Relationships Between Brain Biogenic Amines and Reproductive and Defense Behavior in Honey Bees (Apis Mellifera L.).

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RELATIONSHIPS BETWEEN BRAIN BIOGENIC AMINES AND REPRODUCTIVE AND DEFENSE BEHAVIOR IN HONEY BEES

(Apis mellifera L.)

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

The Department of Zoology and Physiology

by

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ABSTRACT

This dissertation represents an attempt to relate behavioral states of worker honey bees to brain levels of biogenic amines. The first chapter describes the results of experiments which indicated that brain levels of octopamine, dopamine and serotonin were affected by factors including the degree of handling stress, worker age, season of sampling and the source colony from which the workers were sampled. These results helped shape the methods employed in the subsequent chapters. The second and third chapters relate changes in brain dopamine levels to worker honey bee ovarian development. In particular, chapter II showed that an increase in brain dopamine content could be associated with increased ovariole width in worker bees. Chapter III presents experiments which show that both brain dopamine and worker ovary development are reduced by exposure to CO₂. The evidence indicates that dopamine is significantly elevated after the ovaries have begun to develop; therefore, brain dopamine may or may not be directly related to stimulation of reproductive processes in worker bees. The final chapter represents an attempt to modify the buzzing responses of groups of honey bees to the alarm pheromone component, isopentyl acetate (IPA). A laboratory bioassay that measured sound responses by groups of bees after exposure to IPA was used to test the effects various doses of ingested amine precursors (tryptophan, 5-hydroxytryptophan and L-DOPA). Ingested 5-hydroxytryptophan was found to reduce responses to IPA, and ingested tryptophan was found to cause hyperactivity in bees. These results are discussed relative to changes in brain chemistry related to ingestion of the different amine precursors.
INTRODUCTION

The migration of Africanized honey bees (*Apis mellifera scutellata* Lepeletier) through South and Central America, Mexico and now the extreme southern United States of America has created some anxiety among public health officials and agricultural experts. Perhaps the most publicized characteristic of these bees is their often fierce defense of their colonies. The problems related to protection of people from stinging attacks (mass envenomization) are numerable, and the effects of these bees on agriculture are not fully predictable.

Interest in the Africanized bee has lead to the examination of the genetic and pheromonal basis of honey bee alarm communication and defense behavior (Collins and Rothenbuhler, 1978; Collins *et al.*, 1980; Collins and Kubasek, 1982; Collins, 1989). Specific alarm chemicals have been identified from the mandibular glands (Shearer and Boch, 1965; Boch *et al.*, 1970) and sting apparatus (Boch *et al.*, 1962; Maschwitz, 1966), and behavioral responses to these chemicals (2-heptanone and isopentyl acetate, etc.) in lab and field tests can be quantified. Differential responses to a variety of chemicals and repellents and other stimuli have also been tested (Collins, personal communication), but the use of repellents against attacking bees currently offers only limited control for very defensive colonies.

Field and laboratory tests have also been used to determine differences between various genetic lines of bees and to determine heritabilities of various parameters that are
used to quantify colony defense (Collins and Rothenbuhler, 1978; Collins, 1989). These behavioral bioassays have defined the distinctions between Africanized honey bee and European honey bee defense behavior that beekeepers have been aware of for many years.

Although, models have been proposed to relate the activities of individual bees to the overall colony response (Collins et al., 1980), honey bee defense has been evaluated primarily at the colony level. This approach is logical, but I feel that examination of the mechanisms that determine individual bee behaviors might increase our understanding of the colony response. Our particular focus is on the relationship between biogenic amine neuromodulators within the bee brain and specific behavioral responses to an olfactory stimulus (or other stimuli) such as an alarm pheromone. Recent work with vertebrates and invertebrates has indicated that a variety of behaviors are mediated by the actions of biogenic amines within the central nervous system (Kravitz, 1988; Bicker and Menzel, 1989). Indeed, the examination of olfactory learning in bees has shown that memory retrieval and storage (complex nervous system functions) are mediated by biogenic amines (Menzel et al., 1990).

**Africanized Honey Bees**

In 1957, W. E. Kerr, a Brazilian geneticist, accidentally released 26 African honey bee (*Apis mellifera scutellata*) queens with accompanying swarms near the Rio Claro area of Brazil. His intent for importing the African bees was to use them as breeding stock to improve the qualities of honey bees living in the neotropics. For nearly 100 years prior to this introduction, European races of honey bees (*A.m. ligustica, A.m. iberica, and A.m.*
mellifera) had been introduced and used by beekeepers throughout the Americas. Although the bees of the European races are fairly gentle in regard to their colony defense, they experience difficulty in surviving the hot, humid climate of the tropics. European bees seem more susceptible to diseases and mites in the tropics, and in general, honey production from European bees is lower in tropical climates than in moderate climates. On the other hand, African bees have evolved for life in the African tropics, and many of their characteristics make them well suited for survival in neotropical South and Central America. Kerr had hoped to produce a better Brazilian bee by incorporating the mild defensive behavior of the European bees with other characteristics of the African honey bees into a better hybrid bee that could be used by beekeepers.

Subsequent to the initial release of African swarms and through mechanisms not completely understood, Africanized bees established a substantial feral population throughout Sao Paulo by supplanting the pre-existing populations of European bees (Page, 1989; Taylor, 1985). By the middle 1960's the Africanized bees had established themselves throughout Brazil and radiated toward other regions of South America. During the 1970's the Africanized bees moved into Surinam, and by 1980 they became established in Columbia. In 1983, Africanized bees were found in Panama, and by the mid to late 1980's they moved into extreme southern Mexico near Chiapas. Within the past decade, the Africanized bees have moved in two fronts, one along the Pacific coast and the other along the Caribbean coast of Mexico, toward the United States. Deserts and other geographical barriers have caused the Pacific corridor to move slower than the Caribbean front. Finally, in October of 1990, a few frontier swarms appeared in Hidalgo and surrounding counties.
near Brownsville, Texas. Currently, many Africanized swarms have been found in these same areas.

The very characteristics of Africanized bees that allow them to survive a tropical climate, have almost lead to the collapse of beekeeping industries in many South American countries soon after large feral populations became established. For example, the number of modern commercial beekeepers dropped from 18 to just 2 within five years (1976 - 1981) of Africanized bees moving into Venezuela (Rinderer, 1984, 1986b). Honey production dropped from 580 metric tons to less than 100 metric tons during the same period (Rinderer, 1984, 1986b). The long term effect of Africanized bees on the beekeeping industries of South America has not been all gloomy. Beekeepers have been forced to learn new management techniques, and after a transition period, many beekeepers have accepted the challenge of producing a honey crop using the now well established Africanized bees instead of European ones. Some Brazilian beekeepers reported the highest honey yields ever (up to 150 pounds of honey per colony) in 1991 (White, 1991).

One obvious characteristic making beekeeping difficult is the fierce defensive behavior of Africanized bees. Several hundreds of thousands of animals and a few hundred to a thousand people have died as the result of Africanized honey bee attacks during the last 35 years (White, 1991). Africanized bees respond faster to a colony disturbance, and twice as many alerted bees leave the colony to attack the intruder in Africanized colonies than in European ones (Collins et al., 1982; Collins and Rinderer, 1986). Africanized bees sting the intruder eight to ten times more frequently than do European bees (Collins et al., 1982; Collins and Rinderer 1986). Also, Africanized colonies have about five times more guard...
bees than European colonies (Collins et al., 1982). Few beekeepers accustomed to working with European bees tolerate the increased number of stings and the increased time and equipment needed to safely work with Africanized bees.

Another characteristic leading to difficult management of colonies is the Africanized honey bee propensity to swarm (Rinderer, 1986a). These bees swarm many times a year and fly long distances, while European honey bee colonies swarm only once a year during a limited period of time during the spring and fly relatively shorter distances. Swarming in European bees is usually reproductive, while Africanized bees tend to abscond their colonies when nectar resources are low. Increased swarming by Africanized bees leads to reduced honey production for two reasons. Firstly, Africanized bees can invade managed colonies of European bees and kill the queen (Vergara et al., 1989). Once the European queen is dead, the invading Africanized queen will begin egg-laying, and within a few weeks the colony becomes completely Africanized. These Africanized colonies use the majority of their nectar and pollen resources for brood rearing; consequently, less honey is stored as surplus. Secondly, swarming by Africanized bees leads to the establishment of large feral or unmanaged populations of bees (Rinderer, 1986a, 1986b). These bees often directly compete for the same nectar resources that managed colonies must forage. Hence, feral competition leads to reductions in honey production from managed bee colonies.

Some experts believe the fear concerning these bees is unwarranted and stems from sensational journalism and the movies that depict 'killer bees' as searching for unweary prey. Within the scientific community, opinions vary on the behavioral nature of the bees that will eventually become established in the United States. Evidence from mitochondrial DNA
studies suggests that the advancing bees are as African as their predecessors that were first introduced into Brazil in 1957 (Hall and Muralidharan, 1989; Page, 1989; Smith et al., 1989). These data also suggest that modification of behavior in these bees through breeding may be impossible. Other evidence suggests that various characteristics of these advancing bees have been changed through hybridization with the more manageable European bees (Collins, 1989); therefore, the term Africanized bee is more appropriate than African bees. More recent examinations of mitochondrial DNA from bees sampled in the Yucatan peninsula indicates high levels of hybridization (Rinderer et al., 1991). Hybridization suggests that many characteristics (including defense behavior) might be modified through breeding practices.

Even more uncertainty surrounds the potential range and effect of these bees on the United States beekeeping industry. Many bee experts feel that the advanced state of American beekeeping practices will easily offset and control these bees. Others are concerned that American commercial beekeeping will experience a drastic reduction in terms of beekeepers and honey production as a consequence of large feral populations of Africanized bees. Currently, the American beekeeping industry earns $150 million annually, and may be responsible for another $11 - $20 billion a year in agricultural production linked to beekeeping (Rinderer, 1986a).

Although beekeepers may be able to adapt to working with Africanized bees, these honey bees do represent a major public relations obstacle for the beekeeping industry. The American public may be intolerant of Africanized bees in public parks or other populated areas. Hence, "bee-free" zones may become established around residential areas,
amusement parks, and shopping malls. Beekeepers will be forced to place their colonies in more remote and less visible localities. Some rural land owners may refuse to allow beekeepers to place colonies on their land from fear that livestock or family members will be harmed. Legal questions concerning liability related to stinging attacks have yet to be answered, and public perception of honey bees may dictate whether beekeepers can be legally liable for stinging attacks, even if the beekeeper has made every attempt to keep gentle colonies.
REVIEW OF LITERATURE

Measurement of Honey Bee Defense Behavior

Many researchers have attempted to quantify the honey bee defense response at the colony level. Collins (1989) stated that the response of bees to intruders was not an act of 'aggression' since bees respond only after a series of stimuli and not by active search mechanisms (the term “aggression” implies a behavior initiated by the bees). Early measurements of colony defense made by Free (1961) and Maschwitz (1966) used the number of stings received by a target as an indicator of colony defense without consideration of the complex sequential nature of the colony response. Free (1961) found that movement of dark objects (especially blue) in front of colonies provoked attack by bees and that odors and textured surfaces on the target enhanced the number of stings received by the target (cotton ball, etc.). Maschwitz (1966) found that the presence of the sting apparatus released alarm pheromones that could induce an alerting behavior in bees. He described alerted bees as those with raised abdomens, extended stings (presumably to release more alarm pheromones), and waving antennae.

Prior to this description of the alerting posture of bees, Boch et al. (1962) identified isopentyl acetate (IPA) as an alarm pheromone from the sting apparatus of honey bees. Free and Simpson (1968) found that IPA agitated bees at the colony entrance, but the actual number of stings received by an IPA-soaked target were less than targets treated with sting extracts. This result suggested that other compounds probably contribute to the alarm
pheromone blend. Shearer and Boch (1965) found 2-heptanone (2-HPT) to be an alarm pheromone produced by the honey bee mandibular glands, and subsequent evaluation by Boch et al. (1970) showed that 2-HPT was also less effective at eliciting stinging responses than whole sting extracts.

Blum et al. (1978) identified several acetates (n-butyl, isoamyl, n-hexyl, n-octyl, n-decyl, and benzyl) and three alcohols (isoamyl alcohol, 2-nonanol and benzyl alcohol) from volatile extracts of the honey bee sting apparatus using gas chromatography-mass spectrometry. During the same year, Collins and Rothenbuhler (1978) reported a laboratory bioassay using small groups of caged bees for measuring behavioral responses to alarm pheromones (IPA). When caged bees were exposed to IPA, they flicked their wings and increased their locomotion (undirected). Variables measured in the laboratory test included the time to react to presentation of the alarm chemical, the time to quiet, duration of the reaction, and initial intensity of reaction. Measurement of the last variable was determined by the number of bees responding and a subjective feel for intensity of the group response.

Using this laboratory bioassay, Collins and Rothenbuhler (1978) showed that IPA was more effective at eliciting an alarm response than 2-HPT. The IPA was dissolved in paraffin oil, and as IPA concentration was increased the time to react decreased and the duration and intensity of reaction increased. For best results, the authors recommended an IPA: paraffin oil ratio of 1:10 (v/v). They also found that the bioassay could only be used on bees aged 2-7 days, because older bees became too active to allow determination of increased locomotion after administration of IPA. A minimum interval of 15 minutes between subsequent tests can be used, but the researchers recommended a one hour period.
between tests to ensure that results are not biased from previous treatment. A minimum group size of 25 bees per cage was recommended for laboratory tests.

Later work by Collins and Blum (1983) showed that many of the compounds isolated by Blum et al. (1978) and some new compounds (1-butanol, 1-acetoxy-2-octene, 1-acetoxy-2-nonene) isolated from the honey bee sting were effective at eliciting behavioral defense responses by caged worker bees in a laboratory bioassay. These results suggest that the alarm signal released by worker bees is a complex mixture of volatile esters and alcohols, and the exact composition of the pheromone has not been fully determined. The following mixture reported by Collins and Kubasek (1982) was found to be effective at triggering colony defense when presented at the colony entrance prior to a mechanical disturbance of the colony:

**Pheromone blend**

1.5% n-butyl acetate, 32% isopentyl acetate, 14.5% isopentyl alcohol, 4% n-hexyl acetate, 16.8% n-octylacetate, 10% 2-nonanol, 1.5% n-decyl acetate, 15.7% benzyl acetate and 4% benzyl alcohol dissolved in paraffin oil. Mix 1 volume of this alarm pheromone mix with 99 volumes of paraffin oil.

Although the alarm pheromone of honey bees is a complex blend, IPA has been shown to be effective at eliciting a defense response if it follows or precedes a mechanical disturbance. Boch and Rothenbuhler (1974) tested colony defense by breathing into a colony entrance, followed by opening the colony, and counting the number of stings received by a cork soaked with IPA when it was placed at the colony entrance. In their test, IPA was very...
effective at eliciting stinging. A similar approach was used by Moritz et al. (1987). In their procedure, the colony was opened and initially exposed to a filter paper soaked with 1% IPA placed on the top bars of the brood nest. A small suede patch was then waved 1 cm above the top bars, and the total number of stings were counted after 10 seconds. Although these tests provide some quantification of defense behavior, they detail very little about colony behaviors that occur prior to the culminating act of stinging the target.

