be sufficient discussions on urban beekeeping both in the social and economic context of securing safe living for people and in the ecological context of conserving biodiversity.

In the United States, where honeybees have been regarded as a dangerous species, beekeeping in urban areas has been restricted. However, there has been relaxation of these restrictions as the case for urban beekeeping gained momentum [46]. London in the United Kingdom is one of the most active cities in terms of beekeeping [42]. In Japan, however, there are no regulations that restrict beekeeping, and beekeeping can be conducted anywhere in principle [47].

Act on the Promotion of Beekeeping was amended in 2012, and even small-scale hobby beekeepers are now required to notify to the government. However, there is still practically no regulation as to the restrictions on the locations of beehives and guidelines to follow. The width and depth of governance for urban beekeeping vary greatly between countries and regions. Now is the time to establish an adequate governance structure, where measures can be taken depending on the need of a particular country or a region.

3.2 Approaching with the latest technology (eDNA)

3.2.1 Adding value to honey with DNA analysis

3.2.1.1 Research on honey-source plants with environmental DNAs (eDNA)

In the paddy fields in Fukushima Prefecture, beekeeping has been conducted with hairy vetches after their decontamination, with the aim to produce a local

honey specialty for sale. Thorough analysis on radioactivity is in place for food safety and has been verified with the eDNA analysis technique that the product is honey from hairy vetches.

Identification of honey-source plants with eDNA analysis technique has been tried since around 2010 (**Figure 3**). It has some advantage over the conventional pollen analysis, but it is not fully verified that it can demonstrate a level of contribution of each honey-source plant accurately [9, 48].

Although DNA is an effective indicator of honey-source plants, most honey products do not show the results of their DNA analysis. In Gifu Prefecture where beekeeping is active, it is indicated on the label on some of the honey products that they are from cherries and ilexes by their DNA analysis, but this is one of the few examples.

3.2.1.2 Is it honey from acacia trees?

The eDNA analyses were conducted on 14 honey products purchased at high-end supermarkets in Tokyo. None of the products sold as single-flower honey/monofloral was actually from a single source. The DNA of false acacia was detected in all of the seven well-known acacia brands, but it was dominant in only four of them and the second-dominant following other plants in the other three products. In some of those sold as honey from a single source such as astragalus, ilex, amur cork, buckwheat, or manuka, these nominal source plants were not the dominant sources, or their DNA was not detected at all. None of the products analyzed was actually from a single source. Since honeybees visit various flowers, this is not surprising.

Acacia single-flower honey is much sought after in Japan, and it is traded at a high price. Among the seven acacia monofloral/single-flower honey products, however, acacia was found to be a dominant source for honey only in four of them, and the other three products contained honey only from false acacia as its second-dominant source. A product sold as monofloral honey may actually have been from several source plants/multifloral honey. Astragalus honey made in Tokyo is from

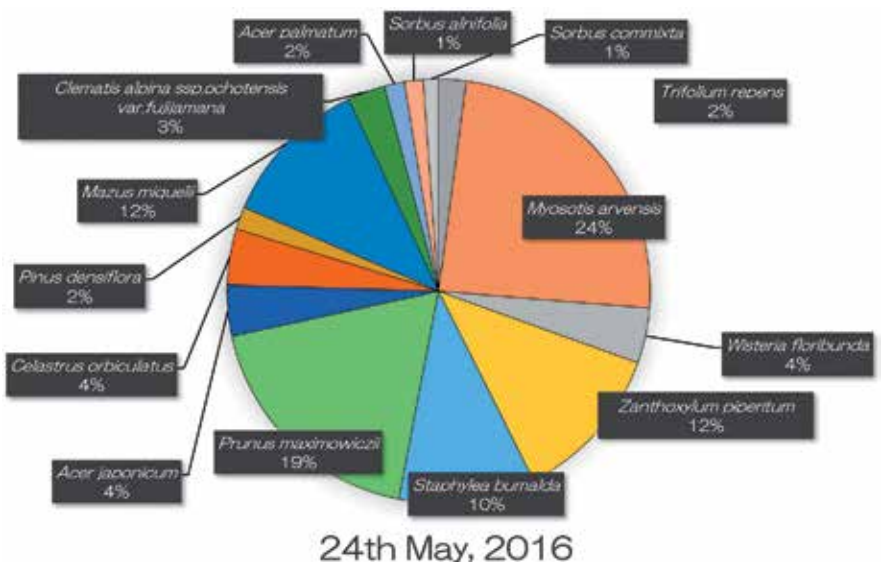

Figure 3.
An example of analysis results of honey we produce. More than 20 kinds of plants were confirmed. With the exception of Prunus maximowiczii, Wisteria floribunda, *and* Trifolium repens*, most species are not famous as honey source plants. Based on data from IDEA Consultants, Inc.*

three sources, while 18 source plants were detected in another astragalus honey made in Fukuoka. Some of the honey brands tested had plants containing toxic alkaloid as their source plants.

3.2.1.3 eDNA would reveal the authenticity of honey

Verifying the quality of high value-added honey is important for the development of a healthy beekeeping industry. Thus, DNA analysis can be a powerful tool to identify origins. If the list on the label contains a plant species that does not grow in the local area or does not contain the DNA that should be detectable as a species on the product label, it may be misrepresenting the honey and its origin.

We analyzed 14 honeys purchased at a luxury supermarket (**Table 1**). More than half of the products contained plant DNA on the label, but some products were not detected at all. Manuka DNA was not detected in the Manuka honey analyzed this time. Manuka honey may have been shipped after aging for several years after harvest, so it is possible that DNA degraded during this aging period. DNA is generally unstable and fragile. Since it breaks down over time, there is a possibility that the labeled honey source cannot be detected by DNA analysis, even if it is correct. However, this sample remains suspicious because it has confirmed several plant DNAs normally growing in New Zealand. Following the accusation that their Manuka honey was fake, New Zealand authority has mandated DNA analysis for their Manuka honey since 2018 [49].

3.3 eDNA analyses can open the door for new scientific findings

3.3.1 Discovering the flora and phenology in surrounding areas

With eDNA analysis, we can better understand the flora of the areas around the hives. This method could only identify plant DNAs that were visited by bees. Furthermore, this method may not show a correlation between the amount of DNA and the volume of existing plants. Despite these disadvantages, honey eDNA analysis is a effective tool to verify the general trends of honey origins.

3.3.2 Honeybees are experts at flower hunting

Bees are much better at finding flowers than humans. Occasionally, plant species that humans are incapable of identifying may be found in the DNA of honey. In the honey produced near Mt. Fuji, the DNA of several types of plants which were not identified in and around the production area were detected. One of them, *Gaultheria pyroloides,* distributes in the alpine area of Mt. Fuji, which was more than 8 km away in terms of horizontal distance and 1500 m in vertical distance from the beehives. This simple result provides two possibilities that this plant grows at low attitudes or the bees fly over a distance of more than 8 km. It is a new scientific discovery in any case.

3.3.3 Importance of woody plants as a source for honey

It is typically thought that honeybees mainly use grassland plants. This may be because we usually observe only house flowers near the ground. The honey made by our bees contains a lot of woody plants, and we can also see such examples in previous studies showing that woody plants are more prominent as nectar plants than previously thought [50].

Type	**Monofloral**												**Mixed**	
Labeling name and Country Specific name	Manuka (*Leptos-permum scoparium*)	*Phellode-ndron amurense*	*Fagopyrum esculentum*	*Robinia pseudoacacia*							*Aesculus turbinata*	—	Forest	Alpine plant
	New Zealand	Tokyo, Japan	Tokyo, Japan	India	Romania	Switzerland	Okayama, Japan	Saitama, Japan	Kyoto, Japan	Tokyo, Japan	Okayama, Japan	Mexico	Switzerland	Switzerland
Asteraceae spp.1	34,767	66,624	54,573	1	596	877	3971	273	497	371	7853	38,068	2642	15
Robinia pseudoacacia	3			13,640	63,643	40,622	18,815	18,583	20,915	14,749				49
Quercus sp.	2413		1	8	890	1223	3558	2784	2121	2809	35,956	208	1600	3
Toxicodendron sp.			187	54		9	3753	28,996	9758	2059	1439		2	21
Rosaceae spp.1	840	589		2014	2422	865	4634	3321	1725	2803	28,530		324	1
Actinidia sp.	1560	18,107	387			6	4070	577	28,172	106	467		1	72
Persoonia spp.	27,832													
Wisteria floribunda				15		12	3438	5863	3205	23,550	8569		21	9
Fabaceae spp.3	790		3	2	1	2	301	1759	1499	2	246		2342	4
Prunus sp.1			1	278	3279	1846	4491	15,956	5396	8330	7245		648	3
Lauraceae spp.	1236												14,884	
Aesculus turbinata						2	490		122	645	14,680		27	
Dalbergia sp.				13,117										
Cryptocarya sp.												1	12,754	
Prosopis sp.				10,885								1	57	

Type	Monofloral										Mixed		
Pterospermum heterophyllum											10,169		4
Prunus sp.4			1		3	5	11	20	16	13	18	1	
Rosa sp.	2		1	1396	9395	6972	2786	2013	6905	4859	392	5092	14
Rosaceae spp.2	1082					1		110	460	583	1275	6039	3
Myrtaceae spp.1		2		1107								7856	
Asteraceae spp.2		1						1			7202		
Weinmannia spp.	6997												
Acer sp.				2	2	1	373	890	285	400	6540	22	
Picrasma quassioides				13		8	1971	6440	339		675	1	
Anacardiaceae spp.1				6403									
Fabaceae spp.1	457				6199	4678	688		7			35	
Prunus sp.2				3			461	1174	2483	6184	4733	10	5
Prunus sp.3				3			461	1174	2483	6184	4733	10	5
Populus sp.	3147			5511		89							
Lysiloma sabicu											5237		
Monimiaceae spp.	5039												
Melicytus spp.	4766												
Asteraceae spp.3			1	1468		13	4763	2920	145	183	1818	1310	

Type	Monofloral											Mixed		
Lopezia langmaniae											4747	318		
Argemone mexicana											4092			
Filipendula vulgaris					3850									1
Cornus controversa				11		2	254	3680	549	824	1561			
Kerria japonica								92	63	6	3583		11	
Podocarpus sp.	3430													
Leucaena leucocephala				3387							159			
Asparagus sp.			1676			100	1346		1926					2
Phellodendron amurense			48			4	1549	379	1692	46	614		1	4
Asteraceae spp.4	20	1158	80									1		
Number of detected species	31	11	16	31	21	36	36	38	42	31	30	23	45	25
Presence of labeling species	N.D.	N.D.	N.D.	1st	1st	1st	1st	2nd	2nd	2nd	3rd	—	—	N.D.

Table 1.
A result of eDNA analysis of honey sold at Supermarkets. All honey contained DNA from multiple plant species. Of the 11 samples sold as single flower honey, 4 actually had the highest amount of DNA. Based on data from IDEA Consultants, Inc.

3.3.4 What does the DNA of animal origin in honey indicate?

We found the DNA of the Varroa mite *Varroa destructor* in our honey throughout the seasons. Most of the time during the study period, we could not find the Varroa mite by visual inspection. In other words, the presence of parasites that humans cannot find can be examined by the eDNA analysis.

In general, the amount of DNA in a sample is correlated with the abundance of organisms. Therefore, by monitoring the amount of the DNA of the Varroa mite in honey, we can predict the level of parasite damage in advance and help as early countermeasures.

The presence of the DNA of aphids and scale insects in honey suggests that the honey is honeydew honey. Honeydew honey is made not from nectar, but honeydew refined by parasite insects on plants. It is widely produced as a specialty product in Germany and New Zealand. In Japan, honeydew honey was not detected until 2019. The bees we keep in the forest make dark honey in August when there are few flowers. We assumed that the honey would contain honeydew. Analysis of whole eukaryotes using the eDNA analysis revealed the presence of aphid DNA. This result suggests that honeydew honey is a constituent in our honey (**Table 2**).

3.4 Beekeeping using the eDNA technique can revitalize communities

3.4.1 Urban beekeeping is spreading

In the 2000s, urban beekeeping began to appear in various cities around the world [7, 51, 52]. In many cases, it does not aim to produce honey but is used as a means to revitalize the community and improve the quality of life. For example, beekeeping at the White House and the Paris Opera has a greater effect on appealing to the environmental friendliness of public venues than its value as a place for honey production.

3.4.2 Honeybees as an indicator of urban biodiversity

On the other hand, it may be used as a simple method for monitoring the quality of the urban environment. In general, it is difficult for civilians to measure and monitor the quality of the living environment by professional and scientific methods. However, eDNA analysis can be the "litmus paper" or "canary" to assess the quality of the environment. It is possible to grasp the flora, phenology, and the safety of the surrounding environment by combining with the analysis of substances that affect health such as pesticides. Such activities can be expected to improve citizens' environmental literacy.

In an apartment house in Tokyo, beekeeping began on the green space on the roof. Residents gathered every week to enjoy harvested honey in a rooftop green area that was rarely used before. In urban areas of Japan, the lack of connections between residents has become a major social concern nowadays, but in this house, a sense of cooperation was born, thanks to the opportunities brought about by beekeeping. Residents planted seasonal flowers in the green spaces managed by each house, cooperated to repel hornets, and voluntarily cleaned the communal space. As a result, residents' autonomy and governance improved. It is also said to have increased the real estate value.

Kasumigaseki in Tokyo's Chiyoda Ward is the administrative center of Japan, where central government ministries are concentrated. An NGO has been beekeeping for several years on the library rooftop in Hibiya Park adjacent to the Ministry of the Environment [53]. Here, government officials, corporate employees, and

Kingdom	Phylum	Class	Order	Family	Genus	Scientific name
Animalia	Chordata	Mammalia	Primates	Hominidae	*Homo*	*Homo sapiens*
	Mollusca	Bivalvia	Venerida	Veneridae	*Ruditapes*	*Ruditapes philippinarum*
	Arthropoda	Insecta	Hemiptera	Aphididae		Aphididae spp.
		Arachnida	Acari	Varroidae	*Varroa*	*Varroa destructor*
			Mesostigmata	Phytoseiidae	*Neoseiulus*	*Neoseiulus womersleyi*
			Opiliones	Phalangiidae		Phalangiidae spp.
			Prostigmata	Eriophyidae		Eriophyidae spp.
						Eriophyidae spp.
Fungi	Ascomycota	Dothideomycetes	Dothideales	Dothioraceae	*Aureobasidium*	*Aureobasidium pullulans*
			Capnodiales	Cladosporiaceae	*Cladosporium*	*Cladosporium cladosporioides*
		Eurotiomycetes	Chaetothyriales			Chaetothyriales sp.
		Saccharomycetes	Saccharomycetales	Phaffomycetaceae	*Wickerhamomyces*	*Wickerhamomyces anomalus*
				Saccharomycetaceae	*Debaryomyces*	*Debaryomyces nepalensis*
					Saccharomycetales	*Saccharomycetales* sp.
					Zygosaccharomyces	*Zygosaccharomyces rouxii*
				Incertae sedis	*Kodamaea*	*Kodamaea ohmeri*
					Starmerella	*Starmerella bombicola*
	Basidiomycota	Basidiomycota	Microbotryomycetes		*Curvibasidium*	*Curvibasidium pallidicorallinum*
	Incertae sedis		Mucorales			Mucorales spp.
Apicomplexa		Conoidasida	Neogregarinorida	Lipotrophidae	*Apicystis*	*Apicystis bombi*

Table 2.
Eukaryotic DNA contained in honey. A list of eukaryotes other than honeybee in DNA. These data are derived from honey that we produce ourselves. There were several types of mites, such as Varroa, aphids, and multiple molds. Clam species, does not live near our apiary, was also detected. Based on data from IDEA Consultants, Inc.

local residents cooperate to improve environmental literacy through beekeeping. They conducted eDNA analysis as one of the methods to assess the biodiversity of the city.

The analysis showed that urban areas also have diverse sources of honey, and some bees flew to the imperial palace, where public access is prohibited. These characteristic results have enough momentum to stimulate the curiosity of the participating citizens and enhance future activities.

Acknowledgements

This work was supported by IDEA Consultants, Inc. I am grateful to Tomoyasu Shirako and Shigeru Tabata. This work was funded by the JSPS KAKENHI Grant Numbers JP20K12398.

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Chapter 6

Beekeeping: Sustainable Livelihoods and Agriculture Production in Nepal

Kedar Devkota

Abstract

Nepal has tremendous opportunities on the beekeeping due to the richness in the honeybee's species and the availability of plenty pasture diversity. There exist four native honeybee's species *Apis florea, Apis dorsata, and Apis laboriosa* are open nesting and *Apis cerena* halfway domesticated types. The beekeeping practices and production of the honey have been increased during the 10 years. Along with this, the natural honey export was also increased in recent years. The beekeeping in Nepal contributes to the economics boost up of the rural and marginalized landless farmers. Besides the economic contribution from the bees' products, beekeeping enhances the pollination services assuring the crop yields and helping to maintain the natural biodiversity from the Terai to the high Himalayans. Beekeeping gives the mutual benefits to both beekeeper and the crop farmers on the economic returns from the selling of the bee products and beehives and also increases the yields of the pollination-dependent crops by ensuring the efficient pollination services. These perspectives of beekeeping enhance the livelihoods of the farmers through the sustainable practices of beekeeping.

Keywords: beekeeping, diversity, economic benefit, livelihoods, pollination

1. Introduction

Beekeeping has been in practice from an ancient time in Nepal. It is one of the potential sectors to generate the employment and increase the income for the people in Nepal. Beekeeping is landless and marginalized based farming provides the economic, nutritional, and ecological benefits.

Beekeeping in the Nepal carried the tremendous potentiality due to the distribution of high diversified bee flora [1–3] and suitable climatic condition for honeybee diversity [4]. Although Apiculture contributes a very small fraction (less than 1%) to Agricultural Gross Domestic Product (AGDP), beekeeping has been considered as a high value income-generating agriculture activity in Agricultural perspective plan (APP), and it has also been mentioned in the tenth plan. The topographical, climatic, and floral varieties spell heaven for beekeeping in Nepal [5]. Five of the world's seven species of honeybee *Apis laboriosa* S., *Apis dorsata* F., *Apis florae* F., and *Apis cerana* F., and one exotic honeybee *Apis mellifera* L. are found from the plain to the high Himalayan in Nepal. The traditional log hives as well as modern beehives were in practices to keep the bees. Especially, the rural farmers from the

Himalayan regions kept the *A. cerena* in the traditional wooden log hives whereas the urban farmers from the lowland Terai kept both the *A. cerena* and *A. mellifera* on the modern beehives. The farmers in Nepal kept the bees to meet the demand of honey in the local, national, and international markets and also for the pollination in some crops like Oilseed crops, Buckwheat, and fruit crops to increase the yield. The bee species plays crucial role in the conservation of biodiversity by pollinating wild flowers in the entire region, and the species for ecotourism development and income generation in the poor, rural, and landless people in Nepal [6]. It helps to enhance agricultural productivity and conserves biological diversity and ecosystem through ensured pollination services [7]. Despite the huge benefits of beekeeping both in the term of economic and ecological aspects, the quantity and quality of honey production over a period of time was satisfactory may be due to the insufficient management of practices and lack of the training [8]. Beekeeping is very important to increase the productivity of the crops and increase the income of the farmers in the Nepal. In this context, the objectives of the study were to figure out the beekeeping situation by exploring the data on the number of hives, honey production and export situation through the electronic sources and the authorized government organizations.

2. Material and methods

This the theoretical work based on the secondary data and literature available about the beekeeping in Nepal. The secondary data on the beehives number and honey production were taken from the Centre for Industrial Entomology Development (CIED), Ministry of Agriculture and Livestock Development, Government of Nepal and the data on the export of honey were taken from the Trade and Export Promotion Center (TEPC), Ministry of Industry, Commerce and Supplies, Government of Nepal. The data were gathered and coded in the MS-Excel. The trend analysis on the different years, number of hives, production and the export situation was carried out using the ggplot in R program [9].

3. Results and discussion

3.1 History of beekeeping in Nepal

Beekeeping is a cultural heritage in Nepalese community, practiced from an ancient time as honey hunting has been dated back to thousands of years [10]. It is reported that little honeybee (*Apis florea*), rock bee (*Apis dorsata)*, Asian bee (*Apis cerana),* and largest honeybee *(Apis laboriosa)* were native honeybees found in Nepal [11]. The exotic honeybee, European bee *(Apis mellifera)* was introduced in Nepal in 1994. Although, the scientific beekeeping in Nepal was initiated in 1989 with the introduction of moveable comb hive of native bee *Apis cerena*, however, commercialization of modern beekeeping geared up with the introduction of high yielding exotic honeybee *Apis mellifera* [12].

In 1980, Beekeeping Development Section (BDS) was formed for the development and extension of the apiculture under Nepal Agricultural Research Council (NARC) with mandated to conduct research on various aspects of applied entomology including industrial entomology [7]. Until 1990, *A. cerena* was the only one managed honeybee and was flourished throughout the country. Then after, *A. mellifera* was imported in large scale replaced the native bee *A. cerena* from the Terai region upto the mid-hills. *A. cerena* now remains with the farmers from the

hilly and mountain regions areas like Dhading, Humla, Jumla, Jajarkot, Kaski, Lamjung, Lalitpur, etc. *A. mellifera* has not arrived in these areas due to road and transportation inaccessibility, and also difficulties in the management practices such as keeping the colonies warm, feeding sugar, and migrating to low hill areas in winter season [1].

3.2 Statistics of beekeeping

Honeybees in Nepal are characterized through greatly variations based on the altitude and topography. More than 50,000 Nepalese households are involved in beekeeping, rearing 125,000 beehives and producing about 1100 t of honey per year [13], in which, 29.86% of honey produced from *A. cerana*, 39.19% from wild honeybees and 33.93% from *A. mellifera*. The honey produced from the *A. cerena* (rear in the traditional wooden loghives), *A. dorsata*, and *A. laboriosa* can be considered as organic, since the bees forages on the natural forests of remote areas in Nepal, where usage of pesticides and agrochemicals are considerably zero. There is rich tradition of beekeeping in different villages of Nepal, which is associated with genetic diversity of *A. cerana*, availability of bee forage plants, and a wealth of indigenous knowledge associated with wild honeybee harvesting. Although, Nepalese people have been rearing honeybees for many years, the scientific and commercial approach to beekeeping has been in practice fairly a decade ago. Therefore, modern commercial beekeeping is still in nascent stage.

In scenario of beekeeping industry, in Nepal, the honeybee industry includes 5700 registered beekeepers operating 55,000 hives. A hive is home to 25,000–70,000 bees, depending on the species [14]. The number of beehives, including those from non-commercial keepers was 280,000 in the 2017/2018 fiscal year, twice as many as 10 years ago, 2009; those hives produced 5500 tonnes of honey, more than six times the yield from 10 years ago [15].

From the data [14], the number of the beehives is increasing from 140,000 in 2009 to 280,000 in 2018. Similarly, as the number of hives increases, the honey production was also increasing during the 10-years period, which is shown in **Figure 1**.

The number of the beehives increased slowly from the year 2009 to 2013. After 2013, the number of beehives increased in higher rate as compared to the before. Likewise, the amount of the honey production was declined sharply in the year 2011, then after it starts to increase. The increase in the honey production as compared to the number of beehives was not satisfactory in the last 2–3 years.

3.3 Market scenario of honey

Honey is one of the important nutritive food produce of the bee containing various kinds of sugar, protein, free amino acids, minerals, trace elements, enzymes, and vitamins with a fairly high caloric value [16]. The production of the other products like wax, pollen, and royal jelly is not in practice in Nepal. Nepal is the only country in the world, where honey is produced between the ranges of 70 and 4200 m above the sea level. Honey produces in the Nepal are of multi floral and unifloral origin with a high medicinal value. The honey produced are of chiuri (Indian butter tree), mustard, buckwheat, rudilo (*Pogostomone spp*), sunflower, and fruits. The *A. mellifera* honey is produced in the Terai region, while *A. cerana* is very common in the hilly and mountain regions of Nepal. About 70% of the honey produced in Nepal comes from wild flora, which is by definition organic.

In Nepal, honey is also classified according to bee species, harvesting season, and geographical location. The average annual honey productivity of *A. dorsata*,

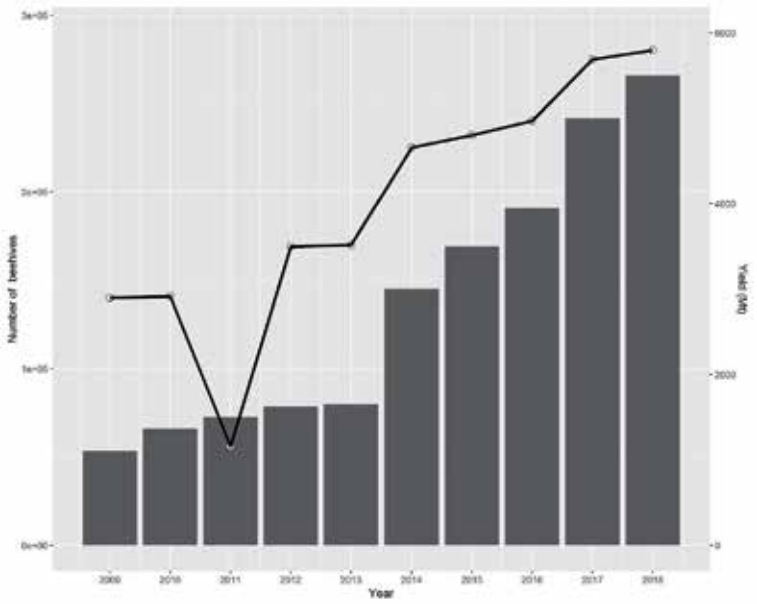

Figure 1.
Scenario of the number of beehives and the production of honey (mt) from 2009 to 2018.

A. cerana, A. florae, *A. laboriosa*, and *A. mellifera* L. is 5–5, 8–15, 1–5, 20–100, and 20–50 kg of honey per hive per year, respectively [17].

In recent years, the consumption of honey in Nepal has increased, particularly in the major cities and urban areas. The honey production depends on the availability of floral resources but it is presumed that approximately 1000–1500 metric tons per annum would be produced. However, around 50% of the honey is sold out in the national and international market, whereas the rest are consumed at village or district level [10]. The total annual domestic demand for honey is estimated to be about 300–350 tons. It is estimated that if honey consumption increased by 100 g per capita, then total demand for honey in the domestic market would be about 2800 tons per year [17].

The Nepalese honey market was extended to the India, UAE, Japan, South Korea, Thailand, USA, and Bangladesh. The imported honey is processed and provided to the other parts of the world [18]. According to [19], industrialized countries such as China and Argentina produce the honey at low unit cost, and export to the world market. However, Nepali beekeepers are unlikely to produce honey at prices that can compete with these major producing countries even though they are of varied resources. The price and volume of Nepali honey is not competitive compared to honey from its neighboring countries and supply of honey is also not consistent. This makes Nepali honey noncompetitive for mass market.

It is estimated that in the honey sector, an important proportion of the exports is happening via informal channels not reflected in official statistics. The key export markets for Nepalese honey are China, Malaysia, India, Japan, European countries, and the USA. Nepal exported 378 tons of processed and unprocessed honey in the fiscal year 2016/17, a majority of which was exported to China and India that accounted for a value of over Rs. 67 million [20]. The trends of the production and exports of the natural honey were shown in **Figure 2**.

This figure showed that the export of the natural honey was lowest in the year 2012–2014. Then after, it increased sharply in the year 2016. In the year 2017, it showed decreasing trend of export. The production of trends is increasing since

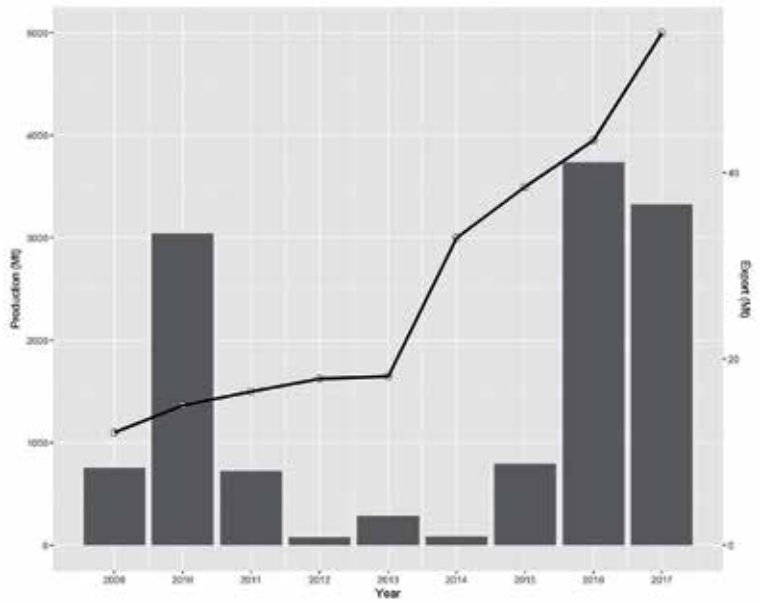

Figure 2.
Situation of the production and export of honey from 2009 to 2017.

the beginning of 2009, which shows the higher potentiality of the honey export from the Nepal.

