CHAPTER I

INTRODUCTION

The honeybee is a social insect living in a colony or hives comprising 20,000 to 80,000 individuals. Within a colony, there are three physically distinct castes including queen, drones and worker bees (Figure 1.1). Each has their own distinctive role to perform in the honeybee society. The queen performs a limited although critically important repertoire of activities for the colony. Her major tasks are egg laying and secreting exocrine hormone analogue called pheromone to act upon the other individuals of the same species. The queen is thus the mother and central authority figure in the nest, but otherwise performs little or no work. The drones have less activity and function of life inside the colony. They have only one task which is mating with queen. The worker bees perform almost all of the work in the colony including brood rearing, construction, defense, foraging, thermoregulation, cleaning and many other tasks. In general, their numerous tasks are very precisely organized and largely dependent age. The younger workers tend to perform within-colony tasks and older workers do outside jobs like guarding and foraging, but there is great variability in this temporally based caste structure (Figure 1.2).

Temporal caste structure in honeybee is closely linked to glandular development and resorption, particularly for the brood food and wax glands (King, 1933; Simpson, Riedel, and Wilding, 1968). The

hypopharyngeal, mandibular and wax gland begin to enlarge shortly after a young worker emerges, reaching and maintaining their maximum sizes at 5 to 15 days of age, coincidental with brood rearing and comb building tasks. All three glands diminish in size and output coincidental with the transition from these jobs to other tasks.