Prior to the development of the laboratory bioassay for alarm chemicals developed by Collins and Rothenbuhler (1978), attempts to quantify the entire behavioral sequences of the honey bee colony defense response were made by Stort (1974) using Africanized and Italian honey bees. As with the early field studies of Free (1961) and Maschwitz (1966), Stort used a small moving target (black leather ball) in front of a colony to elicit the colony defense. Unlike his predecessors, he not only counted the number of stings received by the target, he also measured the time needed before the first bees became alerted, the time before the first sting in the target, the number of stings in the observer's gloves, the time for the bees to calm, and the distance that bees would pursue the experimenter upon withdrawal from the colony. Using these criteria, Africanized bees were found to be significantly more defensive than European bees in Brazil.

Subsequent comparative studies of Africanized and European honey bees in Venezuela by Collins et al. (1982) and Collins and Kubasek (1982) confirmed many of Stort's original results. These field evaluations began with the release of IPA at the colony entrance followed by striking the front of the colony with a projectile fired from a sling shot. Two dark suede patches were waved by a mechanical device in front of the colony
throughout the test. The variables measured were the time to respond to IPA, the time to respond to the mechanically waved targets, the number of stings in the targets, and the number of bees responding at three different times (30, 60 and 90 seconds) during the test. The time measurements were made by counting the number of bees at the colony entrance in photographs that were taken at 30-second intervals after the test began.

Africanized bees reacted significantly faster to IPA and movement of the suede targets than European bees. Africanized bees also stung the target significantly more times (6-8 times more) than European bees, and many more Africanized bees were found on the colony entrance in photographs through 60 seconds. The reaction time was reduced to both alarm pheromones and mechanical stimuli, and the number of bees responding and the total number of stings increased. The number of bees responding was significantly correlated to the total number of stings only for Africanized bees. Collins et al. (1987b) found that Africanized bees did not respond more quickly than European bees, but they did respond in greater numbers and they persisted in their defense longer than European bees. Although the results from these experiments were slightly different, they clearly indicate that Africanized and European bees are different in their response to a colony disturbance. Similar conclusions concerning the nature of defense behavior for Africanized and European bees have been made using the laboratory bioassay (Collins and Rothenbuhler, 1978).

Collins and Rinderer (1988) used the previously mentioned field test of defense behavior to measure colony defense for Africanized and European bees and their hybrids (European x Africanized) in Venezuela. As in the previous work, Africanized bees were significantly more defensive than the European bees. The hybrids were intermediate in the
time before stinging a mechanical target, total number of stings in the target, and number
of bees in the air at 60 and 90 seconds. The hybrids were not significantly different than the
European parent type in terms of time to react to IPA and the numbers of bees at the colony
entrance at the 30 second interval. These results provide evidence that hybridization with
European bees can lead to a reduction in the defense behavior of Africanized bees.

A more recently developed laboratory test for honey bee responses to alarm
pheromones measures metabolic oxygen consumption by groups of bees subsequent to
release of a test chemical (Moritz et al., 1985; Southwick and Moritz, 1985). The test
chamber containing the bees is fitted to a flow through system consisting of a chamber
containing air saturated with IPA at the one end and a flow-through oxygen analyzer at the
other end. A pump maintains a constant flow rate of air, and a system of electronically
controlled valves control the air mixture that the bees receive. Prior to the test, bees are
placed in the chamber and receive only air through the system. The IPA is administered by
briefly (5 seconds) opening the valve connecting the IPA chamber to the system. The exact
dosage of IPA can be determined. Using this technique, Southwick and Moritz (1985)
showed that oxygen consumption per bee increased after administration of IPA. They
concluded that the increased oxygen consumption was not caused by additional release of
alarm pheromones by the bees during the test. They also found significant differences
between colonies, and the results of their metabolic test correlated well with results from
field trials.
Guard Bees

Guarding behavior is an important element of the colony defense response. Africanized honey bee colonies have up to five times more guards than do European honey bee colonies; however, the increased number of guards cannot explain all aspects of the typical Africanized honey bee colony defense response. Usually many hundreds to thousands of Africanized bees respond quickly to a colony disturbance, while the total number of guards may be less than 100 (Breed and Moore, 1989). High numbers of guards may reflect an increased ability to become alerted and to recruit other colony members to participate in defense behavior rapidly.

Guarding also represents a transition from tasks associated with colony upkeep to foraging behavior. Worker honey bees perform a variety of tasks throughout their lives, and the duties performed at any specific age are at least in part determined by juvenile hormone III titer (Robinson et al., 1989). Typically, very young bees have well developed hypopharyngeal glands used to secrete brood food for developing larvae; hence, these bees serve as nurse bees. As a bee grows older she will advance through many tasks that include nest cleaning, comb construction, undertaking, guarding, and ultimately foraging behavior. Many physiological changes accompany these behavioral changes. For example, the hypopharyngeal glands decrease in size and activity and simultaneously the corpora allata volume and JH titer increase as the bees ages.

One of the most visible guarding behaviors is the greeting of returning foragers on the entrance ramp of a colony (Butler and Free, 1952), and the frequency of intercepting foragers by guards was found to vary with food abundance and other conditions. Ribbands
(1954) found that colonies separated by a few inches were not hostile during periods of nectar abundance, but the same colonies were very hostile during nectar dearths. Individual guards typically patrol small areas on the entrance ramp. Incoming bees are greeted or inspected with the antennae, and when an intruding bee is detected, the guard bees bite and pull the intruder away from the colony entrance. Often intruders are mauled to death. Although most of the colony guards are found at the colony entrance, Moore *et al.* (1987) found that guards often take short flights of 3-4 meters near the colony.

More recent descriptions of guarding behavior explore odors important to nestmate recognition (Moore *et al.*, 1987; Breed and Moore, 1989). Environmentally acquired odors (Kalmus and Ribbands, 1952) and genetically determined odors (Breed, 1983, 1985) are involved in nestmate recognition, and guard bees can usually detect foreign bees based on either the absence of particular odors or the presence of foreign odors. Guards become alerted to defend by the same pheromones used to alert other colony members.

Moore *et al.* (1987) also describe the position of guarding behavior within the temporal polytheism of a bee's life. Using marked cohorts of bees, they found that bees begin guarding 14-16 days after eclosion, which represents a transition from colony duties to foraging. Only 4-15% of the bees in a particular cohort were found to act as guards at any one time. For 12 colonies, the mean number of guards found at the colony entrance was 72 (range: 24 - 160). The highest percentage of guard bees occurred during periods of abundant nectar (146 guard bees/1094 marked bees). Also, bees only guard for relatively short periods. Breed and Moore (1989) found that of 741 guard bees observed, 578 guarded only one day before becoming foragers (the remaining bees guarded for six days
or less). They also found that the more days a bee guarded, the longer each guarding bout lasted. Rarely did any single worker guard longer than 5 days.

**Aggression and Defense Behavior in Queenless Bees**

One of the most noticeable characteristics of a queenless group of workers is the development of aggression. The first reports of aggression in queenless colonies came from Sakagami (1954, 1958). He noted increased mauling between workers as laying workers developed in queenless colonies. Velthuis (1976) found that workers being attacked have ovaries more highly developed than their attackers and that worker dominance correlates to elevated vitellogenin (VG) levels in the hemolymph. Moritz and Hillesheim (1985) observed dominance hierarchies in the Cape bee, and Korst and Velthuis (1982) correlated dominance in trophallaxis to ovary development. Velthuis (1985) found that workers receiving the most food during trophallaxis have little pollen in their guts, but have highly developed ovaries; while workers giving food materials during trophallaxis often have large amounts of pollen in their guts with only slightly developed ovaries. These results indicate that protein exchange from submissive bees to dominant bees may play a major role in control of worker oogenesis. Velthuis (1985) found no direct correlation between aggressive behavior and egg laying by workers.

In regard to defense behavior of queenless colonies, Hoffman (1961) reports that although the age-related activities of workers in queenless colonies do not differ from those of workers in queenright colonies, workers in queenless colonies are more irritable and the number of guards are higher. Contrary, Delaplane and Harbo (1987) found a reduction in
defense behavior in queenless colonies. Reasons for the differences between these results are not obvious.

**Biogenic Amines as Neuromodulators of Behavior**

Insect nervous systems contain high levels of octopamine, dopamine and serotonin or 5-hydroxytryptamine (Evans, 1980; 1986). Unlike the classical neurotransmitters (acetylcholine, glutamate, gamma-aminobutyric acid) that rapidly affect ion permeability in postsynaptic cells, these biogenic amines and various peptides act as neuromodulators that indirectly affect ion channels, modifying the neuronal response to neurotransmitters on a longer time scale. In turn, the activities of neuromodulators are altered over long distances and for a longer time by neurohormones (Bicker and Menzel, 1989).

The actions of neurohormones and neuromodulators allow coordination of spatial and temporal neural events of the insect nervous system (Bicker and Menzel, 1989). By altering the activities of neurons and muscles and by regulating certain changes in metabolism, these compounds can generate different behavioral states in organisms by allowing neural circuits to operate differently at different times. This neural plasticity is very important for an animal to effectively deal with various sensory inputs important for survival.

Biogenic amines have been implicated in determining arousal states in animals. A reduction in levels of serotonin in the brain of some vertebrates increases motor activity of the animal (Mason, 1984). Norepinephrine has been shown to increase activity and facilitate memory by increasing selective attention (Bicker and Menzel, 1989). Serotonin and
neurosecretions from the pars intercerebralis have been implicated as regulators of circadian locomotor rhythms in crickets (Cymborowski, 1970; Muszynska-Pytel and Cymborowski, 1978a, 1978b; Renucci et al., 1989). Serotonin also controls seasonal dispersal in the boll weevil (Guerra et al., 1991).

Some of the best examples of biogenic amine regulation of behavior can be demonstrated by crustaceans. Biogenic amines affect behavior not only by influencing arousal state of an animal, but by modulating synaptic transmission in rapid response circuits, such as in the crayfish tail-flip escape behavior (Bicker and Menzel, 1989). For the crayfish tail-flip response, biogenic amines are thought to influence transmission between sensory neurons and interneurons that precede the motor neurons that cause contraction of the flexors. Application of octopamine between sensory neurons and interneurons that connect to the lateral giant fibers (the lateral giant fibers electrically synapse with the motor neurons) of the crayfish tail-flip circuit causes increased flexion of the tail. Application of serotonin decreases tail flexion. The biogenic amines are thought to act on the input circuits because stimulation of the lateral giant fibers leads to the complete act of flexion of the crayfish tail.

In lobsters, octopamine and serotonin modulate both aggressive and submissive postures when applied between motor neurons connected to extensor and flexor muscles (Harris-Warrick and Kravitz, 1984; Kravitz, 1988). Injection of serotonin into lobsters leads to flexion of limbs and abdomen resulting in an aggressive posture, while injection of octopamine causes a submissive posture. Injection of both amines in the peripheral synapses between motor neurons and muscles results in both responses. From this evidence, it is
clear that antagonistic modulation of lobster posture occurs via the central nervous system, and some modulation occurs via peripheral systems. When applied peripherally, serotonin increases spike frequencies in flexor motor neurons, and octopamine decreases these spike frequencies. Opposite effects are seen in the extensor muscles. This antagonism extends to excitatory and inhibitory neurons that innervate each muscle.

**Roles of Biogenic Amines in Insects**

Although many biogenic amines occur in insects (Clarke and Donnellan, 1982), octopamine has been the most widely studied compound because of its relationship to the insect "fight or flight" response. Octopamine regulates lipid and carbohydrate mobilization (Orchard et al., 1981; 1982; 1983; Downer et al., 1984; Pannabecker and Orchard, 1986), modulates neuromuscular transmission and muscle contraction in insect skeletal muscle (Evans and O'Shea, 1978; Evans, 1981) and controls insect visceral muscles in the gut and ovaries (Orchard and Lange, 1987). Octopamine, which has also been implicated in modulation of the locust forewing stretch receptor (Ramirez and Orchard, 1990), regulates flight muscle metabolism (Candy, 1978), initiates light emission from the firefly light organ (Nathanson, 1979) and mediates some insect behaviors (Brookhart et al., 1988; Linn and Roelofs, 1987; Mercer and Menzel, 1982).

Levels of octopamine in the insect central nervous system (CNS) change in response to metamorphic development (Fuzeau-Braesch et al., 1979; Bodnaryk, 1980; Woodring et al., 1988), starvation (Davenport and Evans, 1984a), flight (Goosey and Candy, 1980;
Bailey et al., 1984) and handling stress (Downer, 1979; Davenport and Evans, 1984b; Woodring et al., 1988; Woodring et al., 1989). Serotonin, dopamine and 5-hydroxytryptophan have been shown to be involved in modulation of some insect behaviors. Serotonin and 5-hydroxytryptophan were found to decrease the attacking behavior of ants to a beetle prey, while increasing aggressive interactions between the ants (Kostowski and Tarchalska, 1972). Dopamine has been shown to modulate activity of an inhibitory motor neuron in the cockroach (Pitman and Baker, 1989). Actions of all biogenic amines in insects have been related to specific adenylate cyclase second messenger systems associated with biogenic amine receptors (Bodnaryk, 1982; Evans, 1988; Vaughan, 1988).

Biogenic Amines in Honey Bees

In honey bees (Apis mellifera L.), large amounts of octopamine, dopamine, serotonin and other neuroactive compounds (acetylcholine, gamma-amino butyric acid (GABA), glutamate, tryptophan and kynurenine) are found in the CNS (Mercer et al., 1983; Mercer, 1987; Fuchs et al., 1989). Brain content of biogenic amines differs between castes (Brandis et al., 1990), and Fuchs et al. (1989) found that the levels of various neuroactive compounds were different in brains from adult worker honey bees of different ages. In their study, glutamate and GABA levels were found to increase with worker age (up to a point), while serotonin and dopamine did not.

There are several studies that have examined the effects of various amines on honey bee behavior. Mercer and Menzel (1982) found that dopamine and serotonin injected into the brain reduced the percentage of bees responding to a conditioned olfactory stimulus,
while octopamine enhanced the degree of responsiveness. The electric potentials caused by stimulation of the antennae with air or scents are reduced by dopamine, while octopamine increased potentials formed in the α-lobes of the mushroom bodies in response to light (Mercer, 1982). The roles of serotonin, tryptophan and kynurenine in controlling honey bee behavior have been examined in bees (Lopatina and Dolotovskaya, 1984; Lopatina et al., 1985; for a review of the honey bee eye mutants see Tucker, 1986). These studies showed that serotonin and tryptophan depress neural (CNS and peripheral systems) and behavioral activity while kynurenine stimulates CNS activity.
CHAPTER I

'*EFFECTS OF STRESS, AGE, SEASON, AND SOURCE COLONY ON LEVELS OF OCTOPAMINE, DOPAMINE AND SEROTONIN IN THE HONEY BEE

*(Apis mellifera L.) BRAIN

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Introduction

Insect nervous systems contain high levels of octopamine, dopamine and serotonin or 5-hydroxytryptamine (Evans, 1980; 1986). Octopamine has been the most widely studied because of its relationship to the insect "fight or flight" response. It regulates lipid and carbohydrate mobilization (Orchard et al., 1981; 1982; 1983; Downer et al., 1984; Pannabecker and Orchard, 1986). It serves as a neuromodulator of neuromuscular transmission and muscle contraction in insect skeletal muscle (Evans and O'Shea, 1978; Evans, 1981) and controls insect visceral muscles in the gut and ovaries (Orchard and Lange, 1987). Octopamine also regulates flight muscle metabolism (Candy, 1978). It initiates light emission from the firefly light organ (Nathanson, 1979), and mediates some insect behaviors (Brookhart et al., 1988; Mercer and Menzel, 1982).