3.4 Source of livelihood

The average landholding of small-scale farmers in Nepal is just 0.03 ha and many have less than 6 months of food security. Food security is not possible without income security; honey production through beekeeping could be a useful avenue for improving economy. Poor, marginal, and even landless farmers can benefit from beekeeping to support their livelihoods as it can be started even with limited resources giving income and supplying nutrition to them [21]. Beekeeping provides hundreds of families the chance to earn enough to provide for themselves, lifting them out of poverty for good. Honey sector is considered as one of the income-generating activities for resource poor farmers including women, youth, and underemployed sections of the community. Beekeeping is increasingly recognized as a market for numerous employment opportunities in the rural and urban areas of the Nepal. Beekeeping is also recognized as the gender-inclusive activity, where women also participate in the honey harvesting and collecting process. Over 500,000 farmers are directly or indirectly involved in beekeeping [22].

In the 15 years period of beekeeping history, the average price of honey from the *A. mellifera* has risen from about Rs 65 per kg to Rs 500 and for the honey from *A. cerena* risen to Rs 1200 per kg [23]. Beekeeping is critical for local development as it typically requires minimal investment, generates diverse products, can occur without land ownership or rent, and provide flexibility in timing and locations of activities.

Beekeeping could be the important agribusiness options to the landless and small-holder farmers in order to sustain their livelihood. Beekeeping being as a profitable business earning good income makes more people to engage for their sustainable livelihoods [24]. Beekeeping is attractive business and high proportion of the annual income is secured from beekeeping activities for many farmers in

the world. It helps in diversification of source of incomes for rural communities that help minimize the demands of land and pressure on forests. It requires little capital input, so it does not compete with other aspects of the farm system for the scarce resource [19]. Beekeeping has gained much attention as a means of raising the productivity of farm systems in the developing world. Beekeeping can also form the basis for gaining and transmitting knowledge about ecological processes [25]. Beekeeping can contribute to the pollination services, assuring crop yields, and can also be used to strengthen the livelihoods through commercialization to increase economic revenue. Beekeeping contributes to the provision of pollination services, assuring crop yields, and helping maintaining plant biodiversity in natural ecosystems. Honeybee pollination has been reported to increase seed production in oilseed, rapeseed, and sunflower seed, as well as the oil content in the seed, and beekeeping activity provides benefits in terms of employment, pollination of crops, and conservation of biodiversity [26].

3.5 Beekeeping for the crop production

The ecological importance of bees in crop pollination and the preservation of the biodiversity of both flora and fauna are unquestionable. Pollination is a valuable ecosystem service for the production of fruits, vegetables, nuts, cotton, and oilseed crops among many other agricultural crops [27].

Bees and other pollinators make important contributions to agriculture. Generally, the insect pollination contributes 35% of global yield of the agricultural production of 87 of the leading food crops worldwide [28]. The pollination-dependent crops seem to be five times more valuable than those that do not need pollination [29]. Honeybees are the single most important insect pollinator species for the diverse crop yield, both quantitatively and qualitatively [30].

The loss of insect pollinators has greater potential consequences on human food production directly through reduced crop yields. In these contexts of pollinator's declines, beekeeping contributes to the provision of pollination services, assuring crop yields and helping maintaining plant biodiversity in natural ecosystems [27].

Honeybees are the most efficient pollinators for the self-incompatible and cross-pollinated crops, which ensure the pollination services by maintaining the abundance of the pollinators during the flowering period [31]. The self-pollinated crops may also produce higher yields with good quality seeds showing their hybrid vigor without any alteration in the innate properties of fruits and seeds [7]. As managed insects, honeybee colonies are less vulnerable to several pressures affecting wild pollinators [32], thus can provide alternative insurance in case of wild pollinator losses, and effective service provision where wild pollinator populations are sub-optimal. The value from the crop pollination by the honeybees is much higher than the value of all the hive products.

Both domesticated honeybees species *Apis cerana* and *Apis mellifera* are being utilized for pollinating fruits, vegetables, oilseeds, and cereals crops. Pollination by honeybees increases fruit set, enhances fruit quality, and reduces fruit drop in apple, peach, plum, citrus, kiwi, and strawberry. Reports have also indicated an increase in fruit juice and sugar content in citrus fruits. The beekeeping has higher advantages in the crop production; however, integration of managed crop pollination as a component of agricultural development strategies is missing.

3.6 Future prospects and potential of beekeeping

Beekeeping and honey production in Nepal is still under development stage. Due to the climatic suitability and being a more profitable business, many farmers from

different regions start beekeeping with a small-scale investment to attain the sustainable. Along with the honey, bee products like bee wax, pollen, royal jelly, and queen bee production could also fetch the income for the farmers, which motivated them toward the beekeeping. It is estimated that the floral resources in the country can support over 1 million of beehives with production potential of 10,000 tons of honey annually [33]. The Nepalese honey demand is ever increasing in the international market. Due to this, the Nepal government has prioritized the honey and bee product as high value product [34]. Various ecotypes of indigenous honeybees have enriched Nepal with the excellent potentials for exploiting them for the production of various variable beehive products. There is tremendous scope to produce royal jelly, bee venom, and bee pollen in commercial scale in Nepal and export to foreign countries.

The honey along with other hive products such as propolis, royal jelly, and bee venom are poorly known to Nepalese beekeepers. Therefore, along with low-cost honey production technology with proper beekeeping management, disease, and pest control, research on other hive products, and their harvesting, and processing techniques are also needed [35].

There is an increasing demand for the Nepalese honey in international markets specially based on the honeybee species floral sources thereby indicating a huge potential for beekeeping in Nepal. Beekeeping can be started with very low investment, so that, even the poorest person can go for it with very little support. Thus, the beekeeping enterprise is very useful medium for the alleviation of poverty in Nepal.

A study conducted in Chitwan district [26] showed that beekeeping practices can improve the farmers' livelihood and also have great importance on the pollination, which helps to boost up the agricultural production in the country. The available resources in the country are favorable for production of honey, beeswax, and other bee products [10]. The woodland and natural vegetation managed in the national park and community forests are also providing the suitable conditions for the development of beekeeping. There is strong potential for the production of organic honey as chemical pesticides use is very low. The presence of the wild honeybees hanging in the high mountain and the domesticated honeybees in the wooden log hives managed by existing indigenous knowledge in beekeeping and honey hunting ensures potential for developing bee watch eco-tourism.

4. Conclusion

The varied diversity of ecological zones in Nepal, from the plains to the mountains favors the beekeeping. The beekeeping has been increased during a decade and the competitive market opportunities exist in the honey and honey products from the Nepal developed the domestic and international markets and reaching growing consumer not only in Nepal, but also large adjacent markets in India and China fetching good prices. The price of the honey was increased around 10 times during the 15 years period of beekeeping. Thus, beekeeping helps to earn the income and provides the self-employment opportunities to the poor and rural people, helping in the poverty eradication. Along this, bees are the major pollinator, which increases the crop yield that helps to attain the agricultural sustainable goals and creates the ecological balances by conserving the biodiversity. Beekeeping sectors show the high-growth potential of small enterprises with a holistic service offering, seeking to enable product, process, and business model innovation, thereby accelerating their growth and job creation and providing the pollination services increasing the crop yields. We can conclude that a new perspective relationship between

beekeeping and crop pollination, emphasizing that pollinator deficit can be mitigated through beekeeping, which enhances the livelihoods of farmers through greater crop yields and economic benefit received by selling bee products.

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Chapter 7

American Foulbrood and the Risk in the Use of Antibiotics as a Treatment

Enrique Mejias

Abstract

Honeybees (*Apis mellifera*) crucially pollinate agricultural crops and endemic species, in addition to producing various apiculture products. The most economically relevant and abundant beehive product is honey, a sweet substance made from the secretions of melliferous plants. Honey is a natural food rich in nutrients, including certain bioactive compounds inherited from floral nectar and pollen. Among the most dangerous diseases for bees is American foulbrood. Spores of the causative microorganism, *Paenibacillus larvae,* can contaminate larvae food or the operculum wax in which larval stages of honeybees are kept. Infection is further promoted by common apiculture practices, such as reusing inert material contaminated with spores, even after months of storage. American foulbrood is untreatable, and management implicates completely incinerating the infected hive and all material that could have come into contact with pathogenic spores. The purpose of such drastic measures is to decrease propagation risk for other beehives. While evidence indicates that antibiotics could effectively control and combat this disease; antibiotic use is prohibited in most honey-producing countries due to increased risks to microbial resistance. Antibiotic residues in honey can affect consumer health, since the natural biological attributes of honey can be altered.

Keywords: American foulbrood, *Paenibacillus larvae*, *honey*, beehive products, antibiotics residues

1. Introduction

Honeybees (*Apis mellifera*) and wild bees possess a number of morphological traits that facilitate pollen transport, making bees efficient pollinizers and ensuring the preservation and diversity of agricultural crops and native plant species [1]. However, the continued existence of bees and, by extension, pollinating and honey-producing activities are under threat by a range of hostile conditions. Pesticide exposure [2, 3] is one such condition, but the acute effects of climate change are among the primary drivers for decreases in and the weakening of beehive populations.

Climate-change phenomena have strongly impacted the viability of ecosystems [4]. Prolonged droughts and high temperatures due to intense heat waves have become, in recent years, determining factors in weakened [5] and decreased [6] beehive populations across Mediterranean climates, including western Australia,

southeastern Africa, central Chile, California, and the Mediterranean basin of Europe. The combination of harsh temperatures and shortened flowering periods, as associated with insufficient water, can result in reductions to fat reserves and overall body mass in bees. This status can translate into fewer pollinating and honey-producing activities [7], as well as an increased incidence of specific diseases affecting honey bees weakened by nutritional deficits [8, 9].

Addressing the aforementioned threats to sustainably preserve the apiculture industry requires compliance with strict international regulations and norms to ensure the quality and safety of export products. Sufficiently monitoring residues in honey and adequately controlling diseases affecting bee health and production are constant preoccupations for apiculturists and exporters worldwide. In this chapter, there will be listed the main issues related to the uses of antibiotics as effective treatment against *Paenibacillus larvae* and the risk of potential resistance effects over health of consumers of bee products focused mainly on honey. Also, the development of alternative strategies to control this disease is discussed briefly.

2. Honey and other apiculture products

Besides fulfilling the critical role of pollinizing agricultural crops, *A. mellifera* are responsible for a number of economically valuable products, including propolis, royal jelly, bee venom, beeswax, bee pollen, and honey. Pollination and these apiculture products are made possible by adaptations that facilitate the collection and transport of pollen grains to the beehive. Behaviorally, bees improve pollen-transport efficiency by wetting pollen grains with nectar, thus creating a cohesive surface that increases the amount of pollen that can be carried during flight. Due to its anatomical skills, the characteristic buzz of bees facilitates the collection of pollen grains located on floral structures [10]. Brushes of hairs present on bee legs further favor pollen collection, specifically through the formation of a special cavity known corbiculum, on the hind pair of legs. Plant pollens are first conglomerated in the corbicular structures, but once inside the beehive, flightless bees are able to move and fragment the collected material into a honeycomb cell, where it is further broken up into a powder and accumulated against the interior of the honeycomb cell [11]. This collection and conglomeration process results in bee pollen, the water content of which is between 4 and 10%. These levels ensure good preservation over time (i.e. organic components do not degrade or decompose), thus guaranteeing that the preferably polar nutritional contents of bee pollen are chemically unaltered [12].

In addition to pollen collection, young melliferous bees secrete a liquid from wax glands that, when exposed to air, hardens and forms small flakes that collect on the underside of the bee. This economically valuable natural substance is known as beeswax and is used by bees to construct hexagonal alveoli into honeycombs. The rigid structure of honeycomb cells serves to conserve honey and pollen. Likewise, alveoli serve as a place for the queen bee to deposit eggs and for larvae or pupae to develop [13]. Beeswax contains carbohydrates (present in pollen and nectar) that have transformed into fats due to the presence of enzymes and enzyme precursors secreted by bees. More specifically, beeswax is constituted by water and minerals (1–2%), mono-esters and hydroxyl mono-esters, complex wax esthers, hydrocarbons, and free wax acids [14].

Despite the importance of bee pollen and beeswax, honey is the primary apiculture product. The global honey trade is valued at 2.4 billion dollars annually and involves the movement of approximately 630 thousand tons of honey. Chile accounted for 0.6% of total exports in 2017 and is ranked 30th among export countries. In 2017, the main import markets of honey were the United States and Germany.

Honey has been described as a naturally sweet mixture produced by *A. mellifera* bees from the nectar of flowers and the secretions of melliferous plants. These components are mixed with bee-produced substances and are deposited, dehydrated, and stored in honeycomb cells until later use [15]. The composition of this natural food includes sugars, mostly glucose and fructose, the ratio of which determines the degree of granulation for a honey [16]. Disaccharide and maltose sugars are also present [17]. Components found in lesser quantities include organic acids, amino acids, proteins, enzymes, minerals, lipids, vitamins [18, 19], and hydroxymethylfurfural, which is used as an indicator of freshness [20, 21].

Status as a natural functional food means that honey is the best-characterized apiculture product. Bees selectively use floral resources available in proximity to beehives [22–24]. This is important to consider as the traits of apiculture products, including honey and bee pollen, are inherited through secondary plant metabolites transferred in nectar [25]. Consequently, the attributes of melliferous species are directly related to the biological properties of resulting honeys [26]. Notable among the biologically active components of honey are phenolic compounds [27] and flavonoids [28, 29]. Phenolic compounds and flavonoids have antioxidant capacities, acting through routes complementary to enzymatic antioxidants identified in honeys, such as glucose oxidase and catalase [30–32]. Antibiotic activity, also as related to phenolic acids and flavonoids, has been reported in some honeys globally [33–36].

In addition to affecting biological properties, plant origin also directly influences the market value of honey. Quantitative and qualitative melissopalynological analyses can be used to classify honeys as monofloral, bifloral, or polyfloral. The highest demand is for monofloral honeys, which are primarily constituted (>45%) by pollen grains of the same melliferous species. Therefore, honey quality depends on the presence and concentration of specific chemical compounds and on the botanical origin of said compounds [37].

The elaboration of the aforementioned apiculture products can, under certain conditions, concurrently occur with the production of live material. More specifically, rearing queen bees and colonies are diversification options for national apiculturists [38]. There is a demand for bee packages, nucleus colonies, and, particularly, queen bees in countries such as Canada, France, Mexico, and Italy. This point has driven industry growth in Chile, which, over the last 3 years, has doubled in size, going from more than 10,000 exported queen bees in 2015 to more than 20,000 in 2017 [39].

3. American foulbrood

There are two groups of diseases that can affect beehives—exotic and endemic diseases. Exotic diseases include parasites such as the small hive beetle (*Aethina tumida*) and Tropilaelaps mites (*Tropilaelaps clareae*). Endemic diseases include pathologies that more frequently affect bees, such as nosemosis (caused by *Nosema apis*), varroosis (caused by *Varroa destructor*), and acarapisosis (caused by *Acarapis woodi*). This same group of diseases also includes two Gram-positive microorganisms that cause American (*Paenibacillus larvae*) and European (*Melissococcus plutonius*) foulbrood [40].

The first report of American foulbrood in Chile was in 2001, whereas the first case of European foulbrood was in 2009. According to protocols for the management of apiculture diseases issued by the Chilean Ministry of Agriculture, both foulbrood diseases are classified as endemic and with low prevalence in the country. Nevertheless, the management of European foulbrood is less complex and involves

less drastic sanitary measures than American foulbrood. Indeed, the incidence of European-foulbrood outbreaks has consistently declined since initial detection, with only one incident reported in 2016.

By contrast, American foulbrood is difficult to manage and eradicate. This pathogen has been detected in most regions of Chile, but the number of reported cases has varied since 2005. Notwithstanding, a worrying 44 outbreaks were reported in 2018, and an additional 61 outbreaks have been reported as of June 2019. Most cases have been reported in the Atacama, O'Higgins, and Maule Regions of Chile [41]. Given that antibiotic treatment of this disease is prohibited [42] and that sanitary control measures include the incineration of all live material, it is believed that American foulbrood outbreaks are underreported in Chile out of fear for the total loss of infected beehives. In this way, according to the World Organization for Animal Health (OIE), there are cases reported in the first half of 2019 in Europe (with declared infection in Finland), South Africa, North America, South America and Australia. Despite those data, many countries have no information available for knowing the real state of this disease around the world as it shows **Figure 1**.

The infectious pathway of *P. larvae* is through spores that can survive in the environment for many years, contaminating beehive materials and apiculture products. These spores are particularly resistant to heat and a number of chemical compounds. Once bee larvae have ingested food contaminated with spores, the bacteria, in a vegetative state, proliferate without damaging the stomach lining of the larva. During this infectious stage, bacteria obtain nutrients from food ingested by the larva [43]. American foulbrood affects larvae in any of the three honey-bee castes. The most susceptible, however, are immunosuppressed bees due to exposure to environmental contaminants (e.g. pesticides, metals) or that have suffered any of the aforementioned diseases. During outbreaks, *P. larvae* spores can be found in the honey and beeswax, and pillaging from sick hives, the use of contaminated beekeeping materials, and poor beehive management, among other factors, can contribute to the spread of disease [44].

Bee colonies present a coexistence mechanism with *P. larvae.* This host-etiological agent relationship has existed for more than 2400 years and is a highly specific infection, with germination possible only in bee larvae aged 1 or 2 days [45].

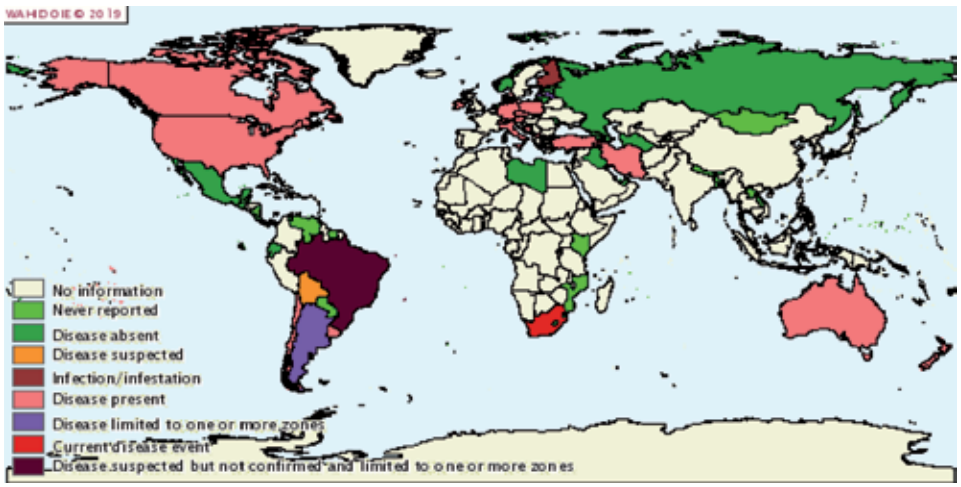

Figure 1.
Dynamic maps showing the presence or absence of American foulbrood at the national and sub-national levels. Information based on 6-monthly reports (first half of 2019) according to the data base taken from World Organization for Animal Health (OIE).

Nevertheless, this microorganism has at least four distinct genotypes (ERIC I-IV) that modulate infection with different degrees of pathogenicity. The ERIC I and ERIC II genotypes were found to be the most aggressive through repetitive-element PCR analyses performed with primers amplifying enterobacterial repetitive intergenic consensus elements [46]. Therefore, American foulbrood infection can, in some cases, mean a total loss of colony larvae. In other cases, hives can survive with the spores and, even, never show visible clinical symptoms [47]. Inadequate management by beekeepers can result in a disease outbreak, specifically by unbalancing the internal equilibrium of the beehive and provoking a violent increase in the load of spores within larvae nests [48].

The symptoms and effects of American foulbrood manifest slowly in beehives and occur while larvae receive contaminated food. In this stage, disease is not visible, but the first signs include the presence of dark, sunken, and greasy cappings that may be perforated by bees removing brood already in the process of putrefaction [49]. Finally, hive death occurs due to the lack of new, live brood and the aging and death of adult bees. The weakened hive then become an easy target for pillaging by bees from stronger hives seeking food reserves. Such pillaging serves to propagate the disease in nearby beehives and, consequently, the entire apiary [43, 50].

3.1 Control strategies

3.1.1 Antibiotic treatments and the analytical methods for detecting residues in honey

The need to control American foulbrood is principally driven by damage caused by infection, which can include the loss of beehives and compromised honey and queen-bee exports. The use of tetracycline prophylactics is widespread in large animals and is allowed for bees in some honey-producing countries. In most countries, however, *P. larvae* expansion is controlled through the total incineration of hives with active infections [51]. The application and uses of veterinary antibiotics have been restricted primarily due to the appearance of antibiotic-resistant *P. larvae* strains. Such resistance could partially be due to the frequent application of veterinary drugs to prevent and control potential infestations, even in the absence of disease diagnosis [52]. In addition to antibiotic-resistance in *P. larvae,* the presence of antibiotics represents a health risk for consumers of contaminated honeys.

Where antibiotic use is allowed, maximum residual limits range between 10 and 50 ppb. These limits are intended to minimize the presence of antibiotic compounds in end-products, such as honey [53]. Antibiotics can, undoubtedly, affect the properties, quality, and, finally, export price of honey. Additionally, some purchasing countries regulate against the presence of antibiotics in beehives, thus impacting beekeepers that export honey [54–56]. This is a particularly relevant point for Chilean beekeepers as the primary export market is Europe, which has zero tolerance for antibiotics in imported honey (**Table 1**) [42]. These strict regulations require the determination of each compound in honey through highly sensitive analytical methods.

Several studies have aimed to develop reliable methods for detecting and quantifying the presence of antibiotics in complex organic matrixes, such as honey. Despite the ban of antibiotics in beekeeping, these substances have been detected in various European honey samples [57]. Liquid chromatography with UV–Vis detection resulted in the isolation of tetracycline, oxytetracycline, chlortetracycline, doxycycline, minocycline, and methacycline in different fortified honey samples

Antibiotic	Maximum residual limit
Oxytetracycline	Forbidden
Tylosin	Forbidden
Lincomycin	Forbidden
Streptomycin	Forbidden
Sulfonamides	Forbidden
Chloramphenicol	Forbidden
Nitrofurans	Forbidden

Table 1.
Maximum residual limits for antibiotics in the European Union.

cleaned by solid-phase extraction [58]. A more recent methodology with good results is QuEChERS solid-phase extraction followed by liquid chromatography tandem mass spectrometry [59].

Antibiotic resistance against tetracyclines by American and European foulbrood strains has led to research of other antibiotics. Sulfonamides have been widely used, but specific methods of determining and detecting these compounds in honey are needed since toxic collateral effects in association with allergies have been observed in humans [60]. To this end, high performance liquid chromatography paired with time-of-flight mass spectrophotometry has detected trace amounts of these compounds through direct injection [61].

Tylosin, a macrolide antibiotic active against many Gram-positive bacteria, has been increasingly used instead of tetracyclines and sulfonamides in beekeeping. Nevertheless, American foulbrood also presents resistance against macrolides. The best methodology for detecting macrolides in honey samples is solid-phase extraction followed by liquid chromatography tandem mass spectrophotometry [62]. Another type of antibiotic used against American and European foulbrood is streptomycin. This aminoglycoside can potentially control foulbrood disease in beehives. Traditional methods of detection include high-performance liquid chromatography with different strategies of solid-phase extraction [63, 64]. The adverse effects to consumers of honeys contaminated by streptomycin include acute otitis and allergic dermatitis [65].

Finally, a number of antibiotics have been fully banned in the control of American foulbrood due to adverse effects to human health. For example, nitrofurans are associated with possible carcinogenic effects while chloramphenicol can cause aplastic anemia, in addition to evidencing possible carcinogenic risks [59, 66].

3.1.2 Nuclear irradiation

One reliable and traceable treatment for efficiently eliminating the highly resistant *P. larvae* spores is the gamma irradiation (15 kGy) of structural components in beehives [67]. Effective treatment would reduce the significant economic losses caused by the destruction of all material contaminated with *P. larvae.* An important advantage of this methodology is that the same procedure can be used to control various diseases at once; i.e., fungal, viral, and bacterial diseases affecting bees can be effectively eliminated through gamma irradiation [68]. Nevertheless, the use of gamma irradiation to control apiculture diseases is restricted only to the elimination of spores in honey, beeswax, and inert material in the hive. Irradiation cannot be used on live individuals within the hive due to previously reported adverse effects [69].

3.1.3 Antimicrobial peptides

An alternative strategy for controlling and combating *P. larvae* has been through peptides that an act as natural antibiotics against this microorganism. Some peptides evidencing infection resistance have already been isolated from adult melliferous bees [70]. More recent studies have established which peptides with antibiotic activity originate from symbiont bacteria present in bees, such as lactic acid bacteria and *Brevibacillus laterosporus* [71, 72].

4. Conclusions

American foulbrood has been present since the beginning of beekeeping and has evolved over time. Nevertheless, the apiculture industry today faces a complex situation. The effects of climate change have modified the availability of nutrients and food for bees, ultimately weakening hive health. Food availability for bees has been further decreased by the use of agrochemicals and the occurrence of extensive, devastating forest fires. These situations have provoked a resurgence of American foulbrood outbreaks, which need to be controlled to mitigate population and economic losses. Researchers specializing in apiculture should focus efforts on the search for new, environmentally friendly control strategies against this disease. Such efforts will help prevent the use of antibiotics, which in addition to inducing *P. larvae* resistance can lead to adverse effects in individuals who consume honeys contaminated by veterinary-use drugs.

Acknowledgements

Funding by CONICYT—PAI/Inserción sector productivo, 1era conv. 2019, Grant number I7819010001.

Conflict of interest

The author declares no conflict of interest.

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Chapter 8

Kikuji Yamaguchi Principles of Natural Beekeeping: A Novel Bio-Method of Natural Beekeeping for High Quality Royal Jelly Production

Kikuji Yamaguchi

Abstract

Many serious problems, such as artificial control and overworking of honey bee colonies, deterioration of bee products due to incorrect treatment and inadequate environments, have been postulated in recent beekeeping which should be resolved for sustainable development of modern highly profitable beekeeping in the future. Thus, a novel beekeeping method, Kikuji Yamaguchi Method of Natural Beekeeping (KYAMENABEE), was established for the natural royal jelly (RJ) preparation and the biological and pharmacological properties were examined for evaluation of the authenticity of royal jelly products. RJ samples prepared by KYAMENABEE and ordinal beekeeping were subjected to the quantitative analyses of 10HDA and MRJP1 multimer, identification of functional substance based on the effective growth and development of queen bees, stability of the functional substance, proliferative activity of human and animal cells. The content of 10HDA and MRJP1 multimer in RJ prepared by KYAMENABEE were significantly higher than that prepared by ordinal beekeeping. The biological and pharmacological activities were also superior for RJ prepared by KYAMENABEE than that by ordinal beekeeping. Thus, it might be important to use a novel beekeeping method, KYAMENABEE, in order to produce high quality RJ for sustainable development of biopharmaceutical beekeeping.

Keywords: natural beekeeping, novel and improved method, high quality Royal Jelly, Kikuji Yamaguchi method of natural beekeeping (KYAMENABEE)

1. Introduction

My practice of beekeeping and study of apidology extending over 54 years starting when my father was close to death due to end-stage liver cancer and cancerous hepatocirrhosis.

On the early morning of 1 day in mid-April 1965, my father vomited a large amount of blood exceeding 2 L because of esophageal variceal rupture. Immediately he was transported to a hospital by ambulance. He also showed strong jaundice

symptoms and fell into a coma. The physician in charge said that he would have 3 months or less to live.

Under these circumstances, one of the visitors said as follows: "I heard that Pope Pio XII was resuscitated with royal jelly (RJ) from critical conditions due to old age [1, 2]. How about testing the effect of RJ?"

At that time, I was completely unaware of RJ. I looked for "beekeepers" all over Japan, found one beekeeper in Gifu Prefecture and purchased some RJ. I rushed back to Tokyo feeling a powerful urge to give it to my father as soon as possible. However, I did not know how to make a patient in a comatose state take the RJ. After consultation with the chief nurse, it was injected into the rectum with a syringe. It was administered at a dose of 5 g twice a day, once in the morning and once in the evening.Two days later, my father came out of the coma in the morning and said, "I am hungry." He again asked for something to eat on the following morning, distressing his wife. It was realized that he was getting better day by day. First, urination became smooth. The jaundice symptoms disappeared, and the skin became tinged with a pink color. The abdominal region, which had been swollen with ascites, gradually became smaller. In the meantime, appetite increased. Even after he became capable of ingesting foods, RJ continued to be administered at a dose of 10 g every day.

