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Chapter

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**).

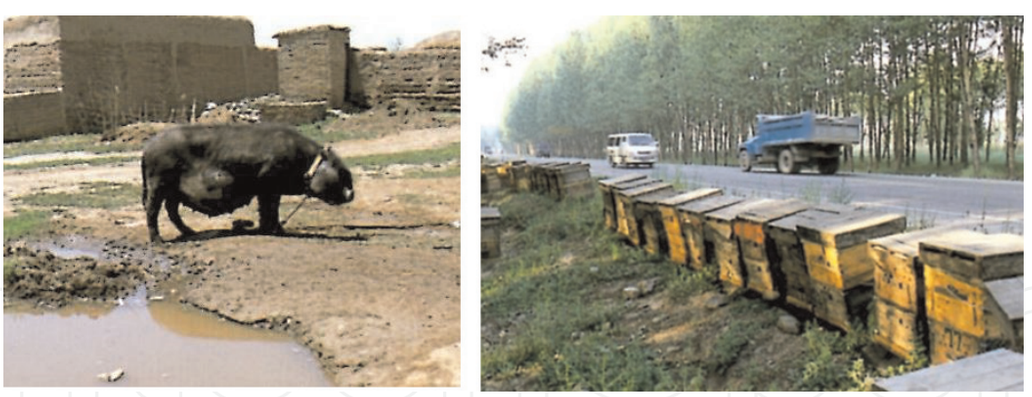

Figure 3.
Location of apiary. Left: The apiary should be located from far away, approximately 10 km, from pig farm, cow farm, and/or chicken farm. Right: When bee hives are set beside a road, the apicultural products may possibly be contaminated with exhaust gas, asphalt dust, and heavy metals. Thus,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

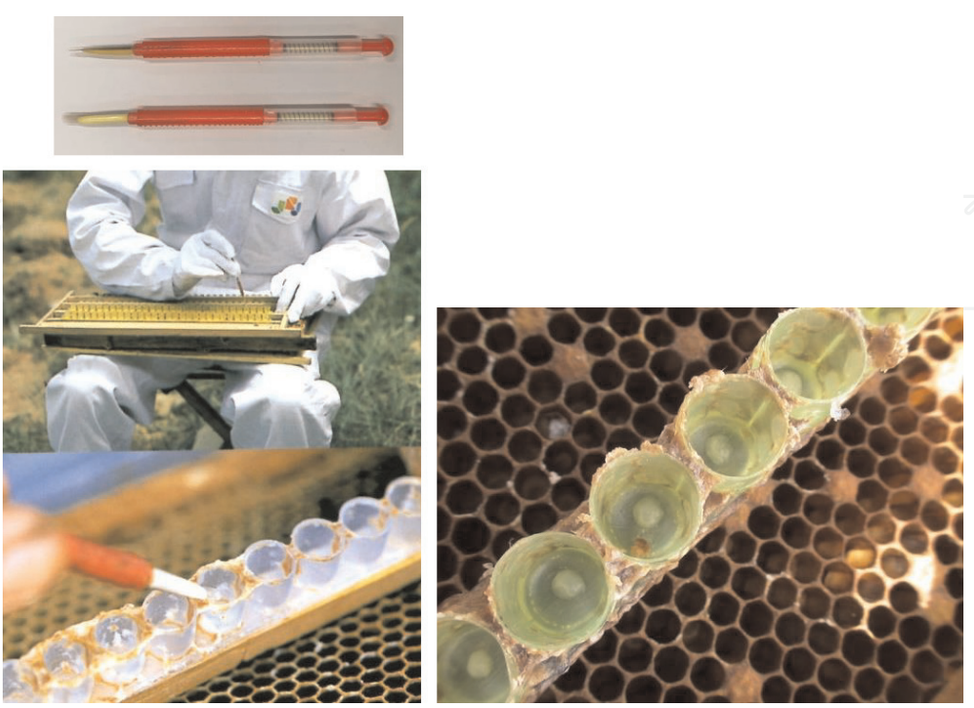

Figure 6.
Transferring of larvae. The larvae are quickly transferred into the artificial queen cell cups using a transferring tool called "larva-transferring needle" paying attention not to hurt the larvae, but this operation needs considerable mastery.