Levels of octopamine in the insect central nervous system (CNS) also change in response to metamorphic development (Fuzeau-Braesch et al., 1979; Bodnaryk, 1980; Woodring et al., 1988), starvation (Davenport and Evans, 1984a), flight (Goosey and Candy, 1980; Bailey et al., 1984) and handling stress (Downer, 1979; Davenport and Evans, 1984b; Woodring et al., 1988; Woodring et al., 1989).

In honey bees (Apis mellifera L.), large amounts of octopamine, dopamine, serotonin and other neuroactive compounds (acetylcholine, gamma-amino butyric acid, glutamate, tryptophan and kynurenine) are found in the CNS (Mercer et al., 1983; Mercer, 1987; Fuchs et al., 1989). Octopamine affects the behavior and responses of bees to odors (Mercer and Menzel, 1982; Mercer, 1982). Tryptophan and serotonin depress neural (CNS...
and peripheral systems) and behavioral activity while kynurenine stimulates CNS activity in bees (Lopatina and Dolotovskaya, 1984; Lopatina et al., 1985).

The present study represents a preliminary evaluation of environmental and genetic factors that affect levels of biogenic amines in the brains of honey bees. My immediate goal is to demonstrate that handling stress, season, worker age and queen mother influence the levels of biogenic amines found in the cerebral cortex of honey bee workers. Consequently, such factors must be considered when designing experiments examining behavioral relationships to these neuroactive compounds.

Materials and Methods

Biogenic Amine Detection and Quantitation

Octopamine, dopamine and serotonin were separated through a C\textsubscript{18} reverse phase column (4.6 x 100 mm Alltech column with Adsorbosphere packing) and detected simultaneously using two coulometric detectors (ESA Model 5000) in series; the first set to a potential of 300 mV (for dopamine and serotonin) and the second to 700 mV (for octopamine). A running buffer consisting of 3.35 g chloroacetic acid, 6.0 g monobasic sodium phosphate, and 0.5 g sodium dodecyl sulphate dissolved in 720 ml water, 200 ml acetonitrile, and 80 ml methanol (pH 3.00 - 3.10) was run isocratically (1 ml/min).

Biogenic amines were identified and quantified by comparison of peak areas with known standards. We used 3,4-dihydroxy-benzylamine (DHBA) as an internal standard for each sample. Spiking samples with standards and altering the running buffer were methods employed to confirm sample peak identities.
Brains (cerebral ganglia) were removed from bee heads in cold bee saline (0.2 g KCl, 0.2 g CaCl₂, 4.0 g saccharose and 9.0 g NaCl per liter). Only cleanly dissected brains with intact connective membranes were used to avoid loss of biogenic amines from damaged tissue. Only 3-5 minutes was needed to remove a brain from a bee. Brains were placed (3 brains/sample) into 50 μl of 0.4 N perchloric acid in 1.5 ml Eppendorf tubes, sonicated for 15-20 seconds, centrifuged at 10,000 g for 15-20 minutes, and filtered through Amicon YMT membrane filters (at 2,500 g for 15 minutes) to remove large proteins. The samples were then frozen (-20 °C) until needed, and tests indicated no loss of amine content in frozen samples over a 3-4 week period. Just prior to analysis, samples were thawed and the supernatant injected directly onto the HPLC column.

Workers of Different Ages

To reduce variation in worker bees that might be related to different drone fathers, a heterozygous cordovan honey bee queen was instrumentally inseminated with a single, unrelated cordovan drone. One expects 50% of the resulting brood to be phenotypically wild type and 50% to be cordovan. Only the cordovan workers were sampled to avoid sampling wild type workers that may have drifted in the colony from other colonies. No other cordovan source colonies were in close proximity to the colony. Brain samples were collected from newly-emerged cordovan bees (≤ 24 hours old) that had been collected from brood combs in the lab and from randomly-aged cordovan bees that were picked with forceps from the top bars of the colony brood nest on the same day that the young bees had emerged. Bees were cold immobilized prior to removing their brains. The total protein
content of brains from newly-emerged and randomly-aged bees was compared using the Biorad protein assay (Bradford, 1976).

**Seasonal Sampling**

Four sampling periods were used: 26 April - 7 May, 25 June - 9 July, 28 August - 10 September, and 27 October - 4 November. During each period, 15 - 20 samples (3 brains/sample) of non-agitated (see below) bee brains were collected from an observation colony maintained in the lab. All samples were collected during the middle of the day (10:00 am to 3:00 pm).

**Establishment of Different Colonies**

All colonies were established from 1.5 lb. packages of a homogeneous mixture of bees that had been shaken from several source colonies. Each new colony was given a queen that had been inseminated with a single, unrelated drone. Bees were not sampled from the colonies until after the queens had laid several complete brood cycles to ensure that all bees from a single colony were genetically uniform and that none of the unrelated bees that had been used to establish the colonies were included in the samples.

**Collection of Stressed and Non-stressed Bees**

An observation colony containing two standard depth Langstroth wax combs, between 3,500 - 5,000 worker bees and a young, naturally-mated queen was maintained in the laboratory. The colony was supplemented with honey during long periods of dearth to
avoid starving the bees. The colony was connected to the outside by two adjacent pieces of Tygon tubing (inner diameter - 13 mm) through holes cut through a window. Non-stressed bees (0 minutes) were collected by disconnecting a length of the exit tubing as bees were following their footprint pheromone path to the outside exit. The tube could be disconnected, and if handled gently, the bees were not visibly agitated before being anesthetized with CO$_2$ ($<1$ minute exposure).

Stressed bees were collected from the exit tubes by grabbing their legs with forceps, and stress was maintained by pinching and holding their legs with an alligator clamp. Bees were stressed for 0, 3, 10 or 20 minutes. We did not stress them beyond 20 minutes because they showed a reduction in stressed behavior (biting, twisting and sting extrusion).

**Statistical Treatment of the Results**

Multiple comparisons were made using the least significant difference mean separation test (SAS) after significant differences in biogenic amine levels for the stress, season and colony data were indicated by analysis of variance (SAS). The Student's t test was used to compare biogenic amine levels and total protein content of brains between newly-emerged and randomly-aged bees (SAS).

**Results**

**Age-related changes in biogenic amine levels**

Brain levels of octopamine ($P<0.03$), dopamine ($P<0.002$) and serotonin ($P<0.004$) were significantly lower in newly-emerged worker bees ($\leq 24$ hrs) when compared to their
randomly-aged sisters (Fig 1.1). Since the total protein content in brains from workers of both age groups were not significantly different ($P>0.82$), the increased biogenic amine levels in older bees must be attributed to something other than growth of the brain.

**Seasonal variation in biogenic amine levels**

Significant seasonal differences for levels of octopamine ($P<0.0001$), dopamine ($P<0.001$) and serotonin ($P<0.0001$) were found in worker bee brains (Fig 1.2). Amine levels were lowest during the spring (April-May) and fall (October-November) and highest during the summer (June-July and August-September). March-June corresponds to high levels of colony activity in regard to population growth and nectar foraging in Louisiana (Harbo, 1986). Winter bees (December-February) were not sampled.

**Source colony variation in biogenic amine levels**

Levels of octopamine ($P<0.001$), dopamine ($P<0.001$) and serotonin ($P<0.035$) varied significantly between the five colonies (Fig 1.3). Levels of octopamine were directly related to levels of serotonin. For example, the highest octopamine and serotonin levels were seen in colonies E and B, and the lowest levels for both amines were seen in colonies C and D (Fig 1.3). Levels of dopamine seemed to be unrelated to octopamine or serotonin levels (no correlation was found between dopamine and the other two amines).
Figure 1.1. Effects of age on biogenic amine levels in the brains of worker honey bees, *Apis mellifera* L. (left axis). The solid column is octopamine; the open column is dopamine, and the striped column is serotonin. Each amine column represents the average (mean ± SE) of 12-15 samples (3 brains/sample). All three amines were significantly lower in newly emerged bees (P<0.05). Protein content of the brain (right axis) did not change with increased age. Each protein column (dotted bars) represents the average (mean ± SE) for 10 individuals.
Figure 1.2. Effects of season on biogenic amine levels in the brains of worker honey bees, *Apis mellifera* L. The solid column is octopamine; the open column is dopamine, and the striped column is serotonin. Each column represents the average (mean ± SE) of 14-20 samples (3 brains/sample). For each amine, columns with the same letter were not significantly different (α=0.05).
Figure 1.3. Effects of source colony on biogenic amine levels in the brains of worker honey bees, *Apis mellifera* L. Five colonies were sampled (A-E). The solid column is octopamine; the open column is dopamine, and the striped column is serotonin. Each column represents the average (mean ± SE) of 6-12 samples (3 brains/sample). For each amine, columns with the same letter were not significantly different (α=0.05).
Stress-induced changes in biogenic amine levels

Handling stress elevated levels of all three biogenic amines in worker honey bee brains (Fig 1.4). Octopamine levels showed the greatest increase; the levels doubled \((P<0.015)\) after 10 minutes of stress and started to decrease after 20 min. Serotonin levels increased to a lesser, but statistically significant amount \((P<0.001)\) after 10 minutes. The apparent elevation in dopamine was not statistically significant \((P>0.095)\).

Discussion

The results clearly indicate that the levels of biogenic amines in the brains of honey bees are influenced by at least four critical factors. These are the source colony, the season of the year, the age of the bee, and the extent to which the bee is stressed. This baseline information indicates that these factors must be considered in any study of biogenic amines, particularly in studying the role of biogenic amines in insect behavior.

Fuchs et al. (1989) found that the levels of various neuroactive compounds were different in brains from adult worker honey bees of different ages. In their study, glutamate and GABA levels were found to correlate with worker age, while serotonin and dopamine did not. The current study differs in that levels of dopamine and serotonin were found to be different between very young bees and older bees.

An increase in the optic lobe octopamine content of recently emerged Manduca and the pharate form of the noctuid Mamestra configurata is thought related to an increased sensory input and behavioral repertoire of the adult moth (Bodnaryk, 1980; Davenport and Wright, 1986; Klassen and Kammer, 1985). The increased levels of all three biogenic
Figure 1.4. Effects of stress on biogenic amine levels in the brains of worker honey bees, *Apis mellifera* L. The solid column is octopamine; the open column is dopamine, and the striped column is serotonin. Each column represents the average (mean ± SE) of 13-15 samples (3 brains/sample). For octopamine and serotonin, columns with the same letter were not significantly different ($\alpha=0.05$). Dopamine levels did not change significantly with stress ($\alpha=0.05$).
amines in the brains of honey bees during periods of high colony activity might also be related to increased behavioral repertoire associated with foraging activities. Insufficient ages of worker bees were checked to determine a possible relationship between biogenic amines and age-related tasks that are mediated by juvenile hormone (Robinson and Ratnieks, 1987; Robinson et al., 1989), but more experiments are in progress.

Alternatively, the changes in biogenic amine levels of bees at different times of the year might be related to the colony's nutritional state, population size or levels of stress related to a variety of factors. Many other physiological parameters are affected by season. Seasonal changes in fat-body stores and blood sugar content have been noted for both workers and queens (Shehata et al., 1981). Worker survival is related to colony size and brood rearing rate (Harbo, 1986), and brood rearing is directly related to seasonal changes in abundance of nectar and pollen (Harbo, 1986; Newton and Michl, 1974). Juvenile hormone III, thought to control age-related behavioral and physiological changes in bees (Robinson et al., 1989; Fluri et al., 1982), continuously increases in the blood of ageing summer bees but remains low in winter bees (Fluri et al., 1982).

Both the increased level of biogenic amines in older bees and the higher levels in bees during the season of high foraging activity support the hypothesis relating biogenic amines levels to behavior. There are several studies that have examined the effects of various amines on honey bee behavior. Mercer and Menzel (1982) found that dopamine and serotonin injected into the brain reduced the percentage of bees responding to a conditioned olfactory stimulus, while octopamine enhanced the degree of responsiveness. The electric potentials caused by stimulation of the antennæ with air or scents are reduced by dopamine,
while octopamine increased potentials formed in the α-lobes of the mushroom bodies in response to light (Mercer, 1982).

The roles of serotonin, tryptophan and kynurenine in controlling honey bee behavior were examined in bees expressing different chemotypes of the snow group of eye-color mutations of the tryptophan-kynurenine-ommochrome pathway (Lopatina and Dolotovskaya, 1984; Lopatina et al., 1985; for a review of the honey bee eye mutants see Tucker, 1986). These studies showed that serotonin and tryptophan depress neural (CNS and peripheral systems) and behavioral activity while kynurenine stimulates CNS activity in bees.

The variation found in colonies with different genetic backgrounds is potentially the most interesting result of this study. It has been clearly established that the Africanized honey bee is genetically (Page, 1989) and behaviorally (Collins et al., 1982) different from the European honey bee races. Our results present some evidence that at the effector level, the genes responsible for augmented defensive behavior may be mediated via biogenic amines in the brain. The increased octopamine levels in the bee brain in response to stressing the bees lends further support to the idea that "fight or flight" responses in insects are mediated by octopamine (Matthews and Downer, 1974). In several species the octopamine titers in both the blood and brain increase in response to various kinds of stress (Davenport and Evans, 1984a; Downer, 1979; Woodring et al., 1988; Woodring et al., 1989), and it is possible that the CNS of Africanized bees has higher levels of various biogenic amines or responds to them differently than less defensive races of bees.
CHAPTER II

'ELEVATED BRAIN DOPAMINE LEVELS ASSOCIATED WITH OVARY DEVELOPMENT IN WORKER HONEY BEES (Apis mellifera L.) FROM QUEENLESS COLONIES

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Introduction


Changes in behavior often accompany changes in reproductive status of workers. For example, social dominance during food exchange can be correlated with high ovary development (Korst & Velthuis 1982; Moritz & Hillesheim 1985; Velthuis 1985). Also, aggressive acts (biting or mauling) increase between workers having different levels of ovary development (Sakagami 1954, 1958; Velthuis 1976). Sometimes, certain laying workers can become the sole egg-layer (false queen) and receive the court behavior that is normally associated with a queen (Sakagami 1954, 1958; Ruttner et al. 1976; Crewe & Velthuis 1980; Crewe 1982; Saiovici 1983).

The changes in reproductive status or behavior associated with queenless bees may be mediated in the central nervous system by biogenic amines. Biogenic amines have been shown to modulate behaviors and metabolism in many arthropods (Bicker &
In honey bees, studies have shown that levels of octopamine (OA), serotonin (5HT), dopamine (DA) and other neuroactive substances vary with worker age (Fuchs et al. 1989), levels of stress, changes in season, and source colony (Chapter I or Harris & Woodring 1992). Biogenic amines in the brains of honey bees have also been shown to change with morphological development, and they may also be correlated to changes in age-related tasks of worker bees (Taylor et al. 1992). For example, foragers have higher brain DA levels than do nurse bees, and pollen foragers have higher levels of DA than do nectar foragers (Taylor et al. 1992). OA and 5HT exhibit antagonistic effects in modulating conditioned olfactory learning and memory in bees (Mercer 1982, 1987; Mercer & Menzel 1982; Michelsen 1988) and in the directional response component of the visual antennal reflex (Erber et al. 1991). The antagonistic effects of OA and 5HT extend beyond behavioral responses in honey bees. In general, OA tends to enhance responsiveness of honey bee nervous system components (interneurons, motoneurons, and receptor cells) while 5HT decreases responsiveness (reviewed by Erber et al. 1993).

