THE PRODUCTION AND INTRA-NEST TRANSMISSION OF HONEY BEE (*APIS MELLIFERA* L.) QUEEN MANDIBULAR GLAND PHEROMONE

by

Kenneth Naumann

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Name: Kenneth Naumann

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Examining Committee:

Chairman: Dr. G.R. Lister, Assistant Professor

Dr. M.L. Winston, Professor, Senior Supervisor,
Department of Biological Sciences, SFU

Dr. K.N. Slessor, Professor,
Department of Chemistry, SFU

Dr. R.C. Ydenberg, Associate Professor,
Department of Biological Sciences, SFU

Dr. J.M. Mackauer, Professor,
Department of Biological Sciences, SFU
Public Examiner

Dr. T.D. Seeley, Associate Professor,
Department of Biological Sciences,
Cornell University, Ithaca, New York
External Examiner

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The Production and Intra-nest Transmission of Honey Bee (Apis mellifera L.) Queen Mandibular Gland Pheromone.

Author: _____

(signature)

Kenneth Naumann

(name)

March 31, 1992

(date)

ABSTRACT

The social cohesiveness of eusocial insect colonies is maintained primarily through the utilization of pheromones, many of which are queen-produced. These compounds direct or influence worker activities such as attraction to the queen, inhibition of queen rearing and worker ovary development, and foraging. The "message" of some of the queen-produced pheromones may be that the queen is present and viable in the colony, with the result that the workforce is inhibited from producing new queens and is otherwise not distracted from acting cooperatively. One aspect of this influence by the queen is the production and transmission of her pheromones to the workforce. This project quantitatively elucidated the production, secretion, and transmission of 3 of the 5 components of the honey bee (*Apis mellifera* L.) queen mandibular pheromone complex (QMP). Each day, mated queens produce approximately 1 queen equivalent (Qeq), the amount found in the glands of a mated queen at any one time. Approximately 10^{-3} Qeq are maintained on the body surface of a queen by an equilibrium between exudation, internalization, tracking on the comb, and removal by workers. Workers attending the queen, especially those making licking contact, remove the greatest amount, and act as messengers, disseminating QMP to the workers and to the wax. Pheromone transfer is influenced by self-grooming behaviors that lead to pheromone translocation on the bodies

of workers. A model evaluating the pathways and relative quantities of QMP components transferred through the nest is presented. The nature and rates of transmission of the 3 studied components are remarkably similar, suggesting that the pheromone complex is transferred through the nest as a unit. The nature of QMP transport in populous, slightly congested colonies is not different from that in less populous colonies. However, fewer workers in populous colonies receive sufficient QMP to be inhibited from rearing queens. It is likely that the increase in population size and colony congestion that comes with colony growth interferes with QMP transport, leading to queen rearing associated with reproductive swarming. Synthetic QMP was found to be as effective as mated queens at pacifying packaged workers during transport and short term storage. "Pseudo-queen" lures of synthetic QMP may thus have a commercial application in the package bee industry. The results of this study have implications for the proper design and use of pheromones in honey bee management, as well as being the first quantitative analysis of pheromone transport in any social insect.

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INTRODUCTION

Among terrestrial invertebrate animals, sociality has reached its highest degree of development in the eusocial insects, which have evolved effective means to integrate the functions of large numbers of individuals into colonies. This social cohesiveness is maintained in part through the utilization of pheromones, communication chemicals that transmit information between colony members. Queens, through their unique position as the dominant reproductive females, are particularly important in influencing the behaviors of their nestmates. This study focussed on aspects of one of these queen pheromones, the production and intra-nest transmission of the honey bee (*Apis mellifera* L.) queen mandibular gland pheromone complex (QMP). This pheromone is involved in numerous activities, including mate attraction, arresting of nearby workers, stimulation of foraging, and the inhibition of queen production. In order for this pheromone to be effective, it must be produced and disseminated to the workforce in such a manner, and in such quantities, that all relevant sectors of the society receive the message. The message may be simply that "the queen is present and viable". As long as such a situation persists, it may be ultimately in the workers' best interest to act cooperatively in the maintenance and growth of the colony. Loss of such a signal implies a change in the reproductive

status of the colony, and potentially in the reproductive options and motives of each worker.

This study consists of a number of sections. A review of the role of queen pheromones in coordinating the activities of colonies of eusocial Hymenoptera is offered as an introduction to the topic. Thereafter, the thesis reports on experiments that investigated different aspects of the intra-nest movement of QMP. A quantitative model of pheromone flux within the colony is presented, based upon the movement of the most abundant component. This component also was used to examine more closely the role of worker self-grooming in pheromone dissemination. Two other components were similarly studied, in order to compare the rates of transfer of the components of the pheromone complex as it is transported throughout the nest. The experiment detailed in Chapter 5 studied the intra-nest transmission of QMP in small, established colonies, and focussed on the role of colony population size and congestion on QMP transport, and the implications for colony reproduction. I conclude with the results of a test of one potential commercial application of synthetic QMP, the short-term replacement of queens during the transport and storage of packaged bees.

CHAPTER 1

QUEEN PHEROMONES AND THE INTEGRATION OF WORKER BEHAVIORS

The great diversity and success of the eusocial Hymenoptera is due, in part, to the successful utilization of semiochemicals to coordinate the activities of many individuals. The central individual in these integrated units is the queen. Queen-produced chemicals are especially important in the maintenance of reproductive division of labor, whereby one or several individuals dominate reproduction in the group and others forego, or are excluded from producing offspring themselves. This inhibition can often be highly effective. In one study, only 19% of the males in colonies of the bumblebee *Bombus melanopygus* were laid by the workers (Owen and Plowright, 1982), and in a survey of honey bees, *Apis mellifera*, only 0.12% of the males were derived from worker laid eggs (Visscher, 1989). But, there are also eusocial species where the workers commonly lay eggs in the presence of the queen (Engels and Imperatriz-Fonseca, 1990), yet queen-produced chemicals are still necessary for proper colony functioning. Thus, the contribution of queen social pheromones goes beyond the exclusion of nestmates from reproduction; loss of the queen leads to a breakdown of harmony within the nest, such that the efficiency of performing tasks is decreased, and colony survival is jeopardized. In many species, the colony is

doomed if the queen cannot be replaced. Dependence upon one or several individuals for colony reproduction and nest functions, has resulted in a variety of mechanisms for dealing with the eventuality of queen loss. In the primitively eusocial groups, such as the Polistine wasps and the Halictid bees, there is often a complete gradation in behavior between of queens and the subordinate adult females, but in the most highly social ants and bees, queen vs worker caste determination occurs in the larval stage. As a result, subordinate adult wasps have the potential to become queens, but adult workers of ants and bees have limited competence to assume queen roles, even in the absence of queen inhibition. These differences influence how queens and their pheromones operate to influence the actions of other colony members. Social parasites have evolved which take advantage of the central role of the queen, and use her pheromone blends to gain access to the machinery of entire working colonies.

Reproductive competition is a key evolutionary factor in social insects, with queens and workers each pursuing strategies to maximize their own inclusive fitness. The result has been colonies wherein a single or a few queens coordinate (or dominate) the activities of the whole group, and/or societies wherein queens and workers pursue very similar strategies. More primitive social insects often show overt behavioral dominance by the queen to limit worker reproduction, whereas a pheromonally-mediated influence

appears to be more common at higher levels of sociality. In small colonies, a single individual can frequently come into contact with all or most of its nestmates, but in species with populous colonies, utilization of semiochemicals allows rapid communication to a large number of individuals, and, by the breakdown of these compounds, the rapid removal of the signal.

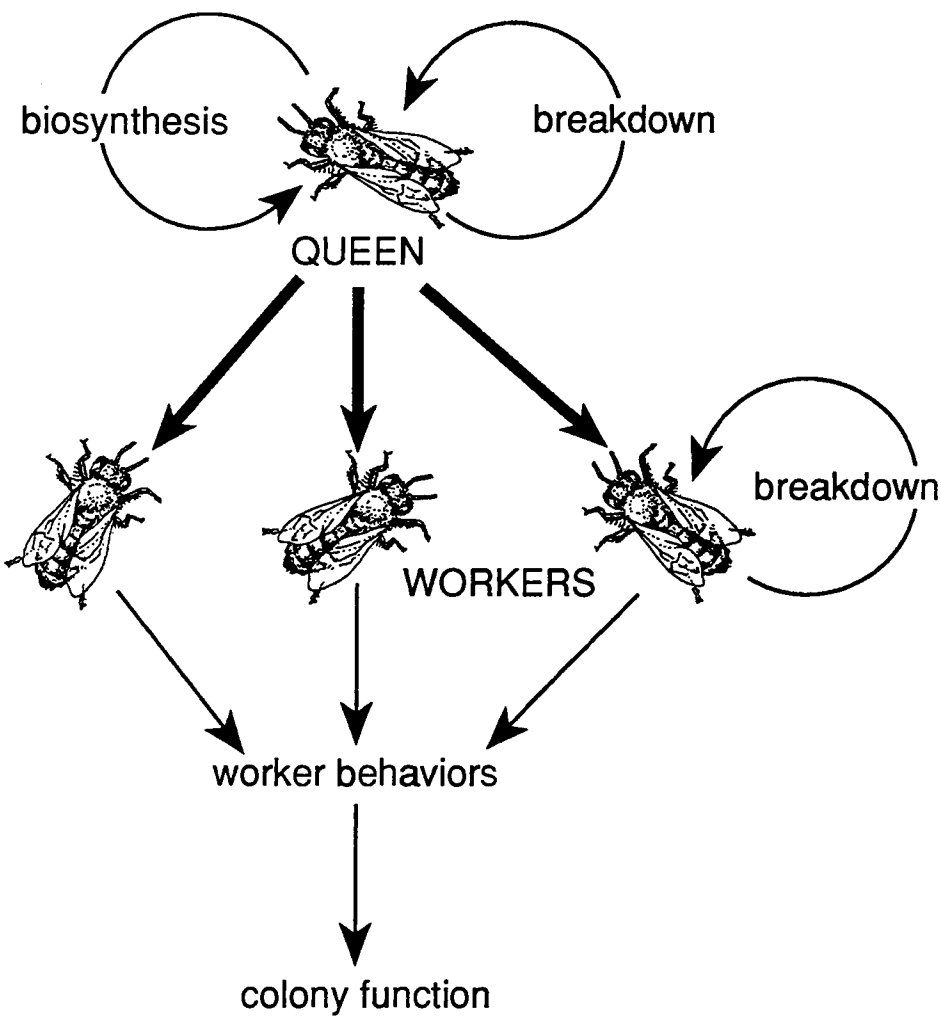
Fig. 1-1 shows the chain of events which lie between queen pheromone production and colony function. At present, very little is known about the modes of biosynthesis or breakdown of queen-produced chemicals. In fact, few such compounds have even been identified. As a result, this chapter will emphasize the nature of queen influence on colony function in the Hymenoptera, i.e. the net effects on workers of different queen semiochemicals, and the physiological mode of action of these chemical messages.

The Origin of Queen-produced Semiochemicals

Semiochemicals likely were originally utilized as defensive and warning compounds by the arthropod ancestors of the social insects, and their communication function evolved secondarily. An examination of the defensive compounds of non-social arthropods shows many compounds identical to those of social insects. The primary defensive compound of the opolionid arthropod *Leiobunum formosum* is 4-methyl-3-heptanone, also an alarm pheromone of several

Figure 1-1

The chain of events lying between queen pheromone production and/or behavior, and colony function. The solid lines indicate the area which is the focus of this study.


biosynthesis
breakdown
QUEEN
breakdown
WORKERS
worker behaviors
colony function

species of myrmicine ants, where it also may have a defensive role (Blum and Brand, 1972). This suggests that the ability to synthesize such compounds was present in arthropod stock before eusociality arose. The biogenesis of new compounds may only have required single mutations for new enzymes which could extend existing biosynthetic pathways. In some groups, sex pheromones may have been coopted for use as social pheromones as well, and utilized to establish the dominant position of the fertile queen.

Social insects are capable of synthesizing many compounds. Over 30 compounds have been identified from the mandibular glands of honey bees (Callow et al., 1964), over 50 volatiles have been isolated from the Dufours gland of the carpenter ant *Camponotus ligniperda* (Bergström and Löfqvist, 1971), and over 30 compounds from the mandibular gland of the weaver ant *Oecophylla longinoda* (Bradshaw et al., 1975). Many such exocrine products appear to have no effects on behavior, and an active compound in one species may not be active in another. In some species, eg. *Apis mellifera* (Slessor et al., 1988), a combination of several compounds is required to elicit a full behavioral response. In fact, most insect semiochemicals have proven to be complex mixtures (Hölldobler and Carlin, 1987; Silverstein and Young, 1976). Many pheromones have multiple functions, depending on the behavioral context in which they are secreted, and as a result, it can be difficult to exclusively assign primer and releaser functions. Releaser

compounds elicit a rapid behavioral response such as attraction, and primers trigger an intermediary mechanism, eg. the inhibition of ovary development. A single response by individuals may be brought about by the products of several glands, eg. the mandibular gland pheromone and a tergite gland pheromone are both involved in eliciting a retinue response from honey bee workers (Vethuis, 1970). Pheromonal parsimony, the use of one pheromone for several functions, is widespread among the Hymenoptera (Blum and Brand, 1972; Free, 1987). Certain compounds also show widespread use across Hymenopteran taxa. For example, 2-heptanone acts as an alarm substance in the Myrmicine and Dolichoderine ants, *Trigona* bees (Blum and Brand, 1972) and the honey bees, *Apis* (Shearer and Boch, 1965).