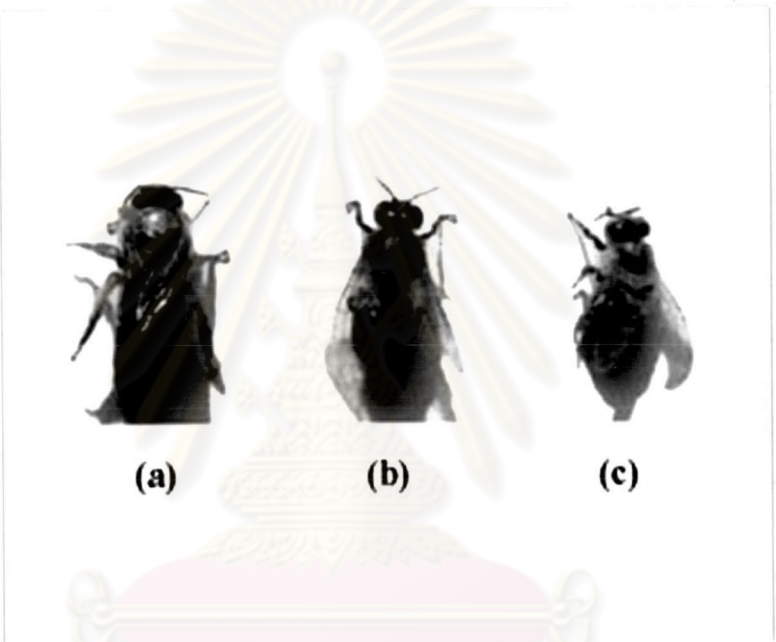

Figure 1.1 Three types of individuals or castes in the honeybee hive

(a) queen (b) drone (c) worker

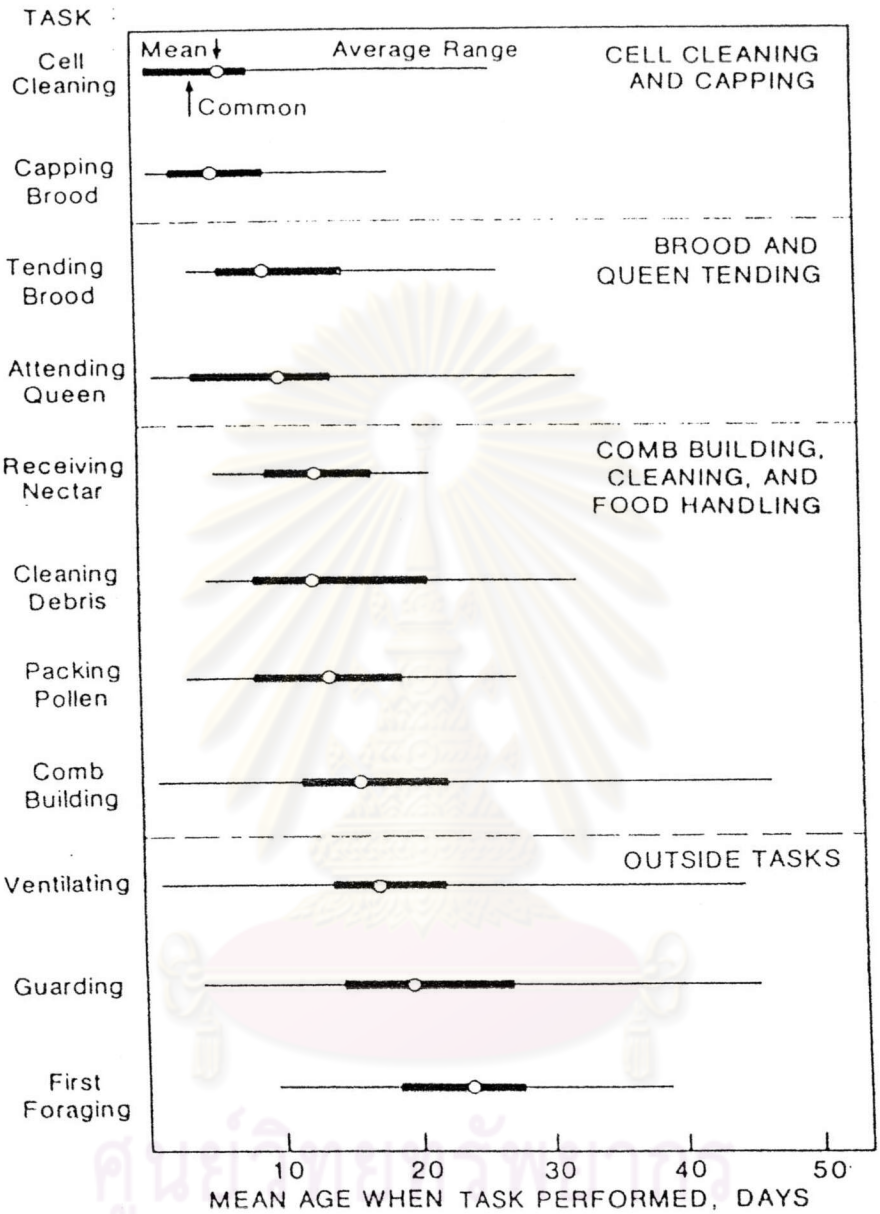

Figure 1.2 Age-related task performance by worker bees

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Royal jelly

Royal jelly (RJ) is a milk-like creamy, whitish, strongly acid material with slightly pungent odor and taste. It is the brood food secreted among the brood rearing by the hypopharyngeal (cephalic) and mandibular glands (Figure 1.3) of nurse bees (worker bees at 5 to 15 days of age) to feed the younger and older larvae and the queen. This secretion is fed only temporarily (no more than 3 days) to the brood of workers and drones, but constitutive food of the queen for the entire span of both her larval and adult lives (Witherell, 1975). The differentiation between queen and worker bees is related to royal jelly feeding amount and period. Thus, royal jelly may consist of some substances involving in differentiated mechanism.

The difference of feeding period between queen and workers has many effects in various aspects such as morphology, development period, life span and behavior (Table 1.1). In morphology, the queen has developed her reproductive organ and lays between 175,000 and 200,000 eggs, annually. Unlike the queen, the workers can not lay eggs and develop organs related to their work such as pollen baskets, stronger mandible, brood food glands and wax gland. The queen develops to adult in 15.5 days while the workers develop to adult in 21 days. The queen has longer life span than the workers. She lives for a few years as compared to a few months for the worker bees.

Moreover, many researchers report on the useful properties of royal jelly. For examples, it could inhibit the growth of some bacterial and fungal strains such as *Paenibacillus larvae larvae, Bacillus subtilis,*

Saphylococcus aureus, Escherichia coli, Streptococus hemolyticus, Enterococcus spp. etc. (Bachanova *et al.*, 2002; Fujiwara *et al.*, 1990; and