In terms of endocrine control of reproduction, DA has been implicated as a modulator of ootheca formation in Periplaneta americana. Dopaminergic neurons in the pars intercerebralis enhance release of neuropeptides from neurons that act directly on the corpora allata to stimulate synthesis and release of juvenile hormone (JH), which subsequently acts to stimulate vitellogenesis by the fat body and yolk protein uptake by the ovaries (Hentschel 1981).
Juvenile hormone seems to be the key factor in controlling age-related polyethism in worker bees. Low JH titres and biosynthesis are found in young nurse bees while higher titres and biosynthetic rates are found in foragers (Robinson & Ratnieks 1987; Robinson et al. 1989; Robinson et al. 1991). Evidence suggests that JH has an atypical effect on vitellogenesis and ovary development in queens and worker bees (Robinson et al. 1991). Hemolymph vitellogenin levels in young worker bees (< 12 days old) can be positively correlated with JH titres (Rutz et al. 1976), but decreasing levels of hemolymph vitellogenin titres in older worker bees cannot be correlated with increasing JH titres (Rutz et al. 1976). Egg-laying worker bees have low JH titres (Robinson et al. 1991).

The purpose of the current study is to determine what changes in brain biogenic amine levels accompany changes in reproductive status of queenless bees. Correlations of ovary development with changes in biogenic amine content of the brain may provide insight to elucidating the neuro-endocrine control of honey bee reproduction. Alternatively, changes in brain chemistry after ovaries have already begun to develop may be more reflective of changes in terms of behavior related to reproductive competition between workers.

Materials and Methods

Biogenic amine detection and quantitation

OA, DA and 5HT were separated as previously described (Chapter I or Harris & Woodring 1992) through a C18 reverse phase column (4.6 x 100 mm Alltech column with
Adsorbosphere packing) and detected simultaneously using two coulometric detectors (ESA Model 5000) in series; the first set to a potential of 300 mV (for DA and 5HT) and the second to 700 mV (for OA). A running buffer consisting of 3.35 g monochloroacetic acid, 6.0 g monobasic sodium phosphate, and 0.5 g sodium dodecyl sulphate dissolved in 720 ml water, 200 ml acetonitrile, and 80 ml methanol (pH 3.0 - 3.1) was run isocratically (1 ml/min).

Biogenic amines were identified and quantified by comparison of peak areas with known standards. We used 3,4-dihydroxy-benzylamine (DHBA) as an internal standard for each sample. Spiking samples with standards and altering the running buffer were methods employed to confirm peak identities. Analog output from our ESA detector was connected to a computer through an analog-digital converter, and peak areas were quantified using the Shimadzu EZChrom chromatography data system software.

Brains (cerebral ganglia minus the optic lobes) were dry dissected from honey bees that had previously been flash-frozen in liquid nitrogen during sampling. Single brains were placed into 100 µl of running buffer in 1.5 ml Eppendorf tubes, sonicated for 15-20 s, centrifuged at 10,000 g for 15-20 min, and the supernatant was stored at -70°C until HPLC analysis.

**Field colonies with and without queens**

Six small colonies were formed by splitting three heavily populated colonies that contained naturally mated queens. Each small colony was given two combs of capped honey, two combs of capped brood, one empty comb, and approximately 7,000 bees.
Three colonies were given a naturally mated queen, and the other three colonies were kept queenless throughout the experiment. Each colony was given 100 newly-emerged worker bees from an unrelated colony on the third day after all colonies had been formed. These newly-emerged workers were marked with paint on their thoraces, and they were introduced to each colony beneath a wire cage placed in the center of the brood nest. This technique was used to avoid heavy losses caused by mauling attacks from the host bees. The young workers were released from these cages two days after their introduction to each colony. Ten marked workers were randomly sampled from each colony after a total of 30 days for HPLC analysis. Levels (pmol/brain) of OA, DA and 5HT were compared between the two groups of brains using ANOVA for a completely randomized design utilizing sub-sampling (SAS).

Caged bees with and without queens

A. Amines measured on days 6 and 12:

An experiment was used to simultaneously test the effects of absence of the queen and worker age on brain biogenic amine levels. Newly emerged worker bees (≤ 15 hrs) were placed into wooden incubator cages (7.5 x 11 x 12.5 cm³) like those used by Kulincčič & Rothenbuhler (1973) containing a two-sided drone comb (8 x 5 cm²) with or without queens. Fifty bees were put into each of six cages; three of these cages were each given a naturally mated queen while three cages remained queenless. Bees were fed water, pollen and 50% sucrose solution (wt/wt) ad libitum.
The brains of a few bees (n=15) were sampled on the first experimental day (day 0) to serve as a reference point for age-related changes in amine content. The brain from several animals (6-8) was taken from each cage on days 6 and 12 for biogenic amine analysis. Levels of OA, DA and 5HT were compared between these queenless and queenright bees using an analysis of variance with a model containing a component for main treatment effects (TRT), a component for effects of sampling day (DAY), and a component testing the interaction of these two effects (TRT*DAY). Subsampling effects (cage effects) were nested sequentially in the main treatment effects for analysis (SAS).

B. Ovariole width and amines measured after 18 days:

To test the effects of absence of the queen on worker honey bee brain chemistry and ovary development, a cohort of newly-emerged worker bees (≤ 18 hrs) were placed into wooden cages with comb in groups of 100 bees. Each of four cages were given a naturally-mated queen (all queens unrelated) and four cages were given no queen. All cages were kept in the same incubator (temperature = 32°C, RH = 50%) for 18 days. The bees were fed water, pollen and 50% sucrose as in the previous experiment.

Ovary development in workers from the two treatments was examined by sampling 8 workers from each cage on days 6, 12 and 18. The ovaries of each worker were examined, and the width (μm) of the widest ovariole was measured using a calibrated eyepiece micrometer. Ovariole width was compared between queenless and queenright bees using analysis of variance with a model similar to the previous
experiment (main effects = TRT, DAY, TRT*DAY; cage effects were nested in main
treatment effects) (SAS).

Brains were removed and prepared for HPLC analysis from a subsample of 5
bees from the 8 bees that were taken from each cage on day 18 for ovariole
measurements. Levels of OA, DA and 5HT were compared between these queenless
and queenright bees using a hierarchial analysis of variance (cage effects nested in
treatment effects) (SAS). Possible correlations between ovariole width and levels of
each of the three biogenic amines were tested using the Pearson's correlation coefficient
(SAS).

Ovary development and biogenic amines in pairs of caged workers

When queenless worker bees are kept in pairs for a few days in an incubator, they
may develop their ovaries (Harbo & Daniel 1992). To further test correlations between
ovary development and biogenic amines, twenty pairs of newly-emerged worker bees
were kept in cardboard cartons (inner diameter = 8.3 cm; height = 8.0 cm) in an
incubator for 10 days. Each carton had a screen top so that water and 50% sucrose
solution could be delivered to the bees in gravity feeding vials. Each carton also
contained a piece of wax foundation. Pollen was supplied ad libitum in vial caps placed
at the bottom of each cage. Ovariole widths were measured, and brains were removed
from bees in cages in which both bees were alive on the tenth day. Possible correlations
between ovariole width and levels of each of the three biogenic amines were tested using
the Pearson's correlation coefficient (SAS). A paired Student's t test was used to test differences in ovariole width and amine levels between members of all pairs (SAS).

**Results**

**Effect of absence of the queen in field test**

A significant difference in DA levels was found between colonies within treatments ($F=5.93; \text{df}=4,54; P<0.001; \text{Fig 2.1}$). Because colonies within treatments were significantly different, the mean square error term for nested colony effects could not be pooled with the random error term as an estimate of a common variance; hence, main treatment effects could only be tested against nested colony effects [see Bancroft and Han (1983) for rules concerning the pooling of variances]. Nonetheless, brains from queenless bees had significantly more DA than did their sisters that had been with queens ($F=12.43; \text{df}=1,4; P<0.03;$ Fig 2.1).

5HT levels were also found to vary significantly between colonies within the same treatment ($F=3.39; \text{df}=4,54; P<0.02$). Main treatment effects (presence or absence of the queen) were not significantly different ($F=0.15; \text{df}=1,4; P>0.7$) (Fig 2.1). The two groups did not differ in the amounts of OA ($F=0.07; \text{df}=1,58; P>0.75$) (Fig 2.1). Unlike levels of DA and 5HT, all nested variance components could be pooled to test main treatment effects. All stages of brood were found in both the queenless and queenright colonies (indicating the presence of laying workers in the queenless colonies).
Figure 2.1. Effects of absence of the queen on brain biogenic amine levels in worker bees from field colonies. Six colonies containing approximately 7000 bees each were kept for 30 days with (WITH-Q) or without (NO-Q) naturally-mated queens (three colonies per treatment). Each colony was given 100 newly-emerged worker bees (< 15 hr old; each marked with paint on the thorax) from a single source colony on day 3 of the experiment. Ten marked workers were sampled from each colony for biogenic amine analysis on the 30th experimental day. Only dopamine levels were significantly different between bees that were with or without queens ($\alpha = 0.05$). Each bar represents the average (mean ± SE) for 30 worker bees.
Effects of worker age and absence of the queen in cage experiments

A. Amine levels on days 6 and 12:

As a reference for age-related changes in biogenic amines, amines were measured in newly-emerged worker bees (n=15) that were aged less than 24 hours. Octopamine levels in these young bees were below our detection limits and could not be quantified. The brains (cerebral ganglia minus optic lobes) of the newly-emerged bees averaged (mean ± SE) 1.26 ± 0.12 pmoles dopamine and 0.42 ± 0.07 pmoles serotonin.

There were no significant differences in OA, DA and 5HT levels between worker bee brains in the two treatment groups on either day 6 or day 12 (Fig 2.2). For OA levels, nested cage effects were not significant (F=2.05; df=4,38; P>0.10); however, the mean square error term still could not be pooled with the mean square error for individual bee effects to test main treatment effects [Bancroft & Han (1983) recommend an α=0.40]. Main treatment effects (F=0.06; df=1,4; P>0.8), effects of sampling day (F=0.01; df=1,38; P>0.9), and the treatment-by-sampling day interaction (F=1.83; df=1,38; P>0.15) did not significantly influence OA levels.

As with OA levels, cage effects did not significantly influence DA (F=2.54; df=4,38; P>0.05) or 5HT (F=1.74; df=4,38; P>0.15) levels in the brains of worker bees; however, for the same reason as above, mean square error terms for cage and individual bee variances could not be pooled for either amine. There was no significant difference in DA (F=0.28; df=1,4; P>0.6) or 5HT (F=0.15; df=1,4; P>0.7) levels between workers with or without queens (Fig 2.2). DA (F=7.27; df=1,38; P<0.02) and 5HT (F=4.27; df=1,38; P<0.046) levels were significantly different between day 6 and day 12;
Figure 2.2. Changes in brain levels of biogenic amines from worker bees caged with (open bars) or without (shaded bars) queens through 12 days. Fifty newly-emerged workers were placed into each of six cages; three cages were given an egg-laying queen while the other three remained queenless. Subsamples (6-8 bees) were taken from each cage on days 6 and 12 for HPLC measurement of biogenic amine levels. Amines did not significantly differ between bees that were with or without queens, but dopamine and serotonin levels were significantly elevated from day 6 to day 12. As a reference for age-related changes in biogenic amines, amines were measured in newly-emerged worker bees (n = 15) that were aged less than 24 hr. Octopamine levels were below our detection limits and could not be quantitated. These brains (cerebral ganglia minus optic lobes) averaged (mean ± SE) 1.26 ± 0.12 pmoles dopamine and 0.42 ± 0.07 pmoles serotonin.
however, the treatment-by-sampling day interaction was not significant for either DA 
\((F=1.26; df=1.38; P>0.25)\) or 5HT \((t=0.8; df=1.38; P>0.35)\).

**B. Ovariole width and amines after 18 days:**

Eggs were found in all cages after 14 days; indicating that laying workers were present in the four queenless cages. Ovariole widths of queenless workers were found to significantly increase with time, while the ovariole widths in workers from cages with queens remained quite small (Fig 2.3).

All nested sampling factors could be pooled with the random error term to test the main effects. Both main factors - presence or absence of the queen \((F=49.9; df=1.186; P<0.001)\) and sampling day \((F=14.56; df=2.186; P<0.001)\) - were significant causes of variation in ovariole width. The interaction of these two variables was also a significant source of variation \((F=13.11; df=2.186; P<0.001)\).

In the day 18 queenless workers, ovariole width and brain DA levels were moderately correlated (Pearson's correlation coefficient = 0.45985; \(P=0.0413; n=20\)). Ovariole width did not correlate with either OA (Pearson's correlation coefficient = 0.28221; \(P=0.2280; n=20\)) or 5HT (Pearson's correlation coefficient = 0.32001; \(P=0.1690; n=20\)).

DA levels were significantly higher in queenless bees than in bees from cages with the queen \((F=9.12; df=1.38; P<0.005)\) on day 18 (Fig 2.4); and cage effects were not significant \((F=0.71; df=6.32; P>0.6)\). 5HT levels were not different between the two groups \((F=0.90; df=1.38; P>0.3)\) (Fig 2.4), and cage effects were again insignificant.
Figure 2.3. Effect of absence of the queen on ovariole width in worker bees kept in groups of 100 bees in incubator cages. Four cages were given naturally-mated queens, while four other cages remained queenless through 18 days. The width of the largest ovariole was measured for each worker in a subsample of eight workers per cage on days 6, 12 and 18. Only queenless workers developed their ovaries. Note: although the ovaries of queenless bees were developed, none of the workers sampled contained developed eggs in the oviduct (fully developed eggs are found in ovarioles measuring 380-500 μm). Each bar represents the average (mean ± SE) for 32 worker bees.
Figure 2.4. A comparison of the brain biogenic amine content after 18 days between caged queenless workers (NO-Q) and their sisters that had shared cages with queens (WITH-Q). These bees represent a subsample (five bees per cage) from the bees used for ovariole measurements in Fig. 2.3. Only dopamine levels were significantly ($\alpha = 0.05$) different between the two types of worker bees. Each bar represents the average (mean ± SE) for 20 worker bees.
(F=0.37; df=6, 32; P>0.8). OA levels were also not different between the two groups (F=0.02; df=1, 38; P>0.8), and cage effects were insignificant (F=0.73; df=6, 32; P>0.6) (Fig 2.4).

Ovary development and biogenic amines in pairs of queenless bees

Only 12 pairs of the original twenty survived through ten days. Both workers died in each of three cages, and the remaining five cages each lost one bee. The 24 bees were used in the analysis.

As in the previous experiment, ovariole width correlated with brain DA levels (Pearson's correlation coefficient = 0.54595; P=0.0058; n=24). Unlike the previous experiment, ovariole width also correlated with brain 5HT levels (Pearson's correlation coefficient = 0.40682; P=0.0485; n=24). Ovariole width did not correlate with OA levels (Pearson's correlation coefficient = 0.23294; P=0.2733; n=24).

The mean differences in ovariole width (t=2.919; df=11; P<0.015) and DA levels (t=3.703; df=11; P=0.005) (Fig 2.5) between bees within pairs were significantly greater than zero. Differences in OA (t=0.846; df=11; P>0.4) and 5HT (t=0.083; df=11; P>0.9) levels between bees of each pair were not significantly different from zero (Fig 2.5).

