CHAPTER I

LITTERATURE REVIEWS

The honey bee is one of the most important social insects in the world of agriculture and food industry, it is wild pollinators which increases yield production. Commercial honey bee products such as honey, pollen, bee venom, bee wax, propolis and royal jelly also have a high economic value in food, cosmetic and medicinal industries.

Figure 1.1 Useful of the honey bees *Apis mellifera.*

1.1 Honey bee castes

The honey bees of the genus *Apis* (Order Hymenoptera) are advanced eusocial insects with three social castes: the queen, the infertile female workers and the male bees or drones. A colony of honey bees has only one queen, a large number of workers and zero to a few hundred of drones depending on different annual period. The queen and worker bees are female, heterozygotes (diploid 2n = 32) grown from fertilized eggs but they are distinctly different in both behavior and morphology. Whereas drones are male, hemizygotes (haploid individuals n = 16) arising from unfertilized eggs (Ruttner, 1988; Wongsiri, 1989).

1.1.1 Honey bee Queen

Being the sole member of the female reproductive caste, the queen is indispensable for the survival of the colony. A mated queen serves the colony in two essential functions: laying fertilized and unfertilized eggs, and secreting substances known as pheromones, required for the stability of the colony's social order.

Within a few days of having mated with several drones, the queen begins to lay eggs, and continues to do so until she is physiologically exhausted. The larvae of female bees (queens and workers) will hatch from the fertilized eggs, while the unfertilized eggs yield drone larvae. During the mating process, the queen store sperm from the drones in a storage organ, the spermatheca, within her abdomen (Wongsiri, 1989). By contrasting the opening and closing of the spermatheca, the queen can allow sperm cells to fertilize her eggs or prevent them from doing so. The fertilized

eggs are deposited in small worker cells, while the unfertilized eggs are laid in the larger drone cells.

Worker bees recognize their queen not by her physical structure but by her scent, given off by the pheromones she secretes. Queen pheromones, 9-oxodextrans-2-ecenoic acid and 9-hydrozydec-2-eonic acid substances, have both direct and indirect effects on the colony's social behavior (Robinson, 1991). During the nuptial flight, they serve as sex attractants, drawing the drones to the queen. Inside the hive, they assist in stabilizing the colony: the workers are aware of the queen's where about by the presence or absence of pheromones. Under certain circumstances, the presence within the hive of pheromones will inhibit the untimely construction of queen cells: they also inhibit the development of the workers' ovaries (Laidlaw, 1992), and during swarming they exercise a direct influence on swarm stabilization.

Before an old queen dies, or departs to start another hive, she lays an eggs in a large queen cell. The nurse bees feed the larva a diet of only royal jelly (RJ, rich mixture of food made from the hypopharyngeal and mandibular glands throughout development.

1.1.2 Honey bee Worker

The workers are an infertile caste of female bees, developed from fertilized eggs (diploid 2n = 32). They are suited by their physiological and anatomical features to perform virtually all kinds of chores except reproduction, to increase the chances of the colony's survival. Factors determining the type of tasks to be executed by a worker include its physiological and anatomical state of readiness, and environmental stimuli, as well as the requirements of the colony to have a particular job done at a particular time.

Soon after emerging from its cell, a young worker receives food, in the form of either nectar or honey, from mature workers, and also helps herself to honey and pollen she finds in the colony's storage cells. In the first few days after she emerges, she is too weak to do anything except inspect and clean empty cells in preparation for food storage by the colony or egg-laying by the queen. During this period she consumes relatively large amounts of honey and pollen, and this directly affects the development of her hypopharyngeal and wax glands.

The secretion from nurse bees' hypopharyngeal glands and mandibular glands is fed to the larvae, those of all ages in queen cells receiving large quantities; for this reason it is referred to as "royal jelly". Larvae in worker and drone cells receive this special diet only during the first days after hatching; during their later larval life they are fed on a mixture of honey and pollen. At about the same time as the hypopharyngeal glands of the nurse bee develop, or shortly afterward, four pairs of wax glands, located below her abdominal segments also develop, under the stimulation of consumption of large amounts of honey. From these glands she secretes flakes of whitish wax, which are manipulated by worker bees, using their mandibles, in the process of comb construction and repair and in capping cells.

Usually, a worker bee when she reaches the age of about 5-15 days is physiologically exhausted from the tasks of secreting royal jelly (Lercker, 1981). After this period, they are spent packing pollen in storage cells, the mouth-to-mouth retrieval of nectar from returning foragers, and occasionally guarding the hive entrance. When she is about three weeks old she stops to be a "house bee" and becomes a "field bee". At this stage her flight muscles are sufficiently developed, and after orientation flight which enable her to locate the hive in relation to surrounding

landmarks, she collects nectar, pollen, water and propolis and carries them back to the hive until she dies (Robinson, 1991; Page and Peng, 2001).

1.1.3 Honey bee Drone

The drones are the male members of the honey bee society reared by the colony shortly before the swarming season begins. In queenless colonies, workers whose ovaries have developed as a result of the lack of inhibiting action by the queen's pheromones can also lay eggs which, begin unfertilized because the worker is unmated, also yield drones.

Drones possess no food-gathering apparatus: their sole biological function is to mate with queens. During the mating season, they are well fed by the workers before taking flight. To ensure successful mating, several thousands of drones must be in the area, although the queen will mate with only about ten. The drone dies shortly after copulation.

When the mating season is nearing its end, the colony reduces its drone-rearing, and when the season is over, the rearing of drones ends completely. The drones remaining in the hive gradually die of old age, negligence by the worker bees or starvation, or they may simply be expelled from the hive.

1.2 Classification of the honey bees

Engel (1999) reported that there are only seven recognized species of honey bee with a total of 44 subspecies. *Apis mellifera*, *A. cerana*, and *A. koschevnikovi* all build nests containing a series of parallel combs. These species usually nest in cavities. *A. florea* and *A. andreniformis* are small species of honey bees whose nests are small, single combs. *A. dorsata* and *A. laboriosa* are large honey bees. Their nests consist of large, single combs.

The taxonomic classification of European honey bee is as follows (Borror *et al.*, 1976; Gojmerac, 1980).

Phylum Arthropoda

Class Insecta

Order Hymenoptera

Family Apidae

Genus *Apis*

Species *Apis mellifera*

1.3 Honey bee body

Honey bee has three body regions: the head, thorax and abdomen. The head contains the sensory organs, and appendages for ingestion. The thorax contains the appendages for locomotion, the legs and wings. The abdomen contains many organs including those for digestion, reproduction and respiration (Figure 1.2).

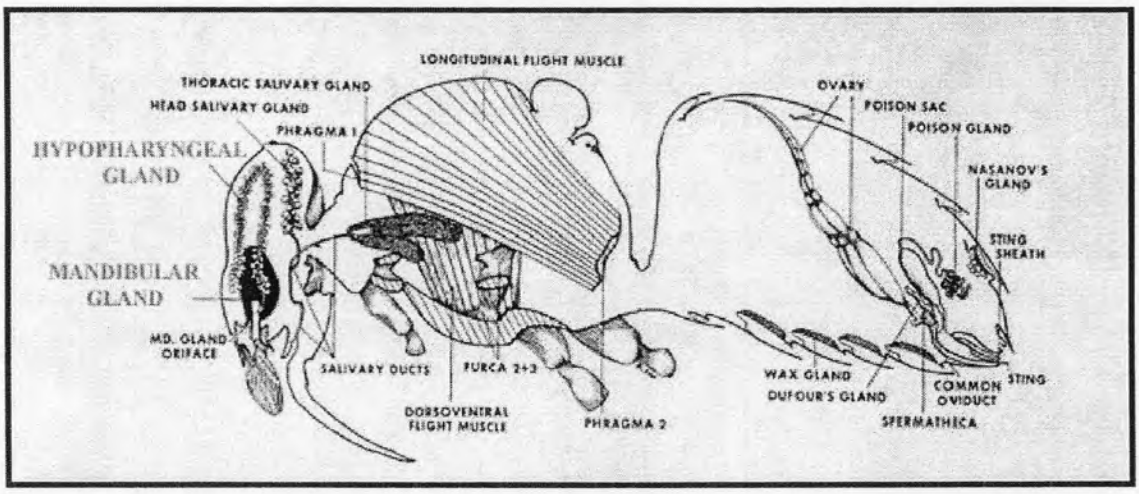

Figure 1.2 Diagram showing the organ systems of an adult female honey bee (Michener, 1974).

Abdomen

The honey bee abdomen is composed of nine segments. The wax and some scent glands are located here in the adult honey bee.

Head

The honey bee head is triangular when scan from the front. The two antennae arise close together near the center of the face. The bee has two compound eyes and three simple eyes, also located on the head. The honey bee uses its proboscis, or long hairy longue, to feed on liquids and its mandibles to eat pollen and work wax in comb building.

1.4 Honey bee reproduction and development

The queen, a mother of all members in the colony, is responsible for laying all the eggs for the colony and can exert marked influence on the behavior of the workers and the drones through the release of chemical signal called queen substances or pheromones. The queen mates with several drones and stores the sperm in her spermatheca for the remaining fertilization. After the queen is mated, the queen begins to lay eggs. The queen lays two types of eggs, fertilized and unfertilized. The unfertilized eggs develop into drones, while the fertilized eggs can develop into workers or queens, depending on what they are fed during development.

Queen and worker bees hatch from fertilized eggs. There is no genetic difference between the egg, which produces a queen, and the egg, which develops into a worker. The difference between the castes is caused by the food given to the larvae by the nurse bees. Two different diets are fed to queen or worker larvae. The queen larvae are always mass-fed a diet of royal jelly throughout her entire life after emergence. Worker larvae are also mass-fed royal jelly for the first three days, after

the fourth day the worker larvae are fed a mixture of worker jelly and pollen (Johansson, 1995).

Due to different dietary feeding which is particular rich in queen, mechanisms between queen and workers in the process of female caste determination and differentiation is observed. In addition, queen attains a larger size than workers and the reproductive organ is well developed to a mature stage and is able to lay several thousand eggs a day. In contrast, workers are smaller in size. The reproductive organ is not well developed but organs that are related with their tasks such as pollen baskets, mandibular, hypopharyngeal and wax glands are fully developed. Basically, the time required for development of the queen larvae to the adult stage is about 15.5 days; that of the worker 21 days; and that of the drone 24 days. The life span of the adult queen is several years, while workers require 21 days for growing up with only a few months of life span (Krell, 1996).

1.5 Suppression of worker reproduction

In honey bee, reproduction is often considered to bee channeled exclusively through the queen. The workers, usually reproductively degenerated and unable to mate. Nevertheless, workers are able to produce unfertilized eggs which can develop into drones but with very small number. Jay (1968, 1970) and Ratnieks (1993) found that in a normal queenright colony, about 10% of workers show some sign of ovarian development. About 7% of a colony's eggs are laid by workers but less than 0.12%

survives to adulthood. However, when a colony losses its queen (queenless colony), many workers developed their ovaries and lay unfertilized eggs.