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

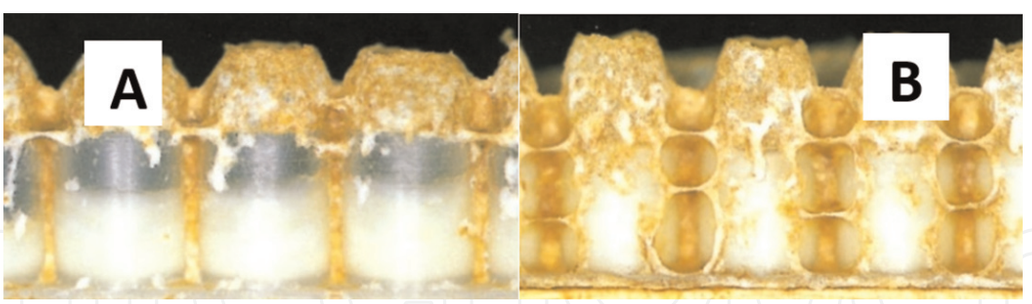

Figure 7.
Harvesting of RJ at 48 h (A) and 72 h (B) after larva transfer. Accumulation of RJ is much greater in the cup 72 h after larva transfer than that 48 h after larva transfer.

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

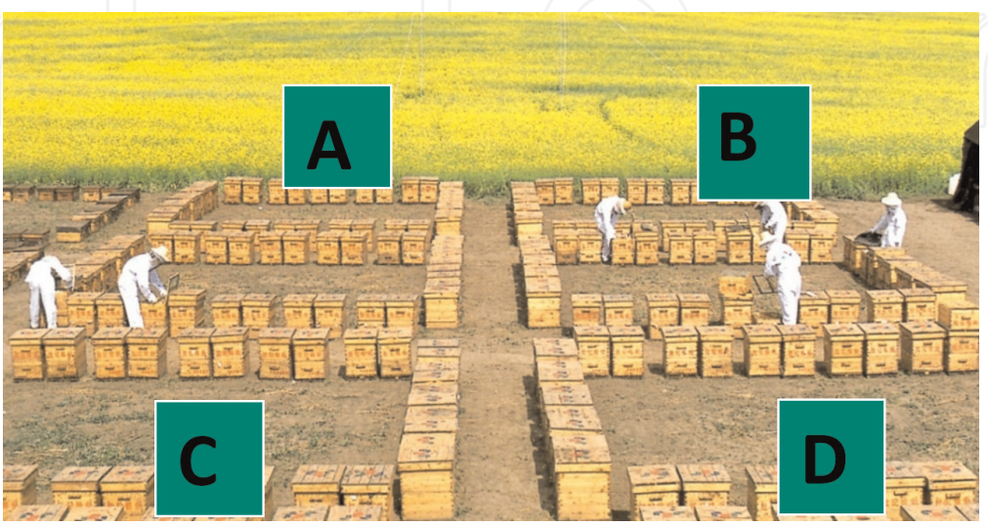

Figure 9.
Rotation of Colony. The colonies are divided into four groups, and the colony used once for larva transfer and harvest is made to rest, and another colony is used for production. This is rotated in sequence at a rate of once every 4 days.