Little is known about the production of colony organizing social chemicals, either in terms of biosynthetic pathways or synthetic control. Research on the latter topic has progressed mainly on the sex pheromones of nonsocial insects. Experiments on several species of moths and cockroaches suggest that production of male attractants by virgin females is under endocrine control in some species but not in others. Barth (1965) hypothesized that endocrine control might be expected in species which are long-lived, and which have repeated reproductive cycles in which there are periods when mating is not possible. Corpora allata control of sex pheromone production has been reported from cockroaches (in Barth, 1965). In the corn earworm,

Heliothis zea, control of pheromone production is via a peptide produced in the brain, and the corpora allata do not work directly in that function (Raina and Klun, 1984). It is difficult to speculate on the factors controlling pheromone production in mated social insect queens, but it seems unlikely that a sensitive system of endocrinal mediation would be required because the inhibitory chemicals are apparently produced constantly throughout the lifetime of the individual. Rather, switching on of genes which code for pheromone-building enzymes may occur during the development of queen-destined larvae.

Queen-produced pheromones can be grouped into two categories, primer and releaser pheromones. The former, which act indirectly by affecting the neuroendocrine system, are the most germane to this discussion because they maintain the distinctions between worker and queen functions and serve to maintain colony harmony. However, releaser pheromones, which rapidly elicit a behavioral response, can also be important, for example, in stimulating retinue behavior which aids in the dispersal of queen substances to the workers.

Queen-produced sociochemicals function in at least five major roles within the nest. They can act as individual and nestmate recognition signals, attract workers to the queen where they can acquire the same or other compounds, inhibit worker or nestmate reproduction, control sexual production, and stimulate workers to perform tasks associated with

colony growth and survival. They also may have some influence on worker temporal caste polyethism.

Queens, Semiochemicals, and the Integration of Colony Functions

Wasps

Social wasp species cover the range of eusocial patterns but share the common pattern of a single female (or several in the case of the swarm-founding Polistinae) that dominates egg-laying in the colony. In the less social groups, aggressive or ritualized behaviors are the most important factor in achieving and maintaining this position, but evidence is accumulating that the more highly social wasps use an integrated system of behavioral and chemical dominance.

Dominant females of the primitively social wasp *Parischnogaster jacobsoni* (Stenogastrininae) usually have the largest ovaries in their colonies and direct the most domination acts towards subordinate females with the largest ovaries (Turillazzi, 1988). It is unknown if these acts inhibit the reproductive potential of the subordinates.

Sphecid wasps of the subfamily Pemphridoninae use nest construction chemicals to mark nest entrances. Such markers are seen, however, both in species where foundresses can be considered solitary, and those with true queens (Wcislo,

1990). There are no reports from that group, of chemicals which are distinctive to the dominant females in a nest.

Wasps of the family Vespidae have been well-studied in terms of reproductive division of labor because of their apparently intermediate position in the evolution of sociality. In the primitively eusocial, independent-founding Polistinae, such as the genus *Polistes*, females are usually arranged in some sort of dominance hierarchy, with a queen at the top. The queen has the most developed ovaries and is the pre-eminent or only egg layer in the nest. Control over reproduction by subordinate females and workers in the colony is achieved by aggressive behaviors, selective eating of worker-laid eggs, a flow of food which favors queens over workers, and the tendency for queens to oviposit rapidly in empty cells (reviewed in Fletcher and Ross, 1985; Jeanne, 1980). The result is that subordinate females show regressed ovaries and a tendency to perform non-reproductive tasks such as brood care and foraging. In the event of queen loss, one of the most dominant subordinates will take her place. Queen removal from nests of *P. fuscatus* depresses the worker activity level and causes episodes of worker activity to become less synchronized (Reeve and Gamboa, 1983), underscoring the queen's role as the coordinator of colony activity. Queens show a high level of activity in that species, frequent interactions with nestmates, and participation in activities other than egg-laying, a suite of behaviors commonly seen among queens of

the more primitively-social species. A cooled, inactive queen was ineffective at stimulating colony activities, suggesting that her behaviors and not a pheromone are the cause of such stimulation.

In the swarm-founding Polistinae, queen removal leads to aggression among young females, and within several days some individuals will mate and begin to lay eggs. A substance produced on the head of *Metapolybia* queens allows workers to recognize queens and may act to suppress worker ovary development, either by directly affecting the young workers or by stimulating older workers to act aggressively towards younger nestmates. Indirect control of worker reproduction by queens has also been reported in *Polybia*, *Protopolybia*, and *Stelopolybia* species (reviewed by Jeanne, 1980).

Queens of the highly eusocial wasps rarely show aggression towards their own workers, but queen removal in colonies of *Vespa* species can lead to an increase in worker - worker aggression, and the formation of new egg layers. When the queen is present, workers will surround her in a retinue and lick her body, especially the head and mouthparts (Ishay et al., 1965). Presumably the workers are gathering a pheromone by this act. Queens of *Vespa orientalis* have been found to contain approximately 6 μg of 9-n-hexadecalactone. This compound functions as a queen recognition pheromone and stimulates workers to construct queen cells (Ikan et al., 1969). Removal of *Vespula* and *Dolichovespula* queens also leads to worker aggression,

worker-laid eggs, and an interruption in foraging and nest constuction. Akre and Reed (1983) found strong evidence for a queen-produced pheromone in *Vespula* that inhibits worker ovary development and stimulates foraging. It is apparently not transferred by licking the queen (Akre et al., 1976), or through the air (Landolt et al., 1977), although in some *Vespula* species the queen is perceived from a distance by the workers (Akre et al., 1982). Limited contact of workers with the queen (queens may have a slightly repellent effect) and infrequent retinue behavior suggests that workers do not act as agents of pheromone dispersal (Akre and Reed, 1983), although the queen may deposit pheromone on the comb during her various activities. Spradbery (1973) suggested that a queen pheromone may be released during oviposition. Another potential route of transfer is in queen feces which are eaten by workers and passed among them by trophallaxis (Akre et al, 1976). There is evidence for a correlation between ovary development and the queen's inhibitory effect (Marchal, 1896).

A number of wasp species exist as social parasites which invade established nests of another social species, and usurp the position of the queen. The usurpers must establish reproductive dominance. Invading females of *Dolichovespula arctica* achieve control by acting very aggresively towards their *D. arenaria* hosts (Greene et al., 1978). Parasitic *Vespula austriaca* females will initially kill the queens of attacked *V. acadia* nests. Thereafter

they inhibit host worker reproduction by what may be an interplay of behavioral and chemical methods (Reed and Akre, 1983). Parasites were observed to perform bouts of gastral dragging in which products of the Dufour's or sternal gland may be directly applied to the comb.

Primitively Eusocial Bees

a) **Sweat Bees** (Halictidae)

In the primitively eusocial bee *Lasioglossum zephyrum.* queens have high levels of activity, are behaviorally less varied than workers, and show behavioral specializations, particularly in the frequency of nudging, and of backing other adult females down to the lower parts of the nest burrows (Mitchener and Brothers, 1974). Queens often sit in locations where they can potentially influence the behavior of nestmates, and actively direct incoming pollen foragers to specific brood cells (Buckle, 1982). These specific aggressive behaviors lead to dominance in egg laying. The queens also occasionally eat worker-layed eggs. In queenright colonies, few, if any, workers will mate while out foraging, indicating that queen inhibition extends to govern worker behaviors outside of the nest (Greenberg and Buckle, 1981). Removal of *L. zephyrum* queens causes a decrease in worker interactions and approximately a one third decrease in colony activity (Breed and Gamboa, 1977).

A queen pheromone may not be necessary in the small colonies of this species; the queen likely is able to coordinate activities by personally influencing the behavior of all of her nestmates.

Nesting queens of *L. figueresi* also mark nest entrances. The source of these chemicals has not been reported but may be associated with the mouthparts (Wcislo, 1990).

There is indirect evidence for a pheromonal component to ovarian regulation in overwintering nests of the Australian species *Exoneura bicolor*. These cues appear to be volatile, and to be produced primarily by inseminated females with large ovaries (O'Keefe and Schwarz, 1990). This pheromone results in reproductive differentiation, whereby the pheromone producers dominate egg laying.

b) **Bumble Bees** (Bombinae) **and Orchid Bees** (Euglossinae)

Bumble bees and orchid bees also have a somewhat intermediate level of social organization, and show examples of both behavioral and chemical 'dominance' by queens. In the orchid bee *Eulaema*, reproductive dominance is achieved through agonistic behavior (Free, 1961; Katayama, 1971; Zucchi et al., 1969), but in *Bombus* reproductive inhibition is achieved via chemicals produced by the queen (Van Honk et al., 1980).

Bombus species produce annual nests that are initiated in the spring by a single, mated foundress. She will

construct and provision the first cells, and care for the first cohort of developing workers. Thereafter, she will cease to forage. Sometime after colony establishment, a number of workers begin to interfere with the queen's oviposition, and later begin to lay their own eggs (van Honk et al., 1980), although the queen eats most of these (van Doorn and Heringa, 1986). The usurpers are usually members of the oldest category of workers (van Honk et al., 1981b).

The mandibular glands of mated *B. terrestris* queens produce a pheromone which prevents or delays worker oviposition (van Honk et al., 1980), and which remains active on the body for only one day after she is killed (Röseler et al., 1981). There is also evidence for a mandibular gland sex pheromone in several species of *Bombus* (van Honk et al., 1978). Extirpation of the mandibular gland does not completely eliminate the ability to inhibit worker ovary development, and inhibition of worker oviposition may also be due to pheromones from other sources and/or behaviors. It is not clear why queen inhibition of worker egg laying breaks down through the season. In *B. impatiens*, queens are, for their size, no more dominant than 23 day old workers (Pomeroy and Plowright, 1990). There may be a "dominance titre" that could be received either from many subordinate bees or a few dominant ones. In this way, the increasing density of workers on the comb could begin to affect the queen's position, even before the appearance of true dominance by some workers. Dominant workers seek

rather than avoid the queen. Pomeroy and Plowright (1990) suggested that these workers may be able to overcome the "dominance" pheromone of the queen by being able to saturate their receptors to the low volatility, queen-produced chemicals. This implies that such workers actively seek out the queen pheromone in order that their receptors become saturated.

Females of the genus *Psithyrus* are able to parasitize colonies of *Bombus* and to prevent worker ovipositions for some time by displaying agonistic behaviors. The parasites do not appear to produce inhibitory pheromones (van Honk et al., 1981a). Like *Bombus* queens, the parasites eventually lose their inhibitory powers, and older *Bombus* workers begin to lay eggs. The successful, if temporary, inhibition of worker egg-laying by agonistic behavior alone is a reminder of the intermediate social organization of bumblebee colonies.

Retinue behavior, in which nearby workers antennate and lick the queen, has been observed in *B. terrestris* (van Doorn and Crambach, 1989) and *B. ignitus* (Katayama, 1971). All emerging workers attend her during the time that the queen is active, but retinue behavior is performed most often by future laying workers. Such individuals are the most active in the nest, and have the highest probability of coming into contact with the queen, and of thus becoming attracted. The apparent paradox between frequent queen

contact and lack of ovary inhibition in these particular individuals has not been satisfactorily explained.

Greenwood and McFarlane (1990) reported a pheromone of low volatility which is deposited on the larvae of *Bombus* species. It is associated with the unesterified long-chain fatty acid fraction, is readily oxidizable, and is detected by the antennae of adults. It has a "calming influence" on the queen and nearby workers and presumably stimulates them to remain in the area and incubate. It is not known if it is a queen pheromone, but it is not produced by the brood. It is also species-specific.

The Highly Eusocial Bees

a)**Stingless bees** (Meliponinae, including tribes Trigonini and Meloponini)

Highly social species of stingless bees usually produce perennial colonies headed by a single, egg-laying queen. There are also a number of polygynous species. Workers are normally obligate participants in nest construction, brood rearing and foraging. However, many will occasionally lay eggs, even in the presence of the queen (Sakagami et al., 1963). Only queens can produce viable worker and queen progeny because unfertilized, worker-laid eggs produce only males. Gynes are always reared within normal brood cycles, rather than being restricted to certain times of the year,

or after queen loss. In fact, queen replacement after loss requires the presence of either emerged virgins or sealed royal cells among the developing brood. Young queens either supersede the existing queen, assume a queenless colony nearby, draw some of the workers away in a swarm, or are killed by the workers.