The suppression of worker reproduction is very common in eusocial Hymenoptera (Hölldobler and Bartz, 1985). In queenright colonies, prevention of ovarian development and egg laying in worker is occurred via:

1) worker policing
2) effect of pheromones

1.5.1 Worker policing

Worker policing behavior is evident in polyandrous eusocial insects for example in *Apis mellifera* (Ratnieks and Visscher, 1989). In *A. mellifera*, worker policing occurs through aggression towards workers that develop functional ovaries, by oophagy of worker-laid eggs (Visscher and Dukas, 1995) and chemical suppression of ovary development (Oldroyd and Osborne, 1999). Worker policing is a well documented phenomena in *Apis mellifera* (Ratnieks, 1988; Ratnieks and Visscher, 1989). In normal colony of *Apis mellifera*, 99.88% of worker laid eggs are removed by worker policing. Workers conduct the policing behavior either by selective removal of other worker's eggs or by aggressive behavior towards ovary developed or laying workers. The ability of workers to discriminate whether eggs derived from queen or workers relies on cues or signals of maternal origin which marked on those eggs. Queen laid eggs were marked by a pheromone from Dufour's gland and this pheromone protects queen eggs from being policed by workers (Ratnieks, 1995).

Oldroyd *et al.* (1994) concluded that in *Apis mellifera*, the ability to avoid policing behavior in workers was genetically derived. However, Oldroyd and his team

found an extremely rare behavior phenotype in *A. mellifera*. Workers in the colony studied developed functional ovaries and layed large numbers of eggs despite the presence of the queen. The phenomena of worker lay eggs and these eggs can escape from other worker policing was termed "anarchistic" and it occurs when the policing fails (Oldroyd *et al.*, 1994; Montague and Oldroyd, 1998). The selective-bred "anarchistic" line of honey bees maintained at The University of Sydney in which about 40% of 10 day old workers have functionally activated ovaries and lays eggs at high frequency (Barron *et al.*, 2001). This mutant line demonstrates that worker sterility has a genetic basis (Oldroyd *et al.*, 1994). Moreover, the patterns of inheritance of the anarchistic phenotype show that there are a small number of genes that regulate worker ovary activation in response to social cues (Oldroyd and Osborne, 1999). Given the strong genetic component to the regulation of ovary activation in honey bees, the gene expression profiles of workers with and without activated ovaries should reveal genes associated with the regulatory control of worker sterility.

1.5.2 The effect of pheromones

It is an advantage for a queen to inhibit worker reproduction so that they can help to rear her offspring. The phenomenon of queens inhibiting the development of worker's ovaries has been frequently documented in European honey bees, *Apis mellifera* (Jay, 1968, 1970, 1972; Visscher, 1989).

Apis mellifera workers are prevented from developing ovaries and egg laying through a combination of brood and queen pheromones (Free, 1987; Winston and Slessor, 1998). Honey bee queens are endowed with caste-specific pheromones, including the mandibular glands (Slessor *et al.*, 1988), Dufour's gland (Katzav-

Gozansky *et al.*, 1997), tergal glands (Wossler and Crewe, 1999a) and queen faeces (Page *et al.*, 1988). Mandibular gland of mated queens produced three aliphatic compounds: 9-keto-2(E)-decanoic acid (9-ODA), 9-(R/E/-(-)-keto-2(E)-decenoic acid and 9-hydroxy-2(E)-decenoic acid (9-HDA), and two aromatic ones, methyl p-hydroxybenzoate (HOB) and 4-hydroxy-3-methoxyphenylethanol (HVA) (Winston and Slessor, 1992). The 9-keto-2(E)-decanoic acid (9-ODA), major component of the queen mandibular gland pheromone (QMP), which acts as a primer pheromone since it inhibits queen rearing by workers (Winston *et al.*,1990), inhibits juvenile hormone III biosynthesis in workers (Kaatz *et al.*, 1989).

Moreover, another queen-specific pheromone produced by the tergal glands, inhibiting worker reproduction in small worker groups was discovered in *Apis mellifera capensis* (Wossler and Crewe, 1999b). However, Jay (1972) found that the ovary activation of workers is not completely blocked by the queen when there was no brood present. Especially in periods around seasoning when there is little or no brood, workers were found to activate their ovaries (Verheijen-Voogd, 1959; Kropácová and Haslbachova, 1970). At present, worker ovary development is believed to inhibit by the combination of queen mandibular gland and tergal gland pheromones, and a mix of fatty esters produced by the brood (Arnold *et al.*, 1994; Wossler and Crewe, 1999b).

Brood pheromone is a blend of ten fatty-acid esters found on the cuticles of honey bee larvae. It was first identified as a kairomone that attracts the parasitic mite, *Varroa jacobsoni* (Le Conte *et al.*, 1989). These ten fatty-acid esters were found on bees during the fourth and fifth days of larval development (Trouiller *et al.*, 1992): methyl palmitate, methyl oleate, methyl stearate, methyl linoleate, methyl linolenate, ethyl palmitate, ethyl oleate, ethyl stearate, ethyl linoleate, ethyl linolenate. Later, it

was found that some components of this blend have releaser-like effects on various aspects of brood care (Le Conte *et al.*, 1990, 1994, 1995). Some components are more active than others, but all ten individual compounds show some releaser activity, leading to their being called, collectively, brood pheromones. Brood pheromone also inhibits ovary development in worker honey bees, indicating a primer effect, which may be involved in the regulation of reproductive division of labor (Mohammedi *et al.*, 1998).

1.6 Honey bee lifespan

A honey bee begins life as an egg. The sex of the new bee is normally determined as the egg passes through the vagina. The queen controls the release of sperm with the so-called sperm pump. If an egg is fertilized, it will develop into a female bee, but if not fertilized, a male bee will result. The result is that male bees have only one set of chromosomes (haploid) acquired from the queen.

The first sign of hatching occurs when an egg is 72 to 84 hours old. Honey bee larvae are fed a nutritious substance called royal jelly by the brood-food glands (hypopharyngeal glands) of young workers. During the first 24 hours, worker larvae are fed lavish amounts of royal jelly by older nurse bees. During the second 24 hours, they get very little additional food and thereafter are cared for by nurse bees of all ages. Pollen and honey are present in the food of older worker larvae.

A female larva fed continuously on lavish amounts of royal jelly and provided a large, peanut-shaped cell will become a queen. Another larva given a mixture of

honey and pollen during the latter half of its larval life and kept in a worker cell becomes a worker. The process that produces the complete expression of sexual characteristics in a queen has not been determined; however, it is considered to be caused by differences in both the quality and the quantity of the larval food provided.

Drone larvae grow larger than either workers or queens and, therefore, require more food. Food given to young drone larvae is nearly devoid of pollen and is milky-white, while that given to older drone larvae is a yellow-brown color and contains considerable pollen. The food given older drone larvae also is higher in pollen content than that given older worker larvae. Thus, both qualitative and quantitative differences distinguish the larval food given queen, worker, and drone.

All castes of honey bees molt about every 24 hours during the first 4 days of larval life. When the ecdysis or molting occurs, the skin splits over the head and slips off the posterior end of the larva. This process normally takes less than 30 minutes. Each new larval stage (instar) is at first only slightly larger than the previous one, but it grows rapidly. The fifth larval instar gains about 40 percent of the total mature larval weight during days 8 and 9 (Table 1.1) (Bertholf, 1925).

By the end of the 8^{th} day after the egg was laid, the cell containing the worker larval is capped. During the 9^{th} day, the larva spins a cocoon using silk from a special gland in its head. On the 10^{th} day, the larva stretches out on its back with its head toward the cell opening and becomes quiescent inside its cocoon. This stage usually is called the prepupa. The 5^{th} molt, which occurs during the 11^{th} day, reveals the pupal form-white in color and motionless. Color develops gradually, first in the eyes (13^{th} day), then in the abdomen (15^{th} day), legs (16^{th} day), wings (18^{th} day), and finally in the antennae (20^{th} day) (Bertholf, 1925).

Table 1.1 Moults of the honey bee (Bertholf, 1925).

Day	Workers		Queens		Drones	
	stages	**moults**	**stages**	**moults**	**stages**	**moults**
1						
2	egg		egg		egg	
3		(hatching)		(hatching)		(hatching)
4	1st larval	1st moult	1st larval	1st moult	1st larval	1st moult
5	2nd larval	2nd moult	2nd larval	2nd moult	2nd larval	2nd moult
6	3rd larval	3rd moult	3rd larval	3rd moult	3rd larval	3rd moult
7	4th larval	4th moult	4th larval	4th moult (sealing)	4th larval	4th moult
8						
9	gorging	(sealing)	gorging		gorging	
10						
11	prepupa	5th moult	prepupa	5th moult		(sealing)
12					prepupa	
13			pupa			
14						5th moult
15	pupa					
16			imago	6th moult (emerging)		
17						
18						
19					pupa	
20						
21	imago	6th moult (emerging)				
22						
23					imago	6th moult
24						(emerging)

Throughout this period, the pupa is encased in a thin outer skin which is shed in the 6th and final molt on the 20th day. Thus, legs, wings, and mouth parts are freed and the pupa becomes an imago (adult) which soon begins to chew its way out of the cell. Because a bee egg hatches into a larva which becomes first pupa and then imago, bees are said to have a complete metamorphosis. The length of the egg stage (3 days) is the same for all three castes, but the larval and pupal stages are shortest for the queen and longest for the drone (Table 1.1) (Bertholf, 1925).

Workers within a honey bee colony engage in various tasks, depending on their age and the needs of the colony. Division of labor by age exists within the worker caste. Bees less than 2 weeks old become involved in cleaning cells and feeding first the older larvae and then larvae of all ages. Workers function as nurse bees during the period that their hypopharyngeal glands are producing brood food.

Older house bees work with honey, pollen, wax, and propolis. Nectar-collecting field bees are met by house bees, usually near the entrance, and are relieved of their nectar loads. The conversion of nectar into honey requires both a physical and a chemical change. The physical change involves the removal of water, which is accomplished by externally manipulating nectar in the mouth parts and then placing small droplets on the upper side of cells and fanning the wings to increase air movement and carry away excess moisture. Nectar is 30 to 90 percent water, but honey should have no more than 18.5 percent water. The chemical change requires the addition to nectar of the enzyme invertase, which the bees produce in their salivary glands. The enzyme breaks the disaccharide sugar, sucrose, into two monosaccharide sugars, glucose and fructose.

Pollen pellets are deposited in empty cells near the brood nest by the pollen-collecting workers. In the cells, the pollen undergoes a maturing process to what is commonly called bee bread. Details of the maturing process are not understood.

When bees are about 12 to 15 days old, their wax glands become functional and comb building is possible. Wax scales are removed from between the ventral abdominal sclerites and positioned into place using both feet and mouth parts. Young house bees in the process of comb building hang in festoons and pass the wax scales from bee to bee.

Propolis-collecting bees also serve as propolis storage reservoirs. Propolis is not stored in combs or elsewhere, but is removed from the corbiculae of these field bees and used as needed. House bees fill cracks and cover rough parts with propolis.

During their third week as house bees, workers take short flights for orientation and defecation. Hives painted different colors aid the bees with orientation and reduce the chance of young bees drifting between adjacent colonies. Some of the oldest house bees also serve as guards at the entrance.