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

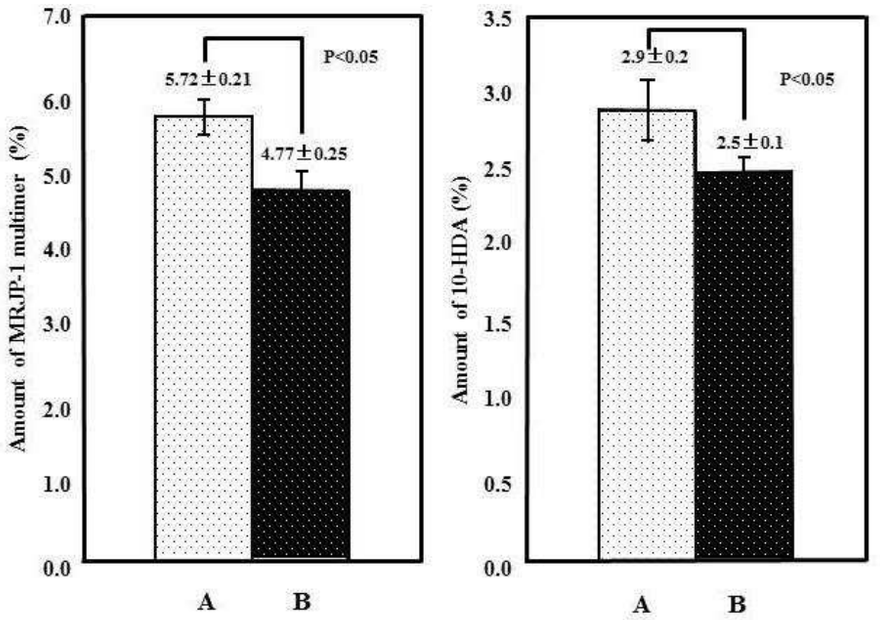

Figure 11.
Comparison of MRJP-1 Multimer and 10-HDA contents in RJ samples between natural beekeeping (A) and ordinal beekeeping (B) [26]. A: Natural beekeeping (harvested after 48 h, with rotation, n = 9); B: Ordinal beekeeping (harvested after 72 h, without rotation, n = 9).

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

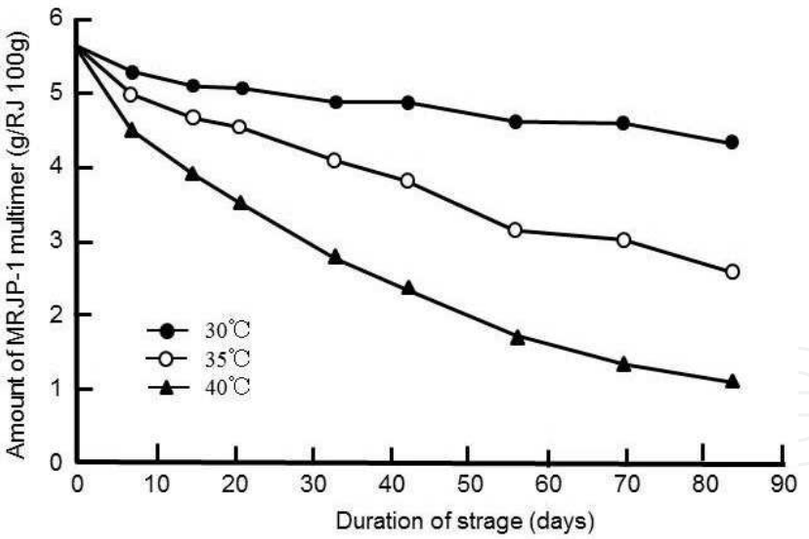

Figure 12.
Transitional change of MRJP-1 multimer contents stored under different temperature [26].

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.