Discussion

Of the three biogenic amines examined, only DA levels seemed to be consistently related to the queenless condition of worker honey bees. In both a 30-day field experiment and an 18-day cage experiment, DA levels were significantly elevated in the
Figure 2.5. Comparison of brain biogenic amine levels (mean ± SE) between worker bees from caged pairs. Each pair of newly-emerged bees was caged through 10 days in an incubator. The bee having the largest ovariole width from each pair is designated Bee A (mean ovariole width = 132.7 ± 26.3 µm), and the other worker is Bee B (mean ovariole width = 63.9 ± 6.7 µm). The difference in ovariole width between bees in pairs was significant (α = 0.05). Of the three biogenic amines tested, only dopamine levels were significantly different (α = 0.05) between bees in a pair. Dopamine and serotonin levels were correlated with ovariole width in this experiment.
brains of queenless bees versus their sisters that had been kept with queens. Additionally, DA levels were correlated with ovariole width measurements from groups of bees caged through 18 days or from pairs of bees caged for 10 days. For the latter experiment, members of 12 pairs of bees were found to have significantly different ovariole widths and DA levels, and both DA and 5HT correlated to ovariole width. 5HT did not correlate with ovariole width in other experiments in this paper.

The elevation in DA may not be directly related to initiation of oogenesis because our measurements of biogenic amines might have been made after the ovarioles had begun to develop eggs. Ruttner & Hesse (1981) found that queenless honey bees could initiate oviposition within 10-14 days of removal of the queen and brood, substantially sooner than the days that amines were measured in the previous experiments (day 18 and 30). Nonetheless, some strains of honey bees need a substantially longer time to lay eggs (as long as 25 days; Harris and Harbo 1991). Our experiments with younger workers (6 and 12 days old) indicated that biogenic amine levels in queenless workers were not significantly different from those in their sisters sharing cages with queens. This result indicates that the increase in DA is probably not the cause of oogenesis if one considers that the ovaries of queenless workers in a similar experiment began to develop within 6-12 days.

Hence, our results indicated that DA levels were changing in queenless bees after the onset of oogenesis, but that DA levels could still be correlated with ovariole width. This result suggests that some aspect of the worker bee that is developing her ovaries increases brain DA levels. Any hypothesis at this point is conjectural at best, but
potential explanations include (1) elevations of DA as a consequence of the stress that might occur in bees that are mauled by competitors, (2) differences in DA levels caused by differences in the nutritional content of food (either quantity or quality) that might be related to trophallactic exchanges between workers having different levels of ovary development, or (3) elevations of DA related to physiological processes triggered by the onset of oogenesis. Harris and Woodring (1991) showed that handling stress could elevate biogenic amines in honey bees, and there is some evidence that bees having high levels of ovary development are mauled in queenless groups (Sakagami 1954, 1958). However, Harris and Woodring (1991) showed that all three amines were elevated in bees that had experienced handling stress, and in this experiment only dopamine seemed affected. In terms of nutrition and trophallaxis, researchers (Korst & Velthuis 1982; Moritz & Hillesheim 1985; Velthuis 1985) have shown workers having different levels of ovary development interact in trophallactic exchanges differently. Bees that specialize as receivers will often have more highly developed ovaries than bees that specialize as givers. Physiological explanations of elevations of DA might involve some sort of feedback mechanism acting on the central nervous system after oogenesis has been initiated. For example, Hentschel (1981) indicated that ecdysone produced by the ovary during vitellogenesis in *Periplaneta americana* L. could increase dopaminergic activity of the pars intercerebralis, which in turn could increase the release of juvenile hormone - the gonadotropic hormone that stimulates vitellogenesis by the fat body and yolk protein incorporation by the ovary in this roach.
CHAPTER III

EFFECTS OF CARBON DIOXIDE ON BRAIN BIOGENIC AMINES IN QUEENS AND WORKER BEES (*Apis mellifera* L.)
Introduction

Although CO₂ has been shown to greatly influence insect behavior and physiology, insects are frequently immobilized with CO₂ during routine studies by biologists. Treatment with CO₂ has been shown to affect insect reproduction (Engels et al. 1976), development (Woodring et al. 1978), feeding (Birkenmeyer & Dame 1970; Woodring et al. 1978) and other behavior (Ralph 1959; Whisenant & Brady 1965; Mardan & Rinderer 1980; Schneider & Gary 1984). Woodring et al. (1978) showed that in crickets (Acheta domesticus) the effects of short-term exposures to CO₂ are associated with a true anesthetic effect on nervous tissue, while longer exposures cause asphyxiation that leads to anoxia of tissues. The anesthetic effect results from the direct diffusion of CO₂ gas to nerve cells followed by a reduction in neuroplasmic pH resulting from the conversion of CO₂ to carbonic acid (Edwards & Patton 1965) and not by the drop in hemolymph pH associated with exposure to CO₂ (Woodring et al. 1978).

In honey bees (Apis mellifera L.), exposure to CO₂ is frequently used during the instrumental insemination of queens (Harbo 1986). Exposure to CO₂ increases vitellogenin titres in the hemolymph and accelerates ovarian development and eventual oviposition in queens (Engels et al. 1976; Engels & Ramamurty 1976). In unmated queens aged 5-20 days old, the rate of vitellogenin synthesis rarely exceeds 30% of total protein synthesis; however, within 24 hours of two exposures to CO₂ and introduction into a colony, the vitellogenin synthesis rate increases to 60% of all protein synthesis (Engels et al. 1976). Virgin queens and instrumentally inseminated queens not given
CO₂ require 30-50 days to begin egg laying. Queens given two exposures to CO₂ and introduced into colonies begin egg laying within 5-10 days (Mackensen 1947).

In worker honey bees, exposure to CO₂ inhibits ovarian development. Fyg (1950) found that 67% of the worker bees in queenless cages developed ovaries after 4 weeks, but if they were given a single exposure or a series of three exposures to CO₂, only 5% of the workers had developed ovaries. Biedermann (1964) and Kropacova et al. (1968) also found that exposure to CO₂ reduces ovary development in workers, and that workers receiving CO₂ eat less pollen than untreated workers (see also Harris & Harbo 1990). Kropacova et al. (1968) found that groups of queenless bees allowed to eat pollen for a 3 day period prior to exposure to CO₂ will begin developing ovaries, but after narcosis, ovary development slows or stops. In contrast, Harris and Harbo (1990) found that a single 15 minute exposure to CO₂ was effective at inhibiting ovary development only in bees that have access to pollen if the narcosis was given during the first three days. If workers are not given pollen until after narcosis, ovarian development remains low.

Although the effects of CO₂ on the reproductive systems of queen and worker honey bees are well known, the mechanism leading to these effects is unknown. These effects on reproduction are, at least initially, most probably mediated by changes in the nervous system triggered by exposure to CO₂. As an example of neuro-endocrine functions affected by narcosis, exposure to CO₂ increases the volume of the corpora allata and the titre of hemolymph juvenile hormone (JH) in worker honey bees (Bühler et al. 1983). Unlike many orthopteran insects in which JH directly stimulates vitellogenesis.
and oogenesis (Hentschel 1981), JH has an atypical, indirect role in reproduction in worker and queen honey bees (Robinson et al. 1991) but has a pronounced role in the control of age-related behavioral patterns in worker bees (Robinson et al. 1989).

Other mechanisms to explain the effects of CO$_2$ on honey bee behavior and reproduction might initially involve changes in biogenic amine neurotransmitters and neuromodulators in the brain. Harris and Woodring (1995) showed that brain dopamine (DA) levels are higher in queenless bees than in their sisters kept with queens, and that brain DA levels were correlated with ovariole width in queenless worker bees. The purpose of the current study is to test the effects of exposure to CO$_2$ on brain levels of octopamine (OA), dopamine (DA) and serotonin (5HT) and some of their precursors [tryptophan (TRP) and tyrosine (TYR)] in worker and queen bees. Because brain DA levels have been related to ovarian development in worker bees, CO$_2$ narcosis could alter levels of DA and/or other neuroactive compounds.

**Materials and Methods**

**Biogenic amine detection and quantification (HPLC analysis)**

OA, DA, 5HT, TRP and TYR were separated as previously described (Harris & Woodring 1991) through a C$_{18}$ reverse phase column (4.6 x 100 mm Alltech column with Adsorbosphere matrix) and detected simultaneously using two coulometric detectors (ESA Model 5000) in series; the first set to a potential of 300 mV (for DA and 5HT) and the second to 700 mV (for OA, TRP and TYR). A running buffer consisting of 3.35 g monochloroacetic acid, 6.0 g monobasic sodium phosphate, and 0.5 g sodium dodecyl
sulphate dissolved in 720 ml water, 200 ml acetonitrile, and 80 ml methanol (pH 3.0 - 3.1) was run with a constant flow rate (1 ml/min).

Biogenic amines were identified and quantified by comparison of peak areas with known standards. We used 3,4-dihydroxy-benzylamine (DHBA) as an internal standard for each sample. Spiking samples with standards and altering the running buffer were methods employed to confirm peak identities. Analog output from our ESA detector was connected to a computer through an analog-digital converter, and peak areas were quantified using the Shimadzu EZChrom chromatography data system software.

Brains (cerebral ganglia minus the optic lobes) were dry dissected from honey bees that had previously been flash-frozen in liquid nitrogen during sampling. Single brains were placed into 100 μl of running buffer in 1.5 ml Eppendorf tubes, sonicated for 15-20 s, centrifuged at 10,000 g for 15-20 min, and the supernatant was stored at -70°C until HPLC analysis.

**CO₂ treatment of queenless worker bees given pollen**

Fifty newly-emerged bees (≤ 15 hours old) were placed into each of 15 wooden cages (7.5 x 11 x 12.5 cm³) similar to those used by Kulincević and Rothenbuhler (1973) during early August 1993. The bees used were the progeny of a naturally-mated queen. Each cage was supplied with 50% sucrose solution and water in gravity feed vials. Pollen was available *ad libitum* in small plastic trays on the floor of each cage. The pollen fed to the bees had been collected two months prior to this experiment (and frozen until needed) from several field colonies. Five cages were randomly assigned to
each of three treatments: (1) controls received no narcosis, (2) cages exposed for 10 minutes to CO₂ narcosis, and (3) cages exposed for 10 minutes to a mixture of CO₂ containing 20% O₂. All cages were kept in an incubator held at 31 ± 0.5°C and 65% RH throughout the experiment.

All narcoses were applied on the third day of the experiment. Each test gas (CO₂ or CO₂/O₂) was administered at room temperature (ca 23°C) by placing the cages in a clear plastic bag and quickly filling the bag with test gas. Timing of the 10 minute exposure began when the first bees fell to the bottom of their cages. After the bag was fully expanded, a flow rate of 50 mL/minute was maintained to keep positive pressure within the bag during the exposure.

Brains were removed for HPLC analysis of biogenic amine content from a subsample of 3 bees from each each cage on the eighth day of the experiment. A hierarchial analysis of variance for a completely randomized design was used to compare amine levels between the three treatment groups. I tested for potential effects of individual cages on variation in amine content within the main treatment effects. Following the rules for pooling variances given by Bancroft & Han (1983), cage effects were pooled with the random error term to estimate a common variance when appropriate, and the main treatment effects were re-tested with more power (SAS). The Tukey's HSD was used for mean separations after significant differences (α=0.05) in amounts of an amine were found between treatments (SAS).
**CO₂ treatment of queenless worker bees kept without pollen**

Each of twenty-four wooden cages (same as in the previous experiment) were given 50 newly-emerged (≤ 15 hours old) bees and supplied water and 50% sucrose *ad libitum* during the middle of September 1993. The bees used in this experiment were from the same colony as used in the previous experiment. The cages were given no pollen throughout the experiment. Twelve cages were treated to a single 10 minute exposure to CO₂ on the third day, while the remaining 12 cages were not treated. Cages were held in an incubator for 4, 6 or 8 days. On each sampling day, the brains from 3 bees were sampled for HPLC analysis from each of 4 control cages and 4 cages that had been treated with CO₂. Levels of OA, DA, 5HT, TYR and TRP were compared between the two different treatments and the three sampling days using an ANOVA that included a main treatment term (TRT), a sampling day term (DAY) and an interaction term (TRT*DAY) (SAS).

**CO₂ treatment of queen bees that were free-running or caged**

During June, 1994, virgin queens of the same age were obtained by grafting larvae from two different lines or stocks of bees maintained at the USDA-ARS Honey Bee Breeding facility in Baton Rouge, LA. A single queenless colony was used to rear the queen cells from both stocks, and the capped cells were removed to individual vials stored in an incubator. A 2x2x2 factorial arrangement of treatments was used in a completely randomized designed to test the effects of (1) CO₂-treatment versus untreated controls, (2) differences between the two stocks, and (3) the effects of caging.
queens versus allowing them to run freely within small colonies on brain biogenic amine content. The statistical analysis included consideration of all possible main factors and interaction terms (SAS).

A total of 23 queens [11 from one stock (A) and 12 from the other stock (B)] were treated with a 15 minute CO$_2$ narcosis on the 11th and 12th days after emergence from the pupal case, and 11 of these treated queens were each placed in a small cage (4 from stock A and 7 from stock B), and all caged queens were stored in a single, queenless colony. Each of the remaining 12 treated queens was introduced into a small colony as a pupa, and after emergence from the pupal case, each queen was captured to be marked and treated with CO$_2$. All colonies were started from a homogeneous mixture of bees shaken from several field colonies on June 3, 1994. Each small colony was provided two frames of capped honey, one frame of capped brood and about 6,000 bees. An additional 21 queens (10 queens from stock A and 11 from stock B) were not treated with CO$_2$. Of these, 12 queens (4 from stock A and 8 from stock B) were caged and placed in the same colony used for the CO$_2$-treated and caged queens. The remaining 9 queens (6 from stock A and 3 from stock B) were introduced into small colonies as queen cells, and they were captured and marked in the same manner as the CO$_2$-treated, free-running queens. The colonies used were from the same mixture of bees used for the CO$_2$-treated queens. All colonies were monitored for the appearance of eggs through a total of 23 days when the brains from all queens were removed for HPLC analysis.
CO₂ treatment of worker bees kept with and without queens

During late April - early May, 1995, an experiment was conducted to test the effects of CO₂ narcosis on the brain biogenic amine content and ovarian development of worker bees kept with or without naturally-mated queens in incubator cages. Fifty newly-emerged bees (≤ 15 hr) from a single source colony containing a naturally-mated queen were placed into each of 12 incubator cages. Each cage was provided a small piece of wax foundation fastened to the wall of the cage, and 50% sucrose and water were provided by gravity feed vials. Six cages were given young naturally-mated queens (all queens were sisters) and the remaining six cages were kept queenless. Within each set of these, 3 cages were treated with a 15 minute exposure to CO₂ (as in the first experiment) on the first day while the remaining 3 cages were not treated. The queens were not treated with CO₂ and were introduced after the workers began to recover from the narcosis. Cages were held in an incubator (at 31 ± 0.5°C and 65% RH) through 10 days.