After approximately 3 weeks as house bees, the workers become foragers, gathering pollen, nectar, water, and propolis for the colony. This period of their lives also averages about 3 weeks. Most foragers collect nectar and pollen, but nectar is collected in greater quantities than pollen. Pollen collection tends to be an activity of younger foragers and nectar collection a function of older foragers. Water collectors may comprise 10 percent of all foragers, but this figure is much higher during periods of heat stress. Propolis collectors are quite rarely observed.

Drones take their first flights at about 8 days of age and are sexually mature at 12 days. Drones fly out on cleansing flights and orientation flights-both of short duration-and also on longer flights to congregation areas in search of a virgin queen.

Drones do not forage and spend about three-fourths of their time at complete rest. Their normal lifespan is 8 weeks or less.

Queens newly emerged from their cells are at first ignored but are touched and licked by workers. This apparently helps prepare the virgin queen physiologically for her mating flight. Mating occurs in drone congregation areas-special locations in the air regularly visited by drones. These occur in the same places year after year.

A queen generally mates 6 to 10 days following emergence. She may go out on several mating flights, mating with several drones on each flight. Additional mating flights are taken until the spermatheca contains an adequate supply of semen (5 million to 6 million spermatozoa). If mating is delayed more than 3 weeks, there is a high risk of her becoming a drone-layer. Egg-laying usually commences within a week after mating, and a queen can continue to lay fertilized eggs throughout most of her life, usually 2 to 5 years. An old queen will not go out and mate again when her original supply of semen becomes depleted, she simply becomes a drone-layer. An old queen and her supersedure daughter sometimes coexist, thus contradicting the commonly accepted idea of one queen per colony.

The protein status appears to be a major determinant of honey bee lifespan (Maurizio, 1950, 1954; Burgess *et al.*, 1996). Of the various proteins involved, the very high-density lipoprotein (VHDL) vitellogenin seems to play a crucial role for several reasons. Being the most abundant haemolymph protein found in both workers and queens it strongly reflects the protein status of the bee (Cremonez *et al.*, 1998). Vitellogenin is also a potent zinc (Zn) carrier (reviewed by Falchuk, 1998), and the amount of haemolymph zinc is strongly correlated with the vitellogenin level in honey bees (Amdam *et al.*, 2003). Zinc is required as a catalytic, structural and regulatory

ion, and zinc deficiency induces oxidative stress and apoptosis in several cell lines in mammals, including nerve and immune cells (reviewed by Mocchegiani *et al.*, 2000).

The protein status of a worker bee is mainly given by the amount of protein present in its fat body, haemolymph and hypopharyngeal glands (HPGs). The fat body consists mainly of thin layers of cells spread against the body wall of the abdomen, where the cells are loosely organized in thin lobes of highly tracheated tissue (Snodgrass, 1956). It builds up during the first days of adult life (Haydak, 1957).

The insect fat body synthesizes a broad range of storage proteins (Haunerland and Shirk, 1995). They typically build up to high concentrations in the haemolymph and fat body cells of the last larval instar, and disappear during metamorphosis to provide a protein and amino acid source for the construction of adult tissues (Ancsin and Wyatt, 1996). Five storage proteins have been recognized in honey bee worker larvae. These are the (1-3) hexamerins 70a, b, c, (4) a 105-110 kDa polypeptide high in glutamine/ glutamic acid and (5) a 160 kDa VHDL (Ryan et al., 1984; Shipman *et al.*, 1987; Wheeler and Buck, 1995; Danty *et al.*, 1998). Storage proteins are also found in adults of the social Hymenoptera. Here they appear to serve as an important nutritional source for the maintenance of brood by workers, as well as egg formation by the queen (Martinez and Wheeler, 1993). Three types have been found in ants: (1) hexamerins, (2) proteins high in glutamine/glutamic acid and (3) the VHDLs (Martinez and Wheeler, 1994; Wheeler and Buck, 1995). Only hexamerin 70a (HEX 70a) is found in adult honey bee workers (Danty *et al.*, 1998).

1.7 The anarchic syndrome

A rare behavioral syndrome, "anarchy", in which substantial worker production of males occurs in queenright colonies. The level of worker reproduction in these anarchic colonies is far greater than in a normal queenright honey bee colony. Usually, worker policing and the pheromonal systems maintain the reproductive division of labor in queenright colonies, but in "anarchic" colonies, these systems break down and worker reproduction is more common (Barron *et al.*, 2001).

Anarchist bees are unusual in at least two ways. First, whereas ovary development is extremely rare among queenright wild type workers (Visscher, 1996), it is relatively common among queenright anarchist workers (Oldroyd *et al.*, 1999; Barron and Oldroyd, 2001). Second, their eggs are policed less (Oldroyd and Ratnieks, 2000). The characteristics of the anarchic syndrome are summarized in Table 1.2. All these characters indicate a general breakdown of the pheromonal system that normally inhibits worker reproduction in queenright colonies, possibly involving changes in the production of pheromones, perception of pheromones, or both.

Table 1.2 Characteristics of normal and anarchic colonies (Barron *et al.*, 2001)

Character	**Queenright wild-type colonies**	**Queenless wild-type colonies**	**Anarchic colonies**
Percent ovary activation in workers	0.1-0.001	5-10	5-10
Brood or queen pheromones	strong	Decline with increasing time since queen removal. The rearing of worker-laid drones reintroduces brood pheromone to the colony	weak
Queen cells	Rare and single supersedure cells	Several 'emergency' queen cells produced in response to queen removal	Common multiple supersedure cells
Production of egg-marking pheromone	Expressed only in queens	Expressed only in queens	Expressed in workers and queens
Worker oviposition	Rare	Frequently seen	Frequently seen
Response of workers to queen and brood pheromones	Strong in most workers, but some variation among subfamily	Strong in most workers, but some variation among subfamily	Weak
Policing behavior	Strong	Policing declines with increasing time since queen removal	Permissive

1.7.1 Ovary activation

The anarchic genotype has a strong influence on ovary activation, since, regardless of their hive environment, anarchist bees are always more likely than wild-type workers to develop their ovaries (Barron and Oldroyd, 2001). But a greater propotion of fostered wild-type workers have active ovaries in colonies from the selected anarchist line than in wild-type hosts (Barron and Oldroyd, 2001) and ovary activation is reduced in anarchist workers fostered into queenright wild-type hosts (Oldroyd *et al.*, 1999). This shows that the genotype of the host colony also influences ovary activation.

1.7.2 Evasion of policing

Honey bee workers will frequently consume any eggs laid by other workers in queenright colonies (Ratnieks and Visscher, 1989; Ratnieks, 1993). This phenomenon, known as 'worker policing', greatly reduces the likelihood that worker-laid eggs will be reared to maturity, thereby minimizing the potential benefits to ovary activation.

Eggs laid by anarchist workers are policed much less efficiently than normal worker-laid eggs (Oldroyd and Ratnieks, 2000). In both wild-type and selected anarchist line discriminator colonies, eggs from anarchist workers are removed more slowly than eggs from wild-type laying workers from queenless colonies, and in selected anarchist line discriminator colonies, eggs from anarchist workers persist as long as queen-laid eggs (Oldroyd and Ratnieks, 2000).

Anarchist workers can counterfeit the queen-produced egg-marking pheromone thereby defeating the egg recognition mechanism on which worker policing relies. Ratnieks (1992) predicted that this strategy would be a way to defeat

worker policing, but he also considered it likely that workers counterfeiting queen pheromones could be detected and would be attacked by their nest mates. Workers often respond aggressively to workers contaminated with queen pheromones (Morse and Gary, 1961; Yadava and Smith, 1971), laying anarchists do not attract excessive aggression from nest mates (Oldroyd *et al.*, 1999; Barron and Oldroyd, 2001).

Eggs laid by anarchist workers are acceptable in normal colonies. Therefore, anarchist workers should be able to reproduce successfully even if the other workers in their colony are not anarchistic. This is a situation that probably occurs in normally managed anarchic colonies when the workers of only one or a few subfamilies possess anarchistic tendencies (Barron *et al.*, 2001).

1.8 Genes use for transcriptional evaluation

The primary experiment from our researcher group used microarray and the database from honey bee genome project gave us the information of the genes suspected to be involved either in ovarian development or in signaling pathways critical for cellular growth or cell differentiation. More over the genes that involved in foraging behavior and the genes in 'ovarian ground plan' (Amdam *et al.*, 2004) were selected to study the level of genes expression in the ovary activated worker honey bee.

1.8.1 Transferrin (Trf)

Transferrins belong to a family of iron-binding proteins that have been implicated in innate immunity and in vitellogenesis in insects (Kucharski and

Maleszka, 2003). *Apis mellifera* transferrin (AmTRF) gene has an open reading frame (ORF) of 2,136 bp spread over nine exons. The deduced protein sequence comprises 686 amino acid residues plus a 26 residues signal sequence, giving a predicted molecular mass of 76 kDa. It clusters with monoferric transferrins, with which it shares putative iron-binding residues in the N-terminal lobe (Nascimento *et al.*, 2004). The primary function of transferrin is binding and transporting of iron (Kucharski and Maleszka, 2003).

Iron is one of the essential elements required by all organisms, but it is also a potent toxin because of its ability to produce free radicals in the presence of oxygen (Crichton *et al.*, 2002; Nichol *et al.*, 2002). In some tissues, such as brain or retina, where anti-oxidative defenses are relatively low and oxygen consumption is very high, iron accumulation in specific regions is associated with a number of neurodegenerative diseases (Crichton *et al.*, 2002). Hence, organisms must balance their nutritional requirements with the necessity to control this potential toxic property. In animals, iron-binding and transporting proteins provide an important way to minimize the reactivity of iron towards oxygen in addition to their facilitating role in iron metabolism. One class of iron-binding proteins that have attracted much attention belongs to a highly conserved family of transferrins. These proteins are well characterized in vertebrates, but Nichol and co-workers (2002) has been some progress in studies on their relatives in insects.

Despite a high level of sequence conservation of transferrins from different lineages there are significant differences in their biochemical properties as well as in their involvements in cellular functions. In vertebrates, transferrins are glycoproteins of approximately 80 kDa with two ferric-binding lobes, most likely resulting from a duplication of an ancient gene encoding a 40 kDa protein (Jamroz *et al.*, 1993; Nichol

et al., 2002). Most insect transferrins bind only one ferric ion because their C-terminal lobes have no iron-binding capacity. One notable exception is transferrin in *Balberus discoidalis* that has been found to contain two functional iron-binding sites (Jamroz *et al.*, 1993).

In addition to binding iron, mammalian transferrin has been shown to act as a growth factor and as a regulator of gene expression at the transcriptional level (Raivich *et al.*, 1991; Espinosa-Jeffrey *et al.*, 2002). A correlation between myelination and transferrin synthesis and secretion in oligodendrocytes has also been demonstrated. In vertebrates, transferrin transports iron in blood, and many cells can access transferrin-bound iron by way of the transferrin receptor pathway. At present it is not known whether a similar pathway exists in insects. The lack of a gene encoding a protein similar to the vertebrate transferrin receptor in *Drosophila* was taken as evidence that insects may use a different transport mechanism or a different receptor (Nichol *et al.*, 2002).