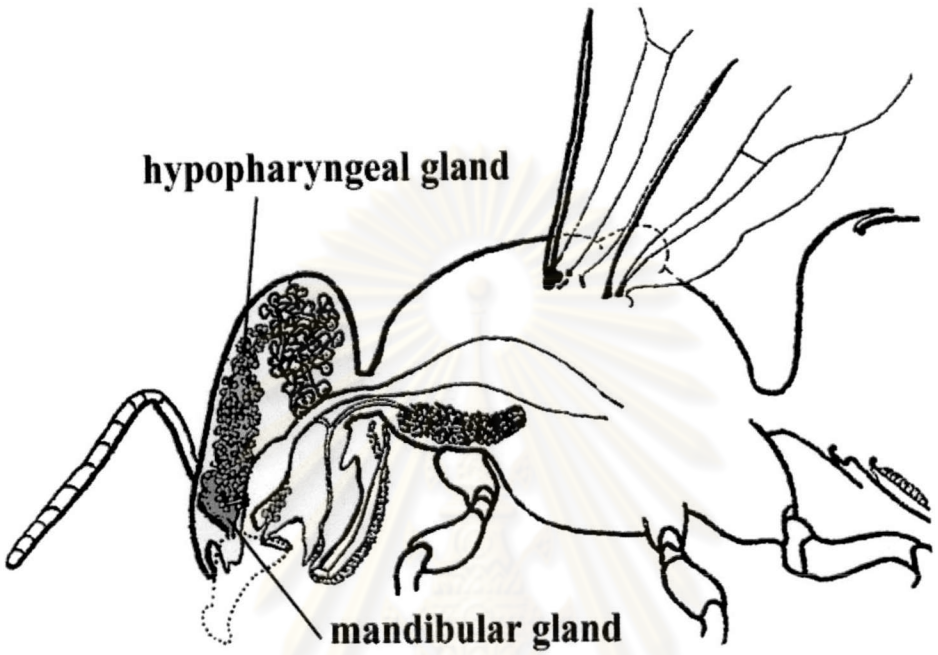

Figure 1.3 The hypopharygeal (cephalic) and mandibular glands

Sanguandukul and Nimachaikool, 1993) and also inhibit tumor growth and increase life span of the patient (Tamura, Fujii and Kuboyama, 1987). In addition, royal jelly could simulate and normalize influence on the function of the adrenal gland (Masterov and Nersesian, 1995). It was found that 50-100 mg per day intake of royal jelly significantly decreased serum and liver total lipids and cholesterol levels ($P<0.01$) and normalized HDL and LDL ($P<0.05$) in rats and rabbits. It also retarded the formation of atheror in the aorta of rabbits, which were fed with a hyperlipemic diet (Vittek, 1995; and Shen, Lu and He,

1995). Oral royal jelly administration showed some anti-inflammatory activity by decreasing exudation and collagen formation using granulation tissue formation in the cotton pellet method (Fujii *et al.*, 1990). Royal jelly also shortened the healing period on desquamated skin lesions (Fujii *et al.*, 1990) Thus, royal jelly was applied to use in clinic and cosmetic.

Table 1.1 Differences of worker and queen bee *Apis mellifera* in various aspects(Kawinsaeksan, 1996)

	worker	**queen**
Royal jelly feeding period	first 3 days of larvar	life span
Weight	86.03 mg	170.06 mg
Life span	10-12 weeks	2-3 years
Reproductivity	cannot reproduce	can reproduce
Egg laying	cannot lay egg	lay 1,000-2,000 eggs/day
Mandibular gland	biosynthesis of royal jelly	biosynthesis of queen pheromone

However, royal jelly has not been a traditional beekeeping product because it is always fed directly to queen and larvae as it is secreted. It is not stored and produced in a few amounts (5-10 g/60,000 worker bee

individuals/day). Therefore, the royal jelly value is high at about 3,000-5,000 baths/kg.

For beekeeping, the harvesting of royal jelly is during queen rearing because the larvae are destined to become queen. All younger female larvae (1 to 4 day larvae) can be the queens if they are cared and fed like queen. The queen larvae are supplied with an over-abundance of royal jelly in their cell. These queen larvae can not consume the royal jelly as fast as it is provided and the royal jelly accumulates in the queen cell and the beekeeper can harvest royal jelly from those queen cells (Figure 1.4).