About 2.5 months after hospitalization, the hospital president visited his room and said: "It is mysterious. I have been a physician for more than 40 years, but I have never experienced a case like yours. There are no concerns as far as judged from the laboratory tests. Next week, we would like to cut your abdomen a little bit to look at your liver by means of abdominoscopy. At that time, cytodiagnosis of the liver will also be performed." After abdominoscopy, the hospital president said as follows with smiling: "Congratulations, Mr. Yamaguchi. This is a miracle. Cancer was not detected anywhere, and metastasis was not seen either. Hepatocirrhosis has also gotten better. It is really mysterious. RJ exerted the effect in the same way as in the case of the Pope, didn't it? We are going to discharge under the condition of visiting us once a week. I am so happy for you. Congratulations."

After witnessing this miracle, I embraced a keen interest in the substance known as "RJ". At this time, I decided to devote my life to research on the substance, RJ, and the mysterious insect, the honeybee, that produces RJ. It was August 1, 1965. At that time, I was 23 years old. I quit my previous job and selected peddler of RJ to convey the surprising effects to other people. Namely, I started a job to purchase RJ from beekeepers and go from place to place to sell it. However, in the 6 months after the start of peddling RJ, I did not experience the dramatic recovery or miraculous result seen in the case of my father, in spite of the recommendations I had made to many people. I reached the serious question of why no effect was seen in other persons.

I decided to visit the beekeeper, from whom the RJ had been obtained to treat my father, to report the results. I asked the beekeeper what the RJ administered to my father was like. The beekeeper answered as follows: "Nowadays, RJ produced using artificial queen cell cups is purchased only by some pharmaceutical companies and does not make much money. Since the purchase price is low in spite of much labor, no beekeepers harvest RJ." I asked further, "So how did you harvest the RJ given to my father?" The beekeeper answered as follows: "Worker bees prepare the natural queen bee's nursing room, which is called a queen cell. When the queen bee lays fertilized eggs therein, the worker bees aged 3–12 days after emergence produce RJ in their cephalic glands by consuming a large volume of honey and pollen, especially pollen, and then they secrete RJ to give the larvae. The number of queen cells sometimes exceeds 10. The RJ pooled in the natural queen cell cups was incidentally harvested and stored in a refrigerator at less than 4°C. When you made

additional orders, it was challenging, but I asked my fellows to collect queen cells, which are usually disposed, and secured the necessary amount."

I learned that the RJ passed over by the beekeeper was the natural RJ collected from the special hive cells called "natural queen cell cups" prepared by the habits of honeybees from artificial queen cell cups [3]. If all the RJ purchased had shown the same effect, interest would not have been so great. Since the story about the miraculous resuscitation of the Pope was heard from someone who had visited my father, RJ was sought for and I encountered the genuine RJ with reliable effects. Since the same effects were not seen when I started peddler of RJ, I started to study apidology.

Thereafter, I became a pupil of Dr. Yoshinobu Tokuda, a world-famous apicultural scientist, and learned in detail about apidology, the modality of correct beekeeping and the methods of harvesting RJ. Dr. Tokuda taught that RJ is vulnerable to the following: oxygen in the air, metallic tools, ultraviolet light, and gastric acid (hydrochloric acid) in humans. Countermeasures were essential in dealing with these four weak points. Dr. Tokuda asserted that RJ should be stored below 5°C, and that cryopreservation below −18°C is necessary for permanent storage [4]. Then I reached the specific "Kikuji Yamaguchi Method of Natural Beekeeping (KYAMENABEE)", which involves not handling honeybees harshly. This was triggered by the following words spoken by one beekeeper when talking about the production of RJ: "Since production of RJ will impair honeybees, I have no intention to do it in mercy of honeybees." When I asked "What is meant by impairs honeybees?" the beekeeper answered "It means the weakening of bee colonies". In other words, he means that when the worker bees are made to produce a large volume of RJ, it goes against the habits of honeybees related to ecology/providence thereof and weakens bee colonies. I made further studies based on this important suggestion and created a methodology to produce RJ using artificial queen cell cups that achieves a certain level of production scale and secures the same level of quality as natural beekeeping [5, 6]. I named this methodology the "Kikuji Yamaguchi Method of Natural Beekeeping (KYAMENABEE)".

2. Problems existing in modern beekeeping

2.1 Various problems existing in modern beekeeping

Apicultural products including RJ have been utilized as health supplements since ancient times. In light of their purpose, these apicultural products must not induce any health injuries and must exert the effects meeting the purpose. In recent years, however, the safety and functionality of apicultural products including RJ have repeatedly been questioned as people have become increasingly health conscious. For example, in Japan, from the viewpoint that it is important to appropriately utilize healthy foods for improvement of people's health, the National Institute of Health and Nutrition is publishing safety/efficacy information through its established material information database for various foods called "healthy foods". According to this database in relation to the "safety of RJ" some descriptions refer to damage to health that is suspected to have a causal relationship with ingestion of RJ. Such instances include the following "Safety is suggested only when orally ingested appropriately for a short term", "Use of RJ should be avoided during pregnancy or lactation, since reliable data have not been sufficiently obtained", "When ingested orally, almost no adverse reactions appear in people without allergic diathesis, but various allergic reactions (pruritus, urticaria, eczema, edema on eyelid or face, arthritis, rhinorrhea, dyspnea, asthma, etc.) occur with a high incidence in people

with a history of atopy or asthma. Use of RJ should be avoided in patients with asthma or atopy, since anaphylactic reactions leading to an asthma attack may be induced, in severe cases leading to death" and "Intoxication may be caused when ingested at a high dose". While it was previously known that such health issues may occur infrequently, there has recently been a trend for increased incidence of these problems. As the background of such increase, serious problems related to the quality of RJ cannot be overlooked. No one can deny the fact that supposedly "high-quality" RJ is now marketed widely after being manufactured by artificially modified beekeeping technology with the post-manufacturing addition of 10-hydroxy-2-decenoic acid (10-HDA), etc. [7–9]. Furthermore, the proteins contained in RJ are denatured by neglecting filtration immediately after harvest and storage in a refrigerator (2°C).

On the other hand, the hive abandonment by honeybees (the Colony Collapse Disorder: CCD) [10, 11], which has often been reported since 2006, is a big surprise to the world of beekeepers [12–22].

It is impossible to deny that the background of this issue includes a serious problem related to the high-level biological functions of honeybees. Honeybees form a highly-socialized population, and the total activities of the honeybee society can be maintained only when the hierarchized honeybees play the roles of each hierarchy layer. It is natural to consider that the serious problem is a result of significant impairing of the high sociality and biological feature of honeybees due to the tremendous stress placed on the honeybee society by the production-first policy and cost-first policy. The consideration emphasizing quantity over quality and pursuing cheapness may weaken bee colonies and reduce disease resistance.

Pollution with agrochemicals, heavy metals and antibiotics accompanying economic development is another serious problem for beekeeping environments. The issue of residual antibiotics in honey began in Europe in December 2001 and subsequently spread around the world. The standards for residual antibiotics in each country were triggered by this issue to change the level from ppm to ppb (1/1000 of ppm).

The low quality of apicultural products manufactured by inappropriate beekeeping and inappropriate processing to supplement the low quality have created a vicious cycle and caused a serious problem that not only disturbs the production and quality control of apicultural products as natural foods with high added values, but also possibly impairs the sustainability of the apicultural industry itself.

I have systemically summarized the immense benefits obtained from beekeeping and apicultural products (especially RJ) as well as an originally-anticipated form of beekeeping through the practices of natural beekeeping and quality control of apicultural products as performed for many years in Japan and China. For the past 54 years, I have focused on ways to improve Japanese beekeeping technology. As a result, I noted many problems needing improvement in the modality of beekeeping and production control of apicultural products such as the absence of basic beekeeping technology in modern beekeeping businesses, the deteriorated quality of apicultural products due to inappropriate processing, contamination with drugs such as antibiotics, and the shortened life span of queen bees and deteriorated bee colonies brought about by excessive artificial inbreeding of seed bees. Based on the above, I have proposed an originally-anticipated form of beekeeping from the viewpoint of natural beekeeping based on the original biological capability of honeybees in order to solve the problems found in the modern beekeeping.

The purposes of this paper are to objectively first grasp the basic problems with modern beekeeping to propose an originally anticipated form of beekeeping for solving the problems by means of theoretical investigation and practical application to verify the functions of the apicultural products manufactured in this way, and to

propose new standards for evaluating the quality of apicultural products with high functionality based on scientific rationales.

In this paper, I would like to consider the problems to be solved for sustainable development of the apicultural industry, based on my experiences and practices.

2.2 Problems brought about by changes in beekeeping environments

Honeybees collect flower nectar and pollen from nectar plants. Originally, flower nectar is secreted by plants to attract insects including honeybees and birds to the flowers for pollination, which is necessary for the preparation of seeds. Secretion of flower nectar depends not only on the climate conditions such as sunlight, temperature and amount of rainfall, but also on the status of soil. Since beekeeping was originally performed for the purpose of collecting flower nectar and pollens, there are deep relations with natural environments in this respect.

In Japan extending from south to north, the flowering likewise spreads from the south towards the north. The apicultural industry of Japan used to develop by pursuing the flowers, i.e., that is the nectar sources. In other words, the beekeeping style was migratory beekeeping. However, in recent years, together with the progress of urbanization, the fields and mountains to be utilized as nectar sources have rapidly disappeared due to land reclamation. Furthermore, there has been increased planting of trees for house construction, such as cedar and cypress, while miscellaneous trees have been cut down, leading to a reduction of the number of nectar plants and making conventional migratory beekeeping impossible. In addition, due to the progress of urbanization, nectar source areas are exposed to contamination from agrochemicals and other various substances derived from human living activities. In particular, the Ministry of Agriculture, Forestry and Fisheries ordered the felling of acacia and pseudo-acacia because these trees are originally non-native species with a high rate of reproduction and adverse influences on the native species, yet these trees comprise one of the nectar sources of the four major transparent honeys.

I had opportunities to see the beekeeping practice sites all over Japan and found that a common problem was the inappropriate location of bee hives. For example, bee hives were often set in a place near a small river along a road. This was a result of consideration of convenience in transportation for migratory beekeeping and securement of daily life water for beekeepers, but this is a fundamentally mistaken policy. When honeybees come into contact with a road, contamination occurs easily with heavy metals on the road from exhaust fumes, etc. Furthermore, in such places, it is highly likely that antibiotics are carried into the hive cells at high concentrations through the waste water derived from livestock farms.

In such current status, it is no exaggeration to say that Japan's apicultural industry has reached a critical moment for its survival. Furthermore, this is closely related to the beekeeping performed in the countries exporting apicultural products to Japan.

2.3 Problems existing in modern beekeeping as an industry

2.3.1 Wintering of Colony and preparation of seed bees

In Japan and Europe, colonies are made to winter. In China, however, honeybees are used only for 1 year at many of the beekeeping industry sites. It can be said that this rearing method is inconsistent with the habits of honeybees. The honeybee is the only insect that can generate heat and hibernate throughout winter. Of course, the honeybee is a heterothermic animal and each individual bee cannot maintain a constant body temperature, but they can maintain a constant hive temperature as a

colony. Honeybees prepare honey by collecting nectar from autumn flowers and store pollen loads by collecting pollen. At the end of autumn, the worker bees form a cluster surrounding the queen bee and generate heat by rubbing their bodies together. Even when the outside temperature is lower than 0°C, the central temperature of the cluster is maintained at a certain temperature. Thus, the bee colony is able to pass the winter.

The wintering worker bees survive even for 5 months. When spring approaches, the queen bee starts laying eggs, and the worker bees fly out of the hive entrance to collect nectar and pollen from the flowers coming out in the early spring. The lifespan of a queen bee eating only royal jelly reaches 5 years, and the queen bee winters four times in its life. In the severe wintering periods, the survival capability of each colony is reinforced for the next season.

However, at not a few apiaries emphasizing efficiency, honeybees are disposed of at the end of the season, since colony management during the wintertime is not cost-effective. As selective breeding for that purpose, inbreeding is performed for the artificial creation of queen bees. When honeybees are disposed of at the end of the season, the colony cannot be reinforced by natural selection, and the queen bee becomes smaller year by year. The worker bees born from such queen bees tend to show a high rate of teratogenicity. The RJ prepared by such worker bees is watery and composed of inferior components.

2.3.2 Weakening of colony by inbreeding

In the natural condition, the queen bee performs a mating flight once or twice in its life, copulates with some of the accompanying drone bees of another colony and returns to the hive after obtaining seminal vesicles. In recent years, a new method was contrived for artificial mating of the queen bee and drone bees using a special device. This method may be effective for the purpose of obtaining high-quality colonies tentatively by selective breeding but tends to lead to inbreeding of bees with excellent characteristics. Some entomologists consider that inbreeding can prepare high-quality strains, and it is said that inbreeding was accelerated by such entomologists. The queen bees prepared in such way cannot avoid decreased vitality. While the lifespan of a normal queen bee is 3–5 years, it has become common for beekeepers lacking resources to dispose of colonies at the end of each season, since wintering requires not only feeding but also accommodation to protect the bees from wind/snow/rain. This is the cause of creating colonies with weak disease resistance. “Heterosis” is an unchanging principle in the living world, and “the Queen bee getting smaller in selective breeding for more production of RJ” in China is exactly the result of ignoring the habits of honeybees. Beekeepers should give sufficient consideration to this issue hereafter.

2.3.3 Harmful effects of overload on honeybees

In the current beekeeping business, there is a strong tendency towards abuse of colonies are in pursuit of economic profits, and the changes occurring in the colonies during the current beekeeping process are depreciated. For example, beekeepers are not particularly interested in the series of problems such as deteriorated quality of colonies, decreased power of resistance, decreased pollen-collecting capability, decreased disease tolerance, and decreased content of active ingredients in apicultural products.

Honeybees have survived a long history of more than 100 million years and have established an orderly society. Honeybees are called “social insects”, and there ought to be pursuit of a modality of beekeeping that is suitable for posterity.

In China, the world's largest production area, there are more than 8 million reared honeybee colonies. Of these, Occidental honeybees account for about 80% and Oriental honeybees about 20%. Occidental honeybees are producing more than about 3000 tons of RJ per year. Originally, RJ is produced for the main purpose of growing queen bees, and we have to say that production of 3000 tons of RJ is performed by ignoring the ecology of honeybees and by abusing honeybees. In the general beekeeping business, more than 200 artificial queen cell cups are set in one bee hive for mass production of RJ. However, RJ production that exceeds the capability of worker bees leads not only to health problem for the honeybees but also to problems relating to the supply to society of inactive RJ with poor nutritive values. For example, the content of 10-HDA in RJ is generally 1.4–1.6% even in the RJ produced in mainland China and Taiwan utilizing the nectar source. These values are too low in comparison with the content of 10-HDA in RJ produced by the author's group in Qinghai province, China (2.5–3.1%) (Japan Royal Jelly Co. LTD., 2008). I consider that the cause of this low content of 10-HDA is abuse of honeybees in excessive production of RJ. Although the beekeepers' wish to obtain a large amount of RJ by using the entire colonies is understandable, it deficiently provides abuse results in weakened colonies, susceptibility to diseases, and eventually to the constant deterioration of colonies.

2.3.4 Problems in harvesting and processing RJ

RJ has been projected as a functional food exerting various active functions and has been utilized since ancient times. It is natural to consider that there are close relations between the functionality of RJ and production methods thereof. It is well known that RJ is stored in the queen cell cups, in which queen bees are reared. RJ is secreted by young worker bees (aged 3–12 days after becoming mature insects), which account for only about 20% of the entire colony. Large amounts of pollen and honey are necessary for the worker bees to secrete RJ. Young worker bees eat them and secrete RJ for the larvae growing into queen bees in the artificial queen cell cups.

Traditionally in Japan, since it would impair honeybees and reduce the size of the colony, many beekeepers harvesting honey for their own use hated the production of RJ. Young worker bees will soon become foraging bees to collect flower nectar and pollen, completing their lifespan of 30–40 days, but their lifespan is shortened when they are made to secrete too much RJ. When the number of such worker bees increases, the colony will naturally become weak.

In addition, when many artificial queen cell cups are used, the quality of RJ suffers marked deterioration, and only watery RJ is secreted.

RJ is projected as a functional food containing various physiologically active substances. On the other hand, one of RJ's weaknesses is that it is a delicate substance deactivated when exposed to oxygen, ultraviolet light, heat, metals, and so on. Therefore, it is extremely important to filter the RJ neat fluid after harvest at the apiary and store at a low temperature. RJ is vulnerable to heat, and denaturation occurs immediately when left at ordinary temperature, since the major components are proteins. However, ordinary beekeeping involves a surprising lack of attention to the processing and storage of harvested RJ. In the conventional general beekeeping activities, the harvested RJ neat fluid is often filled into a plastic bag or container under a tent without filtration and is then stored at ordinary temperature under no protection from sunlight. At the end of the flowering season, the harvested RJ is collected and taken at last to the processing plant, where it is gathered, filtered for the first time, and frozen. Thereafter, freezing and thawing are repeated many times. During this process, 10-HDA is even artificially added in some case in order to comply with quality standards.

In production of RJ using artificial queen cell cups, the attention to be paid to the larva-grafting operations and timing of harvest is also lacking with regards to obtaining RJ of high activity and high purity. Even when there are artificial queen cell cups, these are ignored by young worker bees in charge of RJ secretion. RJ is poured into the artificial queen cell cups only when they contain larvae. Therefore, it is necessary to artificially graft the just-hatched larvae into the queen cell cups. In traditional RJ production, third-instar larvae (3 days after hatch) are ordinarily transferred.

The larvae grow fast, and a surprising body size is achieved in only 1 day. However, the first-instar larvae are too small, making the larva-grafting operations difficult, and not only is the early fluid given already with watery RJ mixed in but also the larva acceptance rate is also unfavorable. On the other hand the third-instar larvae are close to fourth-instar larvae in terms of being large in size, and the larva grafting operations are not difficult. The larvae, however, are too mature and ingest most of the ingredients essential for growth contained in RJ, resulting in low-quality of RJ for harvesting. There is another reason why third-instar larvae are not used. The operation to graft larvae into the artificial queen cell cups has to be performed quickly and carefully using a transferring tool made of feathers (called a "larva-transferring needle"), so that larvae do not collapse. However, since the queen bee is laying eggs successively all day and night, it is fairly difficult to judge the age in days for each hatched larva. The second-instar larvae and the third-instar larvae can be easily distinguished, since the body size is considerably different, but among the third-instar larvae it is almost impossible to determine the exact age in days (whether each larva is at the beginning of Day 3, at the end of Day 3 or at the beginning of Day 4).

Consequently, the problematic point is that the larvae to be reared as worker bees in the worker bee-rearing cells are given a small amount of RJ until Day 3 after hatching and thereafter are given pollen and honey as a mixed weaning food. Since the later third-instar larvae reared in the worker bee-rearing cells have begun receiving the mixed weaning food (pollen and honey), defecation may have started. If such the larvae are grafted into the artificial queen cell cups, defecation also inevitably occurs in the queen cell cups.

2.3.5 Problems in quality control and production history disclosure

Many of beekeepers do not perform filtration at the apiary. In the case of RJ, harvest is normally performed by collecting from the artificial queen cell cups with a bamboo spatula or an ink brush, etc. At this time, contamination from impurities such as hive scum and dust is unavoidable. It is therefore necessary to perform filtration immediately after harvesting. In addition, it is absolutely necessary to store at 2°C after harvesting. Cryopreservation must not be performed together with the impurities. However, at many apiaries, the harvested RJ is not filtered immediately at the apiary and is left for a long period at an ordinary temperature.

I first started contract manufacturing of RJ in Japan 54 years ago. Since that time, the traceability of production history has been given special importance. The word "traceability" is now familiar, and it is originally the responsibility of bee-keepers to consumers to leave correct production records of apicultural products, since safety and functionality thereof was to be strictly assured.

2.3.6 Problems of drug contamination and others

Honeybees are vulnerable to agrochemicals. Honeybees have been seriously affected by the neonicotinoid insecticides that have been used as agrochemicals all

over the world since the 1990s. Neonicotinoids act on the central nervous system of insects, and it is pointed out that neonicotinoids attached to nectar and pollen may cause lethal damage to honeybees.

In 2005, mass deaths of honeybees were reported in Iwate prefecture in Japan. About 70 to 80% of honeybees from each hive suffered and died around each beehive. The cause was an agrochemical. A neonicotinoid agrochemical named clothianidin was used in the same area under the instruction of the prefectural government. Since conventional agrochemicals show neurological toxicity and are dangerous to persons sensitive to chemical substances, clothianidin being a neonicotinoid has come into use, and it was extensively sprayed as a shield bug control, resulting in the mass deaths of honeybees.

Neonicotinoids act on the neurotransmitter functions of living organisms. Acetylcholine (ACh) is one of the human neurotransmitters. It is contained at a high level in the nervous tissues. It is secreted from the ends of parasympathetic nerves and motor nerves in response to stimulation, and it is involved in neurotransmission. A neonicotinoid binds to nicotinic ACh receptors of nerve, shows the physiological effects like those of ACh and continually stimulates the nerves. In both humans and animals, a neonicotinoid is regarded as acting as a neurotransmitter on the autonomic nervous system, neuromuscular junction and central nervous system having nicotinic ACh receptors.

Honeybees are social insects forming a colony and displaying functions based on the entire bee hive. However, when the colony is weakened, that is, when a certain percentage of the bees die or disappear, the entire colony fails to function and ultimately collapses. Ironically, both the prefectural and national governments had instructed the farmers to use a neonicotinoid named dinotefuran which is less toxic than clothianidin. Honeybees became capable of avoiding death even when exposed to dinotefuran, but it was found that mature insects that had grown up from the larvae eating the pollen contaminated with dinotefuran as bee bread (food for larvae) will lose their sense of direction and become incapable of returning to their own hive, since the nerve receptors are impaired by the neonicotinoid.

Also, the use of antibiotics weakens honeybees. In China, since 1990, antibiotics such as tetracycline, streptomycin and chloramphenicol have been mixed into foods and given to honeybees to prevent communicable diseases. Furthermore, agrochemical spraying has been commenced in order to increase the agricultural crops. These agrochemicals are exerting bad influences on honeybees flying over the fields and mountains. Since the farmers and beekeepers did not receive adequate instruction about the antibiotics and agrochemicals, and the amounts used were far more than the limit levels, European countries and Japan have frequently identified this issue as a serious problem every time imported Chinese agricultural products are quarantined.

In all the countries of the world, most of the homeland is contaminated with agrochemicals. In the livestock farms, large amounts of antibiotics such as tetracycline, streptomycin and chloramphenicol are used to prevent infections. Furthermore, in modern beekeeping, the disease resistance of bee colonies has decreased and large amounts of antibiotics are now used for honeybees to prevent infections such as foulbrood. It is unavoidable that apicultural products are contaminated directly or indirectly with these drugs.

3. Proposal for Kikuji Yamaguchi method of natural beekeeping (KYAMENABEE)

As described above, there are many problems in modern beekeeping that ought to be solved for the future of bee industry. Through my experiences and practices of

beekeeping technology extending over many years, I have identified many problems existing in the modern beekeeping such as beekeeping in inappropriate environments, deterioration of colonies due to overloading of production and excessive selective breeding, reduced disease resistance, inappropriate processing, insufficient attention paid to quality control of apicultural products, and so on [5, 6].

On the other hand, there are also problems in the major countries consuming apicultural products, such as Japan. Specifically, the following problems cannot be ignored: low awareness of the quality of apicultural products, insistence on "quantity over quality" and "cheap price", and ambiguous quality standards for apicultural products. On the other hand, beekeeping is an industry, and therefore profit cannot be neglected. In order to solve these problems, difficult countermeasures are required to meet the consumers' needs and harmonize cost performance on an industry-wide basis.

In this paper, I would like to propose the measures to solve the problems based on his past experiences and practices of natural beekeeping.

3.1 Definition of natural beekeeping

In the natural condition, secretion of RJ occurs when the colony in the bee hive propagates and the colony splits (swarm, hive division). For colony splitting, one queen bee is essential for each colony, and when the time of the split approaches, 10–15 natural queen cell cups are prepared in the hive. The queen bee lays fertilized eggs into these natural queen cell cups, and the worker bees secrete RJ. The first queen bee emerging and leaving the brood is tested by the worker bees to check whether it can work sufficiently as a queen bee. When it passes the test, the larvae of other sister queen bees remaining in the queen cell cups are killed by the worker bees. There is a principle that only one queen bee can exist in each bee hive. A new queen bee is reared with the RJ secreted by the worker bees, and the swarm phenomena are seen before the new queen bee emerges. After emergence, the new queen bee flies out of the beehive for a mating flight, copulates with drone bees of another colony and returns to the hive. However, the new queen bee may be attacked often by a foreign enemy and may not be able to return to the hive. In the society of honeybees, the egg-laying bee is the queen bee, and each colony will be in danger of extinction in the absence of the queen bee. When the worker bees get a scent of the danger of extinction, they will take emergency measures by finding third-instar or younger larvae growing in the worker bee-rearing cells and starting the construction work to expand the worker bee-rearing cells to queen bee-rearing cells while giving a large amount of RJ to such larvae. In other words, the larva-rearing policy is changed. The cells prepared when the larva-rearing policy is changed are called "emergency queen cell cups". Utilizing this habit of preparing the "emergency queen cell cups", Inoue invented artificial plastic artificial queen cell cups and filed a patent application in 1963 [3]. On November 19, 1965, the utility model registration (Registration No. 785804, Japan) was filed for dissemination of this technology. This invention of artificial queen cell cups enabled mass production of RJ leading to dissemination in Japan, Taiwan and China. Only about one dozen natural queen cell cups are prepared in the natural hive, but it is possible to set 200 artificial queen cell cups in one bee hive, and about 750 mg of RJ can be pooled in 72 h in each queen cell cup. About 150 g of RJ can be harvested at once, enabling mass production of RJ.

In the period from the 1960s to the 1980s, the beekeeping business was active in Japan. There were 12,000 beekeepers and 320,000 colonies. Along with dissemination of the artificial plastic artificial queen cell cups, the previous beekeeping business targeting only honey production proceeded with production of RJ. However,

the poor knowledge of RJ and RJ production led to the appearance of products inferior in quality or component activities.

In 1967, we organized contracted beekeepers for practice of the Natural Beekeeping in order to realize production of high-quality RJ as proposed by ourselves.

The Natural Beekeeping and ordinal beekeeping are compared below in terms of (i) basic beekeeping conditions, (ii) royal jelly production, so as to clarify the differences (**Table 1**).

3.2 Basic beekeeping conditions

3.2.1 Seed bee rearing

Domesticated honeybees have fixed the characteristics suitable for beekeeping through selective breeding, extending over a long period of time. In addition, there

	Main components of RJ	
Item	**Natural beekeeping**	**Ordinal beekeeping**
Basic beekeeping condition		
Seed bee rearing	Rearing from artificial queen cell cups, hybridization	Inbreeding
Wintering (queen bee)	Used for 3–5 years with wintering	Disposed at the end of season without wintering
Location of apiary	Highland	Lowland
Conditions for bee forage	Limited to agrochemical-free area	Regardless of agrochemical usage
Apiary isolation (from livestock farm)	Complete isolation	No consideration
Apiary Isolation (from road)	Complete isolation	Next to roads
Water source securement	Water tray setting at apiary	No consideration
Filtration at apiary	Performed	Not performed
Antibiotics	Not used	Used
Royal jelly production		
Larva transfer	Second instar	Third instar
Number of Artificial queen cell cups	Not more than 100	200–250
Time before harvest	48 h	72 h
Bee forage	Single nectar plant (rapeseed) artificial feeding	Multiple nectar plants or
Colony management	Rotation of 1/4 of colonies	No rotation
Filtration at apiary	Primary filtration at apiary	No filtration at apiary
Temperature control	Storage at 2°C immediately after filtration	Ordinary temperature
Processing facility	Management at 2°C, filling	Freeze/thaw/mix/re-freeze (cryopreservation)
Transportation	Transportation at 2°C and frozen transportation	Frozen transportation

Table 1.
Comparison of the natural beekeeping and ordinal beekeeping.

has recently been an increase in artificial mating of queen bees. On the other hand, such selective breeding tends to lead to inbreeding, resulting in new secondary problems such as shortened lifespan of queen bees, reduced disease resistance and loss of specific biological capability. In order to solve these problems, it is important to avoid the degeneration of species appearing after artificial inbreeding by means of appropriate interbreeding.

3.2.2 Wintering

Wintering is a harsh experience for colonies, but it is also a rest period. Inside the beehive, the worker bees form a cluster surrounding the queen bee, increase the body temperature by constantly fluttering their wings and maintain the central temperature of the cluster at 31–35°C. However, many of the worker bees die before spring comes. The wintering worker bees are those born in autumn. The bees surviving this harsh season are regarded as having excellent characteristics. The lifespan of worker bees is only about 30–40 days at the peak of nectar collection but reaches up to 5 months after wintering. These wintering bees should be utilized for strengthening the colonies. In order to recover the colony momentum lost during winter, the bee colonies must first be strengthened. Honey harvests should be avoided for a while after the start of bee activities in the early spring. In the early spring, the nectar plants are still not constant, so that the honey is a so-called mixed honey, which is used for the purpose of restoring the colony momentum. By refraining from early harvesting, the production of RJ and honey is promoted as a preparatory arrangement for the start of laying eggs by the queen bee. Harvesting of honey and RJ should be commenced after many worker bees emerged and the beehive is filled with worker bees.