The highest level of AmTRF mRNA is found in the central brain neuropils and in the pigmented eye (Kucharski and Maleszka, 2003). By analogy to mammals, these tissues are likely to have low anti-oxidative defenses (Crichton *et al.*, 2002). Thus, transferrin may play a role in the honey bee central nervous system as a component of a protection mechanism against reactive oxygen intermediates. It has been shown that the vertebrate retina consumes more oxygen per unit weight than any other tissues and the consequent generation of these intermediates is thought to underline several retina diseases in mammals. Light is particularly important in ocular injury mediated by reactive oxygen intermediates (Lu *et al.*, 2002). The high levels of AmTRF in the brain and in the compound eye of mature adults could also be related

to a potential role of transferrin bound iron in flight and directional sensing (Kucharski and Maleszka, 2003).

Lower levels of transferrin make more iron molecules available for reactions that are promoted by transition-metal ions. Iron is required for generating di-oxygen species (O_2) that are critical for many biological processes such as biosynthesis of DNA, serotonin, Fatty acids and other bio-molecules (Kovacs, 2003). Iron is also an essential cofactor of a number of key enzymes needed in energy metabolism. Interestingly, the levels of AmTRF mRNA increase quite dramatically in the adult brain suggesting that transferrin may play contrasting roles in the larval insect nervous system and in the adultbrain (Kucharski and Maleszka, 2003).

In addition to being part of anti-oxidative defenses transferrin may act as a growth factor that can regulate other proteins even at the transcriptional level. In mammals, transferrin is a ubiquitous growth factor that plays a critical role in cellular iron uptake, growth and proliferation (Raivich *et al.*, 1991). Both the high levels of AmTRF mRNA in the adult brain and major fluctuations during developmental stages suggest that transferrin in the bee is an important protein with multiple functions in the nervous system. The changes in AmTRF expression under some conditions, such as light exposure, are more subtle than during development (Kucharski and Maleszka, 2003).

Insect transferrin has been considered an infection-inducible gene (Yoshiga *et al.*, 1997; Thompson *et al.*, 2003) and has been implicated in innate immunity to fight microbial infections by sequestering iron. Kucharski and Maleszka (2003) found that AmTRF is up-regulated following an injection with *E. coli*, but not with yeast. Yet, the level of AmTRF induction is relatively low comparing to that of Hymenoptaecin, which is highly inducible under the same conditions. Hymenoptaecin inhibits viability

of both Gram-negative and Gram-positive bacteria, including several human pathogens (Casteels et al., 1993). This may suggest that transferrin may only be loosely linked to a pathway involved in defense against Gram-negative bacteria.

Treatment with juvenile hormone results in suppression of transferrin expression in the honey bee. This effect is best seen in the abdomen of worker bees and may illustrate the fact that during adult maturation increased titers of juvenile hormone correlate with suppression of vitellogenin in worker bees that in normal colonies are sterile and do not lay eggs (Kucharski and Maleszka, 2003).

1.8.2 Vitellogenin (Vit)

In the presence of a queen, honey bee workers have undeveloped ovaries independent of behavioral state (Butler, 1957). However, the yolk precursor vitellogenin, the major yolk protein in oviparous animals, constitutes the main part of the hemolymph protein fraction in bees that do not forage. The vitellogenin titer increases from emergence to when the bee is 7-10 days old (Fluri *et al.*, 1982). Workers that loose their queen within a week after emergence develop their ovaries to a larger extent than older bees (Lin *et al.*, 1999).

In summer, the worker population consists of a hive bee group performing a multitude of tasks including nursing inside the nest, and a forger group specialized in collecting nectar, pollen, water, and propolis. Vitellogenin is synthesized in large quantities by hive bees. When hive bees develop into forger, their juvenile hormone titers increase, and this causes cessation of their vitellogenin production.

At the physiological level, changes at the onset of foraging include an increase in the juvenile hormone titer (Jaycox *et al.*, 1974), and a decrease in the haemolymph vitellogenin level. Juvenile hormone is produced by the corpora allata complex by the

suboesophageal ganglia, and is part of the hormonal control machinery of oogenesis in solitary insects. Vitellogenin is a conserved yolk precursor protein that it is synthesized by the fat body in invertebrates (Spieth *et al.*, 1991). In many insect species, juvenile hormone is involved in the regulation of reproductive behavior and sensory tuning (Amdam *et al.*, 2004a).

Honey bee vitellogenin has been hypothesized to work together with juvenile hormone in a double repressor network to coordinate behavior (Amdam and Omholt, 2003). In this network, vitellogenin suppresses juvenile hormone and inhibits the worker honey bees' age-associated shift from nest tasks to foraging duties (Guidugli *et al.*, 2005). This shift is a complex behavioral transition characterized by decreasing vitellogenin and increasing juvenile hormone titer (Fluri *et al.*, 1982). It has been proposed also that variation in vitellogenin gene expression early in life is associated with subsequent behavioral specialization that gives rise to a division of labor between nectar and pollen foraging workers (Amdam *et al.*, 2004a). Finally, honey bee vitellogenin can reduce oxidative stress by scavenging free radicals, thereby prolonging lifespan in the facultatively sterile worker castes and the reproductive queen castes (Seehuus *et al.*, 2006).

The hemolymph titer and the transcription level of the yolk protein vitellogenin are significantly different in bees selected for high and low pollen hoarding. Generally, development of vitellogenin synthesis with titers is increasing the first 7-10 days of adult life (Fluri *et al.*, 1982). Throughout this period, the high strain bees have higher vitellogenin titers compared with workers from the low strain. The distribution of the vitellogenin titer at a given age is strongly influenced by the age at onset of foraging, the amount of brood (Fluri *et al.*, 1982), the availability of

pollen (Bitondi and Simões, 1996), and the presence of parasites (Amdam *et al.*, 2004b).

The higher hemolymph titers in the high strain bees result from higher rates of vitellogenin synthesis. However, the complex associations between the titer and the transcription level suggest that the causal relationship is not derived from a simple genetic limitation on the maximum transcription rate of vitellogenin. It seems clear that a regulatory mechanism controls the association, and it can reverse the relationship between the titer and the transcription level within days. An inverse relationship between titer and transcription level suggests that the transcription rate is reduced when the concentration of vitellogenin reaches a certain level (Amdam *et al.*, 2004a). A theoretical outline of this self-governing regulatory feedback loop was developed by Amdam and Omholt (2002) to explain the vitellogenin dynamics of wild type honey bee workers. Within their framework, the dynamic differences between the two strains would result from variation in the transcriptional response threshold to the hemolymph vitellogenin concentration.

Juvenile hormone and ecdysteroids are the key regulatory hormones that control vitellogenesis in a broad range of insect species (Socha *et al.*, 1991; Hiremath and Jones, 1992; Brownes, 1994; Ismail *et al.*, 1998; Sankhon *et al.*, 1999). Juvenile hormone and ecdysteroids affect adult behavior by regulating the growth and central processing of sensory and motor neurons (Cayre *et al.*, 1997; Lin and Lee, 1998; Anton and Gadenne, 1999; Cayre *et al.*, 2000; Gray and Weeks, 2003), and their coordinated regulatory modes have been shown to result in synchronized changes in sensory perception, locomotor activity, and reproductive physiology (Lin and Lee, 1998; Zera and Bottsford, 2001).

In honey bees, both juvenile hormone and acdysone appear to have lost their gonotrophic roles in the adult queens and workers (Hartfelder *et al.*, 2002). Juvenile hormone is not needed for the behavioral shift to foraging (Sullivan *et al.*, 2000) or for the growth of the mushroom body neuropil, a brain region involved in learning and memory that increases in size as the bee gets older (Fahrbach *et al.*, 2003). Juvenile hormone is responsible, however, for a dramatic down-regulation of vitellogenin synthesis in adult workers (Pinto *et al.*, 2000). This negative action on vitellogenin production seems contrary to the norm in solitary insects with a prolonged adult reproductive period, yet the gonotrophic role of juvenile hormone in honey bee pupae may imply that the positive regulatory action seen in solitary adults has been shifted in time to accommodate adaptive production rates of vitellogenin early in life. The initiation of vitellogenin synthesis before emergence may have been instrumental for enhancing the reproductive output of young queens. At the same time, it would provide young bees with a protein source for the production of brood food (Amdam *et al.*, 2003).

Invertebrate and vertebrate vitellogenins (Byrne *et al.*, 1989) constitute a multigene superfamily together with insect apolipophorin II/I, human apolipoprotein B, and the large subunit of mammalian microsomal triglyceride transfer protein (Mann *et al.*, 1999). Honey bee vitellogenin is considered to be a 180 kDa monomer (Wheeler and Kawooya, 1990). In queens, hive bees, and wintering workers (winter bees), vitellogenin is the predominant haemolymph protein (Pinto *et al.*, 2000; Engels, 1974; Fluri *et al.*, 1982) (30-50% of total). The rate of synthesis in a worker is negligible at the time of emergence, but increases rapidly within 2-3 days. Vitellogenin synthesis peaks during the period when the bee normally nurses brood (5-15 days of age) (Fluri *et al.*, 1982; Crailsheim, 1992; Seeley, 1982). In this period,

the rate of synthesis equals the amount needed to provision 30-100 eggs daily (Engels *et al.*, 1990).

The high rate of vitellogenin synthesis in hive bees has been enigmatic for almost three decades since the protein was documented not to be present in jelly fed to the larvae (Rutz and Lüscher, 1974). In the actual study, small amounts of vitellogenin were observed in homogenates of the hypopharyngeal glands (HPGs), the paired acinous jelly-producing glands located in the head of the worker. However, they concluded that this observation was due to contamination and that vitellogenin was not used as brood food. At the same time, it was suggested elsewhere that the activity of the HPGs and the production of vitellogenin were coregulated by the corpora allata brain complex (Engels, 1974). The high rate of vitellogenin production during the nursing phase was thus explained as a regulatory side effect. Based on the observation of vitellogenin in drone haemolymph, it was later proposed that the synthesized vitellogenin was recycled by the fat body as a compensatory strategy (Trenczek *et al.*, 1989). Juvenile hormone and ecdysteroids act as the main inducers of vitellogenin synthesis and uptake (Raikhel *et al.*, 2005). In adult honey bees, however, vitellogenin synthesis is not upregulated by these hormones (Hartfelder and Engels, 1998), and it is only during the initiation of vitellogenin expression in the late pupal stages that juvenile hormone acts as an inducer of vitellogenin production (Barchuk *et al.*, 2002). This unconventional association was partly explained when it was shown that vitellogenin has evolved functions beyond the restricted context of reproduction: putative vitellogenin receptors can be found in the royal jelly producing hypopharyngeal glands of workers, suggesting that vitellogenin is used to synthesize proteins that nurse bees feed to the larvae (Amdam *et al.*, 2003). The conversion of a yolk protein to larval food proteins is compatible with the physiological condition of

nurse bees, which have high vitellogenin and low juvenile hormone titers (Rutz *et al.*, 1976).