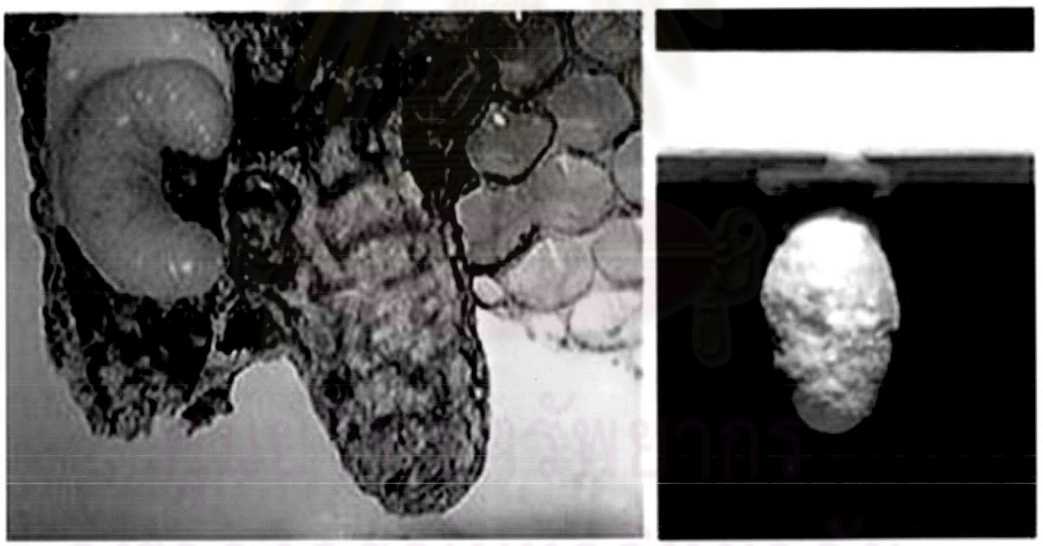

Figure 1.4 The queen cell

The chemical composition of royal jelly

Numerous chemical analyses of the royal jelly have been published over the years. Generally, those analysis are attempted to determine the various nutritional components such as moisture, protein, sugars, lipids, ash and mineral salts. Most of the studies are from the western honeybee, *Apis mellifera*, the European native honeybee, which is fed in commercial hive for beekeeping. Although the analysis results occur with notable variation (Table 1.2), the composition of royal jelly remains relatively constant when comparing different colonies, bee race and harvesting period.

The moisture content is the largest fraction of the fresh royal jelly, but by dry weight, protein and sugar contents are by far the largest fractions. Of the nitrogenous substances, proteins are average 73.9%. Free amino acids are 2.3% (Takenaka, 1983). The protein consists of five major royal jelly proteins (MRJP1, MRJP2, MRJP3, MRJP4 and MRJP5), that contain a relatively high amount of essential amino acids shown in Table 1.3 and Table 1.4. The free amino acids are proline and lysine. A number of enzymes are also present including glucose oxidase, phosphatase and cholinesterase. An insulin-like substance has been identified by Kramer *et al.*, 1982.

Table 1.2 Range of some nutritional composition of fresh royal jelly of *Apis mellifera* (Aeppler, 1992; Haydak, 1943; Townsand&Lucas, 1940; Planta, 1888; Melampy and Jones, 1939; Lercker *et al.*, 1981; Weaver&Kuiken, 1951; Rembold, 1965; and Baker *et al.*, 1972).

Component	Range of amount
Moisture (%w/w)	45-69.9
Crude protein (%w/w)	6.7-30.6
Crude lipid (%w/w)	1.7-8.6
Type of amino acids (μg%)	15-19
- lysine(%)	4.4-25.8
- histidine(%)	1.7-2.8
- arginine(%)	4.8-10
- aspartic acid(%)	9.9-17.7
- threonine(%)	2.8-4.0
- proline(%)	7.3-63
- cystine(%)	0-3.3
- methionine(%)	1.9-2.5
- leucine(%)	7.7-8.0
- phenylalanine(%)	4.1-4.5
- tryptophan(%)	0-1.3
- valine(%)	6.7-7

Table 1.3 Amino acid composition (μg%) of *Apis mellifera* major royal jelly proteins (MRJPs) (Schmitzova *et al.*, 1998)

Amino acid	MRJP1	MRJP2	MRJP3	MRJP4	MRJP5
Ala	3.9	6.2	4.9	4.3	3.8
Arg	**3.4**	**3.8**	**4.9**	**4.1**	**9.0**
Asn	6.9	11.3	15.9	13.8	8.7
Asp	8.6	7.1	7.5	7.5	12
Cys	2.5	1.5	1.1	1.3	1.0
Gln	3.9	5.1	7.1	6.3	3.8
Glu	3.9	3.8	3.8	3.9	2.5
Gly	5.6	6	6.4	4.1	4.0
His	**2.3**	**2.4**	**2.2**	**3.9**	**1.8**
Ile	**6.0**	**5.1**	**4.0**	**3.2**	**4.8**
Leu	**9.5**	**8.2**	**6.8**	**9.7**	**5.2**
Lys	**5.1**	**6.9**	**5.8**	**5.0**	**4.3**
Met	**3.5**	**2.4**	**2.2**	**2.4**	**11.4**
Phe	**4.2**	**4.4**	**1.7**	**2.2**	**2.6**
Pro	3.7	3.1	2.5	2.2	2.6
Ser	8.1	5.8	5.9	8.4	6.2
Thr	**6.3**	**4.6**	**4.0**	**4.7**	**5.6**
Try	**1.2**	**1.3**	**0.9**	**1.3**	**1.1**
Tyr	4.4	3.5	3.1	3.9	3.3
Val	**6.5**	**7.5**	**6.8**	**8.0**	**5.6**
Ess. Aa.*	**48%**	**47%**	**39.30%**	**44.50%**	**51.40%**