Several queen exocrine glands may play a role in queen attractiveness to workers, and physogastric (egg-laying) queens draw either a temporary or almost permanent retinue, depending on the species. It is believed that pheromones are released from glands on the abdominal tergites, and frequent wing beats by the queen as she drags her rear legs over the back of her abdomen suggest that at least some of the components are volatile. Workers do not normally lick the queen, but when they do it usually indicates great excitement. Gyne mandibular glands also are well developed, reaching their highest point in young queens that are attempting a supersedure. Workers appear to 'choose' either the challenger or the old queen in such a contest, and the strength or quality of mandibular gland and/or tergite gland secretions may play a role in the outcome. In *Paratrigona subnuda*, if the old queen loses the contest, she is dismembered by the workers, beginning with the excision of the abdominal tergite glands (Imperatriz-Fonseca, 1978). Truly polygynous species also show control of queen number. Possibly workers attempt to maintain colony-wide pheromone

levels within some optimal range by executing old queens or accepting new ones.

Worker responses to virgin queens usually begin several days after queen emergence, presumably after the maturation of pheromone producing glands. Virgins that survive worker attacks are either more attractive than others in their cohort, or spend time in peripheral areas of the nest or in 'safe cells' constructed or usurped for that purpose. While the increasing attractiveness of accepted gynes is believed to result from products of the tergite glands, drops of yellow liquid are sometimes also offered from the mouths of such individuals, and stimulate the workers to supply them with abundant food (Engels and Imperatriz-Fonseca, 1990).

Mated queens of stingless bees do not appear to inhibit worker fertility or the production of sexuals. Workers are most likely to lay eggs during their tenure as nurse bees (i.e. when young adults)(Engels and Engels, 1977), and frequently place eggs in cells in which the queen has already oviposited (Sakagami et al., 1965). Workers also may produce trophic eggs which are fed to the queen, and which may serve to appease and/or distract her attention from a cell in which a worker has deposited its reproductive egg (Engels and Imperatriz-Fonseca, 1990). In the absence of the queen, irregularities arise in worker reproduction because cell construction and the provisioning of brood cells occurs improperly. Worker fertility is not enhanced, and virgin queens from nearby may enter such nests (Engels

and Imperatriz-Fonseca, 1990). The stingless bees are therefore unusual in that queen pheromones are required to maintain a nest homeostasis in which some level of worker reproduction is ensured.

b) **Honey bees** (Apidae)

Honey bee (*Apis*) queens show distinct physical and physiological differences from workers, characterized by the presence of well developed ovaries, and a spermatheca for storing the semen of several drones. Workers do possess functional ovaries, but the lack of any sort of mating precludes workers from laying anything other than haploid drone eggs. An exception is seen in *A. mellifera capensis*, which will be discussed later. Worker ovaries are usually suppressed from developing to the point where even such unfertilized eggs are not produced. The brood may be mainly responsible for this inhibition (Jay, 1970), however removal of the queen also results in ovarian development in many of the workers, and some of them lay eggs. Queenless workers also differ from queenright ones in related physiological parameters such as vitellogen synthesis by the fat bodies (Engels and Imperatriz-Fonseca, 1990). The queen also influences the extent and timing of colony reproduction by influencing the production of new queens.

Queen mandibular gland pheromone is one of the more important and certainly the most intensively studied honey

bee pheromone. In workers, the mandibular glands normally produce components of brood food early in adult life, then switch to producing an alarm substance, 2-heptanone. The production of queen mandibular pheromone is believed to be mediated by a humoral factor(s), possibly secreted by the queens's brain (Sasaki et al., 1989). It inhibits the production of new queens (Butler, 1954; Butler and Simpson, 1958; Butler et al., 1961), attracts workers during swarming (Butler and Simpson, 1967; Velthuis and Van Es, 1964) and in the nest (Gary, 1961; Zmarlicki and Morse, 1964), and has a stimulatory affect on pollen foraging and food hoarding (Free and Williams, 1974), comb building and brood rearing (Free, 1987). The within-nest attraction produces a retinue of dynamic composition, which almost always surrounds the queen. This attraction has been used in a bioassay that resulted in the identification of this pheromone, the only pheromone of a social insect, with primer functions, which has so far been identified. Slessor et al. (1988) found that the full retinue response could be elicited by a blend of 5 compounds found in the mandibular glands of mated queens: 9-keto-2(E)-decenoic acid (9-ODA)(150 μg), 9-hydroxy-2(E)-decenoic acid (71% R(-), 29% S(+), 55 μg), methyl p-hydroxybenzoate (13 μg), and 4-hydroxy-3-methoxyphenylethanol (1.5 μg)(the μg values are the means found in the mandibular glands of an average egg-laying queen). The synthetic blend also is attractive to swarms (Winston et al., 1989), stimulates pollen foraging (Higo et

al., 1992), inhibits queen rearing (Winston et al., 1989; 1990; 1991), attracts foragers (R. W. Currie pers. comm.), and calms queenless workers (Chapter 5). Butler and Fairey (1963) and Butler et al. (1961) implicated mandibular gland pheromone in the inhibition of worker ovary development, but Willis et al. (1991) found that the synthetic blend had no such affect. Pain (1961) similarly found that 9-ODA did not inhibit worker ovary development. It may be that pheromones produced by the brood are more important in inhibiting worker reproduction than QMP. or other queen produced pheromones. The 9-HDA component of the QMP pheromone blend elicits workers at the nest entrance to release Nasanov pheromone, important for orientation to the nest (Fergusson and Free, 1981).

Virgin queens are as effective as mated queens at inhibiting colonies from rearing queens, but not from producing queen cell cups (precursors to queen cells) (Free et al., 1985). Colonies with virgin queens also forage as much as those with mated queens but collect less pollen. These differences may be explained by differences between the two types of queens in mandibular gland products or their quantities. Slessor et al. (1990) found that the mandibular glands of mated queens contained significantly enhanced levels of the aromatic constituents HOB and HVA over those in virgins. Colonies headed by virgin queens may also collect less pollen because they contain smaller

amounts of brood; brood being known to stimulate pollen collection.

The tergite glands, located dorsally on the abdomen (Renner and Baumann, 1964), produce an unidentified pheromone which is attractive to workers, and likely acts to supplement the mandibular gland pheromone in releasing retinue behavior (Vierling and Renner, 1977). Velthuis (1970) reported that queens without mandibular glands affected retinue behavior and worker ovary development, and suggested that the cause may have been a pheromone produced on the abdomen.

Queen footprints have been shown to inhibit the production of queen cups, precursors to queen rearing (Lensky and Slabezki, 1981), working synergistically with mandibular gland pheromone. The source of the footprints may be the queen tarsal glands, located in the sixth tarsomere of each leg (Lensky et al., 1985). These glands produce an oily exudate. While tarsal gland products have not yet been identified, mandibular gland pheromone is distributed throughout the colony in queen footprints (Chapters 2 and 4).

All colonies of honey bees are able to recognize the queen of their own colony. To date, the source of these discriminating factors have not been determined. It is likely that the source is a combination of a distinctive blend of queen-produced chemicals (Crewe, 1988), elements of the cuticlar wax (Getz et al., 1989), and materials

deposited in and reacquired from the comb wax (Breed et al., 1988).

The feces of virgin queens contains o-Aminoacetophenone, which repels workers and stimulates them to groom (Page et al., 1988; Post et al., 1987). The feces of workers or of virgins older than two weeks elicits no such reaction. The authors suggested that the fecal pheromone may be important in inhibiting rejection by workers of queens by disrupting agonistic behaviors of workers, or that workers may use contamination of a queen with feces as a symbolic determinant of subordination or dominance. In either case, a smooth transition from an old to a new queen is facilitated, and the likelihood of killing a viable new queen is diminished.

The Koschevnikov gland, near the sting base, has been reported to have an attractive effect on queenless bees (Butler and Simpson, 1965).

Groups of honey bees show endogenous circadian rhythms of metabolic activity. Southwick and Moritz (1987) found that workers, entrained to different photoperiods, rapidly coordinate individual circadian oscillations to an overall group oscillation. It was suggested that the control of this oscillating system may be the result of physical interactions between workers (trophallaxis), or a non-volatile contact pheromone. There have been no experiments to investigate the effects of candidate compounds, such as

queen mandibular gland pheromone, on worker metabolic patterns.

Other *Apis* species, eg, *A. dorsata, A. florea, A. cerana*, show individual behaviors similar to those of *Apis mellifera*, and it is likely that many queen pheromones are similar within the genus. 9-ODA has been found in the mandibular glands of queens, and is attractive to workers, of all three Asian species (Akranatakul, 1977; Koeniger and Koeniger, 1980). Butler (1966) reported that extracts of *A. florea* and *A. cerana* queens could partially inhibit queen rearing in small groups of *A. mellifera* workers.

An unusual case of colony organization is seen in the southern African honey bee race *Apis mellifera capensis*, where unmated workers are able to produce female offspring parthenogenically. When such colonies lose their queens, a number of workers are able to quickly begin oviposition, and *A. m. capensis* workers that have drifted into colonies of other races have a great advantage in becoming laying workers after queen loss. Interestingly, queenless *A. m. capensis* workers differ markedly from those of other races in that mandibular gland secretions are dominated by the presence of 9-ODA, the major component of queen mandibular gland pheromone (Crewe, 1988). This compound (and the ratio of other compounds found in *capensis* workers) is only occassionally found in small quantities in workers of other races, even laying workers. 9-ODA production rises as *capensis* workers age, until at 6 days, the bees produce a

signal which is qualitatively similar to that of queens. Emerging virgin queens of this species also show unusually high 9-ODA levels at an early age, possibly because it allows them to establish some form of 'chemical dominance' over a work force which is able to produce its own queen-like signal (Crewe, 1982), a chemical arms race. Such abilities on the part of *capensis* workers are not without a cost at the colony level however, since having too many dominant individuals (more developed ovaries, greater 9-ODA production, earlier oviposition, greater egg laying) decreases queenright colony performance as measured in effectiveness in brood rearing, comb building and hoarding (Hillesheim et al., 1989).

Brood-produced pheromones act in conjunction with queen pheromones in a number of contexts. The presence of worker brood inhibits worker ovary development (Jay, 1970), and stimulates pollen collection (Free, 1967).

Ants

Ant species show a great diversity of colony sizes and lifestyles, and have achieved a high degree of social cooperation. Many special cases of colony organization are found in the ants, including polygynous nests, species without a true queen caste, and slave making.

The attractiveness of queens to their workers has been reported from diverse groups of ants including the

Myrmicinae, *Solenopsis* (Jouvenaz et al., 1974; Vander Meer et al., 1980), *Myrmica rubra* (Brian, 1973), *Pheidole* (Stumper, 1956), *Erebomyra* (Wilson, 1986); the Formicinae, *Lasius* (Stumper, 1956) and the weaver ants *Oecophylla* (Hölldobler and Wilson, 1983); the Ecitoninae, six species of army ants (Watkins and Cole, 1966); and the Ponerinae, the cryptobiotic ant *Prionopelta* (Hölldobler and Wilson, 1986). In *Myrmica rubra*, the mandibular glands produce an attractive volatile (Mamsch, 1967), and a non-volatile attractant is produced on the abdomen (Brian, 1973). The low-volatility compounds remain active for 2 - 3 days after queen death (Cogliotore and Cammaerts, 1981). Queens of the fire ant *Solenopsis invicta* are also known to store substances in the poison sac, including pyranones and dihydroactinidiolide, which are attractive to workers (Glancey, 1986; Vander Meer et al., 1980). These substances are probably not produced in the associated Dufours gland because it is degenerated in queens. Neocembrene acts as a queen-produced attractant in the pharaoh's ant, *Monomorium* (Myrmicinae) (Edwards and Chambers, 1984). Attraction described as retinue behavior has been reported for the army ant genera *Eciton* and *Neivamyrmex* (Rettenmeyer et al., 1978). The source of this attraction in the latter group is the head.

Queen dominance in egg-laying is achieved by different mechanisms in various groups because of the differential ability of workers to lay eggs, but in almost all cases it

is pheromonally based. In some genera, such as *Leptothorax (Myrmicinae)* (Bier, 1954) and *Cataglyphis* (Formicinae) (Cagniant, 1982), workers are able to produce viable eggs, but are inhibited from ovipositing by the presence of the mated queen. There is also evidence for such an inhibitory queen pheromone in *Crematogaster* (Myrmicinae) (Delage-Darchen, 1974). In many ants, the queen stimulates the workers to produce non-reproductive trophic eggs instead of preventing worker oviposition altogether, eg. *Plagiolepus pygmaea* (Formicinae) (Passera, 1965), *Myrmica rubra* (Brian and Rygby, 1975), the weaver ants *Oecophylla longinoda* and *O. smaragdina* (Hölldobler and Wilson, 1983), and *Temnothorax recedens* (Dejean and Passera, 1974). The suppression of fertile egg laying in the weaver ants is mediated by pheromones which persist in the corpses of queens for as long as 6 months (Hölldobler and Wilson, 1983). The degree of queen inhibition of worker oogenesis in *Myrmica rubra*, decreases with time (Brian, et al., 1981), althought this may be due to pheromone transfer being impeded in populous colonies later in the season. In the genera *Solenopsis, Pheidole, and Trematorium* (all Myrmicinae) workers are physically incapable of producing eggs. In *S. invicta* it is virgin queens in the nest that are inibited from becoming reproductive.