From an evolutionary point of view, it is unlikely that the costly production of vitellogenin in nurse bees has no specific biological role. If the trait had not been under positive selection, one would expect that mutational events and subsequent genetic drift would have led to considerable variation of the trait within or between nurse bee populations. However, a high rate of vitellogenin synthesis is one of the key defining characteristics of nurse bees (Pinto *et al.*, 2000; Engels, 1974; Fluri *et al.*, 1982; Engels *et al.*, 1990). Furthermore, failing to detect vitellogenin in jelly does not rule out that the protein is incorporated and rapidly lysed within the HPGs' acini. The consequences of assuming vitellogenin to be a major source for the jelly produced by hive bees were tested by a data-driven differential equations model (Amdam and Omholt, 2002). The model describes the dynamics of the protein in the individual bee as a function of its task profile under various intracolonial regimes, and explains the available empirical data on vitellogenin profiles in workers.

The transport of vitellogenin into ovaries is exclusively reported to be a receptor-mediated process (Mann *et al.*, 1999) and that jelly contains a substantial amount of Zn. Vitellogenin is the dominant Zn-carrier in honey bee haemolymph, and the capacity of this protein to carry Zn over oocyte membranes by receptor-mediated cotransport is documented in several genera (Falchuk and Montorzi, 2001).

1.8.3 Profilin (Prf)

Actin is a major cytoskeleton protein of most eukaryotic cells, and filamentous actin structures are often the primary determinants of cell shape and movement. The polymerization and depolymerization of actin filaments inside nonmuscle cells are

highly regulated, both spatially and temporally, to give the cell the ability to rearrange its cytoskeleton drastically within minutes in response to external stimuli or act certain points in the cell cycle.

One of the dramatic effects of actin-binding proteins in the cell is to inhibit filament formation. There are at least two possible mechanisms for how the cell might limit filament growth: filament nucleation sites might be blocked so that the monomers have nothing to grow off, or actin monomer might be sequestered in a nonpolymerizable form so that filaments have nothing to grow with (Theriot and Mitchison, 1993).

Profilin is a low molecular weight actin-monomer binding protein which seems to have been found in every eukaryotic species where it has been looked for. The amino acid sequences of the profilins are not very highly conserved across the many phyla from those determined (protozoa, mammalian, yeast, insect, echinoderm, plant and even a virus). However, the structure is conserved (Schutt *et al.* 1993; Vinson *et al.*, 1993; Federov *et al*, 1994; Federov *et al*, 1997). Possibly connected with the sequence diversity, the ubiquity and abundance of profilins in eukaryotes is the fact that profilins are major allergens.

Profilin has an unusual property in its ability to bind poly-L-proline, and although the physiological significance of this accidental finding (Tanaka and Shibata, 1985) is in itself very important, this has provided an easy way to purify the protein (Kaiser *et al*, 1989). Profilin binds to poly-L-proline as its actual function is to bind proline rich regions in a number of profilin binding proteins. Not only does profiling bind a series of partners through the praline-rich binding domain but it also binds phosphatidylinositol-4,5-bisphosphate (PIP_2) and phosphatidylinositol-4-phosphate (PIP) (Goldschmidt-Clermont *et al.*, 1990; Machesky *et al.*, 1990). Profilin

can shield PIP_2 against hydrolysis by phospholipase C, unless the lipase has activated by phosphorylation (Goldschmidt-Clermont *et al.*, 1991), and profilin retains its ability to bind actin, polyproline and phosphoinositides. Profilins ability to bind phosphoinositides has caused much interest and it has even been suggested that it is the main job of the protein in cells, since phosphoinositides provide a very important cell signaling cascade transducing messages from outside to inside the cell.

Profilin homologs are present in organisms ranging from fungi and amoebae through trees and mammals. Profilin is unique in having both positive and negative effects on polymerization and that it appears to act both as a sequestering agent and as a desequestering agent (Theriot and Mitchison, 1993).

Profilin was originally identified as a component of cell extracts that inhibited actin filament growth in vitro (Carlsson *et al.*, 1977). For many years, profilin was assumed to be the major sequestering factor in most cells, and sequestering was considered to be profilin's primary function (Figure 1.3).

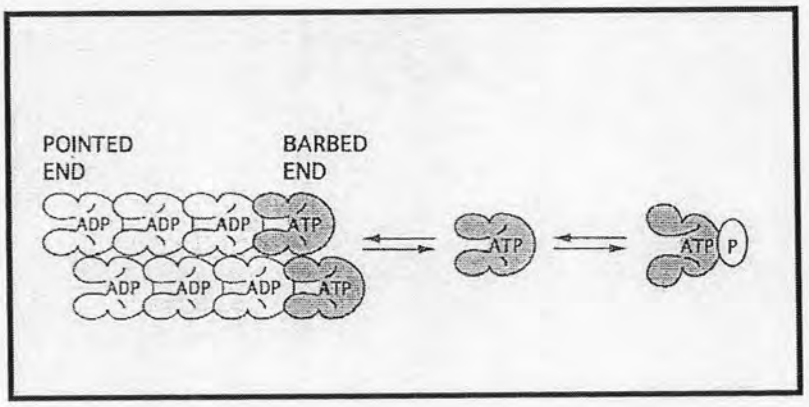

Figure 1.3 Profilin promotes depolymerization by binding to monomer. This is the simplest role of profilin and was the first to be discovered.

Pantaloni and Carlier (1993) has provided evidence for several different functions of profiling besides its monomer-sequestering ability. This highly versatile molecule is able to promote exchange of the adenine nucleotide bound to actin monomer and can effectively lower the critical concentration for polymerization of actin. Furthermore, profilin can interact with several types of signaling pathways and can differentially affect certain types of actin filament structures in vivo. This extraordinary combination of properties places profilin in a unique position to integrate information from external stimuli and regulate the time and place of actin filament growth in cells.

1.8.4 Flotillin (Flt)

Flotillins are integral membrane proteins that have been shown to be present in several subcellular components, including caveolae (invaginated plasma membrane microdomains), lipid rafts (Sphingolipid and cholesterol-rich, detergent-resistant plasma membrane microdomains), and the Golgi apparatus. The molecular function of flotillins remains uncertain. They are probably involved in organizing the structure of caveolae and lipid rafts, and other detergent resistant membrane domains. They may also be involved in signal transduction. Flotillins have been shown to accumulate in brain cells with the development of Alzheimer's pathology (Girardot *et al.*, 2003). Also included in this family are Reggie proteins, which are expressed in non-caveolar neuronal plasma membrane domains.

Flotillins are lipid raft-associated proteins, which have been implicated in neuronal regeneration and insulin signaling. Flotillin-1 is associated with the cytoplasmic face of the plasma membrane. The N-terminus of flotillin contains a prohibitin-like domain (PHB), which shows homology to a number of proteins

associated with raft domains including stomatin, podocin, and prohibitin. The PHB domain of flotillin can efficiently target a heterologous protein, green fluorescent protein, to the plasma membrane. Another PHB-containing protein, stomatin, traffics to the plasma membrane via the conventional secretory pathway.

1.8.5 Take-out-like (JHBP)

Juvenile hormone (JH), together with ecdysone, controls insect growth, development, and reproduction. In the insect hemolymph over 99.8% of JH is bound to juvenile hormone binding protein (JHBP). JHBP from the hemolymph of *G. mellonella* is a single chain, basic glycoprotein containing two disulfide bonds. The interaction between JH and JHBP results in a profound conformational transition of the JH carrier, as judged from changes in electrophoretic mobility, UV difference spectra, sedimentation coefficients, and resistance to proteolysis (Kochman, 2008).

The JHBP gene has four introns. The first, third and fourth introns are in phase 1, whereas the second is in phase 2. Six putative regulatory elements [Hunchback, Heat shock factor binding element, Ultrabithorax, Broad-complex Z3, Elf-1 and Chorion factor 1/ultraspiracle (CF1/Usp)] were found in the distal promoter of the JHBP gene (Kochman, 2008).

1.8.6 Nitric oxide synthase (NOS)

In the nervous system, nitric oxide acts as a signaling molecule with functions very much like those of a neurotransmitter (Dawson and Snyder, 1994; Garthwaite and Boulton, 1995). In addition to its various functions in the development and regeneration of the nervous system (Dawson and Snyder, 1994; Schuman and Madison, 1994), nitric oxide has been implicated in vertebrate learning (Chapman *et*

al., 1992; Hölscher and Rose, 1992; Böhme *et al.*, 1993) and in mechanisms of long-term potentiation (Schuman and Madison, 1991; Haley *et al.*, 1992; O'Dell *et al.*, 1994) and long-term depression of synaptic connections (Bredt and Snyder, 1989; Shibuki and Okada, 1991; Zhuo *et al.*, 1993).

Nitric oxide has been identified as a signaling molecule in the brain of *Locusta*, *Drosophila*, and *Apis* (Elphick *et al.*, 1993, 1995; Müller, 1994; Müller and Bicker, 1994), and a nitric oxide synthase (NOS) gene was cloned in *Drosophila* (Regulski and Tully, 1995). Interestingly, the antennal lobes, the lateral protocerebral neuropil, and the mushroom bodies, all potential sites of associative olfactory learning in the honey bee (Hammer and Menzel, 1995), are also prominent areas of NOS expression (Müller, 1994). It was shown that the nitric oxide system within the antennal lobes is involved in chemosensory information processing in vivo (Müller and Hildebrandt, 1995).

In the honey bee, the proboscis extension response can be conditioned by pairing an odor stimulus with a sucrose reward (Menzel, 1985, 1990). The number of conditioning trials applied to the honey bee induces different memories, which exhibit different properties (Hammer and Menzel, 1995). While a single trial conditioning leads to a medium-term memory that lasts for hours, multiple trial conditioning induces a long-term memory that lasts for days.

The reduced nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase histochemical method allows localization of cells and neuropils in insects that contain NOS, as in vertebrates (Müller, 1994; Müller and Bicker, 1994). The majority of NOS activity (>95%) is localized in insect brain. NOS activity occurs in neurons and glial cells, and there is as yet no indication of nonneuronal NOS activity. In the honey bee, the predominant amount of NOS activity is detected in neuropils

known to participate in the processing of olfactory information, while the visual neuropils exhibit by far the lowest NOS activity (Müller, 1996).

1.8.7 Arginine kinase (ArgK)

In living organisms, ATP is utilized as a convenient store of energy to drive many different chemical reactions that cells need. However, depending on the cell type and its activity, the metabolic half-life of an ATP molecule varies from seconds to minutes. Tissues with a high ATP turnover, such as brain and muscles, have only a few seconds of supply of ATP and rely on a free energy reservoir that functions to regenerate ATP rapidly. In invertebrates, phosphoarginine functions in this capacity (Dumas and Camonis, 1993; Strong and Ellington, 1995). It is synthesized by the reversible phosphorylation of arginine by ATP as catalyzed by arginine kinase (ArgK). Under standard conditions, the reaction: ATP + arginine ⇌ phosphoarginine + ADP, operates close to equilibrium. However, at times of high metabolic activity, when ATP is low, the equilibrium shifts so as to yield net synthesis of ATP Phosphoarginine thereby acts as an ATP buffer in cells that contain ArgK. The physiological importance of this reaction is seen by some authors as providing the metabolic capacitance whereby ArgK (or creatine kinase in vertebrates) allows reduction of peak rates of ATP synthesis in cells that alternate between periods of high- and low-energy consumption. Furthermore, facilitated diffusion of ATP and ADP leads to spatial buffering in addition to temporal buffering (Sweeney, 1994).