3.2.3 Location of apiary

The beehives and the hive frames are important factors in the living environments of honeybees, and it is essential to keep these clean in order to maintain the health of colonies. The cleanliness of colonies is also closely related to prevention of apicultural product contamination. It is therefore necessary to keep beehives clean and old beehives that have existed for 5 years or longer should not be used.

In order to obtain high-quality apicultural products, the apiary should be located in a secluded highland, even though transportation of colonies is expensive. This is because the damage caused by various insects and bacteria can be avoided in locations that are high above sea level (**Figures 1–4**).

The following conditions are desirable for the location of apiaries:

i. high above sea level

ii. dry

iii. not windy

iv. south-facing

v. mild temperature

vi. far away from noisy environments.

Furthermore, the following conditions are also desirable:

i. The target nectar plant grows in a concentrated manner.

ii. The flowering season of the target nectar plant is different from that of other plants.

iii. It is far away from farms growing commercial plants with agrochemicals.

3.2.4 Conditions of bee forage

The bee forage is a substantial base for beekeeping and also a major food source for survival of honeybees and the prosperity of descendants. Therefore, the bee forage should basically be native grass flowers or tree flowers in the agrochemical-free area located in the highland or mountain area at least 2000 m above sea level. It is possible to cultivate a bee forage by seeding, but in such cases, it is necessary to prevent contamination of apicultural products by using a bee forage cultivated

Figure 1.
An ideal location of the apiary for natural beekeeping. It should be in a secluded highland where the target nectar plant grows in a concentrated manner, the flowing season of the target nectar plant is different from that of other plant, and far away from agrochemical-using farms of commercial plant.

Figure 2.
Uncontaminated (agrochemical-free and chemical fertilizer-free) area of Chinese Highland.

using natural fertilizers or home-made organic fertilizers without the use of chemical fertilizers. For the time being, in order to avoid this problem, we should consider beekeeping in areas that remain un-contaminated.

The author's group started RJ production by the natural beekeeping proposed by the author in 1993 in an un-contaminated (agrochemical-free and chemical fertilizer-free) area of Chinese highland 3200–3500 m above sea level (Qing Hai, Menyuan). The honeybees used in this area are of the Occidental species, but the harvested RJ is satisfactory in both quality and quantity. In particular, the 10-HDA value is as high as 2.8% when harvested after 48 h, and no contamination with antibiotics has been detected at all. Such organic areas remain more prevalent in China than in Japan. Beekeeping in un-contaminated bee forages is absolutely necessary for production of high-quality apicultural products (**Figures 1** and **2**).

3.2.5 Isolation of apiary

Antibiotics are used in large amounts on pig farms, chicken farms and fish farms, and the pooled water and waste are possibly contaminated with antibiotics. When an apiary is located near such farm, the honeybees flying for water may carry into the beehive water that is contaminated with antibiotics resulting in possible contamination of apicultural products with antibiotics. (**Figure 3, Left**) In the Natural Beekeeping, such risk is avoided by locating the apiary at a distance of at least 10 km from any culture farm.

Roads have been improved in recent years, and asphalt roads are constructed even in the depth of mountainous regions. Some beekeepers set their beehives beside roads giving priority to convenience in their living activities. When beehives are allocated next to roads, the apicultural products may possibly become contaminated with exhaust gas, asphalt dust and especially heavy metals. (**Figure 3, Right**).

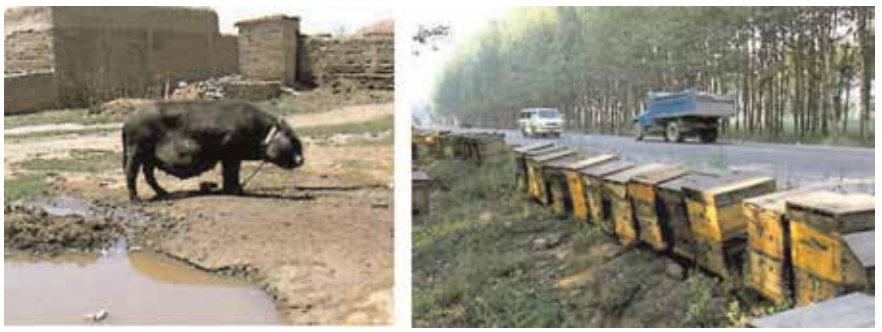

Figure 3.
Location of apiary. Left: The apiary should be located from far away, approximately 10 km, from pig farm, cow farm, and/or chicken farm. Right: When bee hives are set beside a road, the apicultural products may possibly be contaminated with exhaust gas, asphalt dust, and heavy metals. Thus, bee hives should be placed far away from a road.

3.2.6 Securement of water source

Colonies need large quantities of water in order to constantly maintain the intra-hive temperature in the range of 35–37°C and the intra-hive relative humidity in the range of 76–80%. Throughout their long history, honeybees have evolved as an insect that is capable of adjusting the temperature. Even when the outside temperature rises to nearly 40°C, the intra-hive temperature is maintained in the range of 35–37°C all the time. This is achieved as follows. The foraging bees collect water, spit the water as small drops into the hive, bring wind into the hive by fanning with

Figure 4.
Water supply for honeybees. Stainless-steel tray is settled around the hive. The tray is 1 m in length, 1 m in width and 10 cm in depth. Clean water is filled in the tray, and grass is laid therein as footholds for honeybees as a contrivance for easy water taking.

their wings and lower the intra-hive temperature by deprivation of vaporization heat. Water is essential for honeybees to adjust the temperature. Each worker bee carries water weighing approximately the same as its own body weight. They may fly 4–5 km in pursuit of water.

Therefore, a place where morning fog is seen is suitable for beekeeping. A foothold is surely necessary for honeybees to take water. At a brook or babbling stream, honeybees may drown when their wings are caught by water before they manage to take any water back to the hive. Therefore, steaming water is inappropriate as a water source. If there are no appropriate water-taking environments, it is necessary to contrive to assure free water-taking by setting pallets in the apiary yard and around the hive and lying green grass in the pallets so as to secure footholds for the honeybees.

My group usually places a stainless-steel tray around the hive (**Figure 4**). The tray is 1 m in length, 1 m in width and 10 cm in depth. The tray is filled with clean water and grass is laid therein as footholds for honeybees in order to facilitate easy water-taking. These arrangements are made so that honeybees can take water nearby the hive without using excessive amount of energy for flying to a distant place.

3.2.7 Filtration at apiary

I recommend filtering the harvested highly-active RJ immediately at the apiary and storing at 2°C under complete protection from sunlight. When RJ comes into contact with oxygen or carbon dioxide in the air or is exposed to ultraviolet light, it quickly becomes less active. Therefore, in the Natural Beekeeping, the harvested RJ is immediately filtered under a tent avoiding direct sunlight so as to remove foreign matter such as dead bees, hive scum and dust (**Figure 5**). Although denaturation occurs immediately when left at ordinary temperature, it is known that RJ can remain active for a fairly long time when stored at 2°C. The author recommends filtering the harvested RJ on each harvest occasion so as to remove impurities, followed by temporary storage at 2°C under protection from direct light and final cryopreservation below −18°C.

3.2.8 Processing plant

In the Natural Beekeeping, the harvested RJ and honey are filtered immediately at the apiary, so filtration at a processing plant is not necessary at all. However,

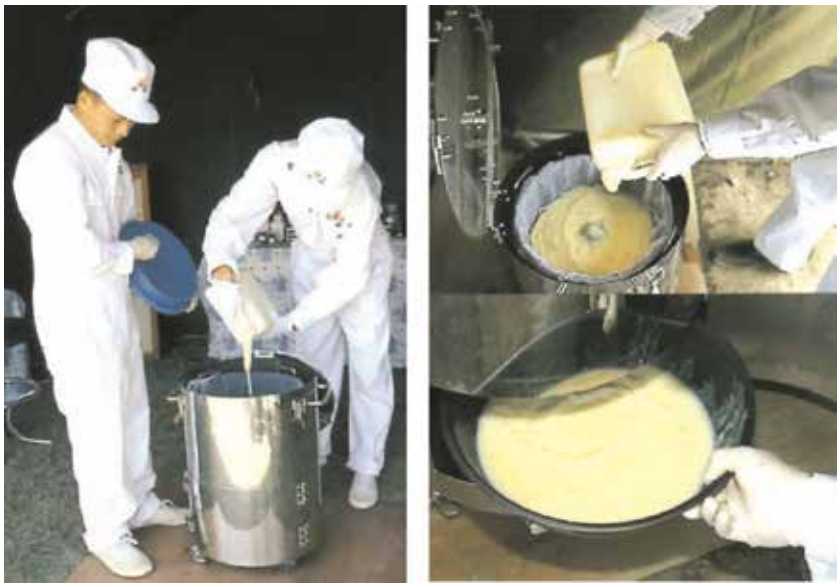

Figure 5.
Filtration of harvested RJ at apiary. The harvested fresh RJ is immediately filtered under a tent avoiding direct sunlight to remove foreign matters such as dead bees, hive scum and dust.

when the apiary is located in a mountain area, apicultural products are generally collected at a processing plant located in an urban area for filtration and packaging. It is problematic to leave the unfiltered apicultural products for a long time. In the meantime, the products become less active due to the impurities contained therein. Honey may be fermented when the Brix degree is low, while RJ shows signs of denaturation.

The apicultural products carried into the processing plant are once frozen, thawed for filtration and mixing performed in turn and then frozen again. The repeated freeze/thaw procedures cause quality to deteriorate.

3.2.9 Antibiotics

The principle of natural beekeeping (KYAMENABEE) is to produce apicultural products using healthy colonies. It is therefore necessary to keep the bee hives clean all the time and protect the colonies from diseases by performing cleaning and fumigation. It is important to avoid artificial mating which leads to the weakening of colonies and antibiotics should not be used to prevent diseases and infections. Methods for improvement of the health of colonies should be adopted before using antibiotics.

3.3 RJ production

3.3.1 Larva transfer

RJ is a functional food containing various physiologically active substances. It is therefore the basic principle in RJ production to harvest RJ with high content of functional components and appropriately ensure that the physiological activities are maintained. In RJ production using artificial queen cell cups, the timing of larva transfer to queen cell cups and the timing of harvest are important.

The influence on RJ production of timing of larva transfer to queen cups was studied extensively to find that it is preferable to transfer second-instar worker bee larvae (early second-instar, larva size: about 1 mm). First-instar larvae are too small and too soft making the larva-transferring operations difficult. The larvae grow quickly, and a surprising increase of body size is achieved in only 1 day.

In the case of early second-instar larvae, the amount of RJ ingested after larva transfer is not so great, and RJ rich in effective components can be harvested. Also, the larva transfer must be performed in a tent to protect it as much as possible from ultraviolet light. The larvae are quickly transferred into the artificial queen cell cups using a transferring tool called a "larva-transferring needle" while taking care not to hurt the larvae (**Figure 6**).

3.3.2 Number of artificial queen cell cups

It is well known that RJ is stored in the queen cell cups where queen bees are reared. In ordinal beekeeping, the number of artificial queen cell cups used is approximately 200–250. On the other hand in the Natural Beekeeping (KYAMENABEE), the maximum number of artificial queen cell cups is limited to 100. It is well known that RJ is secreted by young worker bees (aged 3–12 days after becoming mature insects). However, the number of such worker bees is limited accounting for about 20% of the entire colony. Large amounts of pollen and honey are necessary for the worker bees to secrete RJ. Young worker bees eat these and secrete RJ at full power into queen bees in the artificial queen cell cups for the larvae growing.

Not a few beekeepers who cherish honeybees hate the production of RJ because it impairs honeybees and reduces the size of colony. Young worker bees will soon become foraging bees to collect flower nectar and pollen and complete their lifespan lasting 1 month. However, when they overworked to secrete too much RJ, they die within 21–30 days. The colony is gradually weakened if the number of such worker bees increases.

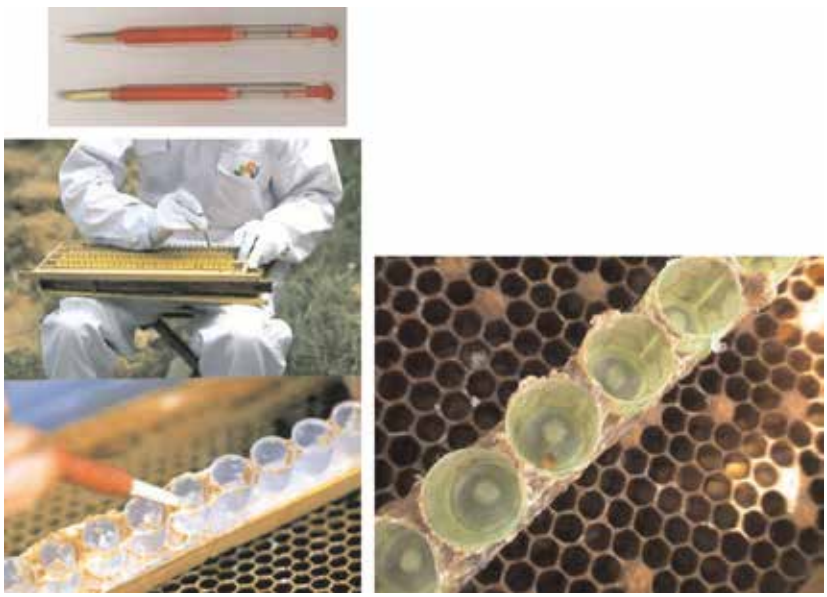

Figure 6.
Transferring of larvae. The larvae are quickly transferred into the artificial queen cell cups using a transferring tool called "larva-transferring needle" paying attention not to hurt the larvae, but this operation needs considerable mastery.

Additionally, when excess number of artificial queen cell cups is used, the quality of RJ thus harvested markedly deteriorates, and the stored RJ becomes watery and exhibits low viscosity, and the quality may be markedly deteriorated. When such RJ is given to larvae hatching from fertilized eggs, they cannot become decent queen bees and most of them become to grow up to worker bees, demonstrating that such RJ is of low nutritive value and is inactive.

3.3.3 Time before harvest

RJ should be harvested 48 h after larva transfer to artificial queen cell cups. This is because the RJ harvested 48 h after larva transfer is very active although the amount is smaller than the amount harvested after 72 h. As described below (Section 3.2.1), the content of 10-HDA in RJ is significantly higher in the harvest after 48 h than that after 72 h. The various effects on humans and experimental animals are also known to be more excellent in the former harvest. In the case of 72 h harvest, the operation efficiency is higher since a larger amount of RJ is pooled in each queen cell cup and the operation can be performed once every 3 days, but such the RJ harvested is far less active compared to that after 48 h harvest (**Figures 7** and **8**).

3.3.4 Bee forage

In the Natural Beekeeping, RJ is manufactured in single bee forage of rape blossoms. The RJ obtained from rape blossoms is highly active. In particular, the pollen of rape blossoms is active and strengthens the colonies after wintering.

On the other hand, the bee forage is not specified in ordinal beekeeping, and RJ is manufactured even in the absence of bee forage by feeding with sugar water and artificial pollen. The rape blossoms growing in the flat plains of mainland China can no longer be used due to contamination from agrochemicals, and my group has

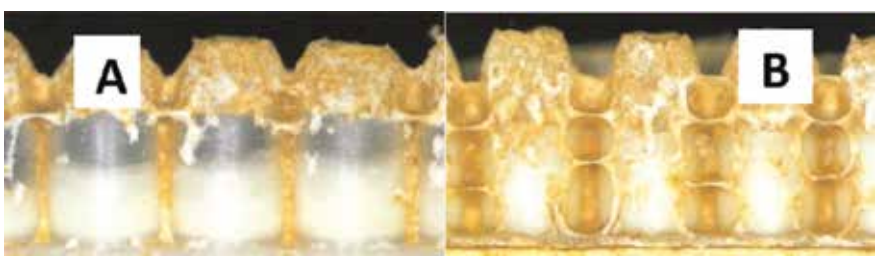

Figure 7.
Harvesting of RJ at 48 h (A) and 72 h (B) after larva transfer. Accumulation of RJ is much greater in the cup 72 h after larva transfer than that 48 h after larva transfer.

Figure 8.
Collection of RJ from the artificial queen cell cup.

been manufacturing RJ since 1993 in a declared un-contaminated area of Chinese highland over 3000 m (3200–3600 m) above sea level (Qing Hai Haibei Menyuan).

3.3.5 Colony control—Rotation of colonies

Originally, the mass production of RJ using artificial queen cell cups became feasible based on the biological and ecological properties of honeybees. Only one queen bee in the hive keeps laying 2000–3000 eggs per day. At first glance, the queen bee appears to be playing the central role in the colony, but the colony is actually controlled by the worker bees. Neither the queen bee nor the drone bees can live unless fed by the worker bees, since they have no habit of procuring foods by themselves. Since the worker bees have the feeding rights, in the absence of queen bee after isolating the queen bee from the hive, the worker bees rush towards rearing a new queen bee. When the worker bees find a third-instar or younger larva in a hive cell, they break the cell by eating, enlarge the cell to a queen bee-rearing cell cup and prepare a temporary queen cell cup (this is referred to as an "emergency queen cell"), and thereafter they start secreting RJ into the emergency queen cell. The larva that up until this point was going to grow as a worker bee is given RJ due to a policy change, and this larva ultimately emerges as a queen bee. In RJ production, utilizing the habit of honeybees to change an abnormal state without a queen bee into a normal state with a queen bee, the worker bees are made to secrete RJ by introducing artificial queen cell cups into the hive in place of the emergency queen cell.

In spite of the industrial policy for mass production of RJ, it must not be forgotten that honeybees are living organisms. Furthermore, they are extremely delicate living organisms due to their high and sophisticated capability and sociality. In order to avoid weakening the colony by reducing its disease resistance due to overloading the honeybees, I have adopted the bee colony rotation system in harvesting RJ. When harvesting, only two queen cell cup frames are inserted and only 60–100 artificial plastic artificial queen cell cups are put into each queen cell cup frame. In this way, it was confirmed that the colony with sufficient feeding becomes powerful: 100 mg of RJ can be harvested from one queen cell cup in 24 h and 300–480 mg in 48 h. This is performed in a rotation system (**Figure 9**). The

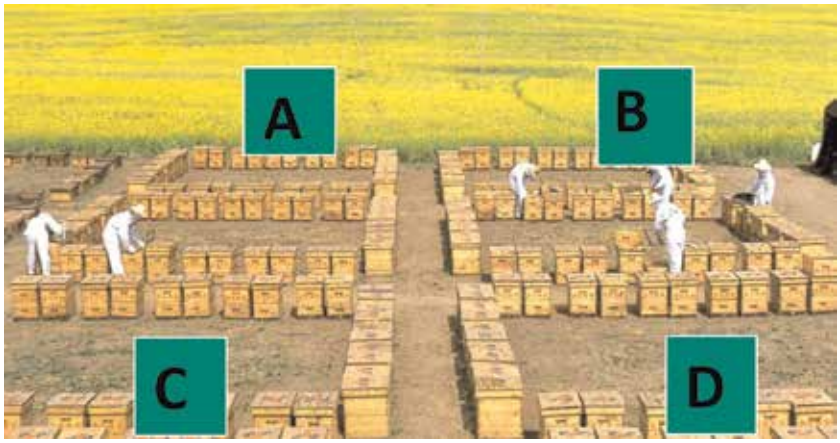

Figure 9.
Rotation of Colony. The colonies are divided into four groups, and the colony used once for larva transfer and harvest is made to rest, and another colony is used for production. This is rotated in sequence at a rate of once every 4 days.

colonies are divided into four groups, and the colony used once for larva transfer and harvest is made to rest, with another colony used for production. This is rotated in sequence once every 4 days. In other words, the colony made to work once will then be given a 2-day rest. Needless to say, a greater amount can be produced when all of the colonies are made to work fully, but this is a fundamentally mistaken policy. Originally, in the method of producing RJ using artificial queen cell cups, the circumstance in which worker bees prepare the emergency queen cell in response to the emergency situation after disappearance of the queen bee was created artificially. In this sense, RJ production is a significant stress on worker bees. Through the rotation of colonies, colony momentum is maintained and the lifespan of worker bees is also prolonged. Most important is that the disease resistance of the colony is increased by this rotation, leading to avoidance of the use of drugs such as antibiotics. Even when the rotation is adopted in RJ production, the colony in the resting state can be dedicated to the collection of nectar and pollen, as the queen cell cup frame is not inserted. Honeybees are insects, and it is impossible to prepare genuine RJ required by consumers unless the ecology and providence of honeybees are followed.

3.3.6 Filtration at apiary and temperature control

The RJ harvested from the beehive should be subjected to primary filtration at the apiary in order to remove bee wings, etc. and is then transported to a processing plant.

It should be recommend to filter the harvested highly-active RJ immediately at the apiary and store it at 2°C under complete protection from sunlight. When RJ comes into contact with oxygen or carbon dioxide in the air or is exposed to ultraviolet light, it quickly becomes less active. It is also known that denature and turn-over of components occur immediately when RJ is left at ordinary temperature. The activities, however, can be maintained for a far long time when stored at low temperature.

Therefore, in the Natural Beekeeping (KYAMENABEE), the harvested RJ is immediately filtered so as to remove foreign matter such as dead bees, hive scum and dust. Although denaturation occurs immediately when left at ordinary temperature, it is known that RJ can remain active for a fairly long time when stored as at 2°C. I recommend filtering the harvested RJ on every harvest so as to remove impurities, followed by temporary storage at 2°C under protection from sunlight and final cryopreservation below −18°C (**Figure 10**).

3.3.7 Storage and transportation

RJ is vulnerable to oxygen and carbon dioxide in the air, while ultraviolet light causes immediate chemical changes leading to loss of activity unless it is shut out immediately after harvest. RJ is also vulnerable to heat and shows denaturation gradually in short period when left at ordinary temperature. Despite the facts, in ordinal beekeeping, at the end of the flowering season, the harvested RJ is finally taken to the processing plant where it is filtered and frozen. In Natural Beekeeping, primary complete filtration is performed at the apiary, and there is no need for a processing plant for filtration.

In ordinal beekeeping, RJ carried from a processing plant to a harbor is once thawed, mixed with old RJ stored in the warehouse, and then frozen again. On exporting to Japan, etc., the freeze/thaw and re-freeze processes are repeated many times over. During these processes, 10-HDA may be added in order to comply with

Figure 10.
Generator and refrigerator. An electric refrigerator combined with gasoline-driven generator was supplied to beekeepers so that the temperature control was easily performed at the site.

the standard value. Under such circumstances, we cannot even hope to receive high-quality RJ. Therefore, in the Natural Beekeeping, the harvested RJ is filtered immediately at the apiary so as to remove foreign matters, and sufficient effort is made to avoid component changes. The RJ is stored at 2°C and cryopreserved at −18°C after packaging. It is frozen only once on this occasion.

Concerning the suitable temperature for storage of RJ (2°C in refrigeration and −18°C in cryopreservation), Smith had already reported in the "Bee World" journal in 1959 that "The harvested RJ must be immediately stored in a refrigerator. One-year storage is probably possible at 2°C. No changes were observed in royal jelly stored at −18°C for several years", and this storage method was established world-wide [23]. Thereafter, the optimal temperature for storage being 2°C was also reported in the German beekeeping journal "Archive Hule Bienenkunde", and Inoue, a Japanese beekeeping researcher [3], stated that "The opinions of the world's researchers are mostly in unison" (cited from "New technology for higher yield of royal jelly").

In 1967, I supplied an electric refrigerator combined with a gasoline-driven generator to Japanese beekeepers so that five beekeepers could share one set. At that time, prevailing wisdom dictated that RJ should be stored at ordinary temperatures under a tent, and the beekeepers resisted the introduction of refrigerators, complaining that they were "troublesome", "owner indistinguishable", "may be stolen", "have questionable security", and so on. I took the following countermeasures: the neat fluids were accommodated in plastic bags with different colors, and the manufacturing site, manufacturer, date and time of harvest, etc. were written on each bag with a magic marker. In other words, attention was paid to perfect traceability even at that point in time. This system was also introduced to the rape blossom bee forages in Qing Hai Haibei Menyuan.

3.4 Major components of RJ obtained by natural beekeeping

Table 2 shows the standard values of major components of RJ proposed by the associations of China, the world's biggest apicultural product-manufacturing country, and Japan. Among the major components for which standard values are specified, 10-HDA is clearly the characteristic fatty acid contained in RJ. However, it is problematic that the quality of RJ is assessed only based on 10-HDA even in the case of poor storage conditions, since 10-HDA is relatively stable regardless of heat and can intentionally be added at a later point in time. I would like to emphasize that MRJP-1 multimer, which is the most abundant protein found in RJ, should be adopted as a new index to assess the quality of RJ in addition to 10-HDA. The reasons for this are that MRJP-1 multimer accounts for the major part (40–60%) of water-soluble proteins (about 75% of soluble proteins) among the entire range of proteins (11–15 g/100 g RJ), it cannot be added artificially, and it is easily decomposed by heating. The RJ obtained by Natural Beekeeping is compared below with that obtained by ordinal beekeeping to show that the former is superior in quality and can maintain the strength of the colony.

3.4.1 Comparison of contents of MRJP-1 Multimer and 10-HDA in RJ between natural beekeeping and ordinal beekeeping

I compared the component contents between the RJ obtained by Natural Beekeeping (performed in the area with good beekeeping environments (Qing Hai Haibei Menyuan, harvested after 48 h) and that obtained by ordinal beekeeping (harvested after 72 h)). This experiment used colonies from which no RJ had been harvested within 1 month before the start of the experiment. After starting the experiment, RJ was harvested by Natural Beekeeping (after 48 h) and ordinal beekeeping (after 72 h).

3.4.1.1 Harvest of RJ

The RJ was harvested according to the method of Natural Beekeeping proposed by me [5, 6]. Basically RJ was prepared in the following manner.

The core of the Natural Beekeeping is respect for the ecology and providence of honeybees. Based on this fundamental recognition, the rearing environments and facilities are arranged in such a way that keeps honeybees healthy and vigorous at all occasions, and the honeybee-rearing methods and apicultural product-manufacturing methods to create RJ and honey of the highest quality are practiced.

The concept of natural beekeeping is as follows: "Specific beekeeping where the nectar plants are natural plants growing wild, the ecology and providence of honeybees are respected and harsh harvesting is not adopted. In addition, no artificial foods are given, and any drugs including antibiotics are not used. Instead, the health of honeybees is controlled with good environments and good rearing management."

The details of the Natural Beekeeping are outlined below:

1. The ecology and providence of honeybees and their society are respected.

2. Honeybees are not abused.

3. Honeybees are fed with natural honey and pollen, and are not given artificial pollen made from sugar water or soybeans.

Item	Natural Beekeeping RJ Specifications[1]	Food Standards and Criteria for RJ[2]	Standards of Japan Royal Jelly Fair Trade Council[3]	National Standards of People's Republic of China[4]	ISO 12824[5]
Description	A yellowish white milky liquid substance with a specific odor, a weak acidic taste and an astringent property	A yellowish white milky liquid substance with a specific odor, a weak acidic taste and an astringent property	Generally, a milky white or light yellow paste-like substance with a specific astringent property and a flavor	**Color tone**: A milky white, pale yellow or pale orange color surely accompanied by a gloss. When frozen, there must be also a cryohydric gloss. **Odor:** In a creamy state, there must be an odor like flower nectar or pollen and an acrimonious odor. The odor must be pure, and there must not be a fermentation odor or a foul odor. **Taste and texture:** In a creamy state, there are a clear acidic taste, a bitter taste, an acrimonious taste and a sweet taste, and the maxilla and throat feel stimulation. When swallowed or spit out, stimulation remains at the throat for a certain period of time. In a frozen state, there is a granular feeling immediately after put into the mouth, but such feeling disappear gradually and the same tastes are felt as in the creamy state. **State:** The creamy royal jelly at ordinary temperature or after thaw has fluidity. It must not be contaminated with foreign matters such as bubbles or wax scum.	Royal jelly is milky white, pale yellow, white luster. It is pasty or jelly-like at room temperature with fluidity and shall be free from bubbles and foreign substances. Minor crystallization phenomena can occur naturally in royal jelly during storage. Odor and Taste: It is pungent, unfermented and shall not be rancescent. It is acerb, spicy and it brings acrid taste to palate and throat.