Fletcher and Blum (1981) reported that the laying queen in colonies of *Solenopsis invicta* inhibits virgin queen dealation (wing loss) and oviposition by means of a non-

volatile primer pheromone produced in her mandibular glands, and even dealated virgins are capable of preventing other virgins from dealating (Fletcher et al., 1983). Removal of the queen's influence leads to the histolysis of alary muscles and to oviposition, but many of the layed eggs are large and inviable (trophic eggs). The queen pheromone acts directly on virgin queens, and not as was previously thought, by somehow causing workers to prevent the virgins from dropping their wings (Fletcher and Blum, 1983). Workers respond to greater than usual amounts of the dealation inhibitor by executing supernumerary queens until the pheromone level is reduced (Fletcher, 1986).

There are differences between various categories of *S. invicta* queens in inhibiting dealation by virgins. In monogynous colonies, highly fecund (high weight) queens have greater inhibitory capacity than low weight ones. In polygynous colonies, inhibition also is related to fertility of individual queens; queens that lay few eggs, overwintered virgins, and sexually mature, spring-reared virgins do not produce enough inhibitory pheromones for it to be detected by a dealation bioassay (Willer and Fletcher, 1986). Such control over virgins has not been reported in other ant species but Fletcher and Blum (1983a) speculate that it may be common in the Myrmicinae.

Queen pheromone-based inhibition of worker reproduction in the slave-making ant *Harpagoxenus sublaevis* (Myrmicinae) is augmented by the behaviors of a subset of dominant

workers (Bourke, 1988). Dominance in these workers is correlated with ovarian development, frequency of trophallaxis, length of time spent in the nest, but not in body size. These workers aggressively compete for egg-laying rights, consume extra food for egg development, and safeguard their reproductive futures by avoiding risks outside of the nest. By inhibiting the reproduction of other nestmates, it seems likely that they are not directly acting in the interest of the queen, but towards their own potential contribution towards male parentage. Competitive dominance hierarchies among workers have also been reported in *Leptothorax* (see Bourke, 1982).

In ants, as in honey bees, all female eggs are totipotent with respect to their ability to yield a gyne or a worker. It is the food and care given by the nursing workers that determines the ultimate fate of each eclosing larva. Many, or most, ant colonies pass through a stage when they do not produce sexuals, and queen pheromones are likely important in the mechanisms that control the timing and production of new queens and males. By controlling the production of new queens, the extant colony mother prevents the production of potential contenders for her position. When production of such individuals does occur, it often is at a time of high worker:queen ratios, suggesting that in large colonies, queen control over worker behavior is more difficult. Perhaps, an inadequate inhibitory signal reaches all of the workers in such a situation.

Queens of *M. rubra* appear to produce both substances that stimulate and suppress larval development. An active, volatile mandibular gland pheromone of some *M. rubra* queens can have a stimulatory affect, increasing larval growth rate (Brian and Hibble, 1963). Carr (1962) showed that dead queens, if replaced regularly, are effective at suppressing the growth of gyne larvae. She concluded that the effective compounds were volatile and scarce. Mamsch (1967) concluded that such compounds do not exist. Brian and Blum (1969) reported that fatty acid extracts of *M. rubra* heads depressed larval growth rate and reduced survival, and that the extracts act directly through the larval cuticle. The inhibitory chemicals are diffused over the body of the queen, are strongest in the spring, and are elutable in ether (Brian et al., 1981). Substances from the queen's head also can cause workers to attack potential gyne larvae at a critical stage of development, scarring them, and causing them to develop into workers (Brian, 1970). It is possible that substances which stimulate at low concentrations become suppressors at higher levels. Alternatively, the stimulatory and suppressive roles of the queen may depend upon different mechanisms and employ different semiochemicals; the former a nonvolatile from the mandibular glands, the second a volatile from the head (Brian and Hibble, 1963). Elmes and Wardlaw (1983) studied 6 species of *Myrmica* and found that, in all the species, queens suppress the development of gynes and tend to

decrease the time between the onset of growth and pupation. Larvae of any of the species can also be suppressed by the queens and workers of any of the others, suggesting common, or at least similar inhibitory chemicals within the group.

There also is evidence for a non-volatile pheromone that inhibits differentiation of queen larvae in *Pagiolepus pygmaea* (Passera, 1980). A pheromone of the fire ant, *S. invicta*, inhibits queen rearing by workers. Vargo (1986) found that abdomens of dead, dealate queens were inhibitory for 10 days while heads and thoraces were active for only three, and therefore suggested that the pheromone originates in the abdomen. The pheromone may, however, be produced elsewhere, and then translocated over the body surface. The abdomen presents the greatest surface area and thus can carry the greatest amount of pheromone. The timing and appearance of sexual forms following colony division also suggests that queen control may be pheromonally mediated and inhibits the growth of sexuals late in larval development (Vargo and Fletcher, 1986). Once individual larvae develop beyond a certain point, they are no longer subject to queen control of development. In *Monomorium pharaonis* (Myrmicinae), removal of queens results in the production of both male and female sexuals from existing queen-layed eggs (Petersen-Braun, 1975). Otherwise this occurs only during normal cycles of repoduction. Berndt (1977) presented evidence that the queen's inhibitory effect is due to a chemical that can be washed from the body with organic

solvents. Edwards (1987) found, however, that queen-layed eggs are the source of inhibition. Dead queens and virgin queens show no effect on stopping sexual production. When eggs are plentiful (i.e. when queens are fecund), only workers are reared, but when egg-laying declines, workers perceive the change, and respond by rearing sexuals. Such a mechanism requires that either workers can gauge the relative abundance of eggs (as Edwards believed), or that workers perceive a pheromone deposited on, or released by, the eggs.

Differences in the abilities of individual queens to inhibit sexual production have been reported for *M. rubra* and *M. scabrinodus* (Brian and Carr, 1960), *Monomorium phraonis* (Petersen-Braun, 1975), and *Leptothorax nylanderi* (Plateaux, 1971). Queens of the Argentine ant, *Iridomyrmex humulis* (Dolichoderinae), have an inhibitory effect on the production of both new queens and males. Part of the mechanism for this inhibition appears to be direct competition between queens and larvae for worker-produced trophic eggs. Male and queen larvae are both larger than worker larvae and therefore require more food (Bartels, 1988). Passera et al. (1988) suggested that there was no queen-produced pheromone involved in the inhibition of male production, but Bartels (1988) reported a frequent, stereotypical behavior, whereby queens smear eggs over young larvae. This is performed by all colony queens and may serve to apply a non-volatile pheromone.

Hölldobler and Wilson (1983) suggested that there may be a fundamental difference in the mechanisms of fertility regulation in monogynous and polygynous ant colonies. If a single queen is in control, the transfer of her inhibitory substances throughout the nest can ensure that the reproductive activities of workers or virgin queens can be regulated relatively simply. In polygynous colonies, however, several or many queens can lay eggs, and as far as is known, are not organized in a dominance hierarchy. Although queens of polygynous colonies produce attractants, these substances do not always function as primer pheromones in suppressing nestmate fertility. Bier (1958) hypothesized that the regulation of worker egg-laying in multiqueen colonies is not regulated by primer pheromones but by a directed flow of 'profertile substances' away from the workers and towards the queens. In contrast, Fletcher and Blum (1983b) suggested that the maintenance of monogyny in *S. invicta* colonies has the following components: i) workers are able to recognize individual queens via different proportions of pheromone constituents (Jouvenaz et al., 1974). ii) Queens produce other pheromones, such as inhibitory pheromones, and the quantities of these pheromones are maintained within optimal ranges. If the queen dies the levels fall, and the workers are stimulated to produce or accept a new queen. If the levels are too high due to supernumerary queens, some queens are executed (Fletcher, 1986). iii) Queens produce different amounts of a

pheromone complex and the amount is positively correlated with fecundity (Fletcher and Blum, 1983b). The poorest queens are recognized and are the first to be destroyed. Queens in polygynous colonies tend to have lower fecundities than those which lead a colony alone, and this may be indicative of lower pheromone production. More queens may therefore be present in a colony before the optimal range of pheromone titre in the colony is surpassed. Queen pheromone transfer in highly populous colonies may also be hindered enough so that not all virgins or developing larvae or workers are fully inhibited from becoming or raising queens. A similar system for controlling queen number also may operate in the Argentine ant, *Iridomyrmex humilis*. In the polygynous nests of that species, 90% of the overwintered queens are executed in May, at the beginning of the reproductive season (Keller et al., 1989). The reduction in the number of queens may decrease the level of pheromonal inhibition on the production of new sexuals, with the result that the differentiation of new male and female sexuals begins shortly thereafter.

The cuticular hydrocarbon profile can potentially act as a colony recognition cue if an ant can recognize small differences in the relative contents of the hydrocarbons. Most species with larger colonies are characterized by more or less homogeneous recognition signals derived at least in part from extrinsic cues (reviewed by Hölldobler and Carlin, 1987). Nestmate recognition in the ant *Lasius flavus*, for

example, originates with workers, not queens, and the basis of the recognition is genetic, not acquired (Anderson, 1970). Nestmate recognition signals in the acacia ant, *Pseudomyrmex ferruginea* (Pseudomyrmicinae) also are not produced directly by the reproductive females (Mintzer, 1982). In *Camponotus* on the other hand, the "colony visa" derives from the queen (Carlin and Hölldobler, 1983; 1988). Yamaoka and Kubo (1990) examined 45 species of ants, and found that the relative amounts of the different hydrocarbons on the workers lost there uniformity after removal of the queen, and the workers gradually lost their tendency to aggregate. Queen effect on colony odor and worker recognition also has been reported in *Myrmica* (Brian, 1986), and *Leptothorax* (Provost, 1986). When several queens are present in a nest, the ability of workers to discriminate nestmates often is diminished, perhaps because of the lack of a specific queen odor upon which they can cue. Polygynous colonies in *S. invicta* show much higher nest densities than monogynous forms, apparently resulting from failure of the nestmate recognition system. The result is the formation of inter-connected super colonies and the collapse of territorial boundaries (in Porter and Savignano, 1990). The above results suggest that the queen is frequently important in maintaining the colony-wide hydrocarbon profile. It is not known how this may be achieved, but nonvolatile, queen-produced compounds with distinctive component ratios may constantly be transmitted

throughout the nest, thereby contributing to a general colony odor.

The queens of slave-making ants are able to circumvent the nestmate recognition system, and/or to overcome the defences of their hosts. In *Harpagoxenus canadensis* (Myrmicinae) attacking queens either aggressively force challenging host workers from the nest or 'sneak' through the nest until a substantial part of the workforce can be adopted to raise her offspring (Stuart, 1984). Attacking queens of *Leptothorax kutteri* (Myrmicinae) and *Polygerus* (Formicinae) release a pheromone from the Dufours gland which causes host workers to attack each other. In the resulting confusion, the parasite queens gain access to the nest (Allies et al., 1986; Topoff, 1990). The *Polygerus* queens also make extensive contact with the host queen, after killing her. This behavior may enable them to assimilate the odors of the old queen, and thereby become more readily acceptable to the workforce (Topoff, 1990).

Temporal division of labor schedules may be under queen influence in some ant societies. Queen removal from colonies of the African species *Aphaenogaster subterranea* (Myrmicinae) causes young workers, which under normal conditions stay in the nest, to forage outside. A return to within-nest tasks follows queen reintroduction (Agbogba, 1989).

An unusual form of colony organization, seen among some species of Ponerine ants, is the apparent absence of a

distinct queen caste. Reproduction is carried out instead by mated laying workers, termed gamergates (Peeters and Crewe, 1984). Such species are, technically speaking, no longer eusocial, yet retain some elements of colony coordination on the part of the reproductive individual(s). In *Ophthalmopene berthoudi*, there can be several gamergates per nest, and there is no evidence for an inhibitory effect of the reproductively active individuals on the others (Peeters and Crewe, 1985). In nests with many gamergates, it also is unlikely that there is a strong influence of the odor of any one laying individual on worker recognition signals (Peeters, 1988). On the other hand, colonies of the queenless ant *Pachycondyla krugeri* contain only one gamergate, suggesting some sort of inhibition of egg-laying by nestmates (Wildman and Crewe, 1988).

Queen Pheromone Transmission

If there exists one common chemical thread which runs across the highly eusocial Hymenoptera taxa, it is the presence of one or several relatively nonvolatile, queen-produced pheromones, which act to attract workers, to sometimes inhibit worker reproduction and/or to inhibit the production of new reproductive individuals. Such nonvolatile chemicals must be physically transferred from the queen to the workers. In honey bees, *A. mellifera*, the queen mandibular gland pheromone is transferred throughout

the nest predominantly via serial physical contacts which begin with workers in the retinue contacting the queen (Seeley, 1979). Although material may be transferred as a result of trophallactic interactions, it is unlikely to be as a result of actual food exchange (Korst and Velthuis, 1982; van Erp, 1960). The comb also can act as a medium for transfer. The initial attraction of the pheromone serves to draw workers into the retinue where they are close enough to acquire the inhibitory message. Some sort of retinue behavior also has been reported from the other eusocial groups, but nothing specific is currently known of how queen pheromone transfer occurs outside of the honey bees. Sorensen et al. (1985) demonstrated, using radio-labelled protein, that physical contact, and trophallaxis could both be efficient modes of transmission for inhibitory pheromone in the fire ant, *S. invicta*. The diffusion of the volatile components of the mandibular gland secretion of major workers of the weaver ant *O. longinoda* has been quantitatively described (Bradshaw, 1981), and such methodology may prove applicable to volatile, queen-produced compounds. Chapters 2 through 4 examine specific aspects of the intra-nest transfer of honey bee queen manidibular gland pheromone.