ArgK is a member of a highly conserved family of phosphagen kinases and is found in invertebrate species where it serves as a function analogous to that of creatine kinase (CrK) in vertebrates (Dumas and Camonis, 1993; Strong and Ellington, 1995). Although ArgK is the major guanidino kinase found in

invertebrates, other members of this protein family have also been reported in annelids, mollusks and arthropods (Muhlebach *et al.*, 1994; Suzuki and Furukohri, 1994).

Kucharski and Maleszka (1998) have cloned and sequenced a 1.68 kb cDNA encoding arginine kinase in the honey bee, *Apis mellifera*. The predicted protein shows a remarkable level of identity to known ArgKs in invertebrates and to other proteins belonging to the conserved family of ATP: guanidino phosphotransferases (phosphagen kinases). Three isoforms of ArgK transcribed from a single gene, are expressed in a characteristic pattern in major tissues of the honey bee. ArgK mRNA is relatively abundant in the brain and in the thorax of the honey bee. In addition, it is also highly expressed in other tissues, in particular in the antennae. However, the highest level of ArgK expression (two to three times higher than in the brain) is found in the compound eye of the bee. By contrast, the levels of mRNAs encoding another metabolically important enzyme, α-glycerolphosphate dehydrogenase (α-GPDH), are low in the eye. The elevated level of ArgK expression in the visual system can be related to a need for an efficient energy system that transfers ATP generated by mitochondria to high energy consuming processes that occur in distal regions of the retina. The visual pigment molecules are inactivated after triggering the excitation of the photo-receptor cell, and they must be regenerated in order to maintain the sensitivity of the sensory cells (Schwemer, 1989; Stowe *et al.*, 1990).

Moreover, a continuous renewal of the light-sensitive components, including the breakdown of membranes and the addition of newly synthesized visual pigment and membranes to the existing light-sensitive structures is also associated with photoreceptor regeneration. These molecular processes are seen as a mechanism to prevent inefficient functioning of the receptor due to aged light-damaged constituents

as well as an adaptation to ambient light conditions (Stowe *et al.*, 1990). A high abundance of ArgK mRNA in the eye provides a molecular solution to such a massive demand of energy. By acting as an energy shuttle, and/or as an energy reservoir, ArgK can provide both spatial and temporal buffering in delivering energy in sensory cells (Kucharski and Maleszka, 1998).

1.8.8 Octopamine receptor (OctR)

Biogenic amines and their receptors regulate and modulate many physiological and behavioral processes in animals. In vertebrates, octopamine is only found in trace amounts and its function as a true neurotransmitter is unclear. In protostomes, however, octopamine can act as neurotransmitter, neuromodulator and neurohormone. In the honey bee, octopamine acts as a neuromodulator and is involved in learning and memory formation.

Octopamine is a monophenolic amine that belongs to a group of neuroactive compounds knows as biogenic amines. Biochemical and pharmacological experiments suggest that octopamine exerts its effects by binding to membrane proteins that belong to the superfamily of G protein-coupled receptors (GPCRs). These receptor proteins share the structural motif of seven transmembrane (TM) domains (Baldwin *et al.*, 1997; Okada *et al.*, 2001). Activation of the receptors may lead to changes in the concentration of intracellular second messengers such as cyclic nucleotides [cyclic AMP (cAMP) and cyclic GMP], inositol-1,4,5-trisphosphate and Ca^{2+}. Octopamine-mediated changes in the intracellular concentration of cAMP and/or Ca^{2+} ($[Ca^{2+}]_i$) have been reported for several protostomian species (Roeder, 1999; Blenau and Baumann, 2001).

Since its discovery in the salivary glands of the octopus, octopamine has been found in high concentrations in neuronal and non-neuronal tissues of many nematodes, annelids, arthropods and mollusks (David and Coulon, 1985). Owing to its regulatory functions, octopamine is considered to be a neurotransmittere, neuromodulator and/or neurohormone (David and Coulon, 1985; Roeder, 1999). Many behavioral and physiological reactions have been attributed to the signaling action of octopamine (David and Coulon, 1985; Orchard *et al.*, 1993), particularly in a number of studies in the honey bee (Braun and Bicker, 1992; Erber *et al.*, 1993; Burrell and Smith, 1995; Pribbenow and Erber, 1996; Schulz and Robinson, 2001; Scheiner et al., 2002). It has been shown that octopamine can modulate the responsiveness of sensory receptors, interneurones and motoneurones, and so affects complex behavioral responses. Octopamine also plays a major role in olfactory learning and memory formation in the honey bee (Hammer, 1993, 1997; Hammer and Menzel, 1998; Menzel *et al.*, 1999).

1.8.9 Major royal jelly proteins (MRJP)

Apis mellifera caste determination occurs when young worker bees in the hive (nurse bees) produce, secrete, and feed a substance called Royal Jelly (RJ) to developing larvae. RJ is a natural source of essential amino acids, lipids, vitamins, acetylcholine, and other nutrients (Colhoun and Smith, 1960; Schmitzova *et al.*, 1998). In the initial 3 days of development, all larvae are fed RJ, but thereafter only larvae designated by workers to become queens receive RJ. In its place, a mixture of honey, pollen, and water is fed to larvae selected to become workers. Since individuals from the previous generation feed the young of the next generation,

determining their developmental fate (fertile queen vs. sterile worker), all major aspects of *A. mellifera* eusociality are considerably influenced by RJ.

Major Royal Jelly Proteins (MRJPs) constitute ~90% of total RJ protein (Schmitzova *et al.*, 1998; Sano *et al.*, 2004; Santos *et al.*, 2005; Scarselli *et al.*, 2005). Eight *A. mellifera* loci encoding MRJPs (MRJP1-MRJP8) have been identified (Klaudiny *et al.*, 1994a, b; Schmitzova *et al.*, 1998; Albert and Klaudiny, 2004), but little is known about the function of these genes or their protein products. Kucharski and his team (1998) reported that the MRJP1 gene is expressed in the mushroom bodies of the honey bee brain, implicating this gene in behavior. This result suggests that the MRJPs can be multifunctional, performing a nutritional role as a component of RJ and executing additional roles in various tissues including the brain. However, the expression patterns of the MRJP genes across development and in different sexes and castes have not been well-characterized (Drapeau *et al.*, 2006).

The water soluble proteins fraction of RJ contains several major proteins (Mr's of 47-80 kDa) that are produced in the cephalic glands of nurse honeybees (Lensky and Rakover, 1983; Hanes and Simúth, 1992; Klaudiny *et al.*, 1994b; Kubo *et al.*, 1996). It is presumed that they have a nutritional function.

Schmitzova´ and co-workers (1998) found that the comparison of nucleotide sequences of cDNAs as well as of the amino acid sequences of proteins MRJP1, MRJP2, MRJP3, MRJP4 and MRJP5 deduced from them showed high sequence identities among cDNAs and protein sequences. The homologies are interrupted by the regions containing repetitive motifs in MRJP3 and MRJP5. From the sequence homologies it is possible to conclude that the MRJPs are members of one protein family.

Although MRJPs are structurally closely related to each other, the expression profiles of MRJPs genes are not identical. The mRNA of MRJP1 is present in higher amounts in the hypopharyngeal gland of both nurses and foragers, while MRJP2-4 are synthesized predominantly in the hypopharyngeal gland of nurses (Klaudiny *et al.*, 1994b; Kubo *et al.*, 1996; Ohashi *et al.*, 1997).

The MRJP1 mRNA was found to be differentially expressed in the heads of early emerged honey bees (Kucharski *et al.*, 1998), nurse and also forager honey bees (Klaudiny *et al.*, 1994b). Its expression was localized to hypopharyngeal glands (Ohashi *et al.*, 1997), and also to a subset of Kenyon cells (intrinsic neurons) of mushroom bodies-presumed centers of learning and memory in the honey bee brain (Kucharski *et al.*, 1998). Therefore, it would seem that MRJP1 not only functions as a component of larval food but also plays a role in the honey bee brain.

Immunoblotting analysis could only detect MRJP2 mRNA in hypopharyngeal gland of nurse bee but not in forager bee (Kubo *et al.*, 1996), whereas microarrays and northern blot hybridization analysis (Kucharski and Maleszka, 2002) was found the expression of MRJP2 mRNA in heads of experienced foragers. MRJP3 mRNA and protein are expressed specifically in hypopharyngeal gland of nurse honey bees (Kubo *et al.*, 1996; Ohashi *et al.*, 1997). The expression level of MRJP4 mRNA in hypopharyngeal gland is very low compared to the expression of the other MRJPs (Klaudiny *et al.*, 1994b; Kubo *et al.*, 1996; Ohashi *et al.*, 1997).

Due to their high relative content of essential amino acid in the structure of some MRJPs, such as MRJP1, MRJP4 and MRJP5, and the presence of the extensive repetitive regions consisting of high amounts of nitrogen-rich amino acids in several MRJPs (MRJP2, MRJP3 and MRJP5) indicates that MRJPs are nutritive components of RJ serving as a supply of essential amino acid and a storage of biologically

accessible nitrogen (Albert *et al.*, 1999a). Besides MRJP3, two other MRJPs (MRJP2 and MRJP5) contain repetitive regions. All repetitive regions in MRJPs contain high amounts of nitrogen-rich amino acids such as asparagines and glutamine. Their presence significantly increases the nitrogen content of the MRJP protein. Therefore, the repetitive regions may be domains storing nitrogen in biologically processable form. Albertova *et al.* (2005) reported additionally that the presence of an extensive repeat region of MRJP3 in four traditional honey bee species (*A. mellifera, A. cerana, A. dorsata,* and *A. florae*) showed size and sequence polymorphisms in all species and correlation between repeat length and nitrogen content, an essential component of biogenic polymers. In another word, the repeat occurred due to a selection for an increase in nitrogen storage for a more efficient nutrition of queens and larvae.

Moreover, biological activities of MRJPs have been reported in various systems. MRJP1 which showed diverse biological activities in several heterologous system was not only informed to enhanced cell proliferation of rat hepatocytes (Kamakura *et al.*, 2001a) but also stimulate the growth of human lymphocytes in a serum-free medium (Watanabe *et al.*, 1996), and showed an anti-exhaustion effect in mice (Kamakura *et al.*, 2001b). Jelleines and antimicrobial peptides were identified in RJ as tryptic digests of the C terminus of MRJP1 (Fontana *et al.*, 2004), suggest that one of the physiological functions of MRJP1 might be to serve as a precursor of jelleines, protecting the RJ against bacterial infections. Futhermore, MRJP3 exhibited potent immunoregulatory effects *in vitro* and *in vivo* (Okamoto *et al.*, 2003).

The MRJPs are secretory proteins with N-terminal hydrophobic regions common to eukaryotic signal peptides. Comparison of the protein sequences of individual MRJPs with the proteins deposited in the Swiss-Prot database gave the highest identity (23.4–27.2%) with yellow protein from *Drosophila melanogaster*

(Schmitzova' *et al.*, 1998). Yellow protein is implicated in the processes of melanin pigmentation in insect cuticle (Geyer *et al.*, 1986; Kornezos and Chia, 1992). The degree of homology between yellow protein and MRJPs indicates that their genes probably evolved from a common ancestral gene (Schmitzova' *et al.*, 1998).