* essential amino acid
bold letter is essential amino acid

Table 1.4 Amino acid composition (μg%) of pure harvested royal jelly and of commercial royal jelly products (*Apis mellifera*) (Howe *et al.*, 1985)

Amino acid	Harvested samples (X±SD) (%)	Commercial samples					
		A	E	F	G	H	I
Lysine	8.06±0.70	7.3	7.9	7.4	10.3	8.1	7.3
Histidine	3.26±0.33	3.0	3.1	3.2	4.7	2.9	3.0
Arginine	6.24±0.46	6.1	5.0	6.3	9.0	5.9	6.1
Aspartic acid	16.14±1.70	18.5	18.6	19.7	15.3	19.5	18.5
Threonine	4.89±0.61	4.9	5.1	4.9	5.9	4.8	4.9
Serine	6.09±0.41	6.2	5.9	5.8	5.7	6.8	6.2
Glutamic acid	10.19±0.59	9.9	9.9	9.8	9.5	11.0	9.9
Proline	6.01±0.86	4.6	5.7	5.6	4.6	5.5	4.6
Glycine	3.68±0.20	3.7	3.8	3.8	3.6	3.6	3.7
Alanine	3.68±0.31	3.9	3.9	3.7	3.4	3.1	3.9
1/2 cysteine	0.14±0.58	-	-	Trace	Trace	-	-
Valine	6.61±0.44	6.7	7.1	6.6	6.3	5.9	6.7
Methionine	1.67±0.63	2.4	0.5	0.1	0.3	0.3	2.4
Isoleucine	5.56±0.15	5.6	5.8	5.5	5.3	5.7	5.5
Leucine	8.18±0.43	7.8	8.0	7.9	7.5	9.1	7.8
Tyrocine	4.30±0.36	4.7	4.3	4.4	3.9	3.4	4.7
Phenylalanine	5.26±0.027	5.0	5.4	5.4	4.8	4.5	5.0

The sugar composition of royal jelly consists mostly of fructose and glucose. At least 90% of total sugar (in relatively constant proportions) is similar to honey. Fructose is prevalent, but sucrose content varies considerably between samples. Other sugars present in much lower quantities are maltose, trehalose, melibiose, ribose and eriose (Lercker *et al.*, 1986).

The total ash content of royal jelly is about 1% of fresh weight or approximately 2 to 3% of dry weight. The major mineral salts are potassium (K), calcium (Ca), sodium (Na), zinc (Zn), iron (Fe), copper (Cu) and manganese (Mn), in descending order, with a strong prevalence of potassium. In addition, royal jelly is extremely rich in vitamins as shown in Table 1.5. Only traces of vitamin C is found while fat-soluble vitamin (A, D, E and K) concerned in fertility of queen bee are not found.

Another interesting feature of royal jelly is lipid content. The lipid fraction consists of 80-90% (by dry weight) free fatty acids with unusual and common structures. In 1982, Lercker *et al.* had identified the component of royal jelly of *Apis mellifera*, especially the organic acids, lipid fraction, hydrocarbons and sterols. They found that royal jelly of *Apis mellifera* contains lipid vary from 3.4 to 13.2% of dry matter. Moreover, results also show that royal jelly mostly contains short chain hydroxy fatty acids (8-10 carbon atoms), fatty acid methyl ester or dicarboxylic acids (Table 1.6), in contrast to the fatty acids with 14 to 20 carbon atoms which are commonly found in animal and plant materials (Lercker *et al.*, 1981). These fatty acids are responsible for most of the recorded biological properties of royal jelly and being signal within the honey hive.