The Mechanism by which Queen Pheromones Operate

Queen-produced chemicals can potentially act upon workers in two ways: via stimulation of antennal receptors, or by being carried to target organs by specific binding proteins. Some pheromones may work both ways. The components of honey bee queen mandibular gland pheromone are able to enter the hemolymph of workers within minutes after topical application (following chapters). There are also antennal receptor cells associated with the poroplates on the antennae of all three honey bee castes which are specialized for 9-ODA (Kaissling and Renner, 1968)(9-ODA is the most abundant component of the queen mandibular gland pheromone). Moritz and Crewe (1988), using metabolic activity as a bioassay, concluded that workers' reaction to 9-ODA is a result of olfaction, and not of contact perception, since offering samples of 9-ODA directly did not lead to an increased activity over odors of 9-ODA. The quantities they used, however (18-19 μl/bee/min), were approximately 100 times the amount found on the entire body surface of a mated queen (Slessor et al., 1988, and see following chapters). Metabolic activity may thus have been maximally stimulated by the lowest of the doses given. A review of the mechanisms of antennal information processing is given by Seabrook (1978), and of pheromone degradation on the antennae by Prestwich et al. (1989).

The translation of the queen's pheromone signal into worker reaction is currently becoming the focus of much attention. It appears that the maintenance of the dominance hierarchy in most, if not all, social insects, is related to the central role of juvenile hormone (JH) in controlling physiology and behavior. Generally speaking, the role of JH after metamorphosis is to ensure ovarian competence, and together with ecdysone, to enable the ovary to deposit yolk (reviewed by Scharrer, 1987). Queens influence the physiological and reproductive status of workers by pheromones that affect messages that are transmitted via the neuroendocrine system. In at least some species, in queenless conditions, the removal of pheromonal inhibition leads to the activation of otherwise quiescent physiological functions, and the formation of individuals of intermediate caste, i.e. with a worker body but more queen-like ovaries. In some species, the source of the reproductive inhibition may be pheromones produced by the larvae, or other individuals in the colony. Pheromonal inhibition could act at many places in the sequence of events which lead to egg production: the neurosecretory cells (NSC), the JH-producing corpora allata (CA), the production of the yolk protein vitellogenin, the deposition of yolk in the developing oocytes, or the competence of the ovaries themselves. Because the physiological processes which govern egg production in the social insects is not fully known, the inhibitory influence of socio-chemicals on these processes

also is only partially understood. In those species with the highest degree of social organization (honey bees and many ants), the canalization of early development constrains the level of 'queenliness' which mature workers may reach. In less rigidly organized societies (eg. wasps), workers are much more competent to become fully functioning queens in the event that inhibitory forces are removed.

The question of how queen pheromones achieve their releaser effects, that is the elicitation of specific worker behaviors, has not yet been answered. Presumably, pheromone perception by antennae send nerve impulses to the brain where other neurons, required for given behaviors, are stimulated. It has proven more rewarding to study the physiological basis of queen's long term effects on worker behavior, i.e. the mechanisms of the primer effects of queen phromones function.

Wasps

In the Polistine wasps, oogenesis is controlled by JH levels, and ovarian development corresponds with behavior (aggression) and social standing. In *Polistes gallicus* (colonies of which have a queen and several sterile auxilliaries), during the period of egg maturation (after winter), the volume of the JH-producing glands, the corpora allata (CA), is correlated with JH synthesis, and oogenesis is controlled by an elevated JH titre (Röseler et al., 1980). The CA are larger and more active in all dominant queens than in subordinates. The female that becomes dominant also has the highest titre of ecdysteroids in its hemolymph (Röseler et al., 1984). These compounds originate in the follicular cells of the ovary and are released into the hemolymph during oogenesis. Removing the ovaries does not cause loss of dominance (Deleurance, 1948; Röseler et al., 1985), but does result in the loss of ability to inhibit egg-formation by subordinates (Röseler and Röseler, 1989). Topical application of JH to subordinates leads to egg maturation and aggressive behaviors in *P. annularis* (Barth et al., 1975), dominance in *P. gallicus* (Röseler et al., 1984), and ovary development in diapausing queens of *P. metricus* (Bohm, 1972). It is thus not the development of potential target organs, the ovaries and their products, that leads to aggressive behavior and dominance, but some other character of the individual queens which causes high

CA activity, and subsequent aggression, dominance, and ovarial activity. The role of ecdysteroids in the hemolymph of adults remains unclear. Workers and future foundresses of *P. exclemans* cannot be distinguished by external morphology but differ in relative development of the fat body, survival at cold temperatures (i.e. ability to overwinter)(Strassmann et al., 1984), amount of body water, and metabolic rate associated with changing temperatures (Solis, 1990). It seems likely that regulation of JH occurs at the site of synthesis rather than by breakdown.

Halictidae

Juvenile hormone titres change in *Lasioglossum* workers without queens, JH application leads to egg maturation in subordinates, and protein ingestion triggers JH secretion (Bell, 1973). The queen may either be able to behaviorally affect worker protein intake, or may use a separate behavioral and/or chemical mechanism to supress worker CA activity.

Bumblebees

In *Bombus terrestris*, queen mandibular gland pheromone inhibits worker CA activity (Röseler et al, 1981). Oogenesis is slowed, but not completely supressed (van Honk et al., 1980). Queenless workers, on the other hand, show

elevated JH levels, have a 5 fold higher rate of RNA synthesis in their trophocytes relative to queenright workers (Röseler, 1974), and are able to produce eggs as their JH titres rise (Röseler, 1977). If JH I is injected into queenright workers, oogenesis occurs the same as for queenless workers (Röseler, 1977). Clearly, the inhibitory affect of the queen acts by affecting JH titres in the workers. As in the wasps, ovarian activity in *B. terrestris* is a consequence of dominance rather than a cause, and task allocation is not dictated by worker ovarian activity (van Doorn, 1989, 1990).

Bumblebees also are similar to wasps in that the JH titre is determined by production, not by breakdown. Röseler and Röseler (1978) found that breakdown of JH takes place in the hindgut (the JH ester was completely degraded to JH acid and JH diol), and that breakdown in the hemolymph occurs at a low rate. Also, the CA of workers remain active after injection of JH I. Because the CA continue to be active for several days, the authors concluded that CA activity is not maintained directly by nervous impulses, but by a neurosecretion. A decrease in activity would be caused by a decrease of neurosecretory material. Röseler and Röseler (1978) found that the amount of neurosecretory granules in fibres from the pars intercerebralis is higher in queenless workers.

Stingless Bees

Almost no study has been directed towards the mechanism by which queen pheromones achieve regulation of nest functions in the stingless bees. Worker oviposition in the presence of the queen, suggests that queen pheromones do not act, as in other groups, to interfere with the processes of vitellogenesis and oviposition. More likely, they act as to elicit attraction, and to stimulate nest constuction and cell provision. Engels (1987) suggested that a common physiological factor may mediate vitellogenin and virgin mating pheromone production.

Honey bees

As early as 1961, Pain suggested that queen pheromones may act via the gonadotropic hormones to inhibit oogenesis in workers. However, the source of the inhibition in honey bees may not be the queen. Regardless of the source, the method by which inhibition is achieved in honey bees may differ from that seen in the other social insects, since the roles of both the CA and JH appear to be different in adult worker bees. The CA of adult workers increase more or less steadily in volume until around the 18th day (Gast, 1967), and the volume of the CA seem to be correlated more or less with their synthetic activity and with the endogenous activity of the JH (Rutz et al., 1976). This development occurs despite the presence of the queen. A correlation

between the volume of the CA and oocytes suggests that oocyte maturation is dependent on the presence of a hormone from the CA (Gast, 1967), and juvenile hormone itself has been shown to stimulate oocyte development (Engels and Ranamurty, 1976; van Laere, 1974). Yet, in the presence of the queen, JH injected into workers does not induce oocyte maturation. In larvae however, ovary development can be induced by implanting extra CA or by applying farnesol (Chai and Shuel, 1970), and JH treatment of larvae reared on worker jelly differentiate into either intercastes (Rembold, 1976) or queens (Dietz et al., 1979). Rachinsky and Hartfelder (1990) reported that enhanced synthetic activity of the CA in future queen larvae results in an increased JH titre, and protects the ovary and inhibits its rudimentation. Initiation and maintenance of vitellogenin synthesis does not depend on the CA, because allatectomy of queen pupae or adults does not prevent vitellogenin or egg production (Engels and Rammamurty, 1976; Kaatz, 1988), and application of JH has no influence on vitellogenin production by queens (Engels, 1978; Kaatz, 1985). Also, vitellogenin synthesis is not linked to simultaneous yolk formation in growing oocytes, as it is in many other insects. In fact, vitellogenin is produced in considerable amounts by non-laying queens, nurse-aged queen-right workers with inactive ovaries, and, in small amounts, even by drones (reviewed in Trenczek and Engels, 1986). JH is not required for the production of vitellogenin in the drone pupae

(Zillikens, 1985). Thus, JH is important in proper honey bee ovary development, but only during larval development. Inhibition of worker ovary development and vitellogenesis does not act by directly affecting JH titres of adults, and it is clear that honey bees differ in this respect from bumblebees and wasps. The role of JH and various pheromones in the deposition of vitellogenin in the developing eggs is a process which has rarely been studied in this context. Kaatz (1988) suggested that a neuropeptide is involved in the modulation of vitellogenin production in queens. Inhibitory pheromones must act to block the production and/or transport of this or other reproductive factors in workers.

Despite the fact that JH is not directly involved in ovary development or vitellogenesis, queen pheromones do apparently affect JH production in adult bees. JH synthesis in young queenright workers is only about one half that for queenless workers of the same age (Hildebrandt and Kaatz, 1990) and this effect can be induced in queenless workers by exposing them to mandibular gland extracts or 9-ODA. In the latter case, JH synthesis of queenless workers is decreased in the same dose dependent manner, to the level of the queenright bees. Hildebrandt and Kaatz (1990) also showed that queenright, 8 day old workers, synthesize two times as much protein in their neurosecretory cells as queenless workers. They suggested that their results may be due to two factors: queen mandibular gland pheromone affect on the

CA may be mediated with polypeptides from the median neurosecretory (NS) cells, since NS factors are presumed to regulate CA activity, or, neuropeptides may be involved directly in the control of the physiological status of workers, as supposed for the stimulation of vitellogenesis in queens (Kaatz, 1988). Besides JH, a factor contained in the neurosecretory material produced in the pars intercerebralis of the brain is also necessary for oocyte maturation. Gast (1967) reported that the growth of the nuclei of the neurosecretory cells is inhibited by the presence of the queen, and that it is doubtful if this is due to the action of mandibular gland pheromone. A double control of oogenesis, by a neurohormonal factor and JH, has been found in several non-social insects (Engelmann, 1968).

Results that contradict a role for JH in worker reproduction have been reported by Robinson et al. (in press), suggesting that JH levels in workers may be more closely related to physiological and behavioral status than to reproductive state. They suggested that high JH levels may partially inhibit ovary development only in young workers, that high JH levels may inhibit ovary development in workers of all ages, but older workers may be less sensitive to JH, or that JH may not play a role in the regulation of worker honey bee reproduction at all. If inhibitory pheromones affect reproduction via an agent other than JH, then the sterility of the workers is guaranteed, even in the presence of high JH levels, and that hormone is

available for the control of other factors which are related to the physiological changes associated with aging (Rutz et al., 1976). One such application in honey bees is the control of temporal division of labor. An increasing JH titre with age is associated with hypopharyngeal gland degeneration (associated with the changeover from within-nest to foraging duties) and the onset of foraging and alarm pheromone production (Jaycox et al., 1974; Robinson, 1985). That the presence of the queen impacts upon JH titres in adults (Hildebrandt and Kaatz, 1990) suggests that the queen also may play a role in regulating the ontogeny of division of labor patterns in the nest. The queen, by dampening JH synthesis in workers, can delay the onset of foraging, thereby retaining some of the workforce in the nest to care for the brood; older workers will act as foragers.

Ants

Palma-Valli and Delye (1981) outlined aspects of the neuroendocrine control of egg-laying in *Camponotus* queens. In the spring, neurosecretory products first leave the corpus cardiacum and then the pars intercerebralis (where they had accumulated over the winter). The corpora allata then become activated, and yolk is deposited in the developing oocytes. There are few neurosecretory products in the corpus cardiacum or pars intercerebralis during laying, and the corpora allata remain active.

Fletcher and Blum (1983a) hypothesized a single primer pheromone for the fire ant, *S. invicta* which regulates the secretion and titre of JH secretion in virgins. Allatectomy of virgins inhibits muscle hystolysis and dealation, and topical application of JH causes an increase in oviposition of allatectomized queens (Barker, 1978). Barker also presented evidence that the brain has a role in oocyte maturation, in addition to the generally accepted role in regulating secretion of JH since the reproductive system of alates with NS cells removed did not respond as well as JH applications to individuals with intact brains. In the absence of a laying queen, there may be a nutritional factor whereby some virgins are more successful at getting food from workers, and these become the dominant egg-layers (Fletcher and Blum, 1983b).