Ten days after narcosis 4 worker bees were sampled from each cage for measurements of brain biogenic amine content and the extent of ovarian development. Brains from the workers were prepared for HPLC analysis before the ovaries were removed. Both the left and right ovaries were classified using a grading scale previously described by Harris and Harbo (1990). Level 1 ovaries were those with the most undeveloped ovarioles; level 2 were those with rounded to bean-shaped eggs; level 3 ovaries were those with more elongated, sausage-shaped eggs. The sum of the grading levels for the right and left ovaries of each bee was used in the statistical analysis of
effects of CO₂ and the presence or absence of a queen on worker ovary development.

Ovarian development and biogenic amine levels were then compared between treatment groups using an analysis of variance that incorporated the 2x2 factorial arrangement of treatment levels with the effects of individual cages nested in the main treatments (SAS).

Results

Effects of CO₂ narcosis on worker bees given pollen

Exposure to CO₂ significantly affected brain levels of two of the three amines measured (Figs 3.1). OA levels were not significantly different between the three treatments (F=1.93; df=2,12; P>0.18), but DA levels (F=5.87; df=2,12; P<0.02) were significantly lower and 5HT levels (F=10.81; df=2,12; P<0.01) were significantly higher in either CO₂ or CO₂/O₂ treated bees versus untreated bees (Fig. 3.1). Levels of both amine precursors, TRP (F=4.58; df=2,12; P<0.04) and TYR (F=23.37; df=2,12; P<0.001), were significantly lower in either CO₂ or CO₂/O₂ treated bees versus untreated bees (Fig. 3.2). Although TRP levels were found to be significantly lower by ANOVA, the Tukey's mean separations test could not differentiate the three treatment means at an α=0.05 confidence level.

Nested cage effects were not significant for OA (F=1.61; df=12,30; P>0.14) or TYR (F=1.38; df=12,30; P>0.2), but according to Bancroft & Han (1983), this nested factor could not be pooled with the random error term for testing main treatment effects. Nested cage effects were not significant for DA (F=1.08; df=12,30; P>0.40), TRP (F=0.64; df=12,30; P>0.7) or 5HT (F=0.41; df=12,30; P>0.9) levels; however, because
Figure 3.1 - Effects of CO₂ narcosis on brain biogenic amine levels in worker bees fed pollen. Fifty newly-emerged bees were placed into each of 15 incubator cages, and each cage was given either [1] no CO₂ (gray bars), [2] a 10 min exposure to CO₂ (open bars) or [3] a 10 min exposure to a mixture of CO₂ and 20% O₂ (striped bars) (5 cages per treatment). The CO₂ treatments were given on the third day of the experiment, and brains were sampled on the eighth day of the experiment. Three bees were sampled from each cage for amine analysis. Octopamine levels were not significantly different between treatments (α=0.05). For serotonin and dopamine levels, treatment mean (± SE) bars having the same letter do not differ as determined by the Tukey's mean separations test.
Figure 3.2 - Effects of CO₂ narcosis on the biogenic amine precursors tryptophan and tyrosine from the brains of bees that were fed pollen. Controls = gray bars; CO₂-treated bees = open bars and CO₂/O₂-treated bees = striped bars (see the Fig 3.1 caption for experimental details). Mean tryptophane levels for the different treatments could not be separated with Tukey's mean separations test. For tyrosine, treatment mean (± SE) bars with the same letter were not significantly different (α=0.05).
cages were a significant source of variation for OA and TYR, the nested experimental error term was not pooled with the random error term to test the main effects.

**Effects of CO2 narcosis on worker bees kept without pollen**

There were no significant differences in levels of OA (F=0.83; df=1,18; P>0.3), DA (F=2.80; df=1,18; P>0.10), 5HT (F=0.61; df=1,18; P>0.4), (Fig. 3.3) or TRP (F<0.01; df=1,18; P>0.95) (Fig. 3.4) in the brains of CO2-treated versus untreated worker bees. However, TYR levels (F=7.18; df=1,18; P<0.02) were significantly reduced by narcosis on day 4 (Fig. 3.4).

Worker age (DAY term) significantly influenced levels of some of the compounds that were measured. OA levels (F=12.15; df=2,18; P<0.001) and 5HT levels (F=4.66; df=2,18; P<0.03) were significantly elevated from day 6 to day 8 (Fig. 3.3). TRP (F=76.18; df=2,18; P<0.001) levels and TYR (F=11.36; df=2,18; P<0.001) levels were significantly reduced through time (Fig. 3.4). The TRT*DAY interaction terms were not significant causes of variation for levels of OA (F=2.87; df=2,18; P>0.08) and 5HT (F=0.59; df=2,18; P>0.5) or TRP (F=0.17; df=2,18; P>0.8) and TYR (F=0.93; df=2,18; P>0.4) levels.

Worker age did not affect brain levels of DA (F=0.85; df=2,18; P>0.4) (Fig. 3.3). As with the previous compounds, the TRT*DAY interaction term was not a significant source of variation for levels of DA (F=0.27; df=2,18; P>0.7).
Figure 3.3 - Effects of CO₂ narcosis on brain biogenic amines in caged bees not fed pollen. Twenty-four cages were given 50 newly-emerged bees, and on the third day of the experiment 12 cages were exposed for 10 min to CO₂ (gray bars) and the remaining 12 cages were untreated (open bars). Cages were held in the incubator for an additional 1, 3 or 5 days after treatment (for a total of 4, 6 and 8 days - respectively). Three bees were sampled for biogenic amine analysis from 4 control cages and 4 CO₂-treated cages on each sampling day. Cages were sampled only once. There were no significant differences between treatment groups for any of the three compounds measured. Octopamine levels were significantly elevated between day 6 and day 8.
Figure 3.4 - Effects of CO₂ narcosis on the precursors tryptophane and tyrosine from the brains of bees not fed pollen. See the Fig 3.3 caption for experimental details. Absence of pollen led to significant reductions in levels of tryptophane and tyrosine in both control (open bars) and CO₂-treated bees (gray bars). Only tyrosine levels were significantly different between the two treatments on day 4. Treatment means (± SE) having the same letter do not differ significantly (α=0.05) as determined by Tukey's mean separations test.
Effects of CO$_2$ treatment on caged and free-running virgin queens from two stocks

All CO$_2$-treated queens that were kept in small colonies produced eggs between days 19-23 of the experiment (n=12). None of the untreated queens that were kept in small colonies produced eggs during this period (n=9). Egg production was not determined for caged queens.

Treatment with CO$_2$ did not have a significant effect on levels of OA (F=0.03; df=1,36; P>0.85), DA (F=3.10; df=1,39; P>0.09) or 5HT (F=2.36; df=1,39; P>0.13) (Fig. 3.5). The effects of caging queens (versus free-running conditions) were not significant for OA (F=1.99; df=1,36; P>0.15) and DA (F=0.04; df=1,39; P>0.8), but 5HT (F=9.56; df=1,39; P<0.004) levels were significantly elevated in caged queens (Fig. 3.5). The differences between the two stocks were not significant sources of variations for OA (F=0.12; df=1,36; P>0.7), DA (F=1.56; df=1,39; P>0.2) or 5HT (F=2.79; df=1,39; P>0.10). None of the two-factor interaction terms (treatment by stock; treatment by caging condition; and stock by caging condition) and the three-factor interaction term (treatment by stock by caging condition) were significant causes of variation for OA and 5HT. For DA, the treatment by caging condition term was the only significant source of variation (F=4.47; df=1,39; P<0.04). This result indicates that the free-running and caged queens were affected by CO$_2$ treatment to different extents (Fig 3.5): for free-running queens, treatment with CO$_2$ reduced DA levels by 45% while treatment with CO$_2$ in caged queens did not reduce DA levels (Fig 3.5).
Figure 3.5 - Effects of CO₂ narcosis on virgin queen honey bees that were caged or kept free-running in small colonies. Treated queens (23 queens from two stocks) were given a 10 minute CO₂ narcosis on each of the first two days of the experiment, while control queens (21 queens from the same two stocks) were not given CO₂. Queens from both groups were then either banked (in a single large colony) or placed individually into small colonies fitted with queen excluders at the entrance to prevent mating flights (12 control queens and 11 treated queens were banked; 9 control queens and 12 treated queens were maintained in colonies). The brains from all queens were removed on the day 23 of the experiment for biogenic amine analysis. Octopamine levels were not affected by CO₂ treatment or banking. Serotonin levels were significantly elevated by banking. Dopamine levels were not significantly affected by CO₂ or banking, but reductions in dopamine levels were more pronounced in free-running queens than in banked queens. Striped bars = free-running, untreated queens; gray bars = banked, untreated queens; dark bars = free-running queens given CO₂; and the open bars = banked queens given CO₂.
Effects of CO₂ treatment on biogenic amines and ovarian development in worker bees caged with and without queens

Eggs were found on the ninth day of the experiment in all three cages of bees that were not given CO₂ and contained no queens. None of the cages containing bees that were given a CO₂ treatment and contained no queens had eggs. All six cages that contained queens had eggs throughout the experiment.

Significant differences in extent of ovarian development were found for both main factors: (1) exposure or no exposure to CO₂ (F=75.00; df=1,8; P<0.001) and (2) presence or absence of a queen (F=75.00; df=1,8; P<0.001). The ovaries of worker bees remained undeveloped after 10 days in all cages containing queens or in cages that had been treated with CO₂. Only workers from untreated cages without queens had developed ovaries. The average (mean ± SE) sum of the right and left ovary scores for these bees was 3.25 ± 0.328 (n=12). A worker bee with both ovaries undeveloped would have the sum of 2.00.

Of the three compounds measured from the worker bee brains, OA (F=0.81; df=1,8; P>0.30) and 5HT (F=0.00; df=1,8; P>0.95) levels were not significantly affected by CO₂ treatment. Only DA levels were significantly affected by CO₂ treatment (F=20.48; df=1,8; P<0.002) (Fig 3.6). The presence or absence of a queen did not significantly affect OA (F=0.21; df=1,8; P>0.65), DA (F=4.97; df=1,8; P>0.05) or 5HT (F=0.77; df=1,8; P>0.40) levels. The CO₂ treatment by presence or absence of queen interaction term was not a significant source of variation for OA (F=1.16; df=1,8;
Figure 3.6 - Effects of CO₂ treatment on worker honey bees caged with and without queens. Twelve cages were kept in an incubator through 10 days when the brains from 4 bees per cage were sampled for amine content (6 cages each with a queen; 6 cages without a queen). Within both groups of bees, three cages were given a single 15 minute CO₂ narcosis on the first day while the remaining three cages were not treated. Only dopamine was significantly affected by CO₂ treatment. Each bar represents the mean (± SE) for 9-12 bees. Striped bars = bees with no queen and no CO₂; dark bars = bees with a queen and no CO₂; open bars = bees without a queen but given CO₂; gray bars = bees with a queen and given CO₂.
P > 0.31) and DA (F = 0.43; df = 1, 8; P > 0.50) levels. This interaction term was a significant source of variation for serotonin levels (F = 9.28; df = 1, 8; P < 0.02).

Discussion

A 10-15 minute exposure to CO₂ or CO₂ containing 20% O₂ led to significant reductions in levels of TRP, TYR and DA in worker honey bees given pollen but had no effect in workers kept without pollen. The present work also showed an age-related reduction in TRP and TYR in worker bees that were not fed pollen. Untreated and CO₂-treated bees with no available pollen had only 5-7 pmoles TRP and 18-20 pmoles TYR in their brains after eight days, considerably lower than the 25-30 pmoles TRP and the 27-30 pmoles TYR found on the fourth day. In bees that were continuously fed pollen, levels of TRP and TYR (although significantly reduced by CO₂) remained high after eight days (30 pmoles).

The reduction in brain TRP and TYR levels in pollen deprived bees is not surprising since many essential amino acids and vitamins are supplied to bees in their pollen (Groot 1953; Haydak & Dietz 1972; Herbert & Shimanuki 1977). However, one might expect a concomitant decrease in 5HT and the catecholamine (OA and DA) levels because TRP and TYR are the dietary precursors to these neurotransmitters. The levels of OA, DA and 5HT levels did not significantly decrease in the absence of pollen. The ability of the nervous system to maintain levels of these neuroactive substances without dietary intake of their precursors seems remarkable, but it is clear that treatment with
CO₂ can cause dramatic changes in levels of important neuroactive substances in the brains of worker bees.

Unlike worker bees, treatment with CO₂ had no significant effect on brain DA levels in queens (TRP and TYR were not examined in the queens). However, CO₂ treatment reduced DA levels more in free-running queens than in queens that were caged and stored together in a single colony. This trend in free-running queens was similar to the effects of CO₂ on brain DA levels in worker bees. These results do indicate that the nervous systems of workers and queens respond to CO₂ treatment differently. Also, the brains of queens had 3-5 times higher levels of DA than those of workers. Similar results were previously reported (Brandes et al. 1990). Although 5HT levels were statistically higher in caged queens than in queens that were free-running in small colonies, the differences were not great.

The current study also showed that brain DA levels in worker bees were reduced by treatment with CO₂, whether the bees were caged with or without queens. Ovarian development in worker bees was reduced either by treatment with CO₂ or by the presence of a queen. The presence of a queen did not significantly reduce brain DA levels in worker bees. At best these results are only correlative, but they suggest a positive relationship between brain DA and reproductive processes in worker bees. How changes in DA levels are related to reproduction remains unclear. However, a similar study by us found that brain DA levels in workers were elevated with increased ovarian development, and the elevated DA levels were correlated to ovariole width (Harris & Woodring 1995). If brain levels of DA are involved in the stimulation of worker honey
bee reproduction, reductions in DA levels caused by exposure to CO₂ may partially explain reductions in ovary development associated with narcosis (Harris & Harbo 1990).

Exposure to CO₂ had no effect on brain OA levels in any of the experiments with workers or queens. Effects of CO₂ treatment on brain 5HT levels were mixed. In one experiment with worker bees 5HT levels were elevated by CO₂ treatment, but in all other experiments levels of 5HT were unaffected. Levels of 5HT in queen bee brains were also unaffected by CO₂ treatment.

The reduction in TRP and elevation of 5HT might retard worker ovarian development independent of changes in DA levels. Because 5HT has been shown to decrease or inhibit many behaviors and metabolic processes in honey bees (Erber et al. 1993), an increase in serotonergic activity within the central nervous system might decrease neuro-endocrine activity related to reproduction. Changes in TRP levels may only indicate an increased biosynthetic conversion to 5HT; however, TRP has been shown to exhibit effects on neural activity in honey bees that is independent of the actions of 5HT (Lopatina & Dolotovskaya 1984; Lopatina et al. 1985). Changes in TYR levels could potentially affect many parts of the nervous system because it is the precursor to the neuroactive catecholamines (DOPA, DA, norepinephrine and epinephrine) and phenolamines (tyramine, OA, synephrine). One cannot discount the idea that changes in levels of TRP, TYR, 5HT and DA may only be symptomatic of disruptive changes in the central nervous system resulting from narcosis or anoxia suffered during long exposures to CO₂ (Woodring et al. 1978) and may not be related to...
worker reproduction. Because the effects of treatment with CO₂ were similar to effects from treatment with a mixture of CO₂ containing 20% O₂, the current study indicates that the effects of a 10 minute exposure to CO₂ in bee brains are probably the result of narcosis and not anoxia. In contrast, Woodring et al. (1978) indicated that a 10 minute exposure marked the beginning of anoxia in the house cricket, Acheta domesticus, and that the major longterm effects of longer exposures to CO₂ are probably caused by anoxia rather than narcosis.
CHAPTER IV

EFFECTS OF DIETARY PRECURSORS TO BIOGENIC AMINES ON THE BEHAVIORAL RESPONSE FROM SMALL GROUPS OF CAGED WORKER HONEY BEES TO THE PRESENTATION OF ISOPENTYL ACETATE
Introduction

In honey bees (*Apis mellifera* L.) colony defense behavior can be quantified in both field and laboratory tests. Various genetic parameters related to the overall colony reaction and the identification of alarm pheromone components have been outlined using field and laboratory bioassays that measure the speed, intensity and duration of the colony behavioral response to a mechanical disturbance after the brief presentation of test chemicals (Blum *et al.* 1978, Boch *et al.* 1962, Collins & Blum 1983, Collins & Rothenbuhler 1978, Collins 1980, 1989; Shearer & Boch 1965). Distinctions between Africanized and European honey bee defense behavior have been determined using these techniques (Collins *et al.* 1982, Collins & Kubasek 1982, Collins & Rinderer 1988, Stort 1974). Another recently developed laboratory test for honey bee responses to alarm pheromones measures metabolic oxygen consumption by groups of bees subsequent to release of a test chemical (Moritz *et al.* 1985, Southwick & Moritz 1985). The common element of all of these tests is that responses to alarm pheromones are only made on groups or whole colonies of bees.