Item	Natural Beekeeping RJ Specifications[1]	Food Standards and Criteria for RJ[2]	Standards of Japan Royal Jelly Fair Trade Council[3]	National Standards of People's Republic of China[4]	ISO 12824[5]
Water content	Not less than 62.5% and not more than 68.0%	Not less than 62.5% and not more than 68.0%	Not less than 62.5% and not more than 68.5% (Acceptance criterion: Not less than 63.0% and not more than 68.0%)	(Excellent product) Not more than 67.5% (Acceptable product) Not more than 69.0%	Min: 62.0% Max: 68.5%
Crude protein	Not less than 11.0% and not more than 15.5%	Not less than 11.0% and not more than 14.5%	Not less than 12.0% and not more than 15.5%	Not less than 11% and not more than 16%	Min: 11% Max: 18%
10-HDA	**Not less than 2.0%**	**Not less than 1.6%**	**Not less than 1.4% (Acceptance criterion: Not less than 1.6%)**	**(Excellent product) Not less than 1.8% (Acceptable product) Not less than 1.4%**	**Min: 1.4%**
MRJP-1 multimer	**Not less than 5.0%**				
Acidity	Not less than 32 mL and not more than 53 mL of 1 mol/L NaOH for 100 g of royal jelly	Not less than 32 mL and not more than 53 mL of 1 mol/L NaOH for 100 g of royal jelly	Not less than 32.0 mL and not more than 53.0 mL of 1 mol/L NaOH for 100 g of royal jelly	Not less than 30 mL and not more than 53 mL of 1 mol/L NaOH for 100 g of royal jelly	Min: 30.0 mL Max: 53.0 mL
Total sugar				Not more than 15%	Min: 7% Max: 18%
Fructose					2–9%
Glucose					2–9%
Sucrose					Type 1: <3.0% Type 2: Na[6]
Erose					Type 1: <0.5% Type 2: Na
Maltose					Type 1: <1.5% Type 2: Na
Maltotriose					Type 1: <0.5% Type 2: Na

Item	Natural Beekeeping RJ Specifications[1]	Food Standards and Criteria for RJ[2]	Standards of Japan Royal Jelly Fair Trade Council[3]	National Standards of People's Republic of China[4]	ISO 12824[5]
Ash				Not more than 1.5%	
Starch				No detection	
Total lipid					2–8%
C13/C12 Isotopic ratio(δ%)					Type 1: −29 to −20 Type 2: −29 to −14
Viable microbe count	Not more than 500 cfu/g	Not more than 500 cfu/g	Not more than 500 cfu/g		Max: 500
E. coli	Negative	Negative	Negative		
Enterobacteriaceae					Absent in 10 g
Salmonella					Absent in 25 g
Heavy metals	Not more than 20 ppm as Pb	Not more than 20 ppm as Pb			
Arsenic	Not more than 2 ppm as As	Not more than 2 ppm as As			
Residual agrochemical	HC, DDT or dieldrin family must not be detected.	HC, DDT or dieldrin family must not be detected.			
Antibiotic	Tetracycline family, chloramphenicol or streptomycin must not be detected.	Tetracycline family, chloramphenicol or streptomycin must not be detected.			

[1]*Specifications of royal jelly harvested by the Natural Beekeeping (acceptance criteria).*
[2]*Japan Health and Nutrition Food Association.*
[3]*From "Practice Rules for Fair Competition Code Related to Display of Royal Jelly" of Japan Royal Jelly Fair Trade Council.*
[4]*From "GB/T9697-2002 Amendment".*
[5]*ISO 12824 (2016/09/15).*

Table 2.
Quality standards and criteria for native RJ.

4. Watering trays for honeybees are set around the apiary and the beehive.

5. Harvested honey is always stored in a cool, dark place.

6. Second-instar larvae are used for larva transfer to artificial queen cell cups for RJ production.

7. RJ is stored in a refrigerator set at 2°C.

8. The number of artificial queen cell cups is to be 100 at most.

9. The work rotation rate of honeybees is to be set at a constant 25%.

10. Both honey and RJ are always filtered at the apiary.

11. The old beehive and hive frame are disposed of, and new ones are used.

12. Absolutely no antibiotics are used.

13. In principle, honey is prepared from a single nectar plant.

14. For harvesting honey, "morning squeeze" is performed instead of "evening squeeze", and honey matured in the hive cells is harvested. Only the fourth and later crops of high purity are released as product.

15. Concentration by heating is not performed.

In Qing hai Haibei Menyuan, RJ was harvested from rape blossoms (almost in full bloom) using Occidental honeybees (*Apis mellifera*) for 15 days from July 10–24, 2010.

For each sample, 60 g was harvested as follows:

1. Natural Beekeeping (rotation harvest giving a rest to honeybees): Using 96 artificial queen cell cups in each colony, second-instar larvae were grafted into artificial queen cell cups. RJ was harvested 48 h after larva transfer. After giving a two-day (48-h) rest to the honeybees, larva transfer was performed, and harvest was performed again after 48 h. Similarly, a two-day (48-h) rest and harvest after 48 h were performed again, and the RJ obtained in the third harvest after 48 h was used for analysis.

2. Ordinal beekeeping (continuous harvest giving no rest to honeybees): Using 96 artificial queen cell cups in each colony, second-instar larvae were grafted into artificial queen cell cups. RJ was harvested 72 h after larva transfer. Immediately after harvest, larva transfer was performed and harvest was performed again after 72 h. Similarly, the same procedures were performed again, and the RJ obtained in the third harvest after 72 h was used for analysis.

3.4.1.2 Analysis of 10-HDA in RJ

10-Hydroxy 2-decenoic acid (10-HAD) belongs to the important physiologically active components of RJ. Analysis of 10-HDA in RJ was performed by the ordinary method (Japan Health And Nutrition Food Association, 2012).

3.4.1.2.1 Preparation of sample solution

After homogenizing the sample RJ, 0.5 g of RJ was weighed accurately into a 200 ml beaker. Water 80 ml was added and the mixture was stirred vigorously until a uniform suspension was obtained. Next, methanol 80 ml was added, and the mixture was further stirred for 20 min and transferred into a 200 ml measuring flask. The beaker was washed with a mixture of water and methanol (1:1), and the washing was added to the 200-ml measuring flask. The mixture of water and methanol (1:1) was added to the measuring flask to make exactly 200 ml. The resulting fluid was filtered with a membrane filter 0.45 μm in pore size, and the filtrate was used as the sample solution.

3.4.1.2.2 Preparation of standard solution

Exactly 0.01 g of 10-HDA Reference Standard was weighed accurately and dissolved in methanol to make 50 ml. Exactly 5 ml of this solution was added with the mixture of water and methanol (1:1) to make 20 ml. The resulting solution was used as the standard solution.

3.4.1.2.3 Quantitative analysis

The test was performed with 10 μl each of the sample solution and the standard solution as directed under the Liquid Chromatography in line with the following conditions. The areas of 10-HDA peaks from both solutions, At and As, were determined, and the amount of 10-HDA in the sample was calculated according to the following formula.

$$\begin{aligned}&\text{Amount (mg) of } 10-\text{HDA in 100 g of sample}\\&\quad=\text{weighed amount (mg) of } 10-\text{HDA Reference Standard}\times \text{At/As}\\&\qquad\times 100\ \text{g/weighed amount (g) of sample}\end{aligned}$$

where As is the area of 10-HDA peak from the standard solution and At is the area of 10-HDA peak from the sample solution.

3.4.1.2.4 HPLC operating conditions

Detector: An ultraviolet absorption photometer (wavelength: 210 nm).
Column: An ODS column (4.6 mm^{Φ} × 150 mm).
Column temperature: A constant temperature of about 40°C.
Mobile phase: A mixture of diluted phosphoric acid (1 in 1000) and methanol (1:1).
Flow rate: Adjusted so that the retention time of 10-HDA was in the range of 14–16 min.
Reproducibility: When the test was repeated six times with the standard solution in line with the above conditions described above, the relative standard deviation of 10-HDA peak areas was not more than 2.0%.

3.4.1.3 Analysis of MRJP-1 Multimer in RJ

MRJP-1 is a major protein of RJ. It is a multifunctional pharmaceutically important protein [24–26] and serves as a marker of the authenticity and quality of honeybee products [27, 28]. The analysis of MRJP-1 multimer in RJ was performed by the method reported previously (partially modified) [29].

The RJ was suspended in water. The supernatant obtained after centrifugation of the suspension was subjected to HPLC analysis.

3.4.1.3.1 Preparation of sample solution

Exactly 3 g of RJ kept at −18°C was weighed, thawed at room temperature and diluted with water to make 100 ml. This suspension was stirred well at room temperature for 30 min and then centrifuged for 30 min at 10,000×g at 4°C. The supernatant was made to 100 ml using a measuring flask, and 1 ml of the resulting solution was filtered with a PVDF filter 0.22 μm in pore size (Ultrafree-MC, MILLIPORE). The filtrate was used for HPLC analysis.

3.4.1.3.2 HPLC operating conditions

Column: TSK-gel G3000SW (7.5 mm^{Φ} × 60 cm: Toso).
Mobile phase: 0.1 M Sodium phosphate buffer (pH 7.0) + 0.1 M Na_2SO_4.
Flow rate: 0.6 ml/min.
Amount injected: 10 μl.
Detector: UV detector (wavelength: 280 nm).
Column temperature: Room temperature.

3.4.1.3.3 Calibration line reparation with MRJP-1 multimer standard solutions

The test was performed with the MRJP-1 multimer standard solutions in line with the "HPLC operating conditions", and the calibration line was prepared from the relation between the peak area and protein concentration (examples of concentration: 0.25, 0.5, 1 and 2 mg/ml).

3.4.1.3.4 Calculation of MRJP-1 multimer content

Content (%) of MRJP-1 multimer in RJ = concentration (mg/ml) of MRJP-1 multimer obtained from calibration line × 1/1000 × 100 (ml)/3(g) × 100.

3.4.1.4 Analytical results of MRJP-1 Multimer and 10-HDA in RJ

As shown in **Figure 11**, the results of comparing the content of MRJP-1 multimer and 10-HDA in RJ between Natural Beekeeping (harvested after 48 h, with rotation) and ordinal beekeeping (harvested after 72 h, without rotation).

The mean content of MRJP-1 multimer was 5.72 ± 0.21% (n = 9) in the RJ obtained by Natural Beekeeping but 4.77 ± 0.35% (n = 9) in the RJ obtained by ordinal beekeeping, being significantly lower in the latter. Even when compared in unfavorable beekeeping environments, it was confirmed that there were the same level of differences (15–20%) (data not shown).

The mean content of 10-HDA was 2.9 ± 0.2% (n = 9) in the RJ obtained by Natural Beekeeping but 2.5 ± 0.1% (n = 9) in the RJ obtained by ordinal beekeeping, being significantly lower in the latter. Even when compared in unfavorable beekeeping environments, it was confirmed that there were the same level of differences (15–20%) (data not shown).

3.4.1.5 Discussion and summary

The results indicate that the quality of RJ may decrease according to the time passage until harvest even in the condition in which fresh RJ is successively supplied

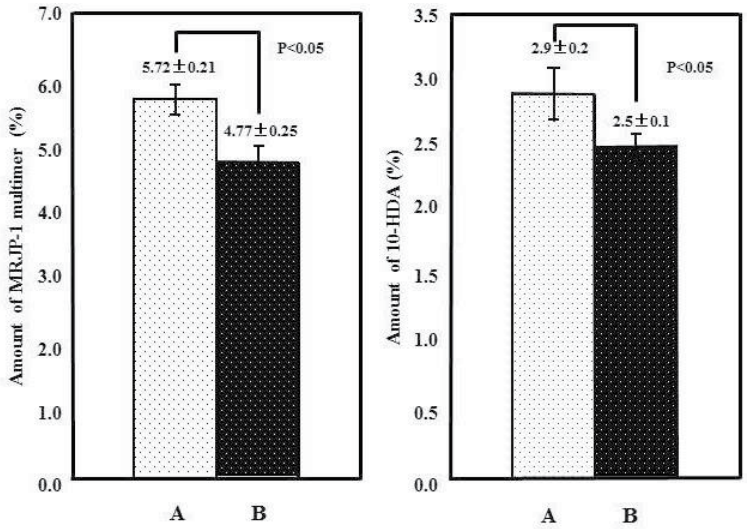

Figure 11.
Comparison of MRJP-1 Multimer and 10-HDA contents in RJ samples between natural beekeeping (A) and ordinal beekeeping (B) [26]. A: Natural beekeeping (harvested after 48 h, with rotation, n = 9); B: Ordinal beekeeping (harvested after 72 h, without rotation, n = 9).

to queen cells by worker bees. The results also support the opinion that RJ harvested on 48 h after queen cell setting provides higher quality than 72 h-harvested RJ.

Moreover, MRJP-1 contents among the RJ samples were compared with or without rotation of RJ production by worker bees and the results are shown in **Figure 11**. The MRJP-1 content was significantly higher in 48-h-harvested RJ ($5.72 \pm 0.21\%$) by worker bees rested 2 days before the RJ production than in 72-h-harvested RJ ($4.77 \pm 0.25\%$), which was successively produced by worker bees without resting rotation ($p < 0.05$). The amount of 10-HDA in these RJ samples were also compared in **Figure 11** and was also found to decrease in 72-h-harvested RJ without resting rotation (48-h-harvested RJ, $2.9 \pm 0.2\%$; 72-h-harvested RJ, $2.5 \pm 0.1\%$; $p < 0.05$). The author has been emphasized in his proposal on natural beekeeping that health of worker bees should be guaranteed by rotation of beehives employed for RJ production, as well as by limiting number of artificial queen cells per colony. The results also support his proposal for production of high-quality RJ.

3.4.2 Reasons why 10-HDA and MRJP-1 multimer are suitable to quality assessment of RJ

10-HDA is the only functional component used for the quality control of RJ (**Table 2**). The author strongly agrees that 10-HDA should be used as the quality control of RJ. Unfortunately, since authentic compound is available and easily added to the product, 10-HDA does not play an important role in the evaluation of RJ. Thus, I proposed one more functional component, MRJP-1 multimer, because this is also a compound only produced by honeybees [28, 30] and it too requires exacting conditions in order to maintain freshness. MRJP-1 is found in a large amount, ca. 60% of soluble protein in RJ, which makes it easy to use as a determining factor. MRJP-1 multimer decomposes easily at high temperature, such as 30°C and higher, over a period of 2 weeks (**Figure 12**) [26].

The transitional change of MRJP-1 multimer contents stored under different temperatures is shown in **Figure 12**. The MRJP-1 multimer contents decreased depending on temperature and period of storage, with a reduction of about 50% in RJ

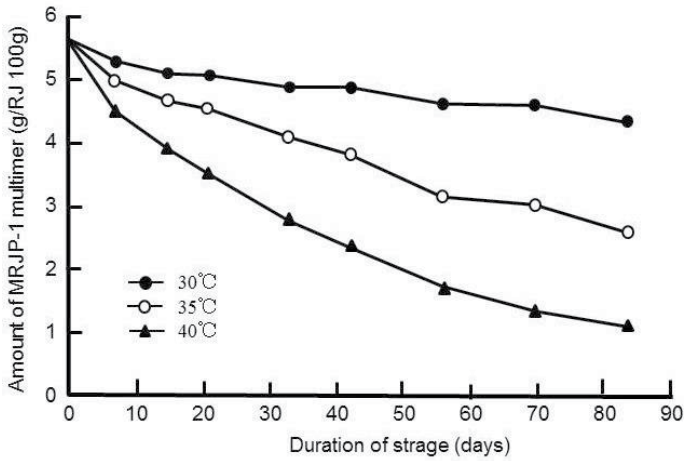

Figure 12.
Transitional change of MRJP-1 multimer contents stored under different temperature [26].

samples stored at 35°C for 90 days and as much as an 80% reduction after storage at 40°C for 90 days. Furusawa et al. [31], also reported that apisin (MRJP-1 multimer) content in RJ was also decreased by approximately 80% at 40°C after 32 days.

Kamakura et al. also proposed 57-kDa protein in RJ as a possible marker for freshness [29]. MRJP-1 monomer has molecular weight of 55 kDa and is the component of MRJP-1 multimer (pentamer or hexamer depending upon the report). Kamakura reported that 57-kDa protein is identical to MRJP-1 monomer.

Among the proteins found in RJ, including MRJP-1 to MRJP-9 and other specific proteins, MRJP-1 multimer and MRJP-1 monomer were the compounds capable of bearing thermolability. Li et al. reported that quantity of MRJP-1 decreased significantly following the temperature trend in 2D-PAGE, MALDI TOF/TOF MS images, but MRJP-2 and MRJP-3 did not increase or decrease following the temperature trend [32]. However, MRJP-4, 5, glucose oxidase, peroxiredoxin, and glutathione S-transferase S1 were clearly absent in all images in samples held at room temperature for 1 year. I indicated that as MRJP-1 multimer was unstable at room temperature within short period, MRJP-1 multimer might be the substance for use as the possible marker for freshness. Although MRJP-4 and MRJP-5 should be studied further, I believe that MRJP-1 multimer is the compound that should be used as the marker for the quality control. Takenaka et al. also reported that the lowering of glucose oxidase activity was found and almost disappeared within 120 days at room temperature in the dark. However, the amount of 10-HDA, 10-hydroxydecanoic acid, and gluconic acid in RJ were constant regardless of the storage condition [33]. The author found that MRJP-1 multimer was stable at 2°C or −18°C. According to the most recent findings, the core structure of the MRJP1 multimer consists of four molecules of MRJP1, four molecules of the peptide apisimin [34] and, surprisingly, eight molecules of 24-methylenecholesterol [35, 36].

Since MRJP-1 multimer or MRJP-1 monomer cannot be chemically synthesized, and MRJP-1 monomer is unstable at even at room temperature, MRJP-1 multimer is exactly the right compound for the quality control of RJ in addition to 10-HDA [37].

3.4.3 Significance of production of natural RJ utilizing artificial queen cell cups

As mentioned above in detail, the modern beekeeping industry ignores the high-level biological functions possessed originally by honeybees; emphasizes quantity

over quality and seeks cheapness with a production-first policy and cost-first policy in pursuit of mass production; and is causing weakened colonies and shortened lifespans through an artificial mating process called "selective breeding". Especially in China, the honeybees dedicated for royal jelly production tend to be used after "selective breeding" and even the 10-HDA content has been decreasing in recent times. In this context, it is apparent that society's confidence in apicultural products including royal jelly will soon be lost and the apicultural industry will enter a course of decline.

Accordingly, I have performed research and practices to solve the various problems for the purpose of contributing to development of the apicultural industry. As a result, I have reached the conclusion that only natural beekeeping producing true apicultural products can protect the quality of apicultural products including royal jelly and can also contribute to the health of humankind. It is needless to say that such natural beekeeping will eventually also improve beekeepers' standards of living.

I have practiced natural beekeeping based on the original ecology of honeybees in Qing hai Menyuan, China over 20 years since 1993. It is necessary to request beekeepers' cooperation for production of excellent royal jelly of a high quality. Especially when harvesting RJ after 48 h, the frequency of harvest operation is increased from once every 3 days to once every 2 days in comparison with harvesting after 72 h. Furthermore, the amount produced when harvesting after 48 h is half of the amount produced when harvesting after 72 h. In other words, the method proposed by me leads to intensification of labor and decreased production efficiency. In addition, in conventional beekeeping, 200–300 artificial queen cell cups are set in one comb for RJ secretion, while in natural beekeeping the number of artificial queen cell cups is limited to not more than 100. The Natural Beekeeping therefore met with stiff opposition due to its resultant low production efficiency. Nevertheless, the author persuaded the beekeepers with a passion for production of the world's leading apicultural products. The philosophy of "high quality, high price" was explained to the beekeepers. Production was encouraged with the basic principle that "only high-quality products can be sold at high prices", and as a result, natural beekeeping has been able to give plenitude to beekeepers' lives and contribute to the improvement of their standards of living. This achievement is of great significance.

Looking at the state of RJ production in 2011, the abnormally dry weather was influential, but the high rate of inflation in China was more influential. Migratory beekeepers have to carry their beehives to distant bee forage areas by chartering trucks, while there was also a steep increase in transportation fees. Furthermore, larva transfer is an important job in RJ production. Since it is difficult for the old beekeepers themselves to perform these operations, young persons are hired and instructed regarding how to perform the operations in the dark under a tent. Beekeepers should be able to live comfortably by realizing the true philosophy of "high quality, high price". Otherwise, it is feared that the apicultural industry will enter a course of decline.

3.5 Biological effect of RJ

3.5.1 Effect of RJ on growth of larvae

The growth of larvae cultured in RJ on 3 and 6 days after the cultivation is shown in **Figure 13** [26]. The graph shows the results with RJ harvested at 48 and 72 h after transfer of queen cells. It was found that the weight of larvae had increased rapidly by more than seven times within 3 days. The increase of body weight, however, became slowed to a factor of only 1.2–1.4 over the following 3 days.

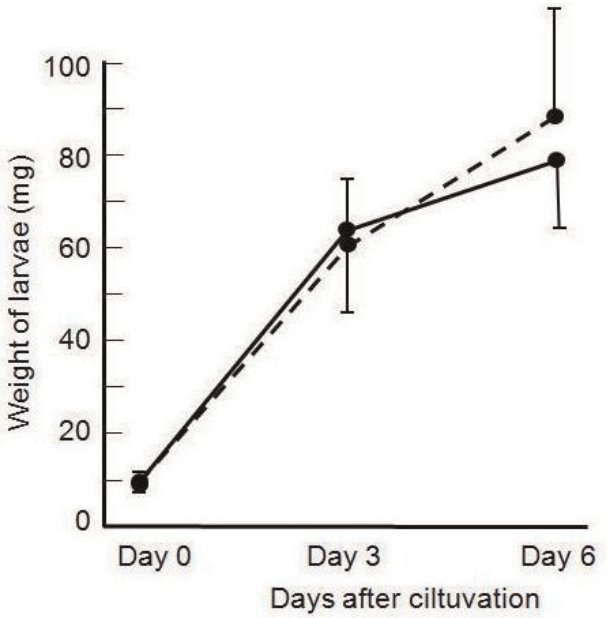

Figure 13.
The growth of larvae in RJ in vitro. Second-instar worker bee larvae were grafted in a 12-well flat-bottomed plastic plate filled with 750 mg fresh RJ. The plate was then set in an incubation chamber and cultured at 31°C for appropriate period. Dotted line: RJ harvested at 48 h. Solid line: RJ harvested on 72 h.

The growth of larvae at Day 0, Day 3 and Day 6 is also represented in **Figure 14**. It was also confirmed that the larvae grew rapidly in the RJ in vitro [26].

3.5.2 Determination of component participating in larval growth

In order to ascertain the RJ component responsible for larval growth [27], compositions of proteins and 10-HDA (considered to be a tool for functional ingredients for activity) were compared during the cultivation.

As shown in **Figure 15**, the contents of total proteins decreased during the cultivation, whereas there was a slight change in the percentage of 10-HDA. These results indicate the possibility that proteins are consumed by the larvae as they grow.

In order to further confirm the result, elution profiles of soluble RJ proteins by size-exclusion HPLC on Superose 12 column were compared during cultivation. As

Figure 14.
Representative data of larval growth cultured in RJ in vitro. Second-instar worker bee larvae were grafted in a 12-well flat-bottomed plastic plate filled with 750 mg fresh RJ. The plate was then set in an incubation chamber and cultured at 31°C for the appropriate period of time.

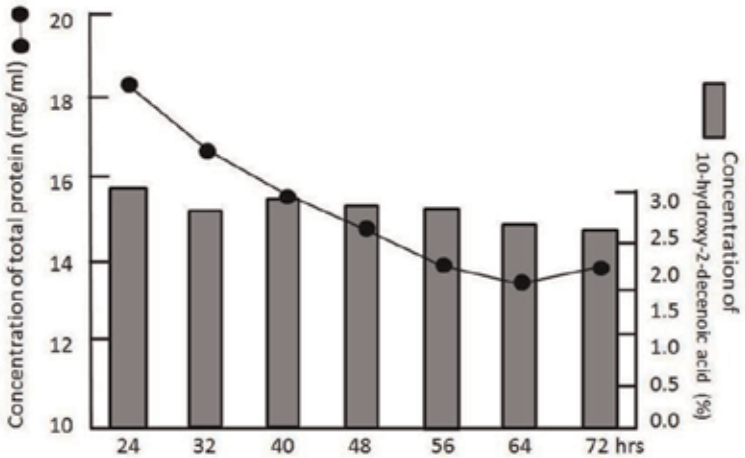

Figure 15.
Changes to major components, total protein and 10-HDA in RJ during the cultivation of larval growth in vitro. The total protein and 10-HDA in RJ were analyzed in a chamber filled with 750 ml of fresh RJ and grafted second-instar worker bee larvae. Total protein and 10-HDA were analyzed for the indicated periods.

shown in **Figure 16**, crude soluble RJ proteins were separated as five peaks, although Peak 1 and Peak 2 overlapped. Each peak was estimated at about 640 kDa (Peak 1), 360 kDa (Peak 2), 100 kDa (Peak 3), 72 kDa (Peak 4), and 4.5 kDa (Peak 5) in their molecular size, respectively. These five peaks were universally detected in all RJ samples examined. The peak 2 protein was considered to be oligomeric form, MRJP-1 multimer (**Figure 17**).

In order to further confirm the participation of Peak 2 protein (MRJP-1 multimer) in larval growth, the body weight of larvae cultured in RJ was compared with RJ samples that had different content of Peak 2 proteins.

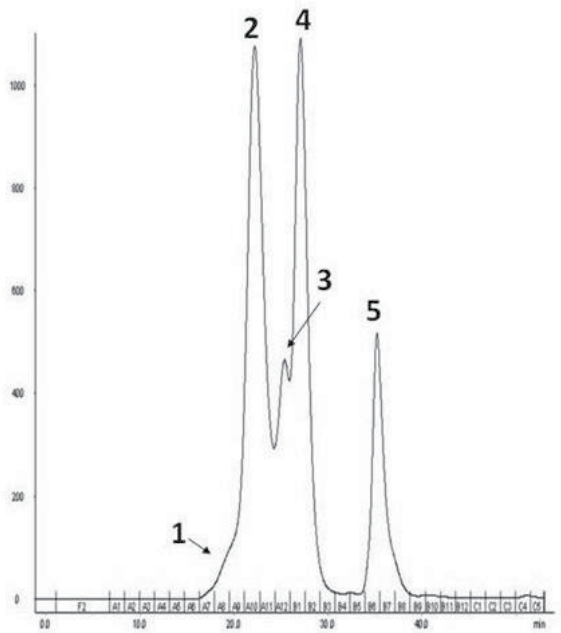

Figure 16.
Elution profile of soluble protein in RJ. A typical elution pattern of soluble RJ proteins. Peak 2 represents MRJP-1 multimer.

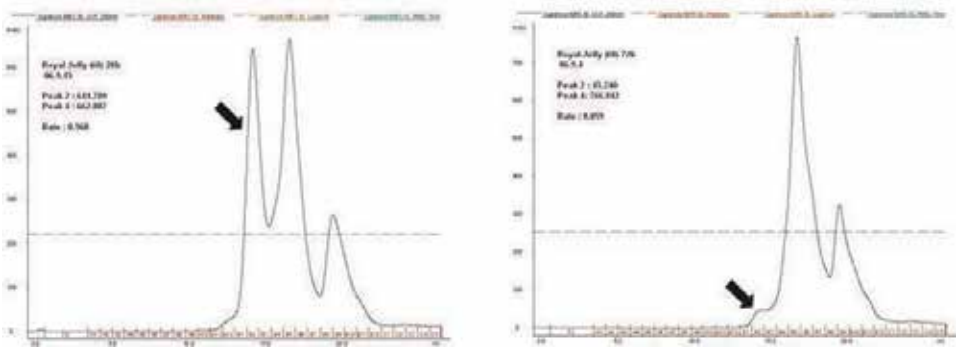

Figure 17.
A case observed where peak 2 disappeared in 72-h cultivation. An unusual case where peak 2 disappeared at 72-h cultivation.

Although crude soluble RJ proteins are distinguished in five peaks, the level of Peak 2 (MRJP-1 multimer) varied greatly in some RJ samples, as seen in **Figure 18A**.

The growth of larvae between RJ samples with high and low Peak 2 level was compared and the results are expressed in **Figure 18B**. The growth of larvae was greater in the Peak 2-rich RJ samples than in samples with poor Peak 2 poor. These results further confirmed that the Peak 2 proteins are a key substance in the growth and development of honey bee queens.

3.5.3 The content of peak 2 protein (MRJP-1 Multimer) in queen cell and its stability

During the study of Peak 2 protein, I confirmed that this protein is a MRJP-1 multimer [26].

Next, the transitional change of MRJP-1 multimer content was monitored in artificial queen cells in order to investigate the relation between MRJP-1 multimer contents and the duration from setting of the queen cells to the harvesting of RJ. As seen in **Figure 19**, MRJP-1 multimer contents decreased gradually over time after setting the queen cells. In the previous experiment in which larvae were cultured in a plastic plate filled with RJ, the author postulated that MRJP-1 multimer was consumed and decreased as larvae grew (**Figure 17**).

Although a remarkable decrease of MRJP-1 multimer as in the previous experiment was not observed, the MRJP-1 multimer content was found to decrease according to the larval growth, that is, time elapsed after queen cell setting, due to the addition of fresh RJ by worker bees.