MRJPs contain high amount of essential amino acids (39.3-51.4%), presumably that MRJPs have nutritional function in honeybee larval food. Amino acid compositions of *Apis mellifera* MRJPs are illustrated in Table 1.3.

Three MRJPs contain high amounts of the 10 essential amino acids in honeybee (De Groot, 1953): MRJP5 (51.4%), MRJP1 (48%) and MRJP2 (47%). MRJP5 is rich in Arg and Met (9% and 11.4%). MRJP3 and MRJP4 have a lower overall content of essential amino acids, but they possess relatively higher amounts of some of them; MRJP3 Arg (4.9%), Lys (5.8%) and MRJP4 Leu (9.7%), Val (8%). The amino acid composition of MRJPs, and also their dominant content in larval jelly, indicate that they together represent a balanced mixture of the amino acids essential for nourishing both honeybee larvae and the queen.

MRJP1

MRJP1, the protein of the dominant band that identical to the protein derived from RJP120 cDNA (Schmitzova *et al.*, 1998), possesses the N-terminal amino acid sequence of "NILRGESLNKS". This protein is the most abundant of RJ protein (31% of the total protein) which shows high amount of the 10 essential amino acids composition of 48%. Molecular weights were rather small about 55 kDa – 57 kDa on SDS-PAGE which may reflect some modification of the proteins during transport, or storage in, the honey bee mouth cavity. MRJP1 was classified to acidic protein

according to Hanes and Simúth (1992); the studied informed that at least eight isoelectrophoretic variants ranging from 4.5-5.0 were found.

For indicating that native 56 kDa glycoprotein (MRJP1), purified 56 kDa protein is treated with N-glycosidase F. The molecular weight of the resulting digestion product is 47 kDa, which is closed to that of the putative protein lacking the signal sequence (46.8 kDa) (Ohashi *et al.*, 1997; Schmitzova *et al.*, 1998).

MRJP1 was reported to have three different forms, 55 kDa of a monomer, and approximately 420 kDa oligomer, and water insoluble aggregates resulted from interaction with fatty acids (Simúth, 2001). In the royal jelly, MRJP1 is able to strongly bind with a small peptide named Apisimin (Bilikova *et al.*, 2002) and possibly with other compounds in a large complex of 420 kDa. The oligomeric form of MRJP1 is water-soluble (Kimura et al., 1996; Simúth, 2001).

MRJP3

The N-terminal amino acid sequence of MRJP3 protein is AAVNHQ (R/K) KSANNLAHS and exhibits a size polymorphism. The apparent molecular masses were between 60 and 70 kDa revealed by SDS. MRJP3 is approximately 26% of total royal jelly protein with lowest essential amino acid content is 39.3% (Schmitzova *et al.*, 1998) and deduced amino acid contains a repetitive regions at the C-terminal part, repetitive motifs of XQNXX, typically with 20 repeated units (Klaudiny *et al.*, 1994a).

Table 1.3 Amino acid composition of *Apis mellifera* MRJPs.

	MRJP1	*MRJP2*	*MRJP3*	*MRJP4*	*MRJP5*	*MRJP6**	*MRJP7**	*MRJP8**
Ala	3.9	6.2	4.9	4.3	3.8	5.8	4.3	4.6
Arg	**3.4**	**3.8**	**4.9**	**4.1**	**9.0**	**3.1**	**3.8**	**3.6**
Asn	6.9	11.3	15.9	13.8	8.7	11.0	9.5	9.1
Asp	8.6	7.1	7.5	7.5	12.0	6.5	8.1	5.5
Cys	2.5	1.5	1.1	1.3	1.0	1.2	1.4	1.7
Gln	3.9	5.1	7.1	6.3	3.8	5.3	5.0	4.3
Glu	3.9	3.8	3.8	3.9	2.5	4.1	4.3	3.6
Gly	5.6	6.0	6.4	4.1	4.0	5.0	5.2	6.2
His	**2.3**	**2.4**	**2.2**	**3.9**	**1.8**	**2.6**	**1.4**	**1.2**
Ile	**6.0**	**5.1**	**4.0**	**3.2**	**4.8**	**7.4**	**7.5**	**7.7**
Leu	**9.5**	**8.2**	**6.8**	**9.7**	**5.2**	**7.9**	**8.6**	**10.8**
Lys	**5.1**	**6.9**	**5.8**	**5.0**	**4.3**	**6.0**	**5.2**	**4.8**
Met	**3.5**	**2.4**	**2.2**	**2.4**	**11.4**	**3.6**	**2.7**	**1.4**
Phe	**4.2**	**4.4**	**1.7**	**2.2**	**2.6**	**3.8**	**4.1**	**4.3**
Pro	3.7	3.1	2.5	2.2	2.6	2.9	2.7	2.6
Ser	8.1	5.8	5.9	8.4	6.2	8.2	6.8	7.7
Thr	**6.3**	**4.6**	**4.0**	**4.7**	**5.6**	**3.4**	**6.6**	**7.0**
Trp	**1.2**	**1.3**	**0.9**	**1.3**	**1.1**	**1.4**	**1.6**	**2.0**
Tyr	4.4	3.5	3.1	3.9	3.3	5.0	4.5	4.8
Val	**6.5**	**7.5**	**6.8**	**8.0**	**5.6**	**5.8**	**7.0**	**6.6**
Ess. aa.	48 %	47 %	39.3 %	44.5 %	51.4 %	45 %	48.5%	49.4%

Percent content of amino acid in native protein was obtained by computer analysis of its sequence (Schmitzova *et al.*, 1998). Essential amino acids are marked in boldface.

* Amino acid composition of AmMRJP6 was obtained by computer analysis employing the program ProtParam (Albert and Klaudiny, 2004).

To detect the polymorphism of the MRJP3 repetitive region, the PCR analysis of genomic DNA result showed highly polymorphic of MRJP3 repetitive region with as many as five alleles found in 10 individuals from the same colony and similar to MRJP2 which appeared a pentapeptide repeat in the C-terminal region (Albert *et al.*, 1999b). In other species, the study of repetitive sequence motifs in Giant bee, *A. dorsata* found that repetitive sequence also existed in MRJP3 gene linked those in *A. mellifera* (Albert and Schmitzova, 2002). The MRJP3 protein was reported to have two different forms, a monomer (70 kDa) and trimer (210 kDa) (Okamoto *et al.*, 2003).

MRJP5

Although, MRJP5 exhibits two different molecular weights (77 kDa and 87 kDa) on SDS-PAGE, they possess an identical N-terminal amino acid sequence of "VTV (R/N) E (N/Q) SPR". The relative content of MRJP5 is 9% of total royal jelly protein with containing 51.4% essential amino acid, dominant in Arg (9%) and Met (11.4%) (Schmitzova *et al.*, 1998).

From MRJP5 cDNA, deduced amino acid shows the extensive repeat region located between 367^{th} and 540^{th} amino acid residues. The consensus sequence (GATAGAATG) which encodes for tripeptide as DRM: aspartic acid (D), arginine (R), and methionine (M) occurred 58 times and interrupted a conserved region at the C-terminal of this protein and invariant in repetitive unit size (Albert *et al.*, 1999a). In addition, the totally different tripeptide repetitive motif of MRJP5 appeared at a different position in MRJP3 (Albert *et al.*, 1999b).

The MRJP5 repetitive region was characterized in *A. dorsata*. The repetitive region was located at the same position as found in *A. mellifera* but smaller in size,

and occurred 23 times compared with 58 times in *A. mellifera* (Albert *et al.*, 2002). From two-dimensional gel electrophoresis, the MRJP5 proteins were found in both the Africanized and the European honey bee RJ. The MRJP5 protein from this two species posses the identical N-terminal amino acid sequence (VTVRENSPRK), however, molecular weight and pI value were different (Sano *et al.*, 2004).

1.8.10 Tyramine receptor (TyrR)

Monophenolic amines such as octopamine and tyramine are found at very low concentration in the vertebrate brain. Their presence is probably associated with noradrenergic or dopaminergic neurons; however, these amines are abundantly present in the nervous system of protostomian invertebrates.

In insects, octopamine is synthesized from tyramine by hydroxylation and tyramine is formed by decarboxylation of tyrosine. Therefore, tyramine is the direct precursor of octopamine and as an analogue it may mimic certain effects of octopamine (and vice versa). In addition, several effects have been ascribed to tyramine (Roeder, 1994). In contrast to octopamine, tyramine has a down-regulating influence on trehalogenesis in cockroaches (Downer, 1979). Downer *et al.* (1993) observed differences in the distribution patterns of octopamine and tyramine in the locust (*Locusta migratoria*) nervous system. There are good indications that tyramine is released from vesicular storage sites. They also report the presence of a specific tyramine uptake system, which is distinct from the octopamine or dopamine uptake systems.

Binding data obtained with [^{3}H] tyramine suggest that there are separate tyramine, octopamine, and dopamine receptors in the locust brain (Hiripi *et al.*, 1994). The unequivocal identification of specific tyramine receptors in the locust supports

strongly the hypothesis that tyramine is not only the precursor amine for the biosynthesis of octopamine, but that it also has a function as an endogenous ligand.

1.8.11 Phosphatidylinositol phosphate kinase (PIP5K)

Phosphoinositol lipids have been postulated to play important roles in various cellular processes including growth, differentiation, and vesicular secretion. The phosphatidylinositol pathway consists of a series of conversions of phosphatidylinositol into singly, doubly, and triply phosphorylated products (Carpenter and Cantley, 1990, 1996; Divecha and Irvine, 1995). An important branching point in the pathway occurs when phosphatidylinositol 4-phosphate (PIP) is phosphorylated to become phosphatidylinositol 4,5-bis-phosphate (PIP2), a step catalyzed by phosphatidylinositol 4-phosphate 5-kinase (PIP5K; Boronenkov and Anderson, 1995; Ishihara *et al.*, 1996).

There are two types of PIP5Ks (PIP5KI and PIP5KII) with distinct biochemical and immunohistochemical properties, but they both catalyze the conversion of PIP into PIP2 (Loijens *et al.*, 1996). The hydrolysis of PIP2 by phospholipase C produces the second messenger diacylglycerol (DAG) and inositol tris-phosphate (IP3). DAG is an activator of protein kinase C, and IP3 plays an important role in the release of intracellular calcium (Rana ang Hokin, 1990).

PIP2 is itself a second messenger that has been implicated in the modulation of the function of cytoskeletal regulatory proteins such as profilin, cofilin, fascin, and gelsolin (Janmey, 1994). There is also evidence that phosphoinositide metabolism is involved in signal transduction and cytoskeleton regulation via interaction with the Rho family of small G proteins (Chong *et al.*, 1994; Ren *et al.*, 1996). Other work has suggested an interaction between phosphoinositides and receptor tyrosine kinases

(Cochet *et al.*, 1991). It has also been suggested that PIP5K function may be associated with, or required for, DNA synthesis and cell proliferation (Divecha *et al.*, 1993).

In *Drosophila*, PIP5KI is essential for cell and organism viability and that it is required for cytoskeletal regulation during sensory structure development, and also is required for germline development (Knirr *et al.*, 1997).