Addition of JH analogs to ant colonies results in the production of sexuals in *S. invicta*, *Pheidole*, and *Myrmica*. It also interferes with queen egg-laying (reviewed in Edwards, 1987). It is likely that the differentiation of sexuals is the result of the direct action of the JH on the development of the larvae, high JH titres acting to stimulate ovary development and other queen-like characters. However, Edwards (1987) speculated that the cause may be fewer eggs having a weaker inhibition over gyne production by workers.

Queenright workers of the carpenter ant *Camponotus festinatus* usually have low vitellogenin titres, however,

queenless workers maintained either in the presence or absence of pupae show increasing vitellogenin titres (Martinez and Wheeler, 1991). Never the less, accumulation of vitellogenin in the haemolymph of workers, even far above the levels detected in some queens, does not result in oocyte development in the majority of these individuals. Thus in *C. festinatus*, the processes of vitellogenesis, and yolk deposition and ovary development may be uncoupled, and it remains unclear at what level queen inhibition is operating.

The Breakdown of Queen Pheromones

The rapid change in worker behaviors that follows queen removal suggests that the social insects have evolved efficient systems for converting active compounds into inactive ones. 9-keto-decenoic acid (one of the components of honey bee queen mandibular gland pheromone) is rapidly (in the order of hundreds of μg per day) converted by honey bee workers to 9-ketodecanoic acid, 9-hydroxydecanoic acid, and 9-hydroxy-2-decenoic acid (Johnston et al., 1965). The conversion to these compounds was reported to be sufficiently rapid to account for the onset of queen rearing by bees that had been separated from their queen for a short time.

Why are queens unaffected by the inhibitory pheromones that they produce? In honey bees, at least, inhibitory

Discussion

The eusocial Hymenoptera are defined by reproductive division of labor, whereby one individual, the queen, largely or completely dominates egg laying. The rest of the females in the nest are constrained from oviposition by queen chemicals and/or behaviors that affect them either in their adult life or during larval development. Nevertheless, in many species some workers are able to produce some male eggs, and, in the stingless bees, queen pheromones may actually be required to allow such worker reproduction to occur. In those species where caste determination occurs during larval development, adult workers have already been prevented from carrying out significant reproduction of their own. Their best remaining fitness option is to aid the queen's reproductive efforts. Such workers are available for other colony activities, and selection has acted to produce colonies which are efficient and well organized at carrying out a large number of activities involving many individuals. Queen-produced pheromones are an effective way of maintaining such organization. Solving the problems of living in groups has resulted in evolution of different forms of colony organization, and evidence suggests that there have been several origins of eusocial behavior (Wilson, 1971), but diverse groups of Hymenoptera have evolved towards the utilization of queen-produced chemicals for the coordination

of activities within the nest. Many of the patterns described here for the social Hymenoptera, including the use of royal pheromones to coordinate colony function, are also reported in the termites.

The roles and mechanisms of queen pheromones in nest homeostasis are diverse, as would be expected for a group of species with so many different and unique lifestyles. Yet, a number of patterns emerge, including the types of species which utilize them (i.e. those with populous colonies), the nature of queen chemicals, the functions which they perform, and the way in which they are probably transmitted, perceived, and affect worker physiology.

Influence in worker reproduction is central to the queen's influence of the workers, but her influence also extends to the timing and extent of sexual reproduction, nestmate recognition systems, worker temporal caste polyethism, stimulation of foraging and nest building, and, in general, the maintenance of harmonious and efficient colony functioning. In other words, the presence of the queen assures a situation in which each worker can best help to pass on its own genes to future generations by aiding in the efficient operation of the colony. Although individuals could increase their direct fitness by producing sons of their own, the presence of several patrilines in the nest would make it likely that workers would not want to aid in raising another worker's offspring. Through worker patrolling or worker (including brood) pheromones, it could

be ensured that cheating is kept at a minimum. As long as a queen can communicate her presence to the workers, they can "know" that their interests are best served by working cooperatively. Loss of the queen (or her signal) communicates to the workers that the status quo is lost, with the result that colony harmony can break down as the remaining individuals begin to compete for opportunites for direct reproduction. Failure to replace a lost queen can also cause colony death. Therefore, all colonies of social insects posess a back-up system for queen replacement. That mechanism is related to the mechanism by which the presence of the queen influences worker reproduction and the production of sexuals. In species with behaviorally dominant queens, there normally are subordinates that are competent to step into the queen's position. In societies with pheromonal regulation, a cut-off in the supply of inhibitory queen substances leads to either the production of new queens, loss of inhibition of existing ones, or eventually, an onset of, or increase in, reproduction by the workers. In the honey bees, for example, queen loss first leads to new queen production, and failing that, a terminal effort to pass on genes to the next generation by male-producing, ovipositing workers.

There is a pattern, across the eusocial Hymenoptera, of the maintenance of queen dominance via personal patrolling in less highly social species, and via pheromones in the most highly social. An integrated system of behavioral and

chemical dominance is seen in intermediately eusocial Vespine species (Reed and Akre, 1983), in bumblebees (van Honk et al., 1980), and probably in *Vespa*, *Dolichovespula* and some Polybiine wasps (Akre et al., 1982; Greene, 1979). The use of queen pheromones is also related to large colony size. These differences can be seen within closely-related taxa; in the slave-making ant genus *Harpoxenus*, *H. sublaevis* colonies are large and the queen controls worker egg-laying with a pheromone, but *H. americanus* colonies are small and have behaviorally aggressive queens (Bourke, 1988).

The physiological mechanism by which queen pheromones inhibit worker reproduction would seem to parallel that by which behavioral domination operates in the more primitive groups of social insects. Primer pheromones work, in general, by affecting the production of JH by the corporal allata, taking advantage of the central role of JH in oogenesis in most insects. Inhibition may actually occur at the neurosecretory cells. Honey bees appear to provide an exception. In this species, other factors, produced in the brain, control oogenesis in adults, and JH titres are utilized for the control of temporal division of labor. Queen attendance is normally performed by honey bee workers of certain ages (Seeley, 1982), demonstrating another way in which colony coordination by the queen is integrated with the other great organizer of worker activities, temporal caste polyethism. Queen influence on worker task

performance has also been observed in at least one species of ant.

Queens influence colony function, but are not in absolute control of worker activities. Both brood and workers have mechanisms for regulating the behaviors of other individuals. For example, honey bee brood-produced pheromones stimulate food gathering, and may suppress worker ovary development (Jay, 1970), and the presence of 4^{th} instar fire ant workers is required to stimulate and maintain oviposition by the queen (Tschinkel, 1988).

Queen-produced primer pheromones tend to be relatively non-volatile. Materials are transferred through the nest because of the tendency for workers to be pheromonally attracted to the queen. In honey bees they are gathered by retinue bees attending the queen, and disseminated either incidentally or via specific behavior patterns (messengers). Outside of the honey bees, little is known about the amounts of pheromone produced by queens, or transferred by workers. Mated, egg-laying honey bee queens produce up to 500 μg's of several different compounds per day. Maintained constantly over several years, this production may represent a substantial metabolic cost. Queen pheromones are probably deactivated by absorption into workers and the nest substrate. Such a system of constant production, transfer by workers, and internalization allows for both rapid transmission and breakdown of a signal, thereby quickly communicating both the presence and absence of the queen.

Volatile pheromones appear to be less important within the nest, perhaps because of problems with uneven air circulation.

The role of queens and queen-produced sociochemicals in regulating integrating the diverse activities of the other colony members is an area of research on the verge of blossoming. The thoughtful development of appropriate bioassays could be combined with advances in analytical chemistry to lead to the identification of more compounds that act as pheromones within the nest. Other important areas that are now being approached are the mechanisms of pheromone perception, and the mechanisms by which chemical messages are translated by receivers into behavioral or physiological responses. The results of these types of studies will have implications for both theory and practical applications; yielding insights into the evolution of eusocial insect societies, and the beneficial use of semiochemicals in agriculture and pest control. One important aspect of the honey bee queen's chemical communication with her daughters is the production and transmission of such compounds within the nest. The following chapters describe research that investigated the production and intra-nest transport of several of the components of the honey bee queen mandibular gland pheromone complex.

CHAPTER 2

THE PRODUCTION AND INTRA-NEST TRANSPORT OF 9-KETO-2(E)-DECENOIC ACID

Odors involved in alarm, orientation, trail following, and similar functions have been identified and intensively studied in many social insects. In contrast, we know little about the chemicals with which queens inhibit worker reproduction, influence the timing and extent of colony reproduction and activities such as foraging and brood rearing, that are integrally involved in colony fitness. Indeed, the honey bee is one of only 2 social insect species for which even some of these substances have been identified (the other being *Vespa orientalis*). Even in honey bees, little is known about the rate of production and secretion, mode of transmission, or site and mode of action for these pheromones.

The substances produced by the mandibular glands of honey bee queens are the only identified, queen-produced, components with which many of the activities that are at the heart of honey bee social organization are influenced. Their diverse actions include mate attraction (Butler and Fairey, 1964), inhibition of queen rearing (Butler, 1954, 1960; Butler and Simpson, 1958; Winston et al., 1989, 1990), attraction of swarming workers (Butler and Simpson, 1967; Velthuis and Van Es, 1964; Winston et al., 1989), and

arrestment (Velthuis, 1976) of workers within the nest (Gary, 1961a; Zmarlicki and Morse, 1964). This latter effect produces a retinue of dynamic composition that almost always surrounds the queen. The retinue consists of up to 12 workers that contact the queen with their antennae, forelegs, and/or mouthparts. After these contacts, the workers generally groom themselves prior to moving throughout the colony and making frequent reciprocal contacts with other workers for approximately 30 min (Seeley, 1979). These retinue contacts facilitate the transport of queen pheromones throughout the nest (Seeley, 1979; Verheijen-Voogd, 1959; Velthuis, 1972).

There is good evidence that queen-produced pheromones are quickly removed from circulation, or made unavailable after being removed from the queen. The development of egg-laying workers follows within weeks of the removal of the queen from the colony, due to the disappearance of both queen and brood-produced substances (Jay, 1972). Changes in other worker behaviors occur within minutes after queen removal (Butler, 1954), and queen cell construction usually commences within 10 hours (Seeley, 1979). The production of virgin queens prior to swarming, and the supersedure of established queens, may be mediated by a decrease in colony-wide levels of queen pheromones (Winston, 1987).

The earliest identified component of the queen mandibular glands, 9-keto-2(E)-decenoic acid, 9-ODA, was discovered after the observation that queenless workers exposed to

queens' mandibular glands acted as if queens were present (Butler and Simpson, 1958; Barbier and Lederer, 1960). However, 9-ODA alone was not as effective as the presence of a mated queen, making it apparent that 9-ODA was not the only component. Slessor et al. (1988) reported that the full mandibular - induced retinue response of workers could be produced by a blend of 5 compounds found in queen mandibular glands: 9-keto-2(E)-decenoic acid (9-ODA; 150 μg), 9-hydroxy-2(E)-decenoic acid (9-HDA; 71% R-(-), 29% S-(+); 55μg), methyl p-hydroxybenzoate (HOB; 13 μg), and 4-hydroxy-3-methoxyphenylethanol (HVA; 1.5 μg) (the μg values are the means found in the mandibular glands of a laying queen, and are defined as 1 queen equivalent, or Qeq). The synthetic blend also is attractive to swarms (Winston et al., 1989), stimulates pollen foraging (Higo et al., 1992), and elicits short term inhibition of queen rearing at higher doses (Winston et al., 1989, 1990, 1991). The effective dose in those studies was between 1 and 10 Qeq per day. Velthuis (1972) found that a 9-ODA source had an inhibitory effect on worker oogenesis in small groups of bees, but Willis et al. (1990) found no inhibition by the full, 5 component blend in whole colonies.

Butler (1954), and Müssbichler (1952) showed that the queen produced pheromones do not act as a vapor, and, while the queen may come into contact with many workers daily, the large number of workers in established colonies and the rapidity with which the chemical message is lost precludes

the queen being the sole vehicle of transfer. Seeley (1979) found that a queen came into contact with an average of only 35% of several thousand workers over a ten hour period. It is therefore likely that the workers themselves are key agents in accepting the queen's odor message and disseminating it throughout the nest (Juska et al., 1981). The behaviors of the workers in the retinue, including antennation and licking of the queen, self grooming, and, after leaving the queen, greater movement and increased frequency of contact with nestmates (Seeley, 1979), are consistent with such a role. However, queen-produced pheromones have not been detected on workers, possibly due to the low level of these chemicals which may be transmitted between workers.

Recently, Webster and Prestwich (1988) succeeded in synthesizing tritium-labelled 9-ODA. This compound can be combined with the other active components of the mandibular glands to produce a powerful tool for studying the movement of these substances through the colony. This chapter reports on experiments to investigate the rate of queen mandibular pheromone production and secretion, and to determine the nature of 9-ODA transfer throughout the nest.