The MRJP-1 multimer content was shown to decrease by about 30% decrease 74 h after larval graft to queen cells. The results indicate that the quality of RJ may decrease together with the passing of time until harvest even under conditions in which fresh RJ is successively appended to queen cells by worker bees. The results also support the view that RJ harvested 48 h after queen cell setting provides higher quality than RJ that is harvested after 72 h. Moreover, MRJP-1 multimer contents among the RJ samples were compared with and without rotation of RJ production by worker bees, and the results are shown in **Figure 11**. The MRJP-1 multimer content was significantly higher in 48 h-harvested RJ by worker bees rested 2 days before the RJ production than in 72 h-harvested RJ successively produced by worker bees without resting rotation ($p < 0.05$). The amount of 10-HDA in these RJ samples was also compared in **Figure 11** and it was found that 10-HDA content was significantly higher in 48-h harvested RJ than in 72 h-harvested RJ ($p < 0.05$). I have emphasized in the author's proposal on natural beekeeping that the health

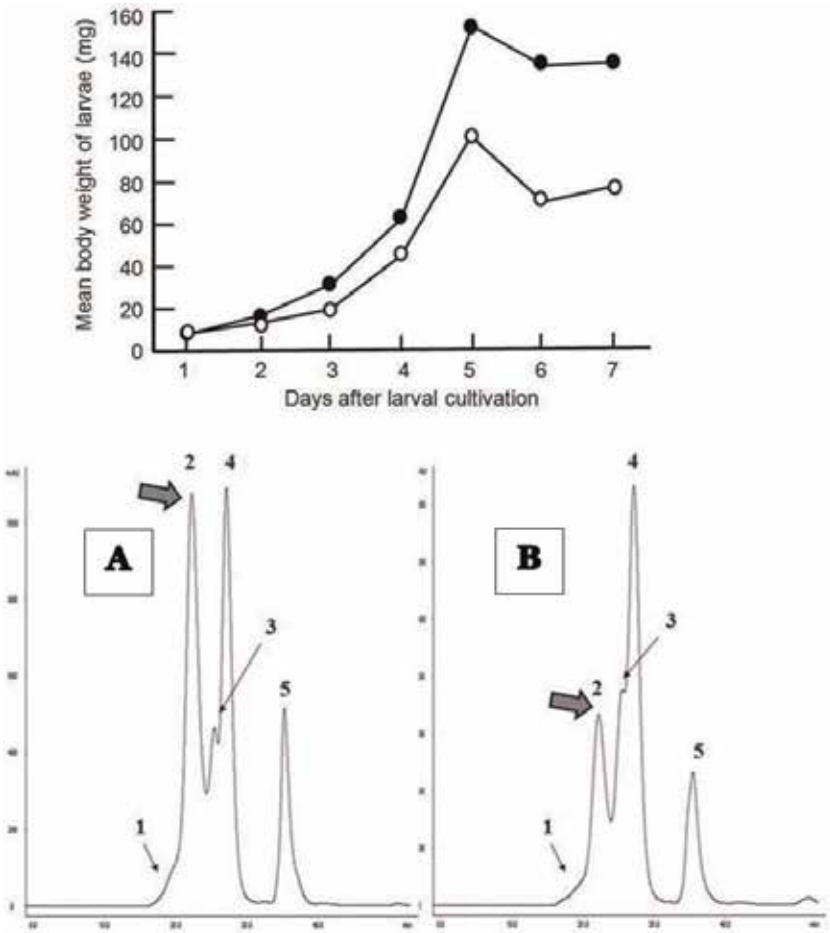

Figure 18.
Larval growth in MRJP-1 Multimer-rich and MRJP-1 Multimer-poor samples of RJ. Larval growth and body weight of larvae cultured in RJ were compared among RJ samples which were different in content of peak 2 proteins. (A) Growth of larvae in MRJP-1 multimer rich RJ (●) and MRJP-1 poor RJ (○), which correspond to the right and left figures in (B), respectively.

of worker bees should be guaranteed by rotating the beehives employed for RJ production, as well as by limiting the number of artificial queen cells per colony. The results also support my proposal for production of high-quality RJ.

3.5.4 Discussion and summary

Since the essential function of RJ is to produce larval growth and ensure differential development of a queen bee, development of larvae in RJ was investigated and components participating in the function were identified. The proteins, especially Peak 2 protein (MRJP-1 multimer), diminished as the larva grew. The growth of larvae was better in the Peak 2-rich RJ samples than in the Peak 2-poor RJ, indicating that the Peak 2 protein (MRJP-1 multimer) might be the most important substance of RJ. The results suggest that the quality of RJ may change by different period for harvesting and size/age of larvae transferred. In addition, the results suggest the possibility that the content of Peak 2 protein (MRJP-1 multimer) differs depending on the size of larvae transferred and the duration from larval transfer to harvest of RJ, and that the quality of RJ is not necessarily uniform among samples produced under varying conditions.

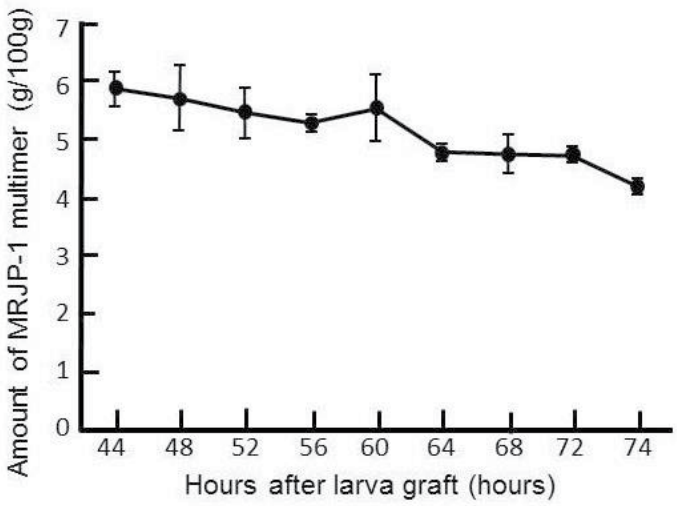

Figure 19.
MRJP-1 Multimer content in queen cells after larval graft.

I demonstrated that the larval growth of honeybees was largely affected by MRJP-1 multimer content. I also proved that the content of MRJP-1 multimer varied predominantly among the soluble RJ proteins by each sample produced under various conditions. The variation of MRJP-1 multimer content is largely affected by the manner of RJ production, such as rotation of bee colonies employed for RJ production, harvesting 48 h after setting queen cells, appropriate storage at low temperature, and so on. The results show that naturally well-controlled bee culture should be promoted for sustainable innovation in modern beekeeping to guarantee high quality levels for RJ and other bee products. Based on these views, the author proposed that the MRJP-1 multimer content in RJ should be used as a new criterion for functional quality evaluation and as a freshness parameter for RJ, in addition to the use of 10-HDA content.

4. Conclusion

Based on my experience and practice of beekeeping for more than 54 years, the author has postulated many serious problems in recent beekeeping, which should be resolved for sustainable development of industrial beekeeping in the future. The core problems found in modern beekeeping include beekeeping in inappropriate environments, deterioration of colonies due to overloading of production and excessive selective breeding, reduced disease resistance, inappropriate processing, insufficient attention paid to quality control of apicultural products, and so on. Other serious problems also include the deterioration of bee products due to incorrect treatment and inadequate environments for beekeeping, which leads to pollution of bee products due to beekeepers' lack of attention to quality control and the added value of bee products.

In order to resolve these problems, the author proposed that it is essential to recourse to natural organic beekeeping using the natural ability of honey bees, and to make efforts to produce high-quality products by means such as maintenance of appropriate apiary location to prevent pollution through nectar source and water supply, strengthening the activity and ability of honey bees by rotation of beehive

employment, and edification for production of quality-added products and related quality control.

On the other hand, beekeeping is primarily an agricultural industry, so it is impossible for beekeeping to ignore the aspect of gaining profits from bee products. From this point of view, the author proposed an ideal situation for natural organic beekeeping, based on the idea that this may result in production of high-quality bee products with added value (albeit with less focus on profitability), thereby increasing revenue and guaranteeing the sustainability of beekeeping that has future potential. Thus, the author proposed a novel method, Natural Beekeeping, based on the principle of natural beekeeping.

The functionality and components participating in the function of RJ products produced by this method were studied, and several interesting results were obtained.

Since the essential function of RJ is to produce larval growth and ensure differential development of a queen bee, development of larvae in RJ was investigated and components participating in the function were identified. The proteins, especially MRJP-1 multimer, diminished as the larva grew. The growth of larvae was better in the MRJP-1 multimer-rich RJ samples than in the MRJP-1 multimer-poor RJ, indicating that the MRJP-1 multimer might be the most important substance of RJ. The quality of RJ may change by different period for harvesting and size/age of larvae transferred. In addition, the content of MRJP-1 multimer differs depending on the size of larvae transferred and the duration from larval transfer to harvest of RJ.

The larval growth of honeybees was largely affected by MRJP-1 multimer content. The content of MRJP-1 multimer varied predominantly among the soluble RJ proteins by each sample produced under various conditions, such as rotation of bee colonies employed for RJ production, harvesting 48 h after setting queen cells, appropriate storage at low temperature, and so on. Naturally well-controlled bee culture should be promoted for sustainable innovation in modern beekeeping to guarantee high quality levels for RJ and other bee products. Also the MRJP-1 multimer content in RJ should be used as a new criterion for functional quality evaluation and as a freshness parameter for RJ, in addition to the use of 10-HDA content.

In conclusion the content of 10HDA and MRJP1 multimer in RJ prepared by Kikuji Yamaguchi Method of Natural Beekeeping (KYAMENABEE) were significantly higher than that prepared by ordinal beekeeping. The biological and pharmacological activities were also superior for RJ prepared by KYAMENABEE than that by ordinal beekeeping. Thus, it might be important to use a novel beekeeping method, KYAMENABEE, in order to produce high quality RJ for sustainable development of biopharmaceutical beekeeping.

Acknowledgements

I am deeply grateful to Prof. Jozef Šimúth and Dr. Katarina Bíliková, Institute of Forest Ecology, Slovak Academy of Sciences, Zvolen, Department of Molecular Apidology, Bratislava, Slovakia, for their help and valuable suggestions, and Professor Emeritus Akira Fujii, Nihon University, Japan, for his suggestion and help in the preparation of this chapter. I would also like express my sincere thanks to my colleagues, especially to my son Mr. Yoshihisa Yamaguchi, and Dr. Kiyoshi Murata, Ms. Michiyo Takahashi, Mr. Masaki Sakai, Mr. Masakuni Shiomi for their endless help.

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Chapter 9

Diagnostic Radioentomology

Mark Greco

Abstract

Apart from the Neotropical flesh eating Trigona species, all existing bees are pollen feeding. Approximately 5% of these form colonies. In honeybees, colony health is evaluated by measuring seasonal hive weight increases and by visual inspections. However, rather than indicating good colony health, hive weight increases can be attributed to increases in stores from foragers feeding precociously during times of colony stress. Additionally, the subjective nature of these methods, leads to large errors. Visual inspections with stingless bee colonies are particularly invasive. Many bees die during inspections because they drown in spilt honey. Re-sealing the hive also kills bees, and the queen risks being squashed. Nevertheless, studies on bees continue as new, improved methods emerge to replace the old. Diagnostic Radioentomology is an innovative, non-invasive, imaging method for studying insects. Since development, it has been adopted by universities, synchrotron facilities and CT scanners to study morphology, physiology and behaviour of insects and has been hailed as the 'Gold Standard' for honeybee monitoring. In 2008, it was described as an emerging non-invasive technique for behavioural, evolutionary and classical biologists who choose to study animals without harming them. This chapter describes methods and includes examples of research conducted using Diagnostic Radioentomology.

Keywords: Diagnostic Radioentomology, bees, non-invasive imaging, X-rays, anatomy, physiology, behaviour, nest architecture, CT scanning, tomography

1. Introduction

Nearly all existing species of bee are pollen feeding, aculeate (with a stinger) Hymenopterans (membranous wings). There are only three species that do not collect or eat pollen and these are the necrophagic (eat dead or decaying flesh) Neotropical, Trigona species [1–4]. Approximately 5% of all bees are highly social. The highly social bees include species of bumble bees, honey bees and stingless bees. The other species are either semi-social, which occur in aggregations or are solitary. For a full description of these terms, see [5, 6]. With honeybees, colony health has been traditionally evaluated by simple visual inspections and/or by measuring changes in hive weights over time. However, these methods are estimates and subjective. Typically, beekeepers and scientists look for behavioural signs which indicate healthy individuals or colonies, where foragers are regularly bringing in resources. In contrast, they look for weak colonies, where there are usually fewer foragers. These foragers typically exhibit a more lethargic and less purposeful behaviour. However, there are also situations where increases in hive weights can be attributed to increases in pollen and nectar stores due to hyper-collection by foragers that exhibit precocious feeding during times of colony stress [7].

These colonies give the false impression that all is well. In these situations, an increase in hive weight can be misinterpreted as a sign of good colony health yet the colony could be under considerable stress from infection or disease rather than being in good health.

Visual inspections on colonies of stingless bees is particularly invasive because of the central location of the brood and many species are less than 3 mm in size. In stingless bees, colony health can be assessed by manually splitting the hive box apart to view internal structures and any evidence of queen activity [8]. Opening the hive for such inspections invariably damages honey storage pots. This causes honey to spill and many hundreds of bees die because many species are diminutive and will drown in their own spilt honey. Closing the hive after visual inspection also kills bees, and places the queen at risk of being harmed because they can be squashed in the process.

Therefore, the subjective nature of visual inspections and hive weight estimations often leads to errors when assessing colony health. Issues such as these were not so important in previous decades however, with the continued pressure from large scale agriculture, loss of bee habitat and the global increase in bee pathogens and pests [9] it has become paramount that new and more accurate methods are developed.

It is vital that behavioural, morphological and physiological studies on bees continue. However, because they have propensities to live in cavities and traditional methods are often invasive and prone to large errors, new methods for studying them are emerging. These new methods will add accuracy to current estimates on individual bees and colony health parameters which will, in turn, enable better solutions for scientists and beekeepers to improve bee health globally.

This chapter describes one new method termed 'Diagnostic Radioentomology' and includes examples of research conducted using this method.

1.1 Non-invasive imaging

On the 8th of November 1895, Wilhelm Conrad Röntgen (accidentally) discovered an image cast from one of his cathode ray generators. He later repeated the experiment by taking an X-ray photograph of his wife Anna Bertha Ludwig's hand **Figure 1**, which revealed the bones in her hand and her wedding ring on one of her fingers.

The photograph initiated great scientific interest in the new found radiation and because Röntgen did not know what type of radiation it was, he called it 'X-radiation', hence the modern term, X-rays. In general, non-invasive imaging is associated with X-rays or medical imaging, which is a non-invasive method for evaluating anatomy and physiology. Although it is now known that X-rays can be invasive (and can damage biological tissues) at the higher energies, the term non-invasive is based on the fact that, at the lower energies that are used in modern imaging methods, X-rays do not create any damaging biological effects.

1.2 Techniques available for non-invasive imaging

As a field of scientific investigation, non-invasive imaging constitutes a subdiscipline of biomedical engineering, medical physics or medicine depending on the context. Methods such as nuclear medicine use radioactive materials to diagnose or treat various pathologies and are generally considered to be invasive. Many of the techniques developed for non-invasive imaging such as X-rays, nuclear magnetic resonance imaging (MRI) and ultrasonography (U/S) also have industrial applications, although the energies used in industrial applications are extremely high

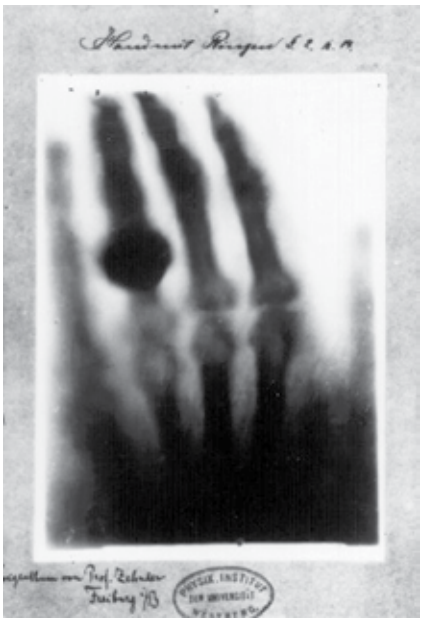

Figure 1.
First medical X-ray by Wilhelm Röntgen of his wife Anna Bertha Ludwig's hand. Wilhelm Röntgen [public domain].

and would be considered to be highly invasive for biological samples. In the case of U/S, the probe emits the beam which consists of ultrasonic pressure waves that return echoes from the various tissue interfaces. The echoes show details of the internal structures. U/S waves do not travel through large interfaces or air and thus limits its use in biological tissue. In the cases of X-rays and MRI, either X-radiation or a magnetic field respectively pass through the tissues to identify, separate and quantify different tissue types such as bone, cuticle, muscle or fat. In general, MRI is the best modality for discerning muscle or fat, is non-invasive and has very long image capture times whereas X-rays are better for discerning smaller structures and have much faster image capture times.

It is the ability to see smaller structures and the fast capture times that led to the development of Diagnostic Radioentomology (DR) to study insects non-invasively. DR is performed on insects using X-ray Computer Tomography Scanners (CT Scanners).

1.3 Diagnostic Radioentomology

The term 'Diagnostic Radioentomology' first came to be used in 2003 during a pollination experiment on the behaviour of the Australian stingless bees *Tetragonula carbonaria* and *Austroplebeia australis* [10]. The term was used because the new method is diagnostic, it uses X-radiation (radio) and is used for studying insects (entomology). **Video 1** gives a brief overview of the methods.

Therefore, DR became the term for an innovative method for studying insect morphology, physiology and behaviour using non-invasive imaging. Since its development, DR has been adopted by the Museum of Natural History in London, Universities, synchrotron facilities and research associations globally.

In 2008, DR was described by The International Bee Research Association (IBRA) as an emerging non-invasive technique for behavioural, evolutionary and classical biologists who need to study insects without harming them.

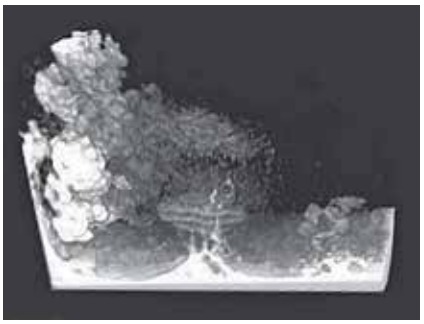

Figure 2.
*The first DR image of a bee colony (*Tetragonula carbonaria*) in a wooden box [11].*

Nowadays, synchrotron beamlines can completely scan and reconstruct 3D images in a matter of seconds and CT scanners can complete a 1-cm scan in as little as one-third of a second, and recent techniques have been developed to enable scanning software to produce 3D images such as in **Figure 2** and 4D movies and physical 3D models which can be downloaded at this address: http://www.radioentomology.com/.

It is generally accepted that for DR studies, the term MacroCT applies to the CT scanning of large items using human body CT scanners and that MicroCT applies to laboratory or Synchrotron CT scanners to study small items at the microscopic level. In recent years, DR has been adopted to visualise macroscopic characteristics of insects and their behaviour [11–15]. Also, with the improvements in spatial resolution and tissue differentiation that are occurring with MacroCT, conventional micro-focus and synchrotron based MicroCT, new methods for the non-invasive imaging of insects are emerging. For an overview of these methods see [16–22].

Historically, traditional methods for colony health, bee behaviour and the morphological classification of bees have been conducted on apiary hives and with the aid of observation hives and dissecting light microscopes. These techniques are, understandably, limited. The inspection of apiary hives disrupts normal bee behavior, observation hives offer only a view of one side of one frame within an entire hive, dissection obviously kills the bee and the use of light microscopy when used for amber inclusions [23–32], particularly with specimens preserved in opaque amber pieces [33, 34] are grossly limited by the specimens opaqueness. In [35] the authors attempted to address methods of examining insect inclusions within pieces of opaque amber and to supplement traditional light microscopy studies of transparent amber. Those researchers and [36–38] produced traditional radiographs which provided the first, albeit limited, steps toward enhanced visualisation of cryptic bee behaviour and fossil material. More recently, detailed information for the study of bees has been obtained with the use of scanning electron microscopy (SEM) as in [39, 40] and transmission electron microscopy (TEM) as in [41]. While SEM and TEM studies currently provide the highest level of detail, sample preparations are laborious and are often invasive to completely destructive [41]. SEM and TEM can be used for the investigation of amber inclusions as in [39–42] however, these methods are generally not suitable because they require destruction of the material. The development of non-invasive imaging methods such as DR, therefore, offers promise to scientists and beekeepers who need to preserve their specimens or observe behaviour non-invasively.

In 2013, DR was hailed as the 'Gold Standard' for honeybee monitoring [43] and the non-invasive path detailed in the following sections will demonstrate that DR is an ideal method which can be used to study bees and other insects in the most natural of settings. It is also important to mention that the results from the following

experiments can be directly applied to beekeeping husbandry to enhance modern beekeeping methods and enable beekeepers to play a more active role in improving global bee health.

2. Describing an ancient social bee in amber using DR

To help demonstrate the non-invasive, diagnostic advantages of DR it is worthwhile detailing the following experiment on one of the oldest bees known, *Proplebeia adbita*. In the following experiment, we examined the external and internal morphology of an Early Miocene (Burdigalian) stingless bee (Apinae: Meliponini) from the Dominican Republic using non-destructive X-ray microtomography analysis (MicroCT). The study shows the accurate reconstruction of features otherwise obscured or impossible to visualise without destroying/damaging the sample and enables diagnosis of the specimen as a new species of bee [44].

2.1 Materials and methods

Bees have several characteristic morphological attributes such as branched or plumose body setae and broadened metabasitarsi [5, 45]. The highly eusocial stingless bees, the Meliponini are within the corbiculate Apinae for example in [46–49]. In addition to extensive morphological and molecular data such as in [48, 50], the corbiculate apines belonging to a single group has been supported by studies investigating their internal anatomy. For example [39], noted that the proventricular morphology of Euglossini and Bombini consists of long columnar plates, triangular apices in Apini, while the Meliponini have slender and elongated plates. Accordingly, the proventriculus can be used as an important diagnostic structure for bee taxonomy [21], among a suite of other internal anatomical features [45]. The examination of such characters often requires considerable manipulation, dissection, sectioning or even complete destruction of the specimen. Thus, the practical application of such data is at times hampered by the methods employed. In the following experiment, the internal and external morphology of an ancient social bee trapped in amber using non-invasive and non-destructive DR techniques is described in detail.

2.1.1 About the bee

The bee selected for this experiment was collected from the La Bucara mine in the Dominican Republic. This stingless bee was trapped at the widest end of a semi-clear, brown piece of amber that contained many other inclusions **Figure 3**.

The posterior of the bee is at the extreme periphery of the piece's thick end, and the apices of both forewings have broken away from the sample over time.

Age estimates of Dominican amber vary considerably in literature nonetheless, most data indicate that the age of most Dominican amber, including the material in this study, is 16–19 Ma [45–47]. The sample had been polished prior to this study and therefore required no extra preparation.

2.1.2 Non-invasive imaging of the bee

Traditionally, the morphological classification of bees has been conducted with the aid of dissecting microscopes which use light. The technique is understandably limited when used for amber inclusions, particularly with specimens preserved in opaque pieces. Light microscopy was used in this experiment in an attempt to describe its limitations with opaque specimens.

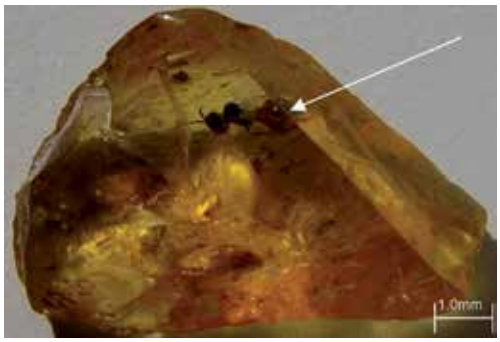

Figure 3.
A piece of semi-clear, brown amber from the Dominican Republic (Early Miocene: Burdigalian), with many inclusions. The stingless bee is at the widest end (arrow) [44].

Figure 4.
Air bubbles, fractures and general thickness of the amber prevent adequate visualisation of the metasoma, posterior mesosoma and wings. Image taken under optimal optical conditions (increasing or decreasing light intensity further degraded image quality) [44].

2.1.3 Light microscopy

For light microscopy, the bee was viewed using a Leica MZ12 stereomicroscope, Leica Microsystems GmbH Ernst-Leitz-Strasse 17–37 35578 Wetzlar. The Leica MZ12 has distortion-free 109 eyepieces with a resolution of 375 line-pairs per mm.

Ideally, because of the thickness, air bubble inclusions and **Figure 4**, it would have been better to slice the fractures present in the amber piece prior to light microscopy examination. However, the sample was intentionally preserved to enable visualisation of the other biological inclusions using DR in future studies.

The colour of the bee was brown to dark-brown; however, it is possible that the bee was black when alive and that the cuticular melanin was altered over time. Moreover, newly moulted adult stingless bees are often lighter in coloration and so the more brownish colour of the specimen cannot be considered diagnostic. Gross external morphological features of the bee such as the chaetae, coxae, trochanter and tibiae were visible to about the level of the mesothorax. The air bubbles, fractures and general thickness of the amber piece prevented adequate visualisation of the more posterior including a lack of detail of the wings **Figure 4**. Increasing light intensity created image degradation due to light diffracting from cracks, air bubbles and generalised opacity of the amber. Decreasing light intensity made it difficult to optically visualise the bee's morphological features.

2.1.4 DR scanning

We need to keep in mind that this experiment was conducted during early testing phases for the potential applications of X-rays to insect morphology. Therefore, to assess their potential, three different apparatuses were used. X-ray MicroCT scans were performed a commercial benchtop system, a custom designed X-ray scanner and the facility for MicroCT available at the SYRMEP beamline of the Elettra Light Source Synchrotron in Trieste (Italy).

For a full description of these methods see [45]. Prior to scanning, the sample was placed in a 20.5 mm cylindrical sample holder between the X-ray source and the image detector **Figure 5**. This simple positioning procedure for the scanning phase of a DR examination can be adapted for all X-ray apparatuses. Scanning produces 2D images which are then converted to 3D images with specialised software.

As with light microscopy, gross external morphological features of the bee such as the Chaetae, the articulations of the coxae, trochanters, tibiae, and tarsi, including the corbiculae of the metatibiae and the broadened metabasitarsi, were well visualised in the 3D reconstructions **Figure 6**. In addition, gross internal structures, such as the brain (including details of its anatomical regions), direct and indirect flight muscles and a loaded rectum were accurately represented, **Figure 7**. **Video 2** (Ancient bee Proplebeia abdita trapped in amber approximately 20 million years ago) will highlight these features and also provide an understanding of what can be achieved during DR, 3D processing. Considering the specimen's age (16–19 Ma), the brain of this bee was

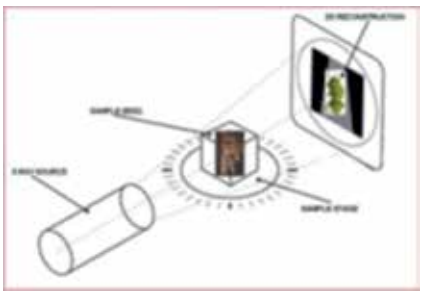

Figure 5.
A schematic diagram of sample positioning for DR. Essentially, the only preparation required is that the sample (bee) is positioned securely on the sample stage so that it remains motionless during the scan [44].

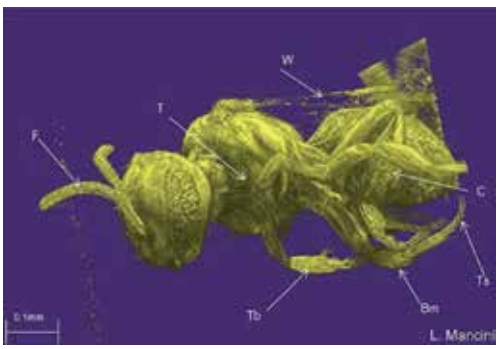

Figure 6.
Volume rendering image of the holotype worker of Proplebeia abdita in Early Miocene (Burdigalian) Dominican amber. Wings (W), flagellomeres (F), base of trochanter (T), tibiae (Tb), tarsi (Ts), the corbicula (C) of the metatibia and the broadened metabasitarsi (Bm) are all well visualised [44].

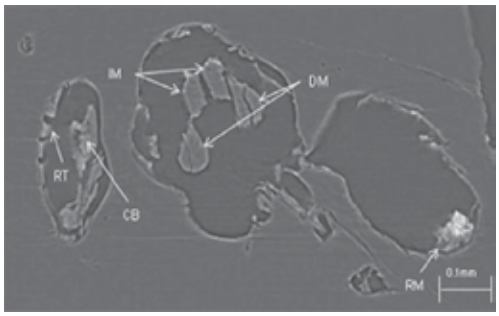

Figure 7.
A 2D view of Proplebeia abdita. Gross internal structures such as the central body of the brain (CB), retinal zone of the compound eyes (RT), direct (DM) and indirect (IM) flight muscles and a loaded rectum (RM) were accurately visualised [44].

particularly well preserved. The optic and antennal lobes were well reconstructed along with the dense central body and the protocerebral lobes. The retinal zone was also well preserved. Adhesion of the retinal zone to the proximal surface of the compound eyes and the corresponding region on the distal surface of the medullae was evidenced by a thin, dense film of tissue.