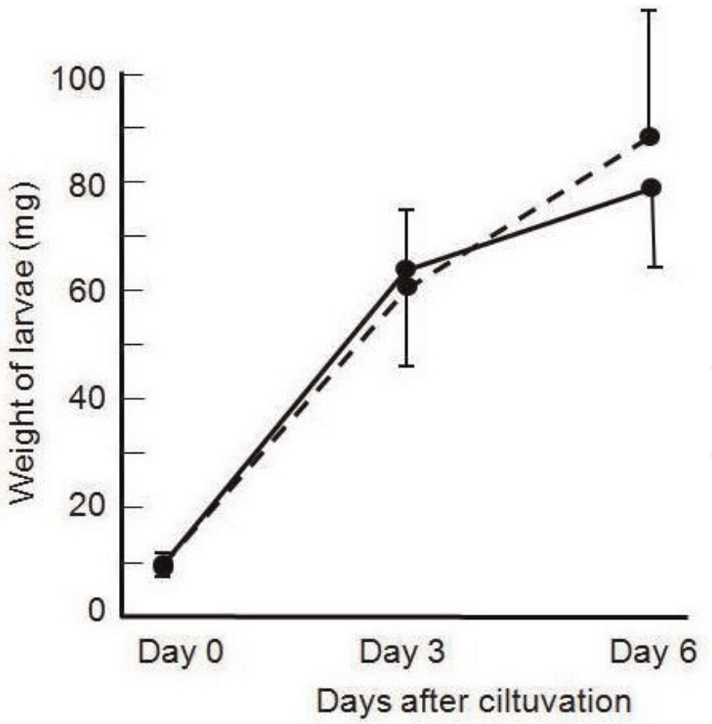

Figure 13.
The growth of larvae in RJ in vitro. Second-instar worker bee larvae were grafted in a 12-well flat-bottomed plastic plate filled with 750 mg fresh RJ. The plate was then set in an incubation chamber and cultured at 31°C for appropriate period. Dotted line: RJ harvested at 48 h. Solid line: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

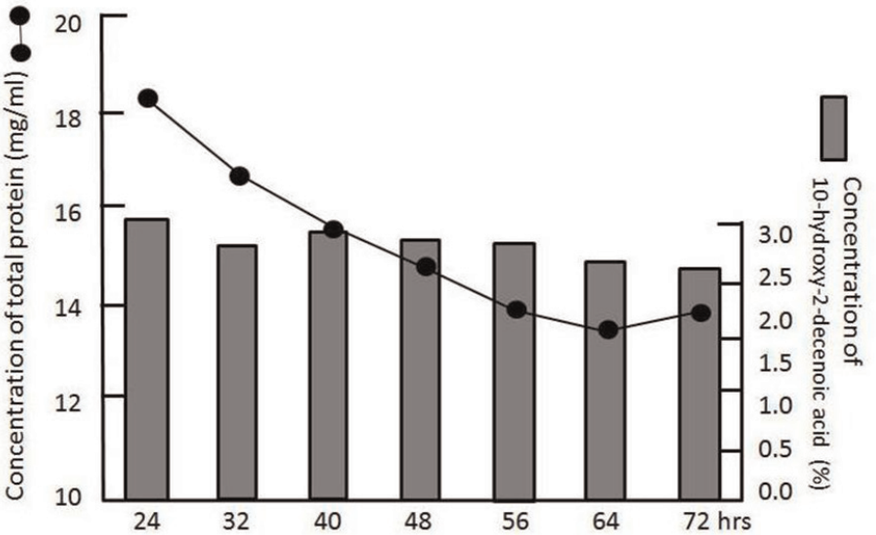

Figure 15.
Changes to major components, total protein and 10-HDA in RJ during the cultivation of larval growth in vitro. The total protein and 10-HDA in RJ were analyzed in a chamber filled with 750 ml of fresh RJ and grafted second-instar worker bee larvae. Total protein and 10-HDA were analyzed for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