MATERIALS AND METHODS

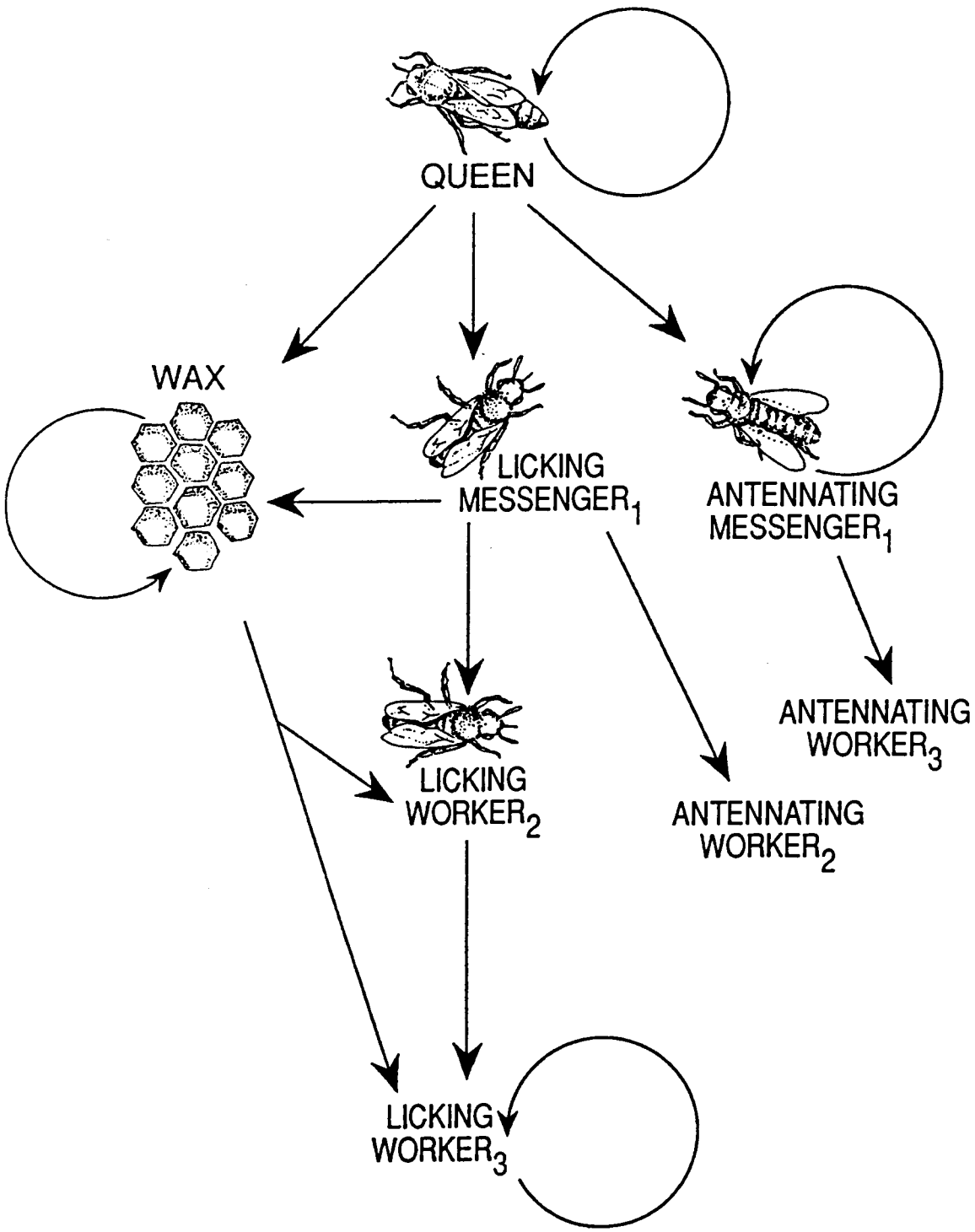
The potential routes of pheromone movement from the mandibular glands of queens to workers are outlined in Figure 2-1, and the specific experiments undertaken to follow and quantify this movement are described below.

Pheromone Production and Exudation

Laying queens of mixed stock (primarily *A. m. ligustica*) were removed in June at about 10:00 A.M., from established colonies in which they had overwintered. Ten queens each were randomly assigned to 4 treatments which consisted of isolation for 0, 2, 4, or 12 h in 60 x 15 mm plastic Petri dishes, kept at 31° C, and given a small amount of honey and water. Each queen was quickly anesthetized with CO_2 following her isolation, and washed by dribbling methanol over the body as it was held by an antenna tip. Two hundred to 300 μg of wash were recovered per queen. The mandibular glands were extirpated and crushed in approximately 100 μg of methanol. The Petri dishes also were washed with solvent. Quantitative analysis of the polar compound 9-ODA was accomplished through gas chromatography (Slessor et al., 1990), and confirmed with the analysis of selected samples by combined gas chromatography - mass spectrometry utilizing

Figure 2-1.

Potential routes of queen mandibular gland pheromone within the nest. Pheromone becomes available for circulation after being secreted onto the queen's body surface.


QUEEN
WAX
LICKING MESSENGER$_1$
ANTENNATING MESSENGER$_1$
LICKING WORKER$_2$
ANTENNATING WORKER$_3$
ANTENNATING WORKER$_2$
LICKING WORKER$_3$

single ion monitoring of 4 pre-selected fragments of the trimethylsilylated molecule.

Internalization and Deposition on Wax by Queens

To determine the rate of absorption and/or binding of 9-ODA by the cuticle of queens, 10^{-3} Qeq of synthetic queen mandibular gland pheromone (synthetic QMP) containing 14 ng of [^{3}H]-9-ODA (with an activity of 90,000 dpm), was topically applied, in 10 μg of methanol, to the heads of 40 mated queens. Slessor et al. (1988) found the greatest proportion of externally carried synthetic QMP could be found on a queen's head and on the feet, presumably as a result of grooming. The queens were isolated in 60 x 15 mm plastic Petri dishes, on a thin layer of wax, for 0, 5, 30, or 60 min. They were then anesthetized with CO_2, and washed by dribbling 10 portions of 100 μg of methanol over their bodies. Two μg of haemolymph were removed, and their alimentary canals excised.

After the queens were removed from the isolation dishes, 3 workers were sequentially admitted for 5 min each to determine if workers can pick up pheromone left on the wax by queens. The sides and tops of the Petri dishes were then washed with methanol. The queen body washes, alimentary tracts, haemolymph samples, wax substrates, dish washes, remainders of the corpses, and the workers were individually analyzed for amounts of [^{3}H]-9-ODA by liquid scintillation

counting (L.C). The samples were first placed into plastic scintillation vials and topped with either Scintiverse II^{R} (Fisher Scientific Co., Fair Lawn, N.J.) or Beckman Readysafe^{R} (Fullerton, CA) scintillation cocktail. Scintillations were measured, in this and the other experiments which follow, for either 30 s with a LKB Wallac 1218 Rackbeta liquid scintillation counter or for 2 min in a Beckman LS 5801 liquid Scintillation counter (Irvine, CA). Recovery efficiency of tritiated 9-ODA was generally greater than 90 %.

Pheromone Removal by Messenger Bees

For all experiments involving the transfer of pheromone, test workers were collected in groups of 15 from a queenright colony, placed into 150x25 mm plastic tissue culture dishes, and given a source of 1:1 sugar syrup (Kaminski et al., 1990). Workers were gathered from frames containing unsealed larvae, and were therefore likely to be of an age, and task group, which would normally come into contact with the queen (Seeley, 1982), and to act as messengers. They were then left undisturbed for a minimum of 1 h before taking part in any test. Unless otherwise stated, ten replicates of each treatment were completed for all experiments.

Removal from a Lure

The nature and amounts of pheromone removal by retinue workers (messengers) were determined by allowing workers to contact a pheromone-treated lure placed into each dish of 15 bees. Lures consisted of sister workers, killed by freezing, and treated topically with 10^{-3} Qeq of QMP containing 250 ng of [^{3}H]-9-ODA, on the dorsal surface. Workers showed a typical retinue response to such a lure. Individuals were allowed to contact a lure for 0, 5, 30, or 60 s, then were removed and dissected to yield antennae, mouthparts (mandibles, maxillae, and labium), head, front legs, middle and hind legs, thorax, and abdomen. Untreated workers were used as controls. Sustained lure contacts (greater than 5 s) almost always included licking. To compare [^{3}H]-9-ODA pick-up by workers making only antennal contact with a lure carrying QMP and 8.5 ng [^{3}H]-9-ODA, a total of 21 antennating workers were removed from the retinue after 5 s and analyzed whole by L.C.

A further experiment examined the possibility that retinue bees may swallow QMP. The protocol was identical to that of the lure contact experiment with the exceptions that only 30 s contacts were used and the alimentary tract of each selected retinue worker was removed and counted by L.C. separately from the rest of the abdomen.

Transfer From Messengers to Other Workers Via Comb and Direct Contact

Dead-bee lures treated with 10^{-3} Qeq of [^{3}H]-9-ODA-QMP were placed into dishes containing 15 sister-workers. When one of these workers had contacted the lure for 30 s, she was removed to either a 100 x 15 mm plastic Petri dish with a floor of wax foundation and 15 sister workers, or a similar dish with no workers. After 5 min, the bees and the wax from the first group of dishes were analyzed by L.C. For the second group, the lone bees were removed for counting and replaced by 15 workers. These bees and the wax were counted after a further 5 min. Untreated workers and wax were used as blank controls. The wax-only treatment allowed pheromone transfer only via the wax; the second treatment allowed transfer via both the wax and direct contacts.

Movement of 9-ODA Through Beeswax.

Synthetic QMP (10^{-4} Qeq) containing [^{3}H]-9-ODA, in 10 μg of methanol, was spread over 8.1 cm^3 discs of beeswax on the bottom of 30 x 10 mm plastic Petri dishes. These were left undisturbed at 32^{o} C (nest temperature) for 0, 30, 60, or 120 min (10 replicates of each treatment). At that time, 2 workers were introduced to the dishes for 5 min. Each was

quantitatively analyzed for [^{3}H]-9-ODA by L.C. Untreated workers on untreated wax were used as controls.

The Fate of 9-ODA on Workers

To quantify the internalization of synthetic QMP and/or the movement into the cuticle, individual workers were treated topically with 10^{-4} Qeq (containing [^{3}H]-9-ODA) on the abdomen. This amount was approximately the mean quantity of material picked up per worker per 30 s in the lure contact experiments. After application, workers were isolated for 0, 5, 30, or 60 min. This was followed by 10 washings with 100 μg of methanol, to dissolve any QMP on the body surface, and the excision of the crops and guts. The washes, excised organs, and the corpses were counted by L.C.; the wash represented pheromone still on the cuticle, the corpse count indicated how much had passed into the body or was bound in the cuticle. Ten replicates of 1 worker each were used per treatment, including a control group of workers which were untreated but otherwise subjected to the same protocol.

A similar experimental design was used to study movement of synthetic QMP into bees which were already dead, the only change in behavior being that the washes consisted of a one minute soak in 1 ml of hexane.

Individuals in all experiments were assigned randomly to experiments, and, except where indicated, 10 replicates of each treatment were performed. Data, occasionally transformed, were analyzed by one-way analysis of variance (ANOVA) with a completely randomized design, and, where significance was found, by a Student Newman Keuls (snk) or Bonferroni multiple comparison test for differences between treatments (Zar, 1984). In some cases where data did not conform to the assumptions of the ANOVA model, statistical inferences were made based on the Kruskal-Wallis test and the nonparametric comparison test of Conover (1980).

A model of pheromone flux in a honey bee colony was constructed using the data from the various specific transfers. Details of the model are outlined below, in the Results section.

RESULTS

Estimating Pheromone Production and Exudation by Queens

The quantity of 9-ODA detected in the glands of the mated queens increased with time spent in isolation, and was assumed to be increasing linearly (Fig. 2-2). The mean production of 9-ODA/24 h predicted by this regression is 204 ug, or 2.4 ng/s; however, 95% confidence limits extend from 12 to 397 µg/day.

The quantity of 9-ODA washed from the bodies of laying queens, and therefore available to workers, was highly variable, but was in the order of 1,000 ng (Fig. 2-3). In isolation, the amount found in methanol (MeOH) body washes did not increase significantly with time (ANOVA; df = 3, 36; $F = 2.02$; $p = 0.13$). QMP components were also deposited on the substrate but could not always be distinguished by GC analysis from the messy background of queen deposition; 1048 ± 315 ng (n = 6) and 730 ± 137 ng (n = 5) of 9-ODA were detected after 2 and 4 hr of isolation respectively.

Figure 2-4a shows the pattern of [^{3}H]-9-ODA internalization from the cuticular surface of live queens. Some material appeared to be swallowed, while a lesser amount accumulated in the haemolymph (beginning at 5 min, the amount of radio-label in the haemolymph was significantly different from blank control counts; t-test; t

Figure 2-2.