2.1.5 Discussion and results

Diagnostic Radioentomology permitted the comprehensive examination of this ancient specimen, where other methods were (in the case of light microscopy) and would be (in the case of SEM or TEM) found to be less reliable or unsuitable because of their limitations and/or destructive nature. The bee's anatomical characteristics were accurately assessed and precise morphometric measurements were performed with on-screen linear measuring callipers. As a result, details of a previously undescribed species, *P. abdita* Greco and Engel were described [44]. This experiment demonstrated that all three apparatuses were appropriate for accurately visualising the bee. Thus, entomologists can consider which facility would provide the best option for them. In addition to the application of DR to this particular bee, its more extensive use on historical type material (e.g. the holotype of *P. dominicana*, other amber preserved bees or even unique specimens of rare modern species) will permit a more complete characterisation of these bees and comprehensive comparisons between them and their modern counterparts. Improved anatomical understanding of these bees will greatly enhance phylogenetic reconstructions utilising paleontological data and potentially revise our paleoecological perspectives of early pollinators. It is hoped that by highlighting the utility of DR for characterising an ancient social bee that these techniques might be more broadly applied to social bee biology and anatomy, much in the tradition of [37] earlier applications of novel imaging methods and in the way it has been applied to the study of termites and living stingless bees [10, 13], as well as solitary bee species [11, 21].

3. Discovering new bee behaviour via DR methods

As mentioned above, DR offers new ways of studying known behaviours and features of bees. As it turned out in the following experiment, DR also introduced us to some new behaviours that were totally unexpected. We know that decision making in honeybees is based on information which is acquired and processed in

order to make choices between two or more alternatives [51]. These choices lead to the expression of optimal behaviour strategies such as floral constancy [52]. Optimal foraging strategies such as floral constancy improve a colony's chances of survival, however, there has been no research on decision making based on optimal storage strategies.

The following DR experiment describes how decision making in storer bees is influenced by nectar sugar concentrations and that, within 48 hours of collection, honeybee workers store carbohydrates in groups of cells with similar sugar concentrations in a non-random way. We can surmise that this behaviour, as evidenced by patchy cell distributions, would help to hasten the ripening process by reducing the distance between cells of similar sugar concentrations [52]. Therefore, colonies which exhibit optimal storage strategies such as these would have an evolutionary advantage and improved colony survival expectations over less efficient colonies and it is plausible that beekeepers could select colonies that exhibit these preferred traits.

3.1 Materials and methods

During an unrelated DR experiment, in an attempt to mark and track Varroa destructor within a honeybee colony, an unexpected pattern appeared on the honey comb images. Bees from several different colonies were fed marked and unmarked sucrose solution ad libitum. The bees then stored this sucrose freely without any restrictions.

3.1.1 The interesting discovery

Soon we discovered patterns that were previously unreported appearing on the honeycomb. Some colonies formed these patterns and some did not. **Video 3** (Flying through an apidea hive) and **Figure 8** show examples of these patterns.

Now, for these marking experiments, there are only two possible pathways that a cell can have only 50% sucrose solution or 70% sucrose solution in it. For a full description of these pathways see [52] and **Figure 9**.

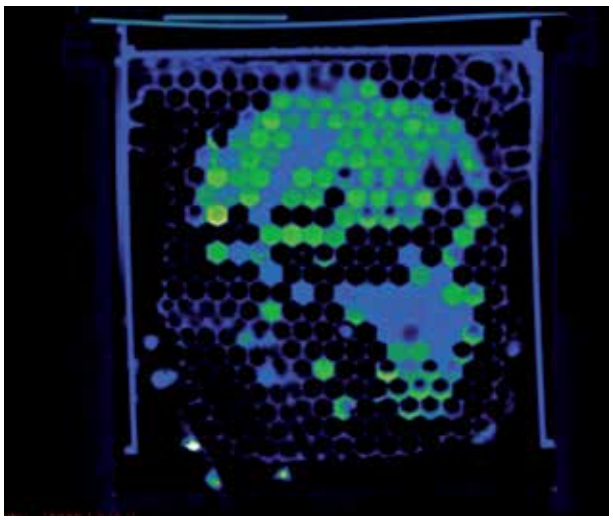

Figure 8.
A DR scan of a honey comb showing patchy distribution of cells containing honey with differing sugar concentrations. The marked 'green' cell patches contained only 70% sucrose syrup and the 'blue' unmarked cell patches contained only 50% sucrose syrup [52].

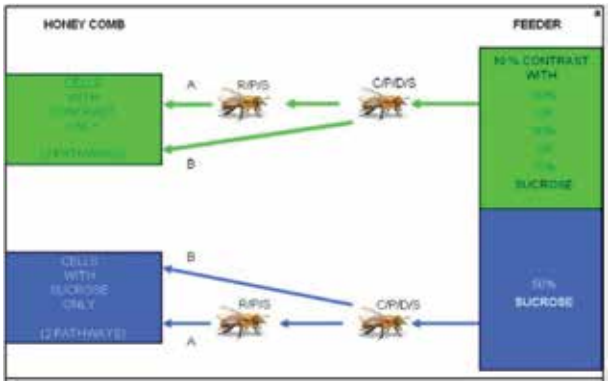

Figure 9.
A schematic diagram of the two possible pathways for either marked (green) or unmarked (blue) sucrose solutions to enter a cell unmixed [52].

3.1.2 Discussion and results

The data from the experiment, showed significant differences in the green/blue/mixed patch ratios. This implies possible behavioural influences on patch ratios. These behavioural influences are likely to be actions by bees making decisions on where to place honey of similar sugar concentrations. As in [53], it is likely that bees from some colonies deposit nectar according to contextual information, such as the location of other cells in the hive containing honey of similar sugar concentrations, and that bees from other colonies do not.

3.1.3 Some honeybee colonies are more efficient than others

These behaviours are influencing the honey storage patterns and are probably based on achieving optimal storage strategies. In this experiment the data indicate, as do those of [54–56], that honeybee colonies show a preference for storing honey according to sugar concentrations in the nectar. Therefore, one optimal storage strategy would be for storer bees to return to cell patches containing cells with similar sugar concentrations until all the cells in those patches were full. This strategy would reduce search time and thus increase storing behaviour efficiency. The DR images in this study clearly show that honeybees are producing these similar sugar concentration cell patches [52].

3.1.4 Can this behaviour help save the colony?

Storing honey in cell patches has benefits other than for those of ripening honey. Nectar collected by honeybees from different foraging patches (either natural or agricultural patches) will have differing sugar concentrations simply because the plants in these patches are growing under different local ambient conditions. In light of the current trend in global colony losses, it is crucial to mention here that the nectar from these plants might also contain other differences in constituents such as lethal or sub-lethal levels of toxins from agrichemicals and other sources [57–59]. Honey storage strategies, like those shown in this experiment, would be based on information such as sensing the sugar concentrations in incoming nectar and that of the ripening honey in the cells. Although it is not clear whether honeybees can detect agrichemicals in nectar or honey, as was shown in this experiment, they might store toxin-containing nectars separately from toxin-free nectars indirectly by way of sensing the nectars' different sugar concentrations. This would be an effective way to prevent all the honey from being contaminated and it would

reduce widespread toxin contamination in the hive and thus help prevent bee losses. The data from [60] also supports that honeybees store pollen with high levels of chlorothalonil separately in entombed cells which is a phenomenon similar to the patchy honey storage pattern behaviour shown in this experiment.

We should also consider that there are plants in several genera from at least 11 families [61, 62] that naturally produce nectar which contain constituents that have varying degrees of toxicity to bees and humans. There are also plants that produce toxic pollen [63, 64]. Forager bees bring these naturally occurring nectar and pollen back to the hive. In evolutionary terms, these naturally occurring toxins in pollen and nectar have provided the selective pressure for honeybees to improve their food storage strategies. Thus colonies that exhibit storage strategies which separate toxic from non-toxic food would have an evolutionary advantage over colonies whose bees store food indiscriminately [52].

3.1.5 How is this recent discovery relevant to beekeepers?

These storage behaviour efficiencies will have important implications for the long term survival of honeybee colonies. The data from the above experiment show that bees from some colonies exhibit efficient selective storage strategies and that bees from other colonies do not. These strategies have the potential to directly or indirectly separate toxins and pathogens with the hive. If beekeepers can determine which bees exhibit more effective storage strategies they will be able to select colonies that exhibit such preferred traits.

The DR experiments above and the in the following section were conducted using non-invasive, state of the art 'High Tech' Science. They have shown behaviour that is not apparent to the naked eye because humans cannot visually detect different sugar concentrations in honey comb cells. The next section will describe a simple method for beekeepers to select bees/Queens from one colony in preference over bees from another colony. This simple method will place beekeepers at the forefront of protecting their colonies at the grassroots level by improving honeybee husbandry.

3.1.6 A simple selection method for the modern beekeeper

The DR experiment above, indicated that honey bees show preferences when storing food and importantly, when feeding other bees via trophallaxis. As a secondary effect, some honeybees might also 'preferentially' spread pathogens/medication, which is contained in nectar/syrup, to other bees within their hive. The experiment below demonstrates that bees from certain colonies show 'preferences' while feeding other bees and that bees from other hives do not. The simple method, developed during the experiment for assessing food and pathogen transmission in bees, will help beekeepers to select and breed bees that have a higher propensity for spreading damaging pathogen or if required for spreading invaluable medication within a hive. This will help place beekeepers in a position to select more efficient bees and use their own breeding programs to help mitigate global bee declines, at the grass roots level.

3.1.7 Testing and selecting bees

To test whether bees show preference when feeding other bees, a simple segregation cup was developed, **Figure 10**.

Collection cups have been used previously to study bees [66] however this new system is the first system to segregate bees within the cup. Segregating bees within the cup enables one group of bees to interact bees from another section and prevents them from interacting with bees from a third section. The mode of

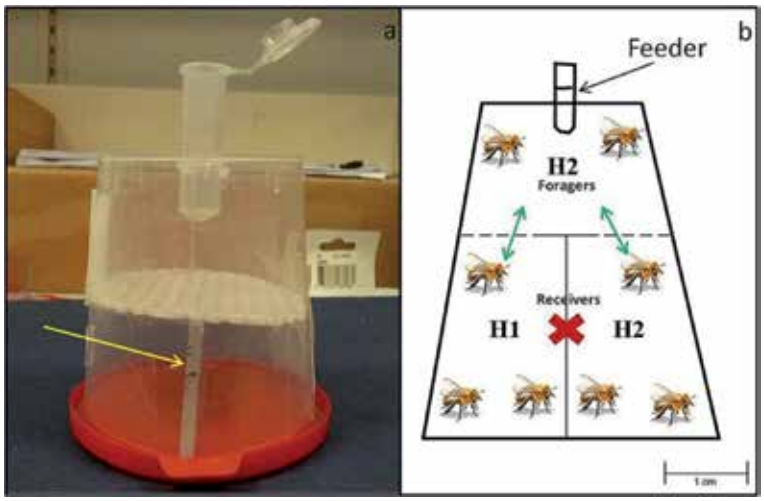

Figure 10.
(a) A segregation cup featuring the unique 'T' piece (yellow arrow) which separates three groups of bees. (b) The 'T' piece allows trophallaxis between bees in upper chamber and lower chambers (green arrows) and prevents all contact between bees in the two lower chambers (red cross) [65].

segregation is provided by the unique 'T' piece shown in **Figure 10**. The 'T' piece is inserted in the cup before the bees are collected. The horizontal portion of the 'T' piece is a 3 mm mesh and the vertical portion is solid Perspex. For a full description of the new segregation cup system see [65].

The segregation cup system enables beekeepers to collect one, two or three different groups of bees. **Figure 10** shows a schematic diagram of bees from hive 2 (H2) in the top section and bees from hive 1 (H1) and hive 2 (H2) in the bottom sections. The top section has a syrup feeder which means that the bees in the bottom sections can only receive food from bees in the top section through the mesh via trophallaxis. Bees that do not receive food from the top group commence starving within a few hours.

This simple system quickly demonstrates to the beekeeper which bees the top group prefers to feed via trophallaxis. The group of bees in the section that does not starve are the preferred bees.

3.1.8 Selecting bees for improved treatment

The recent finding that lithium chloride could be used as a medication added to syrup to treat Varroa destructor infestations [67] would be a good example of improved treatment by utilising better distribution of medication within the hive.

The new segregation cup system showed that when bees from H2 were placed in the top section, they had a significant trophallactic preference for H2 bees and tended to ignore H1 bees which subsequently starved. However, when bees from H1 were placed in the top section, they did not show a trophallactic preference. Bees from H1 fed both bottom section groups equally and bees from both H1 and H2 in the bottom sections survived for as long as the bees in the top section.

It has been established that, due to bees drifting in an apiary, there are commonly bees from other hives present in all hives. In fact, there can be as many as 38% at any given time [68].

As gauged by the level of H1 bee mortality [65], H2 foragers preferentially fed H2 bees over H1 bees. H1 type bees showed fewer preferences and will feed more

bees within the hive trophallactically. Therefore, beekeepers can choose to breed from H1 'type' bees that will spread medication in syrup via trophallaxis to more bees within the hive. After breeding these colonies, beekeepers can then assess whether there are also greater survival rates with those colonies.

3.1.9 Selecting bees for improved health

In light of the current trend in global colony losses, it is crucial to mention here that nowadays, nectar brought in by forager bees might also contain constituents such as lethal or sub-lethal levels of toxins from agrichemicals or pathogens [9]. Although it is not clear whether honeybees can detect agrichemicals or pathogens in nectar, H2 type bees would pass on toxins/pathogens that are in nectar preferentially via trophallaxis. This would be an effective way to prevent up to 38% of bees [68] receiving toxins/pathogens and it would reduce widespread contamination in the hive and thus help prevent bee losses. In addition, there are plants in several genera from at least 11 families [61, 62] that naturally produce nectar which contain constituents that have varying degrees of toxicity to bees. Foragers bring these naturally occurring nectars back to the hive. Thus colonies containing H2 type bees that show more preference would have an evolutionary advantage over bees such as those with H1 type bees.

During times when the environment is less conducive to colony health, such as when agrichemicals are used on crops or when EFB, AFB and Nosema are prevalent, H2 type bees would bring these back in the nectar and spread them within the hive via trophallaxis with less efficiency than H1 type bees because H2 type bees show preferences for H2 bees only. H1 type bees show fewer preferences and are likely to feed all bees within the colony via trophallaxis. This behaviour will spread the incoming nectar more rapidly throughout the colony. The new segregation cup system can test for this behaviour and beekeepers can select H2 type bees for better colony health/survival over H1 type bees.

It is important to mention that on some occasions beekeepers might select H1 type bees and on other occasions they might want to select H2 type bees. The new segregation cup system can enable beekeepers to make these choices. Once the choice is made, beekeepers can then develop their own breeding programs by breeding queens from those colonies to propagate the desired behaviours in subsequent colonies.

4. Conclusions

This chapter described new DR methods and how they can help beekeepers make informed choices. The chapter detailed how current knowledge can be studied in novel ways non-invasively and how DR methods brought to light unexpected new knowledge that is beneficial to the preservation of honeybees globally.

Descriptions and examples were given to describe DR at the cutting edge of state of the art technology. DR methods helped to discover a new species, previously undescribed bee behaviour and a new selection system to help beekeepers improve their hive management knowledge.

DR is an applied science that has a direct impact on modern beekeeping. Although it is very high tech, it also provides links to new methods which help beekeepers make their own informed decisions to improve bee husbandry methods and colony health at the grass roots level.

Video materials

The video materials referenced in this chapter are available at: https://bit.ly/2BV3DnO.

Acknowledgements

The Eva Crane Trust has partly funded DR research conducted in this chapter. This chapter was written with the kind assistance from Marisa Greco, Christina Belcev, Alex Belcev, Nell Greco, Pedro Velasco and Isabella Belcev.

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Chapter 10

Southeast Asian Meliponiculture for Sustainable Livelihood

Atsalek Rattanawannee and Orawan Duangphakdee

Abstract

Stingless bees (Apidae: Meliponini) are one of the most important pollinators of native plants and economic crops in tropical and subtropical parts of the world. They not only establish large perennial colonies with complex social organization but also have a diverse nesting biology. The economic utilization of a total of 60 stingless bee species in Asia has been reported. The current status of meliponiculture in Southeast Asia is mainly focused on pollination utilization and honey and propolis production. This chapter shows that small-scale beekeeping of stingless bees, which is suitable for the flowering pattern in the tropics, is one of the best potential alternative opportunities. The cost-effectiveness analysis based on production yield, investment cost, and profit-return rate is reviewed. Finally, a sustainable utilization of stingless bees is considered to be an enhancer of pollination services both in an agricultural crop and natural ecosystem.

Keywords: stingless bee species diversity, stingless bee beekeeping, products and utilization, marketing and demand, ecological impacts

1. Introduction

Among the 19 tribes of the subfamily Apinae, only Apini (honey bees) and Meliponini (stingless bees) show highly social behavior or eusociality [1]. In contrast to the mono-genus tribe of Apini that consists of 11 valid species [2], the Meliponini demonstrates the most diverse group, not only of the number of species but also of the morphology, nesting habitats, structures, and behavior among species [3, 4]. Meliponini has a wide distribution and is found in the tropical and subtropical regions of the world (**Figure 1**). The highest diversity of stingless bee species is found in the Neotropical with about 391 species and 32 genera, indicating that this area might be the center of origin and dispersal of stingless bees [4, 6, 7]. By contrast, 60, 10, and 50 species have been reported in Asia, Australia, and Africa, respectively [4]. However, the advance of molecular methods has increased the studies on species complexity, and new species of stingless bees are being added [8].

Like eusocial honey bees, stingless bees form colonies with a single female queen, a few hundred to several thousand female workers, and a few hundred males (drones) [1]. The nests of stingless bees show a large variation in the size, substrate used, habitats, and landscapes [9]. In nature, different stingless bee species nest in various cavities, such as hollow tree trunks and stems, under the ground, crevices within rocks, and the nest of other insects [3, 10]. All stingless bees use the same basic material, cerumen, to construct the nest. The worker bees make cerumen by mixing the wax they produce in the wax gland located on the tergites of their

abdomen, with resins that are collected from plants [4]. In spite of high variation in size and ornaments found in the different stingless bee nests, the basic components are remarkably homogeneous across species [4], as shown in **Figure 2**. The nest connects to the outside through the entrance tube made of cerumen. Among the different species of stingless bees, the entrance tube is quite varied in shape and size (**Figure 3**), and it can be used as the characteristic to identify some of the Indo-Malayan stingless bee species.

In contrast to the Asian honey bee (*Apis cerana*), stingless bee colonies are typically long-lived [3, 4] and have low absconding behavior. Some species of stingless bee continuously occupy the original nest, and the nest lives more than

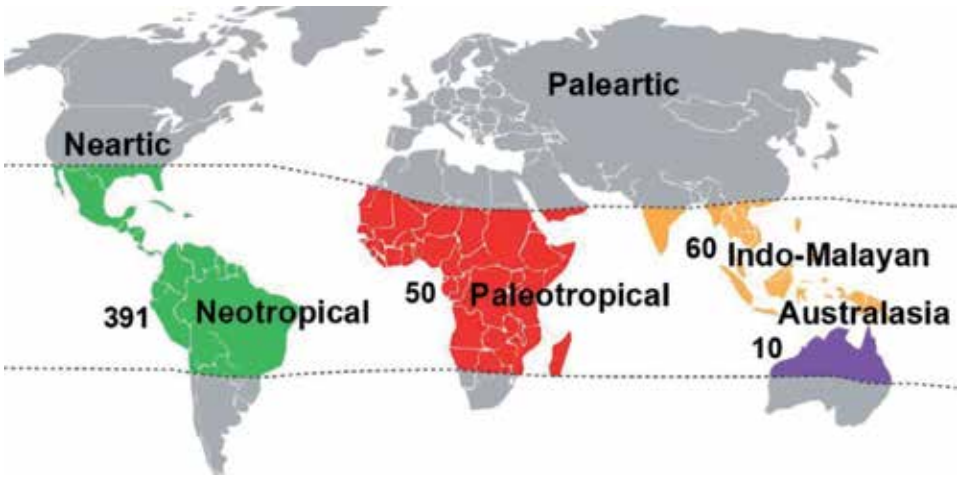

Figure 1.
Geographic distribution of stingless bees (amended in accordance with [4, 5]).

Figure 2.
Basic interior structure of a stingless bee nest: (A) a vertical brood cells, (B) honey and pollen pots, and (C) involucrum.

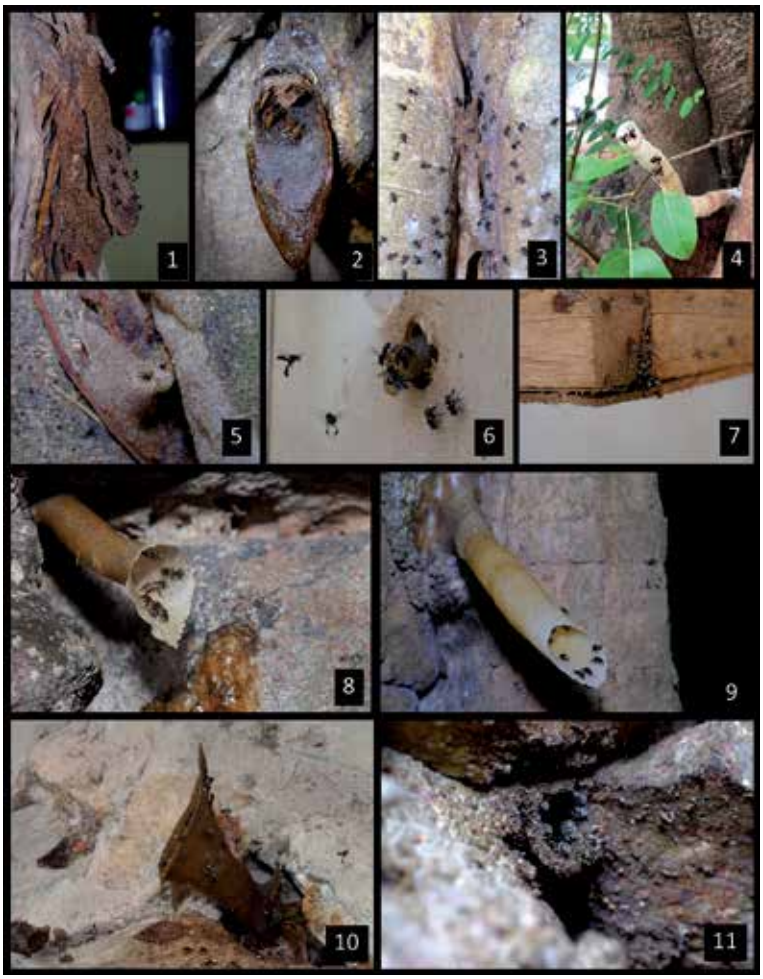

Figure 3.
Variation of entrant tube in some native stingless bee species found in Southeast Asia: (1) Geniotrigona thoracica*, (2)* Homotrigona fimbriata*, (3)* Lophotrigona canifrons*, (4)* Tetragonilla collina*, (5)* Pariotrigona klossi*, (6)* Heterotrigona itama*, (7)* Tetragonula fuscobalteata*, (8)* Lepidotriogona terminata*, (9)* Tetrigona melanoleuca*, (10)* Tetrigona apicalis*, and (11)* Tetragonula pagdeni.

20 years. These aspects of their biology make stingless bee species successful in meliponiculture in Southeast Asia, including Thailand. At least six of Thailand's native species have had nests successfully transferred into a wood box to pollinate orchard crops. Two species (*Geniotrigona thoracica* and *Tetragonula pagdeni*) have been used for honey production. Additionally, management costs have been lower than in apiculture. The meliponiculture might be useful to improve household income in the countryside of Southeast Asia, where there is a very high diversity of flora [11, 12].

2. Stingless bees

2.1 General features

Stingless bees are one of the most diverse groups of corbiculate bees. Unlike Apini, Meliponini shows great interspecific variation not only in shape but also size,

color, pattern of wing venation, and size and shape of the corbiculate [4]. However, all stingless bee species have the same basic morphological body patterns as other Hymenopterans (**Figure 4**). Thus, the body of stingless bees can be separated into three main segments: the head, thorax, and abdomen.

On the head or prosoma of a single bee, the main organs are eyes (compound eyes and dorsal ocelli), antennae, and mouth parts. All structures on the prosoma are used to interact with their environment. For stingless bee identification, the size, shape, and number of teeth on the mandibles have been used as the primary key characteristics [4, 13].

On the thorax or mesosoma, two pairs of wings and three pairs of legs are attached and are involved in the locomotion of the bee. These appendages are moved by groups of thorax muscles [14]. Two types of forewing can be observed in Indo-Malayan stingless bee, two-tone (darker at base and clear white at apex) and mono-tone (clear white entire of wing), as shown in **Figure 5A**.

In worker bees, hind legs which are modified for pollen collection are called pollen baskets or corbicula (**Figure 5B**). The tibia is broadly expanded and slightly concave with curved hairs along the edge for keeping the pollen load. Moreover, the inner surface of the hind basitarsus segment is covered with short bristles that are used for grooming the pollen from the body and transferring the pollen to the pollen baskets [4].

The abdomen or metasoma of adult stingless bees consists of nine segments, but only second–seventh segments are externally visible [14]. Unlike honey bees, wax glands located on the tergites (dorsal plates) of the abdomen are active and produce wax in younger adult workers. Most internal organs and systems are found in this body part, including digestive organs, ventral nervous system, circulatory system, and reproductive organs [4, 14].

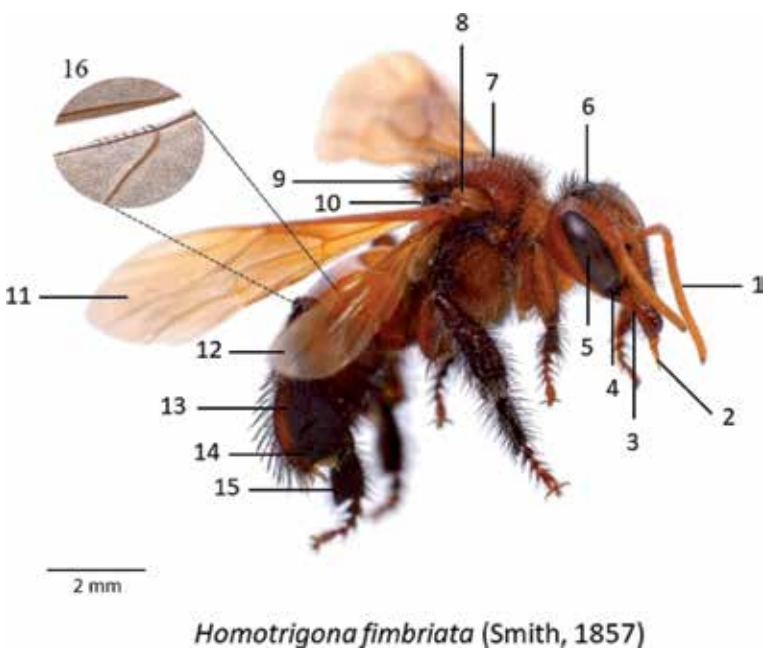

Figure 4.
External morphology of Homotrigona fimbriata *(Smith, 1857) worker: (1) antennae, (2) proboscis, (3) mandible, (4) malar space, (5) compound eye, (6) ocelli, (7) pronotum, (8) tegula, (9) scutellum, (10) propodium, (11) forewing, (12) hind wing, (13) hind tibia, (14) pollen basket or corbicula, (15) hind basitarsus, and (16) hamuli on hind wing.*

2.2 Caste and colony function

In social hymenopteran colonies, there is a division of labor between females of the colony [15]. Two castes (queen and worker) are found in a colony of stingless bees. Like other corbiculate bees, stingless bee colonies consist of two sexes (female and male), which are different in size and shape of external morphology (**Figure 6**). A haplodiploid sex determination system has been described to explain how female and male are produced in all stingless bees [4]. Both the female queen and the worker, called diploid females, develop from fertilized eggs laid by the mother queen, so they have two sets of chromosomes. In contrast, stingless bee males are produced from unfertilized eggs which is known as arrhenotokous parthenogenesis [16], meaning they carry only the mother's genetic materials [4].