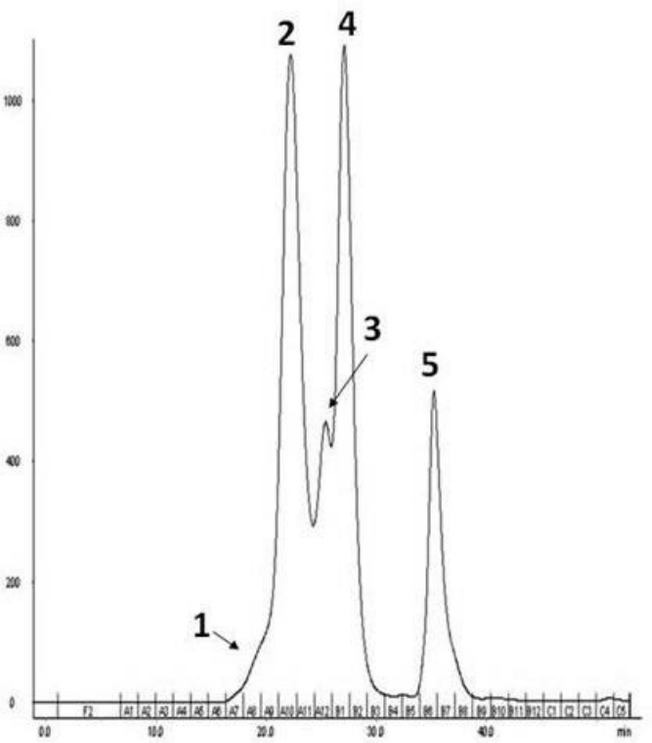

Figure 16.
Elution profile of soluble protein in RJ. A typical elution pattern of soluble RJ proteins. Peak 2 represents MRJP-1 multimer.

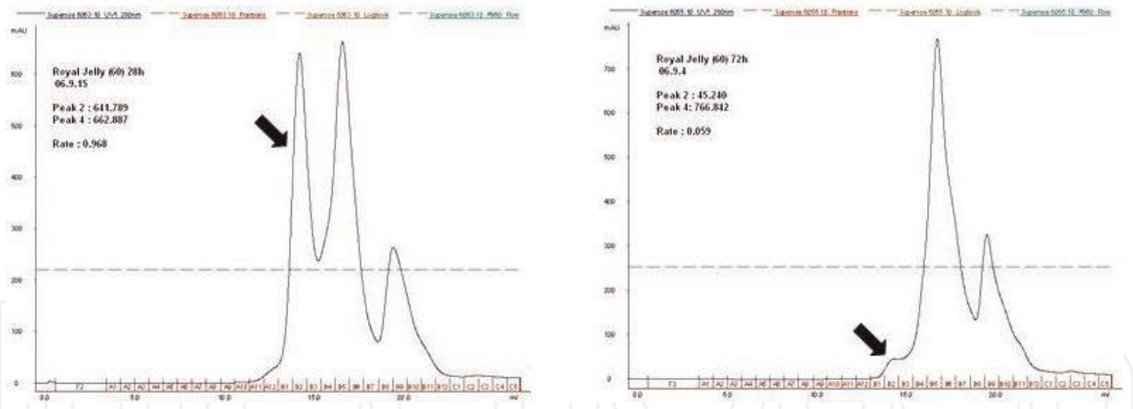

Figure 17.
A case observed where peak 2 disappeared in 72-h cultivation. An unusual case where peak 2 disappeared at 72-h cultivation.