1.8.12 Phosphoinositide-3-kinase 68D (PI3K)

Phosphoinositide have been implicated in a variety of cellular processes as diverse as vacuolar protein sorting (Schwarzer *et al.*, 2006; Martín-Peña *et al.*, 2006), cytoskeletal remodeling (Orme *et al.*, 2006) and mediating intracellular signaling events through which growth factors, hormones and neurotransmitters exert their physiological effects on cellular activity, proliferation and differentiation (Wood *et al.*, 2006; MacDougall *et al.*, 1995, 2004), enzymes catalyzing the addition of phosphate to inositol. Eukaryotic cells contain a variety of inositol derivatives phosphorylated to different extents.

Phosphoinositide-3-kinases (PI3K) are classified into three distinct groups being designated to an individual class by their in vitro substrate specificity, biochemical characteristics and, in examples where a definitive function has been assigned, the nature of the biochemical activity regulated by the specific kinase.

PI3K class I polypeptides have a broad spectrum activity, phosphorylating inositol lipids phosphatidylinositol, phosphatidylinositol-4-phosphate (PIP), and phosphatidylinositols-4,5-bisphosphate (PIP2).

Class II PI3K has a restricted substrate specificity phosphorylating phosphatidylinositol, PIP but not PIP2. Each of the kinases of this class is

characterized by a conserved C2 domain in the carboxyl terminal region of the protein. The presence of conserved motifs within the C2 domain indicates that this region may confer regulation via calcium and/or phospholipids. A comparison of the murine and *Drosophila* class II kinases mop 170 and PI3K~68D respectively reveals a high degree of homology in the kinase domain of these proteins. Significant divergence occurs at the amino terminal regions of these polypeptides suggesting that adaptor proteins interacting with these variable domains may regulate kinase activity.

The third class of PI3K, class III PI3K, is related to the *S. cerevisiae* gene Vps34 (Schwarzer *et al.*, 2006). This kinase was originally isolated as a gene involved in regulating vesicle mediated membrane-trafficking in yeast.

1.8.13 Phosphoinositolglycan peptide (PIG-P)

A novel phosphoinositolglycan-peptide (PIG-P) from the yeast *Saccharomyces cerevisiae* potently mimicks insulin action on glucose transport and metabolism in rat muscle and adipose tissue. Rapid onset and reversibility of PIG-P action on glucose transport were observed in isolated adipocytes with a half-time of transport stimulation of 6-8 min (insulin less than 5 min). Combined treatment with PIG-P and insulin indicated additive stimulation of glucose transport at submaximal concentrations and non-additive action to both agents at maximal doses. The tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) was markedly increased in response to PIG-P in rat cardiomyocytes without any effect on the tyrosine phosphorylation of the insulin receptor β-subunit. PIG-P action in these cells was accompanied by phosphorylation/dephosphorylation of several proteins with molecular masses of 15-30 kDa, a response not detected with insulin (Kessler *et al.*, 1998).

The activation of a specific phospholipase C that hydrolyses glycosylphosphatidylinositol (GPI) lipids in the plasma membrane, leading to the production of soluble phosphoinositolglycan (PIG) molecules is less well defined pathway of insulin signaling. These compounds are thought to function as second messengers of insulin action. However, molecular targets of PIG action and the precise relationship to insulin signaling has remained poorly understood (Vila *et al.*, 1990; Farese *et al.*, 1994).

Compositional analysis of PIG molecules suggested that they consist of a core structure of phosphoinositol, glycosidically linked to glucosamine which is coupled to an oligosaccharide of varied composition. PIG molecules, the polar headgroups of GPI structures, may therefore be generated in response to insulin by lipolytic or lipolytic/proteolytic processing of the corresponding precursors (Varela-Nieto et al., 1996).

1.8.14 cGMP-dependent protein kinase (foraging)

Protein kinases play key roles in the activity-dependent modulation of neuronal activity and morphology. Interest in the cGMP-dependent serine/threonine kinase, or PKG, has grown with the awareness of the diversity of biochemical pathways that involve cGMP (Koesling *et al.*, 1991; Garbers, 1992; Sheth *et al.*, 1997; Wang and Robinson, 1997; Moon *et al.*, 1998; Simpson *et al.*, 1999). PKG has been shown to influence characteristics involved in both functional and developmental plasticity of neural circuits (Zhuo *et al.*, 1994, 1999; Lev-Ram *et al.*, 1997; Wu *et al.*, 1998b; Calabresi *et al.*, 1999; Lewin and Walters, 1999; Renger *et al.*, 1999; Yawo, 1999).

In *Drosophila*, one form of PKG (known as *dg2*; Kalderon and Rubin, 1989) is encoded by the foraging gene (Osborne *et al.*, 1997), which takes its name from a behavioral phenotype, the degree of locomotion while feeding, indicated by larval and adult foraging trail lengths (Sokolowski, 1980; de Belle and Sokolowski, 1987; de Belle *et al.*, 1989; Pereira and Sokolowski, 1993). Two naturally occurring variants, for^R ("rovers", with long foraging trails) and for^S ("sitters", with short foraging trails), have high and low PKG levels, respectively (Osborne *et al.*, 1997). The genetic dissection of learning and memory in the fly *Drosophila melanogaster* has given significant insights into molecular and cellular mechanisms that underlie neural and behavioral plasticity (Dudai, 1988; Griffith *et al.*, 1994; Tully *et al.*, 1994; DeZazzo and Tully, 1995; Heisenberg *et al.*, 1995; Davis, 1996; Wolf *et al.*, 1998; Wu *et al.*, 1998). At least two classes of molecules, second messengers and ion channels, have been implicated Wu *et al.*, 1998a).

1.8.15 Niemann-Pick type C2 protein (Npc2)

Cholesterol, an essential component of eukaryotic cell membranes, also serves as the precursor of many steroid hormones and thus plays vital roles in many developmental processes (Farese and Herz, 1998). Cells in the body maintain proper cholesterol levels through elegant homeostatic regulatory systems. Defects in cholesterol homeostasis and metabolism have been linked directly or indirectly to many disease conditions.

Niemann-Pick type C2 (Npc2), a small, is a secreted protein that binds cholesterol strongly. The crystal structure of Npc2 has been determined and found to contain a cavity that genetic analyses show to be the likely binding site for cholesterol (Friedland *et al.*, 2003; Ko *et al.*, 2003). Npc2 may serve as a lysosomal cholesterol

transporter, rapidly transporting cholesterol to acceptor membranes (Cheruku *et al.*, 2006).

Drosophila and all other insects are unable to synthesize sterol from simple precursors. In order to synthesize the molting hormone 20-hydroxyecdysone (20E) and to sustain the growth and reproduction of the fly, sterol has to be obtained from food (Clark and Block, 1959). 20E plays crucial roles in insect oogenesis, embryogenesis and metamorphosis (Thummel, 1996). Mutation of Npc2 results in abnormal sterol distribution in many cells. Npc2 functions in regulating sterol homeostasis and ecdysteroid biosynthesis, probably by controlling the availability of sterol substrates (Huang *et al.*, 2007).

1.9 Northern blot hybridization

Northern analysis remains a standard method for detection and quantitation of mRNA levels despite the advent of powerful techniques, such as RT-PCR, gene array analysis and nuclease protection assays. Northern analysis provides a direct relative comparison of message abundance between samples on a single membrane. It is the preferred method for determining transcript size and for detecting alternatively spliced transcripts.

The Northern blotting procedure is straightforward and provides opportunities to evaluate progress at various points (e.g., integrity of the RNA sample and how efficiently it has transferred to the membrane). RNA samples are first separated by size via electrophoresis in an agarose gel under denaturing conditions. The RNA is then transferred to a membrane, cross-linked and hybridized with a labeled probe.

Northern hybridization is exceptionally versatile in that radiolabeled or non-isotopically labeled DNA, in vitro transcribed RNA and oligonucleotides can all be used as hybridization probes. Additionally, sequences with only partial homology (e.g., cDNA from a different species or genomic DNA fragments that might contain an intron) may be used as probes.

Despite these advantages, there are limitations associated with Northern analysis. First, if RNA samples are even slightly degraded, the quality of the data and the ability to quantitate expression are severely compromised. Thus, RNase-free reagents and techniques are essential. Second, a standard Northern procedure is, in general, less sensitive than nuclease protection assays and RT-PCR, although improvements in sensitivity can be achieved by using high specific activity antisense RNA probes, optimized hybridization buffers and positively charged nylon membranes. A third limitation of Northern blotting has been the difficulty associated with multiple probe analysis (Applied Biosystems website: http://www.ambion.com/techlib/basics/northerns/index.html).

1.10 Real-time PCR

Normal reverse transcriptase PCR is only semi-quantitative at best because, in part, of the insensitivity of ethidium bromide. Thus real-time PCR was developed because of:

- The need to quantitate differences in mRNA expression
- The availability of only small amounts of mRNA in some procedures

There are a variety of methods for the quantification of mRNA. These include Northern blotting, ribonuclease protection assays (RPA), *in situ* hybridization, and PCR. PCR is the most sensitive method and can discriminate closely related mRNAs. It is technically simple but it is difficult to get truly quantitative results using conventional PCR. Northern blotting and RPAs are the gold standards, since no amplification is involved, whereas *in situ* hybridization is qualitative rather than quantitative.

Techniques such as Northern blotting and RPAs work very well, but require more RNA than is sometimes available. PCR methods are therefore particularly valuable when amounts of RNA are low, since the fact that PCR involves an amplification step means that it is more sensitive. In contrast to regular reverse transcriptase-PCR and analysis by agarose gels, real-time PCR gives quantitative results. An additional advantage of real-time PCR is the relative ease and convenience of use compared to some older methods (Microbiology and Immunology online: http://pathmicro.med.sc.edu/pcr/realtime-home.htm).

The procedure follows the general principle of PCR its key feature is that the amplified DNA is quantified as it accumulates in the reaction in real time after each amplification cycle. Two common methods of quantification are: (1) the use of fluorescent dyes that intercalate with double-stranded DNA, and (2) modified DNA oligonucleotide probes that fluoresce when hybridized with a complementary DNA. Frequently, real-time PCR is combined with reverse transcription to quantify mRNA in cells or tissues.

Cells in all organisms regulate gene expression and turnover of gene transcripts (mRNA), and the number of copies of an mRNA transcript of a gene in a cell or tissue is determined by the rates of its expression and degradation.

In order to robustly detect and quantify gene expression from small amounts of RNA, amplification of the gene transcript is necessary. The polymerase chain reaction is a common method for amplifying DNA, for mRNA-based PCR the RNA sample is first reverse transcribed to cDNA with reverse transcriptase.

Development of PCR technologies based on reverse transcription and fluorophores permits measurement of DNA amplification during PCR in real time, i.e., the amplified product is measured at each PCR cycle. The data thus generated can be analyzed by computer software to calculate relative gene expression in several samples, or mRNA copy number. Real-time PCR can also be applied to the detection and quantification of DNA in samples to determine the presence and abundance of a particular DNA sequence in these samples (http://en.wikipedia.org/wiki/Real-time_polymerase_chain_reaction).

1.11 Objectives of this dissertation:

1) To identify the genes that differentially expressed between wild type and anarchist worker honey bees.

2) To determine the effect of pheromone and carbon dioxide narcosis on ovary activation in workers.

3) To identify the genes associated with ovary activation in workers in response to pheromone and carbon dioxide narcosis.