A stingless bee colony consists of a single female queen (fertile female), several hundred to several thousand unfertile female workers, and a few hundred males [17]. Similar to the honey bee of the genus *Apis*, stingless bee workers perform most activities both inside and outside the nest, including cell construction, taking care of the queen and larvae, defending the nest, as well as foraging for food and other

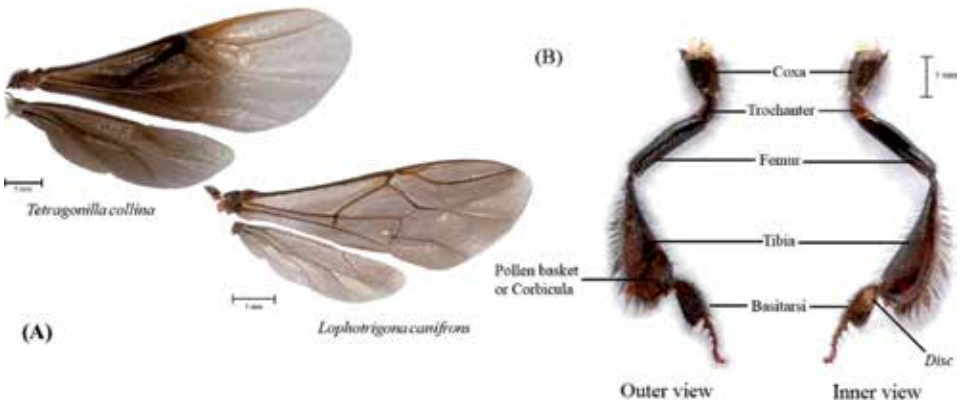

Figure 5.
(A) Fore and hind wings of two Indo-Malayan stingless bee species, Tetragonilla collina *and* Lophotrigona canifrons*, show the color tone of forewing. (B) Outer and inner views of hind leg of* Tetrigona apicalis *worker showing pollen basket on the tibia.*

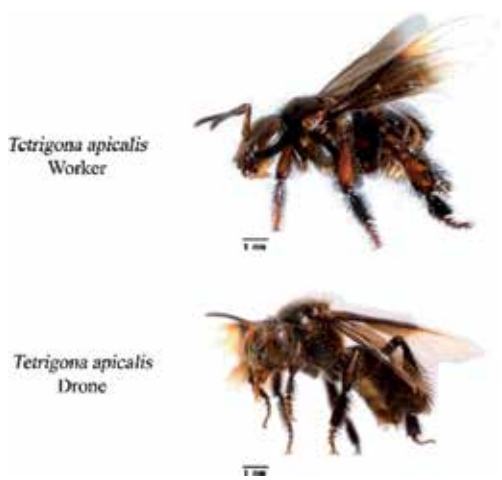

Figure 6.
Sexual dimorphism in Tetrigona apicalis*.*

colony needed materials [17, 18]. Interestingly, the progression of different duties of worker bees corresponds to age after adulthood, known as age polyethism [19]. This phenomenon is also seen in honey bees [19, 20].

Generally, five major stingless bee worker activities are recognized, namely, (1) cleaning blood chambers and feeding larvae, (2) constructing the nest, (3) receiving and processing nectar, (4) guarding in front of the nest entrance, and (5) foraging for food sources and other materials [21, 22]. Similar to *Apis*, young stingless bee workers work inside the nest. They have active wax glands on the tergites, and their main roles are constructing brood cells and cleaning the brood area. After 2 weeks, the ovaries of workers become active, and they produce trophic eggs [4] for feeding the mother queen. They also receive the nectar from foragers and dehydrate it to become honey. Older workers (3–4 weeks old) perform activities related to guarding. At this age, the workers perform short-distance flights for nest orientation [4]. In the final stage (about 1 month old), the worker becomes a forager. However, age polyethism in stingless bees is flexible, meaning that workers can continue or revert across different activities, depending on colony needs [4, 23].

2.3 Nest structure

Although a high variation in size is found in nests of different stingless bee species, the basic materials and patterns are observed [3] as shown in **Figure 2**. Cerumen, the mixture of wax that workers produce, plus resins collected from various plants by workers is the basic material used for nest construction [3]. The outermost part and cover of the interior of the nest are made from bitumen: solid cerumen mixed with propolis. Stingless bees use bitumen to line the cavity and to protect the nest from environment variation. The bitumen also helps to limit the volume of the nesting cavity. It can be removed to permit growth during a blooming period and decrease during a dearth period [4]. Inside the nest of many species, the brood area is separated from the food storage area by using a thin cerumen layer called involucrum [3]. This nest component helps to control the temperature in the brood area. The brood area contains the cells with individual developing larvae. Unlike honey bees, the brood cells of stingless bees are constructed in vertical form and are used only once [3, 4]. The brood cells of stingless bees are connected to each other by small cerumen threads forming brood clusters (**Figure 7A**). In some species, the cells may also be attached wall to wall forming a horizontal comb (**Figure 7B**).

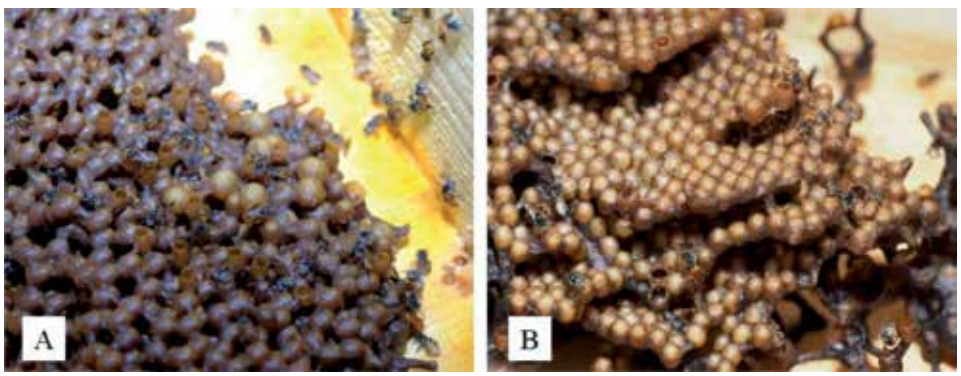

Figure 7.
Two basic type of brood cell arrangement in Southeast Asian stingless bees. (A) Brood cell clusters found in Tetragonula pagdeni *and (B) cell arrangement as a horizontal comb in* Tetragonula laeviceps.

Like honey bees of the genus *Apis*, stingless bees collect nectar and pollen and store it as food for the colony for long periods. For storing food, stingless bee workers build special cerumen containers called pots where honey and pollen are stored [3]. Honey and pollen are kept separately, so there are honey and pollen pots [4]. The size and shape of honey and pollen pots are similar in most stingless bees. Usually, both types of food pots are ovoid in shape, but this may also vary across species [3, 4].

3. Transferring a wild colony to an artificial hive box

There are several methods for transferring stingless bee colonies from their natural habitat to artificial hive boxes. This is one of the most important features of meliponiculture. In Southeast Asia, there are several models and sizes of commercial hive boxes available for stingless bees. However, two basic models of boxes, vertical and horizontal, are used depending on the species' arrangement of brood and food pots. For vertical boxes, the brood cluster is usually placed in the bottom section of the boxes, with honey and pollen pots built on the top of the hive. For instance, a vertical commercial hive box has successfully kept a nest of *Geniotrigona thoracica*. This type of hive box is easy to manage and harvest the honey. Horizontal boxes are the more popular for the small stingless bee species. The horizontal model is normally used for species that build the honey and pollen pots next to the brood clusters, such as *Tetragonula pagdeni* and *Tetragonula fuscobalteata*.

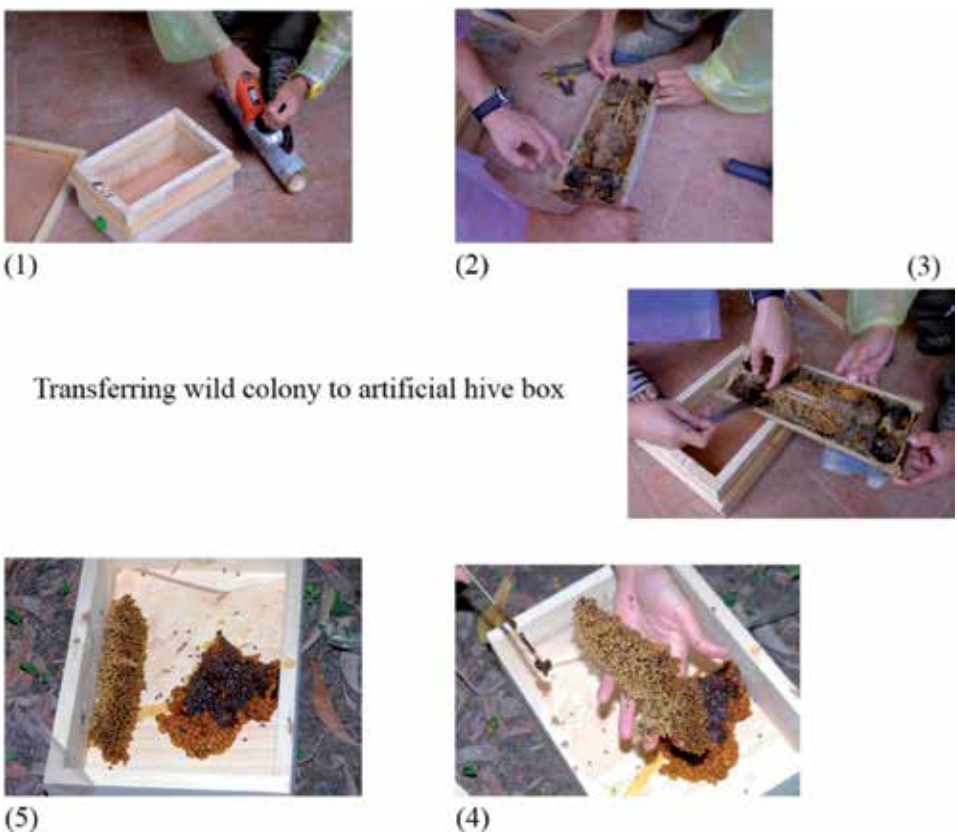

Figure 8.
Step of transferring the natural colony of stingless bee to artificial wood box.

Figure 9.
A horizontal artificial hive of Tetragonula pagdeni *is stacked on a four pod stand.*

The steps for transferring a natural stingless bee colony in a log to an artificial hive box are as follows (**Figure 8**):

1. Two opposite longitudinal incisions of the log using a chainsaw are made. This must be done carefully, because the sawing can injure the brood and food pots inside the log.

2. The brood cluster is the first part to remove and transfer, because the mother queen and young workers are found in this area. The brood area is carefully cut and separated from the original nest by using a knife with a sharp, thin blade.

3. The honey and pollen pots are carefully transferred and put on the floor of the new hive box next to the brood area.

4. Finding the mother queen is necessary to increase the success of transferring the colony.

5. The lid of the new hive box should be replaced to inhibit the workers from flying out, and then the new hive box is put near the original log to let the flying workers move to the new hive box. This procedure may take several hours.

6. After all bees are in the new box, the hive is moved to a suitable location. The box should be put on shelves or four pod stands to deter predators (**Figure 9**).

4. Economics of meliponiculture

Traditional uses for stingless bee honey have been documented for a century, but the selling of stingless bee honey has become cost-effective only during the recent

decade in many parts of the world. The price of stingless bee honey, compared to the honey from *Apis*, is relatively high at around US $40 per liter in Brazil [24], US $80 in Malaysia [25], and US $45 in Thailand (Duangphakdee, unpublished data). Recently, meliponiculture has expanded in many parts of Southeast Asia where there are 45 potential stingless bee species [24]. Currently, the primary purpose of stingless bee beekeeping in Southeast Asia (SE Asia) is for pollination services. They are only now beginning to take root for honey production in Southern Asia (in India) and in SE Asia (Malaysia and the Philippines). Commercial meliponiculture has been intensively developed in Malaysia and the Philippines. In Thailand, as in Vietnam, local people still only use stingless bee honey for "medicinal purposes." Meliponiculture is just at an early state of commercialization. No standard practices have developed for meliponiculture yet. The major difficulty is that of collecting honey from a tree or subterranean nest. The honey harvest technique is still being developed, and it appears still that keeping stingless bees in hives is worth the trouble and difficult to propagate in a large scale [26].

As demand for stingless bee honey is increasing, meliponiculture is getting more interest [25]. The following economic analysis is based on current markets in Malaysia. Two types of stingless bee hives were considered, based on logs (natural) and hives (artificial). Authors examined the investment cost and pricing of a small start-up with 30 colonies. The challenge to new investors was the increasing price of colonies and unpredictable return due to stolen logs, threats from overheating, pests, and parasites. The equivalent annual uniform cost (EAUC) index compares different investments in log and hive system. The study shows that revenue and operational cost are the same in both systems. Because the log type is a 40% cheaper investment, this has 22.7% higher EAUC value than a hive system for the 10 years of life cycle considered. However, both the log and the hive systems offer very good return with a margin exceeding 55%. In addition, the system reached break-even after 8.35 months and 13.56 months with log and hive system, respectively (**Figure 10**). Meliponiculture is therefore economically viable enough to justify investment in Malaysia [25] and other Southeast Asian countries.

The standard size for stingless bee hives has not yet been determined properly. The number of stingless bee keepers in Thailand has been expanding during the past two decades from 700 in 2014 [27] to 1500 in 2018 [28]. Most meliponikeepers are small-scale farms ranging from 20 to 50 hives. Chanthaburi and Trat provinces

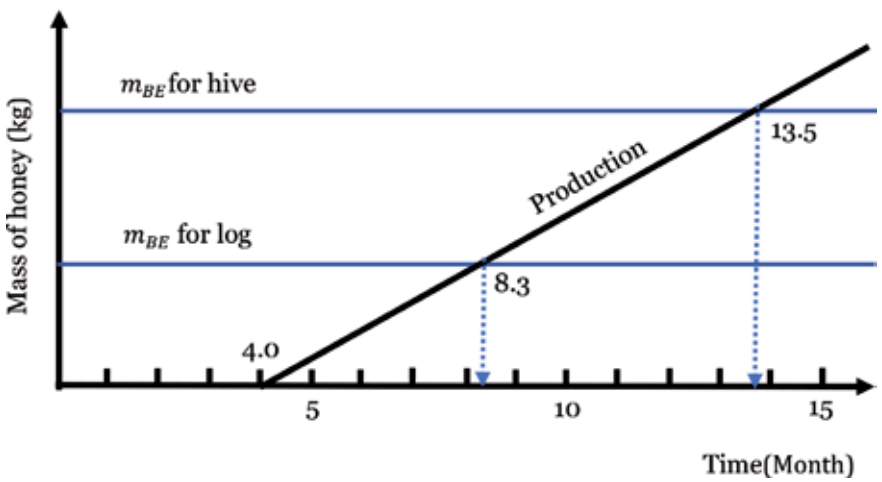

Figure 10.
Breakeven comparison of log and hive system [25].

of eastern Thailand have the most developed commercial meliponiculture for pollination and honey production at approximately 5000 hives [27, 29]. The selling price of Thai stingless bee honey is 1200–1500 THB ($37–$47 USD) per kilogram which 10 times and 3 times higher in price of honey from Thai produced *A. mellifera* and wild *Apis* (*A. florea*, *A. dorsata*, and *A. cerana*), respectively. The propolis and wax cerumen are additional active markets in Thailand with per kilogram returns of 1500–2000 THB ($47–$62 USD). In total, [29] evaluated that stingless bee hive products added 5.76 million THB ($177,500 USD) to the regional economy in 2014. Because of the increase in meliponikeeping, a number of new stingless bee beekeepers in Thailand are increased gradually to support in-country and international markets in other SE Asian countries.

5. Pollination for agricultural productivity and ecological services

As human population grows, the demand for food is increasing every year. The increase in productivity to improve food security without harming the natural environment, and making the improved productivity sustainable for future generations, is a major challenge [30]. Farmers try to improve the quality of their produce to obtain optimum prices. Pollination is one approach to achieve that goal. Incomplete flowers need pollinators for their fruit sets. Even for self-fertile flowers, cross-pollination is still needed for improved production and better quality of seeds and fruits [31]. Thus, beekeeping not only can improve income but also can increase food security [32].

Stingless bees are candidates for commercial and natural pollination. They are highly diverse and abundant and inhabit the tropical and subtropical parts of the world [33]. In SE Asia there are 68 species from 14 genera [34]. Stingless bees form perennial colonies from which they forage year-round with a variety of body size, nest structure and position, and ecological habitats allowing for selection of the most suitable stingless bee species for a given crop species and crop breeding system [33]. Stingless bees are true generalists that visit a vast array of plants [35]. However, at the individual level, stingless bees tend to specialize in a single flower [23, 36]. Indeed, this combination of traits between generalist and specialist is a characteristic that makes them one of the best contributors to pollination for many crops and wild plants [37].

Native tropical plant crops such as coffee and cacao show a mutually beneficial interaction with stingless bees. The genus *Coffea* (Rubiaceae) is native to tropical and subtropical Africa. The two coffee species, *Coffea arabica* L. and *Coffea robusta*, are grown throughout India and Southeast Asia. Even though *C. arabica* is tetraploid and self-compatible [31], the benefits of cross-pollination are still distinctly shown by producing 30% higher fruit set than autonomous self-pollination [38] and 25% higher fruit weight [39]. During the mass flowering season of coffee, the honeybees and stingless bees are dominant. However, they are often absent in the scattered flowering which occurs frequently in tropical plant blooming behavior. Coffee plantations on the edge of forests are more likely to receive a significant benefit from stingless bees which are more abundant within 600 m of the forest margin than other social bees [40] and other insect pollinators. The study of pollinators in coffee plantations in Sulawesi, Indonesia, found three honey bee species and four stingless bee species as the main pollinating species [40].

For strawberries, it is also reported that the yield is increased using stingless bees. Because the strawberry flower seems not to be attractive to honeybees, stingless bees are the preferred choice for strawberry pollination in greenhouses. Most strawberry cultivars are hermaphrodite and self-fertile, but the anther maturation

and stigma receptivity may vary highly in spatial segregation [31] which makes pollination helpful to increase productivity. In Asia, most of the studies of stingless bee pollination have been conducted in Japan [37, 41]. Strawberry pollination with stingless bees has also been seen in Nan, Thailand (O. Duangphakdee, personal communication). In many instances, the fruit and seed crop orchards at the edges of forests noted that natural pollinators are considered in increasing crop yield. Since 1990, SE Asia has suffered deforestation of 33.2 million hectares or 7.6% of the land area [42], which is the highest relative rate of deforestation of any major tropical region. As a consequence, deforestation and forest fragmentation may contribute to declines in crop pollinator populations. Several studies have been conducted to examine the effect of forest proximity on plant pollination ecology. The evaluation of flower visitor diversity, frequency, and fruit set for three crop species has been conducted in mixed fruit orchards of rambutan (*Nephelium lappaceum* L.), durian (*Durio zibethinus* L.), and mango (*Mangifera indica* L.) [43] in southern Thailand. This study compared 10 pairs of orchards that are located at <1 km and >7 km away from the forest edge. Stingless bees were the main visitors for 70.9% of total flower visits on rambutan (**Figure 11**). The distance from forest edge and location of natural stingless bee colony influenced the fruit set of rambutan significantly [43]. Stingless bees were significantly (two times) more frequent on rambutan flowers nearer to the forest.

In the case of durian and mango, even though stingless bees have been observed as flower visitors, the number of fruit sets was significantly influenced by bats (durian) and flies (mango).The distance to the forest did not affect the fruit yield of these two crops [43, 44]. The evidence suggests that the forest is an insect pollinator reservoir. The conservation of natural habitats surrounding a crop orchard is strongly recommended to maintain a population of forest-insect pollinators in natural habitats [45].

As generalists, stingless bees forage in vast array of plant taxa. A study of pollen foraging and resource partitioning of stingless bees throughout year-round

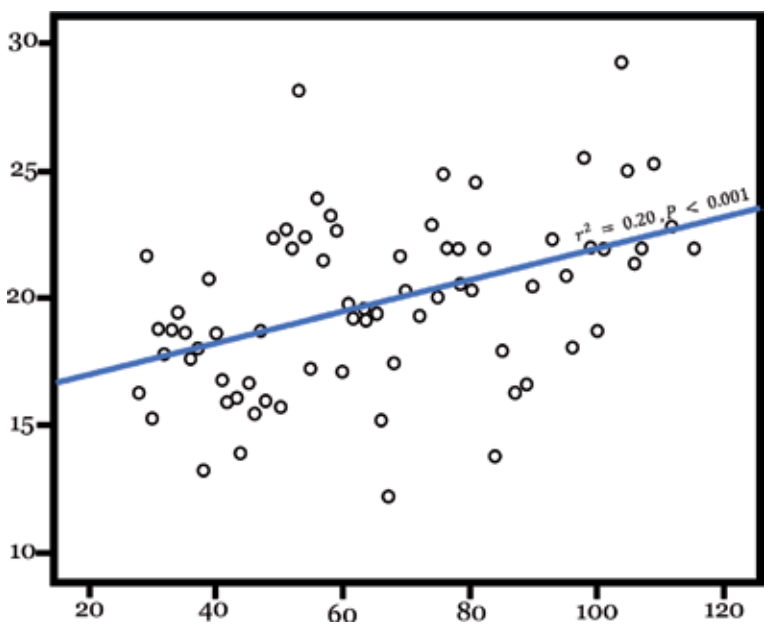

Figure 11.
A linear regression plot for the number of rambutan fruit sets and insect visitation frequency to rambutan flowers in a mixed fruit orchard in Southern Thailand [43].

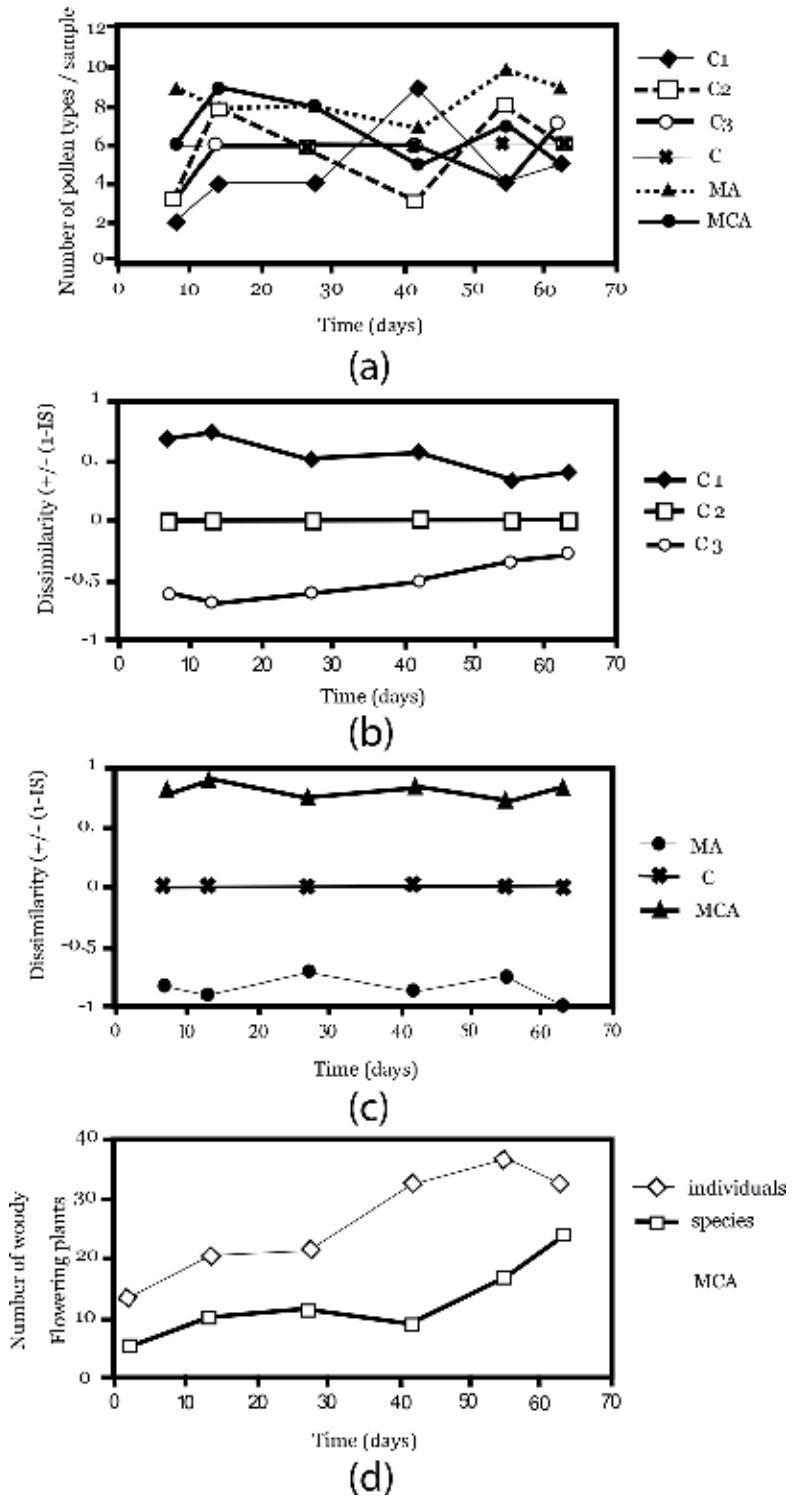

Figure 12.
Pollen resource partitioning of stingless bees: (a) pollen-type richness of samples between different colonies of stingless bees (C = T. collina *(four colonies); MA =* T. melina*; MCA =* T. melanocephala*), (b) dissimilarity of pollen samples within the monospecific* collina*-aggregation over time, (c) dissimilarity of pollen samples within the mixed aggregation, and (d) flowering activity as a function of time in the habitat [46]. Dissimilarity is calculated as (1-Sørensen-index); Sørensen-index* is index *of similarity base on the equation of Sørensen [47].*

flowering dynamic in northern Borneo rainforest [46] unveils some interesting results. They compared pollen foraging within one monospecific (three colonies of *Trigona collina*) and one mixed nesting aggregation (one colony of *T. collina*, and one colony of each of the close relatives *T. melina* and *T. melanocephala*) in lowland tropical rain forest in Sabah, Malaysia. The results suggest that stingless bees, *Trigona collina*, show specificity of pollen source judged by the pollen similarity among the same species in the same aggregation sites. Within the two aggregations of *T. collina*, the similarity of pollen samples showed a strikingly different pattern over time, and there was no similarity at all between colonies in the mixed nest aggregation (**Figure 12**). Nevertheless, the resource partitioning occurs in their geographic location, with those other species of *T. melanocephala* and *T. melina* showing their highly adaptive trait in pollen foraging.

Stingless bees also show interspecific differences in foraging behavior such as the speed of detecting new food sources. Observations at an artificial feeder revealed that *T. melanocephala* arrived at honey baits quicker than *T. melina*, whereas *T. collina* was reluctant to visit the feeding site [48]. Agriculture in SE Asia is frequently multicultivar system. Multiple plant species are cultivated together. For pollination of several flower phenotypes in a mixed plantation, stingless bees are certainly good choices. The diverse crops in those mixed orchards provide a high-quality foraging habitat for pollinators [40]. Much evidence showed that stingless bees are highly adaptive species that are able to contribute to eco-services in SE Asia.

6. Conclusion

Nowadays, meliponiculture (stingless beekeeping) in SE Asia is significantly increased. The promotion of stingless beekeeping as an additional activity for rural villages, together with high stingless bee species diversity [34], stimulates a revival of this activity. For instance, in Thailand, at least six species (*Tetragonula pagdeni*, *T. laeviceps*, *T. fuscobalteata*, *Lepidotrigona terminate*, *Heterotrigona itama*, and *Geniotrigona thoracica*) are commonly managed for commercial pollination services and honey production. Of these, *T. pagdeni* and *G. thoracica* can be the most easily transferred natural colonies to artificial wooden hive boxes. In addition, there are not only short time to colony recovery after dividing colony (4–8 weeks after dividing) but also show high yield of colony production—honey and pollen [29]. Stingless beekeepers increase colony number of these two stingless bee species for both colonies selling and producing honey in short time periods. Colony of *T. pagdeni* and *G. thoracica* can be sole in price of 800–1500 THB ($25–$47 USD) and 4000–5000 THB ($125–$157 USD) per colony, respectively. Therefore, *T. pagdeni* and *G. thoracica* are more suitable to promote and develop for meliponiculture in SE Asia. However, the comparisons of honey and propolis yields from common domestic species of stingless bee of SE Asia are highly suggested. The evaluation of status of a potential industry with the stingless bees with regard to honey production and yield, its commercialization, and management should be also taken into account. Unlike *A. mellifera*, meliponiculture in SE Asia is particularly suggested to the small-scale beekeeping with regard to the flora providing source from multi-cultivar systems that are commonly found in this region. The competitive situation of the prices of honey and other products between *Apis* and Meliponini is also a further issue to be determined. The species-based problems and solution that stingless beekeepers faced should be standardized precisely [49–51]. Finally, the management and production scenario should be developed to collectively improve a substantial quantity and quality of stingless bee products as significant competitive items on the international market are suggested.

Acknowledgements

We are grateful to the Kasetsart University Research and Development Institute (KURDI) and National Research University Project (NRU) for the financial support.

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Edited by Ramón Eduardo Rebolledo Ranz

Beekeeping worldwide has seen remarkable development in the face of the growing demand for products from bees by consumers who demand increasingly innocuous products that do not harm the environment. However, it should be noted that, recently, problems have arisen in beekeeping production that could become restrictive factors for the worldwide development of beekeeping. This book includes, in simple and accessible terms, very relevant topics such as the effect of pesticides, the impact of diseases and their management, production and analysis of pollen present in honey, DNA analysis, and sustainable management, among others. This book is answering an expected need for accurate and international information for the productive sector.

Published in London, UK

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ISBN 978-1-78985-493-0