Table 1.5 Vitamins and Minerals contained in royal jelly of *Apis mellifera* (Justin and Stephen, 1992)

Component	microgram/gram of royal jelly
Vitamins	
Thiamine	6
Riboflavin	9
Pyridoxine	3
Niacin	50
Pantothenic acid	100
Inositol	100
Biotin	1.5
Folic acid	0.2
Vitamin C	4
Vitamin A	0
Vitamin D	0
Vitamin E	0
Vitamin K	0
Minerals	
K	5500
Mg	700
Na	600
Ca	300
Zn	80
Fe	30
Cu	25
Mn	7

In lipid fraction, the predominance is hydroxy acids. These functionalized acids are implicated in the regulation of activities of the hive, and metabolic pathways, some of which are specific to the queen (Plettner *et al.*,1997; Plettner *et al.*, 1996). Their effect in human nutrition is not well known, partly due to the small number of studies. Of the particular interest has been the occurrence, in the lipidic fraction. A main component, 10-hydroxy-2-decenoic acid (10-HDA), appears to be a fatty acid specific to the royal jelly. Some publishes reported that 10-HDA can reduce the content of triglyceride and beta-lipoprotein (HDL), which show preventive and therapeutic effects on hyperlipoidemic (Xu, Mei and Xu, 2002). Also, 10-HDA would be the constituent responsible for the antibacterial action. Currently, it has been proposed as a freshness parameter of royal jelly (Antinelli *et al.*, 2002).

Although both queen and worker mandibular gland produce functionalized 10-carbon fatty acids, but those acids are differ in the position of functionalization. The acids characteristic of queens have the functional group at the penultimate (ω-1) position, whereas the acid found in workers have the functional group at the last (ω) position. While the biological function of acids from queen makes her presence and reproductive dominance throughout the nest, it also makes the queen highly attractive to the workers, causing them to form a retinue around the queen and to cluster around her during swarmimg (Winston and Slessor, 1992), the worker mandibular acids appear to involved in food storage and larval nutrition. Barker *et al.* (1959) found that 10-HDA

Table 1.6 Organic acids present in royal jelly (Lercker *et al.*, 1982).

Compound
Diester
Hydroxyester
Methyl hexenadioate
7-hydroxyoctanoate
3-hydroxydecanoate+ 6-hydroxydecanoate
methyl-octanedioate
n-nonanedioate
8-hydroxyoctanoate+ p-hydroxybenzoate
methyl-octenedioate
9-hydroxynonanoate
isophthalate
9-hydroxydecanoate
n-decanedioate
10-hydroxydecanoate
9-hydroxy-2-decenoate
palmitate
hydroxy-ester
n-decenedioate
hydroxyester
10-hydroxy-2-decenoate
methyl-tridecenedioate
11-dyfroxyundecanoate
octadeconoate
3, 10-dihydroxy decanoate
Others

and the similar acids presented in royal jelly also present as the major component of worker mandibular gland of *Apis mellifera*. In addition, the chemical study of Japanese honeybee (*Apis cerana japonica*) showed that 10-HDA and 10-HDAA (10-hydroxy-2-decanoic acid) were the major components in both royal jelly and young worker mandibular gland (Matsuyama, Suzuki and Sasagawa, 1998). However, workers also have the diacids corresponding to the 10-carbon hydroxy acids, 2-(E)-decenedioic acid and decanedioic acid (Pain *et al.*, 1962 and Weaver *et al.*, 1968). Those of publish showed that the fatty acids in royal jelly are produced from worker mandibular gland.

Study on the biosynthesis of mandibular fatty acid, Plettner, Slessor and Winston (1998) studied by labelled the worker mandibular glands with 1-^{13}C acetate. The results showed incorporation of acetate into 9-HDA, 8-HOAA, 10-HDAA and 10-HDA, which suggest their intermediate precursors as shown in Figure 1.5. They found that the mandibular fatty acid biosynthesis is similar to many fatty acid-derived semiochemicals, all of these biosynthetic routes consist of three processes: (1) synthesis of the precursor fatty acid; (2) functionalization and chain shortening or elongation; and (3) carboxyl group modification. The first process, the precursor of fatty acid is synthesized from acetate. The second process, the fatty acid is introduced of a second functionality such as a double bond or a hydroxyl group. In many cases, the chain length of the precursor fatty acid does not correspond to that of the final product, because the precursor fatty acid is chain shortened or elongated before or after functionalization. The last process in the biosynthesis of fatty acid is

the modification of carboxyl group. For example, fatty acid alcohols are formed by reduction of the corresponding keto-fatty acyl coenzyme A (CoA) esters (Jurenka and Roelofs, 1993), lactones are formed by cyclization of hydroxy acyl CoA esters (Vanderwel *et al.*, 1992), and hydrocarbons are formed by the reduction of a fatty acyl CoA ester to an aldehyde, followed by decarbonylation (Mpuru *et al.*, 1996). However, the enzymatic system was not mentioned in Plettner *et al.*(1998).