Although honey bee colony defense has been investigated by measuring a group response, it has been difficult to relate the physiology of individual bees to the overall colony response. My particular focus is on the relationship between biogenic amine neuromodulators within the honey bee brain and specific behavioral response parameters to an olfactory stimulus, such as the primary alarm pheromone component, isopentyl acetate (IPA). Recent work with vertebrates and invertebrates indicated that a variety of behaviors are mediated by the actions of biogenic amines within the central nervous
system (Kravitz 1988, Bicker & Menzel 1989). Indeed, the examination of olfactory learning in bees has shown that memory retrieval and storage (complex nervous system functions) are mediated by biogenic amines (Menzel et al. 1990). The first three chapters of this dissertation indicate that brain amine levels are correlated to major changes in worker honey bee behavior associated with changes in reproductive status, the effects of carbon dioxide narcosis, changes in season and to changes in amount of dietary pollen. Recent work by Burrell and Smith (1994, 1995) showed possible modulatory roles by octopamine on muscular and motor-neural components of the honey bee sting extension motor program within the ventral nerve cord in isolated honey bee abdomens. A recent preliminary report showed that European honey bees had significantly higher levels of the neurotransmitter beta-alanine in the brain than do Africanized honey bees (Last et al. 1994). This latter report presents the possibility that differences in stinging behavior between Africanized and European honey bees may indeed be related to chemicals within the CNS.

The purpose of the current study is to test the potential effects of ingested biogenic amine precursors on behavioral responses to the presentation of IPA. In particular, the laboratory bioassay used in this study involved the measurement of variables related to the “buzzing response” that small groups of bees within screened cages will produce when exposed to a swab or applicator stick soaked with a concentrated solution of IPA in paraffin oil. Such buzzing responses were monitored using a small microphone located within the screened cages, and the electrical signal from the microphone was continuously recorded and stored on a computer that allowed
for easy examination of the data. The variables measured were (1) the time delay or onset of the buzzing response after presentation of the IPA chemical stimulus, (2) the duration of the buzzing response and (3) the overall intensity of the response. Initially, the responses of groups of bees to 5% IPA in paraffin oil versus a paraffin oil control were compared to show the utility of the method. The effects of various doses of ingested biogenic amine precursors (L-DOPA, tryptophan and 5-hydroxytryptophan) on the buzzing response to IPA were investigated. Finally, the brains of individual bees that were fed the same doses of the various precursors used in the behavioral study were analyzed to indicate possible correlations between the behavioral studies and the relative levels of biogenic amines and amine precursors within the CNS of individual bees.

Materials and Methods

Behavioral Responses to IPA

The behavioral experiments were conducted during May-July, 1995, at the Life Science building of Louisiana State University. Forager honey bees were collected with forceps without use of an anesthetic as they returned to their observation colony. These bees were placed within clear plastic cylinders (3.25" x 6.0") that were screened on both ends with an acetate mesh. The top screen of the cylinder could be removed for adding the bees. Bees were collected between 8:00 am and 10:00 am on each day that tests were to be performed.

Each group of bees were initially held without food for the first 30-40 minutes after capture. After this initial period, each group was fed 2 M sucrose solution ad
*libitum* through the next 5-6 hours. The syrup was pipetted into a small vial cap placed on the bottom screen of each cage through a small access hole drilled in the side of each cage. The volume delivered during each refill was approximately 700 µL, and cages received 2-3 refills throughout the entire experiment. Each cage of bees was secured to a ringstand using a clamp so that the bottom screen was accessible for the presentation of test solutions soaked on an applicator swab. A small tie-clip type of microphone was anchored to the inside top screen of each cage, and the analog signals from the microphones were conducted to separate channels on a MacLab 4S analog-digital converter. The raw waveform data generated from the MacLab were converted to frequency data using the MacLab Chart program on a Macintosh computer. Prior to each test session, all microphones were calibrated using a frequency generator and a speaker at a set distance. Baseline noise from resting bees was found to be around 12 Hz, and maximal responses to any disturbance from these groups of bees was found to be between 200 and 350 Hz; therefore, the microphones were calibrated at frequency signals of 200 and 350 Hz of the sound generator to ensure that all microphones responded the same. The temperature of the test room was 83-85°F and the overhead lights were kept on throughout the experiment. Groups of bees were placed at least 4 feet apart to avoid possible influences of one group of bees on a neighboring group.

To test the effectiveness of IPA at eliciting buzzing responses from caged bees, 20 cages of bees were assembled and fed sucrose as described above. After a 5 hour acclimation period, groups were exposed to a 30 sec presentation of either a swab soaked in paraffin oil or one soaked in paraffin oil containing 5% IPA (10 groups per test
chemical). The swabs were placed close to the center of the bottom screen of each cage without touching the mesh. Each group was only tested once. Response measurements were made relative to a mark placed on the data trace with the simultaneous presentation of the test swab. The variables measured were (1) the onset (in seconds) of the response, (2) the duration (seconds) of the response and (3) the intensity of the response reported here as the maximum value (Hz) of the frequency data trace. If a group of bees did not respond, the group was assigned an onset value of 30 sec, and duration and maximal response values of 0. Differences between the two groups (paraffin controls versus 5% IPA) were tested using the Student's T test procedure.

**Ingested Amine Precursors and the Behavioral Response to IPA**

The effects of ingested tryptophan (TRP), 5-hydroxytryptophan (5HTP) and L-DOPA on the behavioral response to the presentation of IPA was tested during June - July, 1995. A procedure identical to the above was used for all experiments, except that the control groups of bees were fed 700 μl of 2 M sucrose and each test group was fed 700 μl of 2 M sucrose containing any one of four doses (1.0, 2.0, 4.0 or 8.0 mM) of one of the above three precursors (10 groups of bees tested for each dose and drug combination; 120 total groups were tested) exactly five hours before performing the IPA bioassay. After the test solutions were totally consumed, each group of bees was fed 2 M sucrose to keep them alive throughout the experiment. Because of the logistics involved in testing many groups of bees, tests were conducted over a two week period. Hence, on any test day equal numbers of controls (groups only fed 2 M sucrose) and of
drug-fed groups were tested. For simplicity, equal doses of all three drugs were tested on the same days. The data were analyzed using an analysis of variance with a model including major terms for the test drug treatment and dose of drug. Also included was the interaction term for these two sources of variation.

**Ingested Amine Precursors and Brain Amine Content**

The effects of the same ingested amine precursors used in the IPA behavioral response study on amine levels within the brains of individual bees were tested during late summer 1995. Individual foragers were captured from the observation colony and placed singly into a screened cage identical to those used for the IPA tests. Each bee was starved for 30-40 minutes before being fed 45 µl of test solution. The solutions tested were the same as above: controls = groups fed only 2 M sucrose; all other groups received one drug (TRP, 5HTP or L-DOPA) and dose (0.5, 1.0, 2.0, 4.0 or 8.0 mM) combination dissolved in 2 M sucrose. The brains from the test bees were removed for amine analysis five hours after the experiment began. For simplicity, controls and all doses of a single drug were tested on any particular day or series of days. Hence, three different experiments were actually conducted (one to tested the effects of each precursor). Therefore, the statistical analysis consisted of separate analyses for each experiment. An analysis of variance tested the effects of dose of ingested precursor on resulting levels of octopamine (OA), serotonin (5HT), dopamine (DA), tryptophan (TRP), N-β-alanyldopamine (NBADA), kynurenine (KYN) and 5-hydroxtryptophan (5HTP).
Results

Effects of 5% IPA Versus Paraffin Oil Controls on Buzzing Responses of Bees

There were significant differences between those groups of bees exposed to 5% IPA in paraffin oil and those exposed to only paraffin oil for all three variables measured (Fig. 4.1) Paraffin controls required an average (mean ± SE) onset of 25.37 ± 3.09 sec to react, while groups treated with IPA reacted four times more quickly (8.10 ± 1.20 sec) (Student’s t = 5.206; P<0.001; df=12.253, unequal variances). Groups exposed to IPA buzzed above baseline levels (12 Hz) for an average (mean ± SE) of 39.53 ± 9.00 sec, while controls only responded for 2.03 ± 1.38 sec (Student’s t = 4.12; P<0.003; df=9.4, unequal variances). The maximal response in groups exposed to IPA was 233 ± 17.83 Hz, while controls only averaged (mean ± SE) a maximum of 20 ± 13.66 Hz (Student’s t = 9.483; P<0.001; df=18, equal variances).

Effects of TRP, 5HTP and L-DOPA on the Response to IPA

All doses of TRP produced a hyperactive condition in the groups of bees that did not permit measurement of the three behavioral parameters (onset, duration and maximum). As previously mentioned, the baseline noise level for quiet control bees never exceeded 12 Hz. For all doses (0.5, 1.0, 2.0, 4.0 and 8.0 mM) of TRP the baseline noise level often exceeded 20-30 Hz and sporadically exceeded 200 Hz throughout the test phase of the experiment. The irregular and unpredictable buzzing from the TRP-fed bees made evaluation of an IPA response impossible. Hence, TRP was excluded from any subsequent statistical analyses.
Figure 4.1 - Comparison of sound traces produced by groups of bees exposed either to 5% IPA in paraffin oil or control oil. Each trace is a record of the sound produced by a group of 15 bees within a small plastic cylinder to the presentation of test solutions on saturated cotton swabs. Each stimulus (swab soaked in 5% IPA in paraffin oil or paraffin oil alone) was presented at the mark indicated by ON and was removed at the mark indicated by OFF on each trace. Bees reacted much faster and for longer periods of time and with greater intensity when exposed to 5% IPA than to paraffin oil alone.
Neither L-DOPA or 5HTP significantly affected the onset of buzzing responses versus untreated controls for any dose tested (Fig. 4.2). None of the model terms were significant sources of variation: main drug treatment term (F=2.58; df=2, 108; P>0.08), dose term (F=1.13; df=3, 108; P>0.33) and the treatment-by-dose interaction term (F=1.12; df=6, 108; P>0.35). Although there were no significant differences in onset between drug treatments, those groups of bees fed the highest dose of 5HTP required twice as much time to respond as did controls (Fig. 4.2) - hinting that still higher doses of 5HTP may have an effect on the onset of the IPA response.

Only 5HTP had an effect on the duration of the IPA response, and the highest doses (4.0 and 8.0 mM) were most effective at reducing the total duration of the IPA response (Fig. 4.3). Groups fed L-DOPA were not significantly different from controls regardless of the dose. All model terms were significant sources of variation: drug treatment term (F=6.68; df=2, 108; P<0.002); dose term (F=5.23; df=3, 108; P<0.003) and the drug treatment-by-dose interaction term (F=4.01; df=6, 108; P<0.002).

Only high doses of 5HTP significantly reduced the maximal response produced by groups of bees exposed to IPA (Fig. 4.4). L-DOPA had no effect. All model terms were significant sources of variation: drug treatment term (F=13.17; df=2, 108, P<0.001), dose term (F=3.35; df=3, 108; P<0.03) and the drug treatment-by-dose interaction term (F=4.38; df=6, 108; P<0.001).
Figure 4.2 - Effects of ingested L-DOPA and 5-hydroxytryptophan (5HTP) on the onset of the buzzing response elicited from small groups of 15 worker bees by exposure to 5% isopentylacetate (IPA) in paraffin oil. Returning forager bees were captured at the entrance of their observation colony and placed into small cylindrical clear plastic cages that were screened on both ends. A small microphone was mounted at the top center of each cage, and the sound data was continuously recorded. Each group of bees was then fed 700 µl of either (1) 2 M sucrose or (2) 2 M sucrose containing L-DOPA in various doses or (3) 2 M sucrose containing 5HTP in various doses; and all groups of bees were fed 5 hours before being exposed to IPA. Each bar represents the mean (± SE) for 10 groups of caged bees (total = 120 groups). The timing of the buzzing response began with the 30 sec presentation of an applicator swab soaked in the 5% IPA at the bottom screen of each cylinder. There were no significant differences in the onset of the buzzing responses between controls (black bars), DOPA-fed bees (open bars) and 5HTP-fed bees (striped bars) for any of the doses tested.
Figure 4.3 - Effects of ingested L-DOPA and 5-hydroxytryptophan (5HTP) on the duration of the buzzing response elicited from groups of 15 worker bees by exposure to 5% isopentylacetate (IPA) in paraffin oil. See Figure 4.2 for the experimental details. Only high doses of 5HTP (striped bars) significantly reduced the duration of the buzzing response to 5% IPA; bars with the same letter are not significantly different as determined by the Tukey's mean separation ($\alpha = 0.05$). Controls (black bars) and DOPA-fed bees (open bars) were not significantly different at all doses tested. Each bar represents the mean (± SE) duration for 10 groups of bees.
Figure 4.4 - Effects of ingested L-DOPA and 5-hydroxytryptophan (5HTP) on the maximal response during the buzzing response elicited from groups of 15 worker bees by exposure to 5% isopentylacetate (IPA) in paraffin oil. See Figure 4.2 for the experimental details. The maximal response (Hz) reflects the intensity of a particular response and can be correlated to the sound intensity produced by the buzzing bees. Only high doses of 5HTP (striped bars) significantly reduced the maximal response to 5% IPA; bars with the same letter are not significantly different as determined by the Tukey's mean separation ($\alpha = 0.05$). Controls (black bars) and DOPA-fed bees (open bars) were not significantly different at all doses tested. Each bar represents the mean ($\pm$ SE) maximal response for 10 groups of bees.
Effects of Ingested Amine Precursors on Brain Amine Levels in Individual Bees

Levels of all three precursors (L-DOPA, 5HTP and TRP) were found to increase in the brains of bees with an increase in the dose ingested by bees (Figs. 4.5 - 4.7). This result would be expected if all three could cross the insect “blood-brain barrier” to be absorbed by cells within the brain. Also, in the case of all three precursors, the levels of at least one metabolite was found to increase with increasing dose of ingested precursor.