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

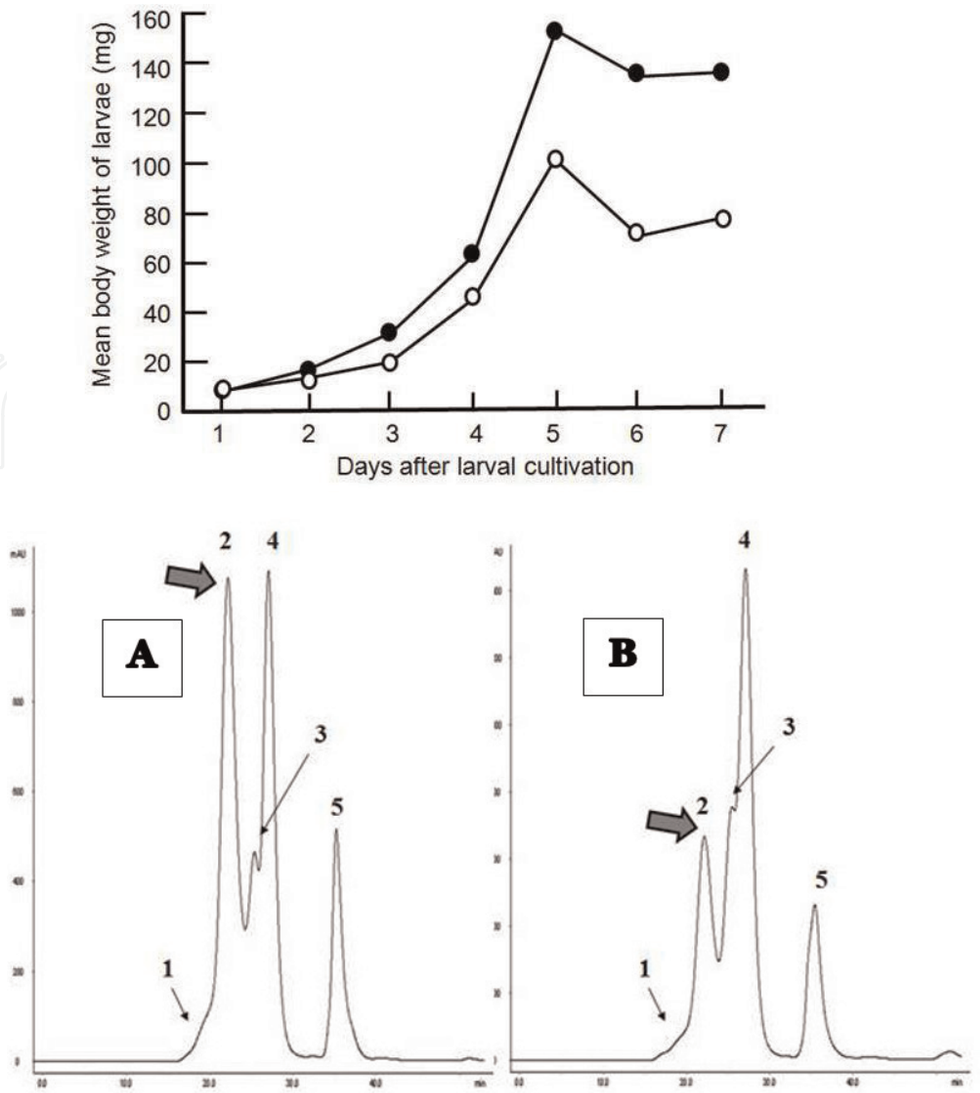

Figure 18.
Larval growth in MRJP-1 Multimer-rich and MRJP-1 Multimer-poor samples of RJ. Larval growth and body weight of larvae cultured in RJ were compared among RJ samples which were different in content of peak 2 proteins. (A) Growth of larvae in MRJP-1 multimer rich RJ (●) and MRJP-1 poor RJ (○),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

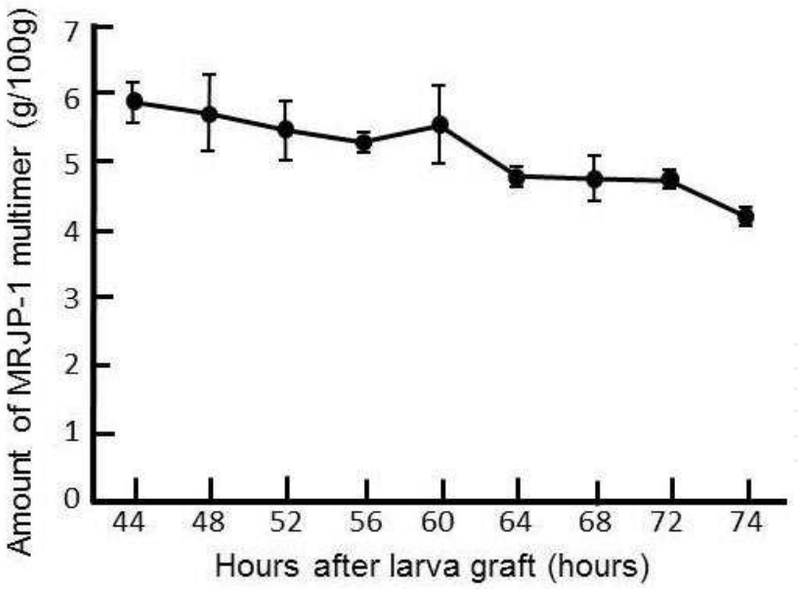

Figure 19.
MRJP-1 Multimer content in queen cells after larval graft.

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.

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