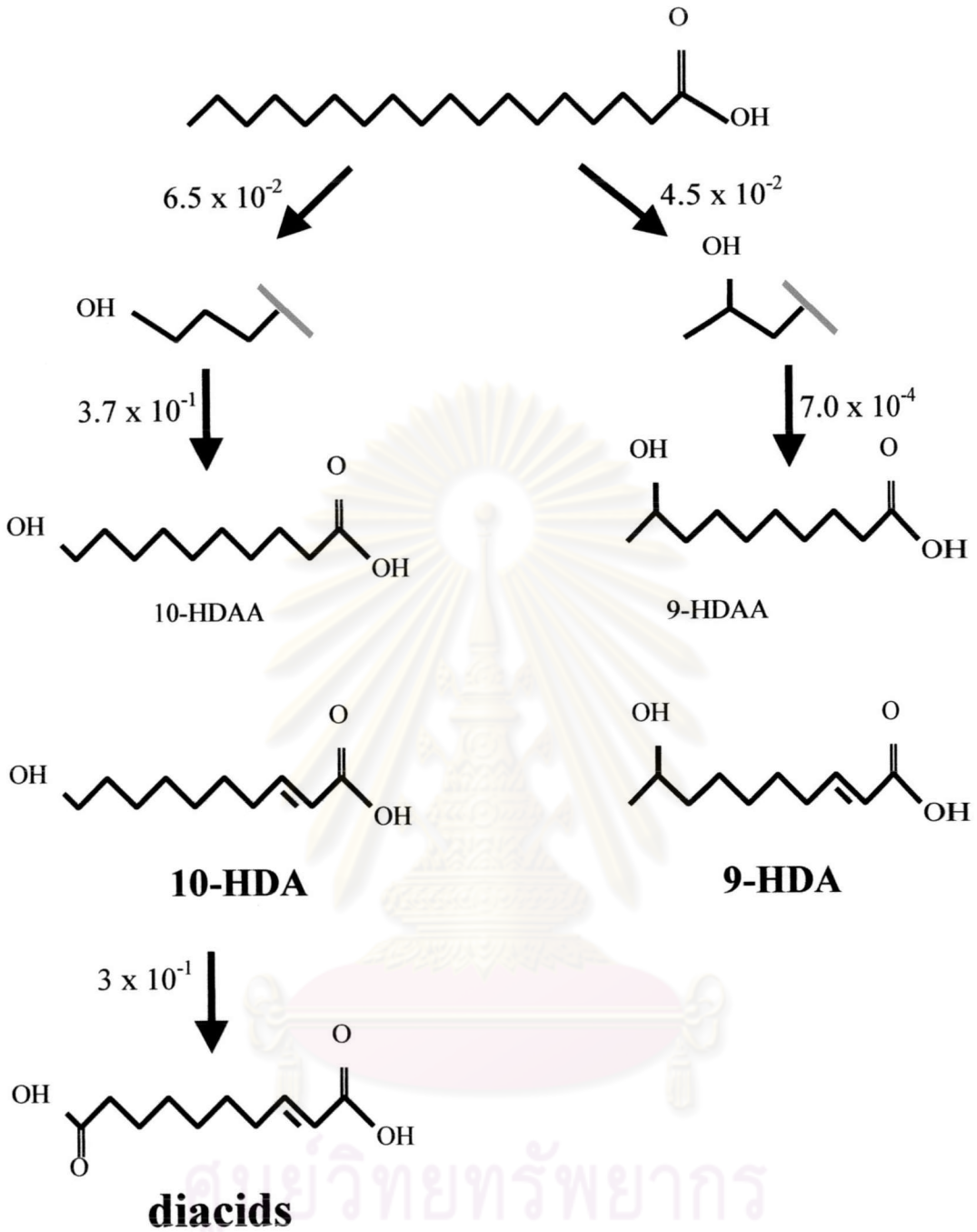

Figure 1.5 Caste-specific biosynthesis of mandibular acids in workers. Estimated rates (nmol/min/gland) for the three steps of the pathway are indicated next to the arrows and the major component of the blend is shown in bold type (Plettner, Slessor and winston, 1998)