For DOPA-fed bees, dopamine (F=5.86; df=5,48; P<0.001) and DOPA (F=8.15; df=5,48; P<0.001) levels in the brain significantly increased with increased dose of DOPA that was fed (Fig. 4.5). Octopamine (F=0.95; df=5,48; P>0.45) and serotonin (F=1.63; df=5,48; P>0.17) did not increase with increasing dose of ingested L-DOPA. For example, bees fed only sucrose (n=19) had 9.21 ± 1.87 pmols/brain octopamine and 7.97 ± 0.26 pmols/brain serotonin, while bees fed 8.0 mM L-DOPA (n=7) averaged 12.65 ± 4.63 pmols/brain octopamine and 8.87 ± 1.10 pmols/brain serotonin (mean ± SE).

For 5HTP-fed bees, brain levels of serotonin (5HT) (F=19.64; df=5,43; P<0.001) and 5HTP (F=30.22; df=5,43; P<0.001) were significantly elevated with increasing dose of ingested 5HTP (Fig. 4.6). Levels of octopamine (F=2.42; df=5,43; P>0.06), dopamine (F=1.65; df=5,43; P>0.16) and tryptophan (F=1.03; df=5,43; P>0.40) were not significantly different between controls and 5HTP-fed bees. For example, bees fed only 2 M sucrose (n=10) averaged (mean ± SE) 5.54 ± 0.56 pmols/brain octopamine, 11.04 ± 1.45 pmols/brain dopamine and 94.66 ± 15.31 pmols/brain tryptophan. Bees fed
Figure 4.5 - Effects of various doses of ingested L-DOPA on DOPA (black bars) and dopamine (open bars) levels in the brains of worker honey bees. Forager bees were captured at the entrance of an observation colony and held individually in vials for 30-40 minutes before being fed 45 μl of either 2 M sucrose (dose 0; n = 19) or various doses (n = 7 bees per dose) of L-DOPA in 2M sucrose. Each bee was provided 2 M sucrose *ad libitum* after the test solution was totally consumed (usually within an hour of being offered). The brains were sampled for biogenic amine content 5 hours after the experiment began. Both brain DOPA and dopamine levels significantly (α = 0.05) increased with increasing dose of ingested L-DOPA. Octopamine and serotonin levels were also measured from these brains, but their levels were not affected by ingested DOPA (see Results).
Figure 4.6 - Effects of various doses of ingested 5-hydroxytryptophan (5HTP) on 5HTP (black bars) and serotonin (open bars) levels in the brains of worker honey bees. Forager bees were captured at the entrance of an observation colony and held individually in vials for 30-40 minutes before being fed 45 μl of either 2 M sucrose (dose 0; n = 10) or various doses (n = 6-9 bees per dose) of 5HTP in 2M sucrose. Each bee was provided 2 M sucrose *ad libitum* after the test solution was totally consumed (usually within an hour of being offered). The brains were sampled for biogenic amine content 5 hours after the experiment began. Both brain 5HTP and serotonin levels significantly (α = 0.05) increased with increasing dose of ingested L-5HTP. Octopamine, dopamine and tryptophan levels were also measured from these brains, but their levels were not affected by ingested 5HTP (see Results).
Figure 4.7 - Effects of various doses of ingested tryptophan on tryptophan (black bars) and kynurenine (open bars) levels in the brains of worker honey bees. Forager bees were captured at the entrance of an observation colony and held individually in vials for 30-40 minutes before being fed 45 μl of either 2 M sucrose (dose 0; n = 19) or various doses (n = 7-8 bees per dose) of tryptophan in 2M sucrose. Each bee was provided 2 M sucrose ad libitum after the test solution was totally consumed (usually within an hour of being offered). The brains were sampled for biogenic amine content 5 hours after the experiment began. Both brain tryptophan and kynurenine levels significantly (α = 0.05) increased with increasing dose of ingested tryptophan. Octopamine, dopamine and serotonin levels were also measured from these brains, but their levels were not affected by ingested tryptophan (see Results).
8.0 mM 5HTP (n=8) had 3.55 ± 0.79 pmols/brain octopamine, 9.67 ± 1.14 pmols/brain dopamine and 98.97 ± 6.95 pmols/brain tryptophan.

For TRP-fed bees, brain levels of kynurenine (F=17.90; df=5,35; P<0.001) and tryptophan (F=24.91; df=5,35; P<0.001) increased with increasing dose of ingested TRP (Fig. 4.7). Brain levels of octopamine (F=2.14; df=5,35; P>0.08), dopamine (F=1.24; df=5,35; P>0.31) and serotonin (F=1.41; df=5,35; P>0.24) were not significantly affected by ingested TRP. Bees fed 2 M sucrose (n=6) averaged (mean ± SE) 1.89 ± 0.44 pmols/brain octopamine, 6.07 ± 1.12 pmols/brain dopamine and 2.91 ± 0.48 pmols/brain serotonin. Bees fed 8.0 mM TRP in sucrose (n=8) averaged 2.70 ± 0.68 pmols/brain octopamine, 8.96 ± 1.40 pmols/brain dopamine and 2.27 ± 0.18 pmols/brain serotonin.

**Discussion**

Of the three ingested amine precursors (TRP, 5HTP and L-DOPA), 5HTP was most effective at reducing the total duration and maximal response of the buzzing event produced by a 30 sec exposure to 5% IPA. The reductions in these two variables were dose-dependent. Also, it was shown that both 5HTP and serotonin levels in the brains of bees increased in a dose-dependent manner with increasing dose of ingested 5HTP.

These experiments cannot conclusively show that reductions in the behavioral variables were related to either 5HTP or serotonin, but the evidence suggests that some factor related to serotonin metabolism within the honey bee CNS may be responsible for reduced reactivity to the presentation of IPA. More work is needed to locate and
identify the exact mode of action, but it has been shown that serotonin often reduces the responsiveness of neural components (Erber et al. 1991, 1993).

Ingested tryptophan was shown to produce a hyperactive state in groups of bees, and it appears that this behavior may be related to metabolic routes involved in the conversion of tryptophan into kynurenine (and not to serotonin - serotonin levels in the brains of bees did not increase within the first 5 hours after ingestion of any dose of tryptophan). It seems likely that the hyperactive state in bees is more related to the increased kynurenine than to the increased brain tryptophan levels. This conclusion is based on studies by others that have indicated that tryptophan in the CNS tends to depress neural activity and that kynurenine stimulates CNS activity (Lopatina & Dolotovskaya 1984, Lopatina et al. 1985).

Although ingested L-DOPA was found to elevate dopamine levels in the brain, it had no obvious effect on the buzzing responses bees make in response to IPA. This result suggests that dopamine may not be involved in neuromodulation of the olfactory response to IPA. It is possible, however, that more time is needed for dopaminergic neural elements to respond to increased L-DOPA levels in the hemolymph.

A simple but effective procedure for evaluating the effects of the honey bee alarm pheromone component IPA was used to test the effects of various ingested biogenic amine precursors on behavioral responses. The use of small microphones and a computer to record the buzzing responses was an easy reproducible method to distinguish between the exposure of a group of bees to a swab soaked in a control oil versus the same oil containing 5% IPA. One shortcoming of this current study is that the
buzzing responses of small caged groups of bees to the presentation of IPA were not correlated to the defense response of colonies in the field. This goal was not the focus of this chapter, but to be complete, such a study will need to be conducted.
SUMMARY

The two most important results from this study were (1) that whole brain dopamine levels were significantly correlated to ovarian development in worker bees and (2) behavioral responses to isopentyl acetate (IPA), a primary component of the honey bee alarm pheromone, were reduced within a few hours of ingestion of the biogenic amine precursor 5-hydroxytryptophan (5HTP). Both results indicate that studies of whole brain chemistry can suggest the direction in which subsequent research aimed at elucidating mechanisms controlling behavior or physiology should go. However, this work alone cannot detail cause and effect mechanisms. Also, it should be noted that lack of significant differences in the levels of other brain chemicals between bees that have different levels of reproductive development or that respond differently to alarm pheromones does not mean that these chemicals are not involved in controlling these behavioral or physiological states. It is quite possible that the total brain content of a particular compound remains unchanged while it is elevated in some brain regions and reduced in other regions. Given the shortcomings of the whole brain approach, relationships between whole brain chemical content and physiology or behavior are indicative of neural changes that do reflect mechanisms that lead to or maintain changes in physiology or behavior. For example, elevated brain dopamine may indicate a gonadotropic feedback loop between the brain and fatbody or ovary. Reductions in behavioral responses to an alarm pheromone several hours after ingestion of 5HTP also
suggest that serotonin (5HT) plays some kind of inhibitory role in the alarm process within individual bees.

Evidence supporting the idea that brain dopamine is involved in the regulation of worker honey bee ovary development stems from several experiments presented in this work. In particular, brain dopamine levels and ovary development increased in worker bees that had no queen versus worker bees that were with a queen. Also, for the bees without queens, dopamine levels were positively correlated to ovariole width: bees with the largest ovaries had the highest levels of brain dopamine. Because evidence of increased ovarian development was apparent within 12 days after removal of the queen and increased levels of brain dopamine were apparent only after 18 days, dopamine biosynthesis may only be stimulated after oogenesis has begun. However, this does not exclude a gonadotrophic role for brain dopamine during early oogenesis. It is possible that undetectable changes in dopamine levels in different brain regions are involved in initial stimulation of gonadotropic substances from the brain during the first 6-12 days. Again, these changes in specific regions might be masked by examination of whole brain content. Alternatively, elevations of brain dopamine levels after the ovaries have manifested detectable growth may be indicative of dopamine’s involvement in positive feedback stimulation of gonadotropic substances or other substances involved in maintenance and growth of the ovaries. A similar role of dopamine in reproductive development in roaches has been proposed (Hentscel, 1981). In this case, increased activity by dopaminergic neurons modulates release of juvenile hormone-stimulating peptides, allatotropins, from neurons that project onto the corpora allata. Increased
allatotropin release leads to increased biosynthesis and release of juvenile hormone which acts as a gonadotropin to stimulate vitellogenic activity by the fat body and increased yolk incorporation by the ovary. Ecdysone released by the ovary as it grows acts to further increase dopaminergic activity within the brain, completing the positive feedback loop.

Additional evidence that dopamine levels in the brain are related to ovary development in worker bees came from experiments in which exposure to carbon dioxide was used to reduce worker ovary development. Within 10-14 days of exposure to carbon dioxide, brain dopamine levels were concomitantly reduced with ovary development in worker bees that had been fed pollen. If workers were not fed pollen, exposure to carbon dioxide had no significant effect on brain dopamine levels but ovary development was reduced. Reasons for the differential effects of carbon dioxide on bees fed or not fed pollen are unclear, but these differences do not necessarily suggest that loss of brain dopamine is unrelated to reduced ovary development. It is possible that absence of pollen in the diet may have led to changes in the brain that inhibited worker oogenesis independent (or in combination with) of changes in the brain related to exposure to carbon dioxide. For example, brain levels of tryptophan and tyrosine were greatly reduced by absence of pollen in the worker bee diet.

The results from this dissertation which have the most immediate potential for practical application is that some aspects of honey bee behavior were modifiable by feeding worker bees precursors to the biogenic amines. In particular, the serotonin precursor 5HTP reduced the duration and intensity of the “buzzing” response produced
by bees when briefly exposed to the volatile alarm pheromone component IPA. In these experiments small groups of bees were fed 700 µl of a single dose (4-8 millimolar) of 5HTP dissolved in 50% sucrose, and five hours later, the "buzzing" response to a 30 second presentation of IPA was significantly reduced. Other amine precursors were added to the diets of groups of bees (tryptophan and L-DOPA) a few hours before exposure to IPA, but only 5HTP reduced the response to IPA. Ingested tryptophan was found to produce hyperactivity in bees, and ingested L-DOPA produced no observable changes in the "buzzing" response produced by the presentation of IPA.

In a subsequent experiment individual bees were fed known volumes of the same test solutions used in the IPA responsiveness tests, and the brains were sampled from these bees for biogenic amine analysis five hours later. In all cases the precursor that was fed to the bees (tryptophan, L-DOPA and 5HTP) and at least one metabolite were significantly elevated in a dose-dependent fashion within these brains. When tryptophan was fed, brain levels of tryptophan and kynurenine were elevated. Serotonin levels in the brain were unaffected by ingested tryptophan. When L-DOPA was fed, brain levels of L-DOPA and dopamine were significantly elevated. When 5HTP was fed, brain levels of 5HTP and serotonin were significantly elevated. Combining the results from the behavioral study with these results, it is very likely that increased serotonergic activity in the nervous system of worker bees led to the reduced "buzzing" responses to IPA. Similarly, the hyperactivity produced by ingested tryptophan may be related to an increased conversion of the amino acid to kynurenine.
The practical utility suggested by these experiments is that responsiveness to alarm chemicals (a very important component in the alerting behavior of bees in response to a colony disturbance) of very defensive bees might be temporarily subdued by treatment of such colonies with amine precursors such as 5HTP. To be effective, high doses of amine precursors would have to be delivered specifically to bees (versus other organisms in the environment). Bait stations have been used to deliver insecticides to honey bee colonies in the field for the purposes of killing unwanted feral nests, and these same bait stations could be used to deliver amine precursors to colonies a few hours before the colonies are manipulated. It would be hoped that reduced responsiveness to the alarm chemical IPA would also confer an overall reduction in the stinging response by bees. The advantage to beekeepers of this technique over poisoning bees with insecticides is that very defensive bees could be handled safely without having to kill them. However, many questions must be answered before such a technique could be safely used. For example, will changes in the brain chemistry of bees from such a treated colony be transient or permanent? If permanent, will important economic variables be significantly affected.

As is often the case with scientific work, more questions stem from this study than have been answered. The role of dopamine in worker honey bee oogenesis is hypothesized only from studies where ovarian development could be correlated to brain dopamine content. To bring this work to the next level of inquiry, specific brain regions and neural pathways will have to be shown to change with oogenesis. The complete gonadotropic axis in the brain will have to be identified and mapped, and if dopamine is
involved, its effects will need to be explained relative to these other pathways. For the behavioral studies involving IPA, special techniques will be needed to follow changes in levels of neuroactive chemicals within specific brain regions (e.g. the antennal lobes) on a second by second basis while a bee is exposed to the alarm pheromone. Such experiments might show which putative substances and which brain regions are most important in controlling the response to olfaction of the alarm pheromone.
REFERENCES


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DLD/fsl
May 14, 1996

Jeffrey W. Harris
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Rights and Permissions Assistant
Jeffrey W. Harris was born on May 23, 1963 in Madison, Wisconsin. He is one of four children from the marriage of Msgt. James Curtis and Barbara Lorraine Harris who now live in Wetumpka, Alabama. As part of a military (U.S. Air Force) family, Jeff and his siblings traveled frequently while growing up, and they lived in various northern states prior to settling in the Deep South. Jeff attended Auburn University at Montgomery for his undergraduate training in physical science. Next, he spent two years in a doctoral program within the chemistry department at the University of Alabama before leaving the program in 1989 to pursue a Masters degree in entomology at Louisiana State University. His study of reproductive abilities of worker bees led to his interest in insect physiology, which has resulted in the completion of this doctorate degree during August 1996 from the Department of Zoology and Physiology at Louisiana State University. Jeff has received the departmental teaching award for excellent instruction within freshman introductory zoology labs, and he considers his teaching abilities to be better than average. He enjoys teaching as much as he enjoys conducting research.
DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Jeffrey W. Harris

Major Field: Physiology

Title of Dissertation: Relationships Between Brain Biogenic Amines and Reproductive and Defense Behavior in Honey Bees (Apis mellifera L)

Approved:

[Signatures]

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

[Signatures]

Date of Examination: November 29, 1995

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