Comparing with *Apis mellifera*, The studies of royal jelly and mandibular gland of *Apis cerana* are very limit. Only few publishes are reported. Hoshiba, Ichiji, and Akio (1981) have reported trials with *A. cerana* queen rearing in *A. mellifera* colonies and a trial with *A. cerana* queen rearing on royal jelly produced by *A. mellifera* in the laboratory. *A. cerana* queen could not grow sufficiently in a colony of *A. mellifera*. So, they suggest that the royal jelly from each species is different. In 1996, Takenaka and Takenaka have compared the chemical composition between *Apis cerana japonica* and *Apis mellifera* (Table 1.7). *A. cerana japonica* royal jelly contained more protein and less carbohydrate than *A. mellifera* royal jelly. MRJPs of *A. cerana japonica* and *A. mellifera* have different molecular weights, isoelectric points and immunological characteristics. Matsuyama, Suzuki and Sasagawa (1998) have presented 10-HDA was major component in both mandibular gland of young *Apis cerana japonica* and royal jelly. This data supports the relation between fatty acid and mandibular gland as those in *A. mellifera*.

Table 1.7 Chemical composition of Royal Jelly produced by *Apis mellifera* (MRJ) and *Apis cerana* japonica (CRJ) (Takenaka and Takenaka, 1996)

Chemical Component	MRJ (n =18)	CRJ (n = 5)
Moisture (%)	68.3 ± 1.4	65.3 ± 2.5
Protein (%)	12.7 ± 0.8	16.4 ± 2.5
Carbohydrate (%)	11.9 ± 0.7	9.4 ± 0.6
fructose (%)	(5.3 ± 0.4)	(4.8 ± 0.5)
glucose (%)	(5.0 ± 0.5)	(3.6 ± 0.4)
others (%)	(1.6 ± 0.4)	(1.3 ± 0.7)
Lipid (%)	6.1 ± 0.4	7.4 ± 0.6
10-HDA (%)	(2.4 ± 0.2)	(0.9 ± 0.2)
Ash (%)	1.0 ± 0.2	1.5 ± 0.2
pH	3.7	3.8
Acidity (%)*	42.2 ± 2.1	39.3 ± 3.1

* 1 N NaOH, ml/100 g of fresh royal jelly

In Thailand, there are 2 species of honeybee can be fed or managed in the commercial hive: *Apis mellifera* and *Apis cerana*. However, the data about royal jelly is mostly studied on *A. mellifera* species because the beekeeping for industry is mainly from *A. mellifera*. This species is not native species of Thailand. Then, in this work, the native species, *Apis cerana* is studied.

Sihanuntavong (1999) have studied on the distribution of honeybee in Thailand using PCR-RFLP technique. Three mtDNA regions: (1)

srRNA; (2) lrRNA; and (3) intergenic COI-COII were amplified and completely digested with *Dra*I restriction endonuclease. Two, four and seven haplotypes were obtained from srRNA, lrRNA and intergenic COI-COII, respectively (Appendix A). These three mtDNA regions employed in this study generated thirteen composite haplotypes. Genetic distances among populations were used for phylogenetic reconstruct using UPGMA approach. Three genetically distinctive groups: (1) northern; (2) southern; and (3) Samui island were separated. In this study, the *Apis cerana* individuals were investigated the population group following Sihanuntavong (1999) and the chemical composition of their royal jelly were investigated and compared with *Apis mellifera* royal jelly. Moreover, to understand the biological roles of mandibular gland of nurse bee (*Apis cerana*), the gene expression of mandibular gland was also studied using EST (Expressed sequence tag) technique. This technique is convenient to screen and identify the high numbers of genes expressed at the moment because it uses mRNAs, which present in sample only in the expression, to construct the cDNAs. And the nucleotide sequences of cDNAs were analyzed. Only 200-300 bases from 5'-terminal datas can be identified the present gene in the studying period by compared the datas with DNA database. Thus, after sampling and mRNA extraction, the mRNAs was converted to cDNAs and the cDNAs were cloned to bacteria *E. coli* using plasmid vector system. Then, the plasmid was extracted from the *E. coli* and sequenced for the nucleotides and the sequence datas were compared with GenBank database. These basic datas may lead us to know the enzymes or

proteins involved in metabolism of royal jelly acid of *A. cerana* that may advantage for further studies.