Amounts of 9-ODA (mean ± SE) in the mandibular glands of mated queens that had been isolated for different times. Linear regression; $y = 90.3 + 8.5t$; $r^2 = 0.13$

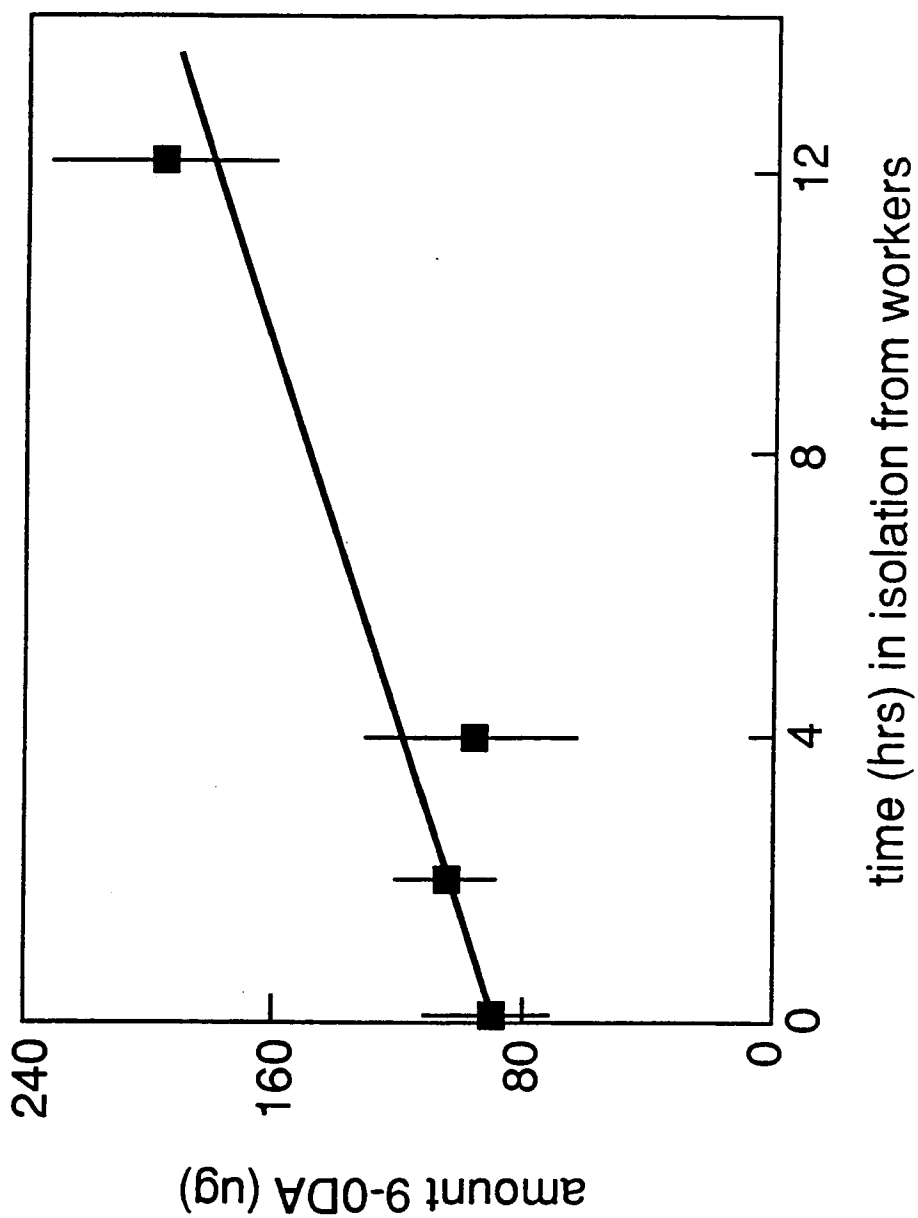

amount 9-0DA (ug)
240
160
80
0
0
4
8
12
time (hrs) in isolation from workers

Figure 2-3.

Amounts (ng) of 9-ODA (mean ± SE) on the body surfaces of mated queens after different times of isolation. The curve demonstrates equilibrium amounts of 9-ODA calculated from the model (see end of "Results") for the body surface of an isolated queen.

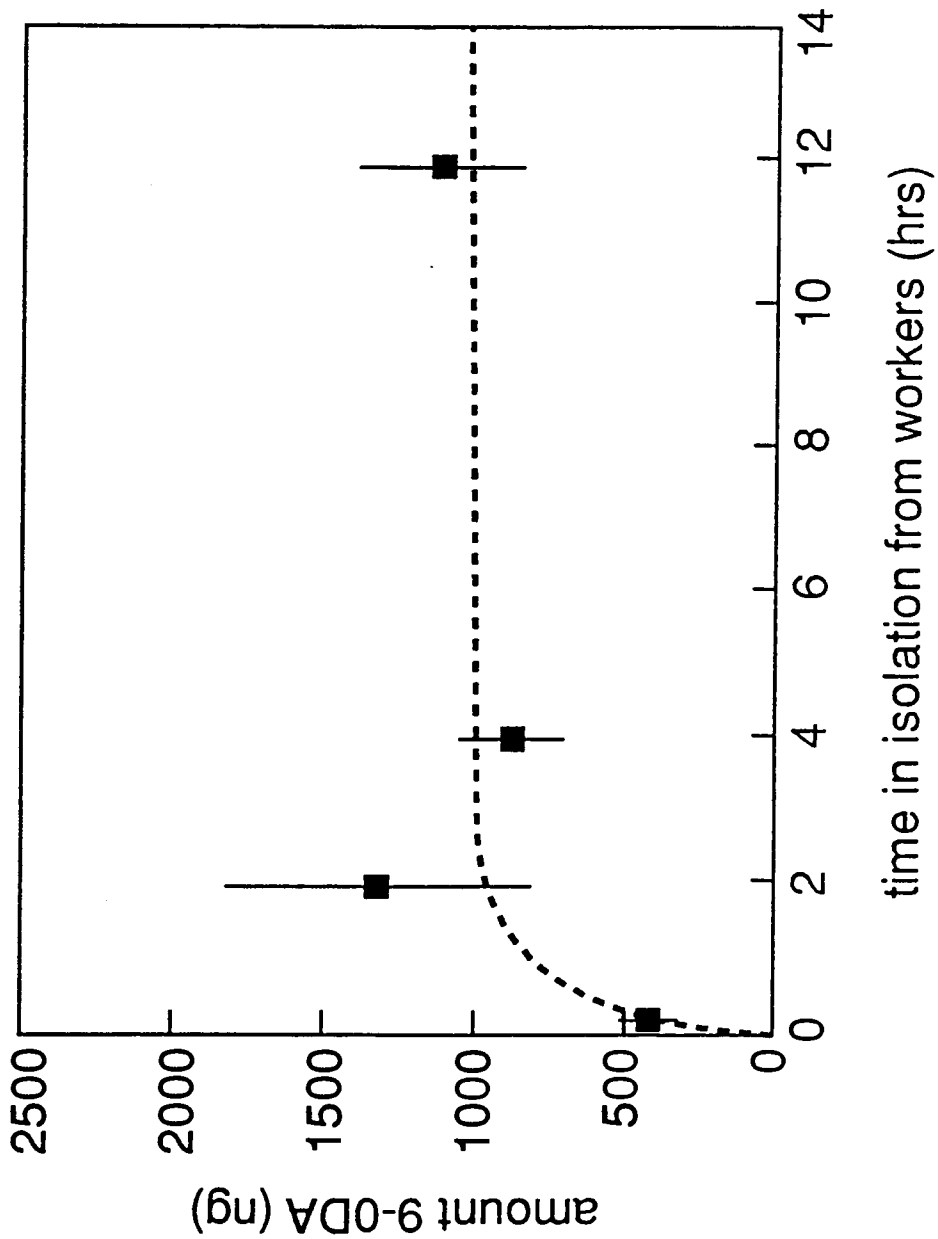
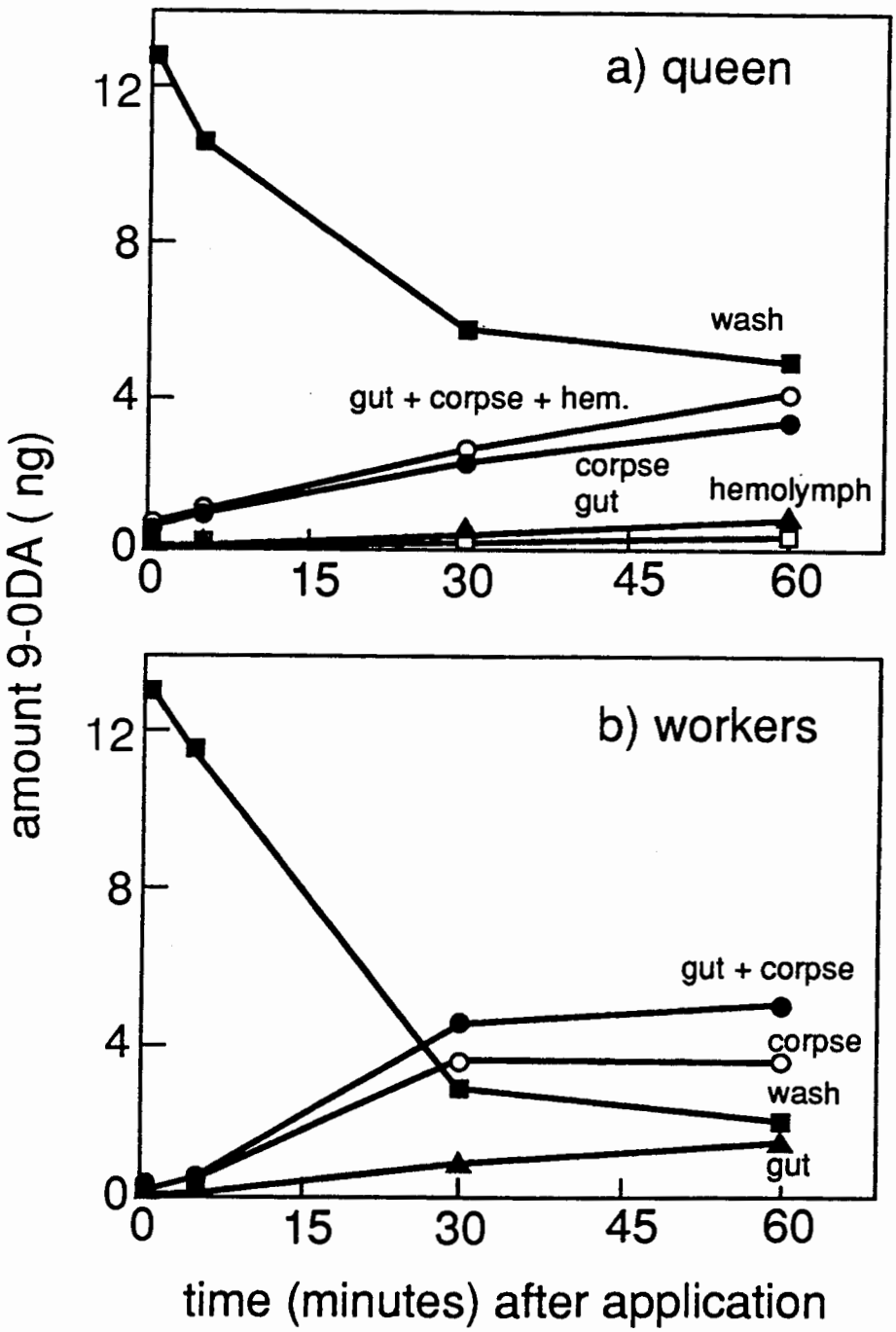

amount 9-ODA (ng)
2500
2000
1500
1000
500
0
0
2
4
6
8
10
12
14
time in isolation from workers (hrs)

Figure 2-4 a, b.

Fate of 9-ODA on the body surfaces of queens and workers. Both were isolated for different times after topical application of synthetic QMP (queen mandibular gland pheromone) containing 14 ng [^{3}H]-9-ODA. Sample size at each time = 10.


a) queen
12
8
4
0
wash
gut + corpse + hem.
corpse
gut
hemolymph
0
15
30
45
60
b) workers
12
8
4
0
gut + corpse
corpse
wash
gut
0
15
30
45
60
amount 9-0DA (ng)
time (minutes) after application

= 2.24, d.f = 9; p = 0.047). A substantial amount, increasing with time, became associated with the remainder of the corpse, so that it could no longer be washed off with methanol.

Pheromone Acquisition by Workers From a Lure

The quantities of [^{3}H]-9-ODA detected on workers making direct, sustained (licking) contact with synthetic QMP-treated lures increased with the duration of contact (Fig. 2-5; r^2 = 0.46, p < 0.001; log transformed data). A substantial amount (9.3 $\pm$ 3.7 ng; mean $\pm$ S.E.) of [^{3}H]-9-ODA was gathered from the lures after only 5 s of contact. In 60 s, 28.9 $\pm$ 10.2 ng, or approximately 11% of the total applied dose, was gathered by individual bees. The actual amounts gathered by individuals were highly variable, covering two orders of magnitude.

The pattern of pick-up was consistent, regardless of the duration of lure contact. The greatest amounts of radio-label were detected on the abdomen and in the gut, followed generally by either the mouthparts, thorax, or head (Fig. 2-6). Less was found on the legs, and only trace amounts were detected on the antennae. Differences were generally significant between these 4 groupings of body parts

Figure 2-5.

Acquisition of 9-ODA by retinue bees. Values are ng of [^{3}H]-9-ODA gathered from dead bees to which synthetic QMP containing 250 ng of [^{3}H]-9-ODA had been topically applied. Values are means ± SEs. Sample sizes were 0 s, n = 10; 5 s n = 1; 30 s, n = 60; 60 s, n = 10.

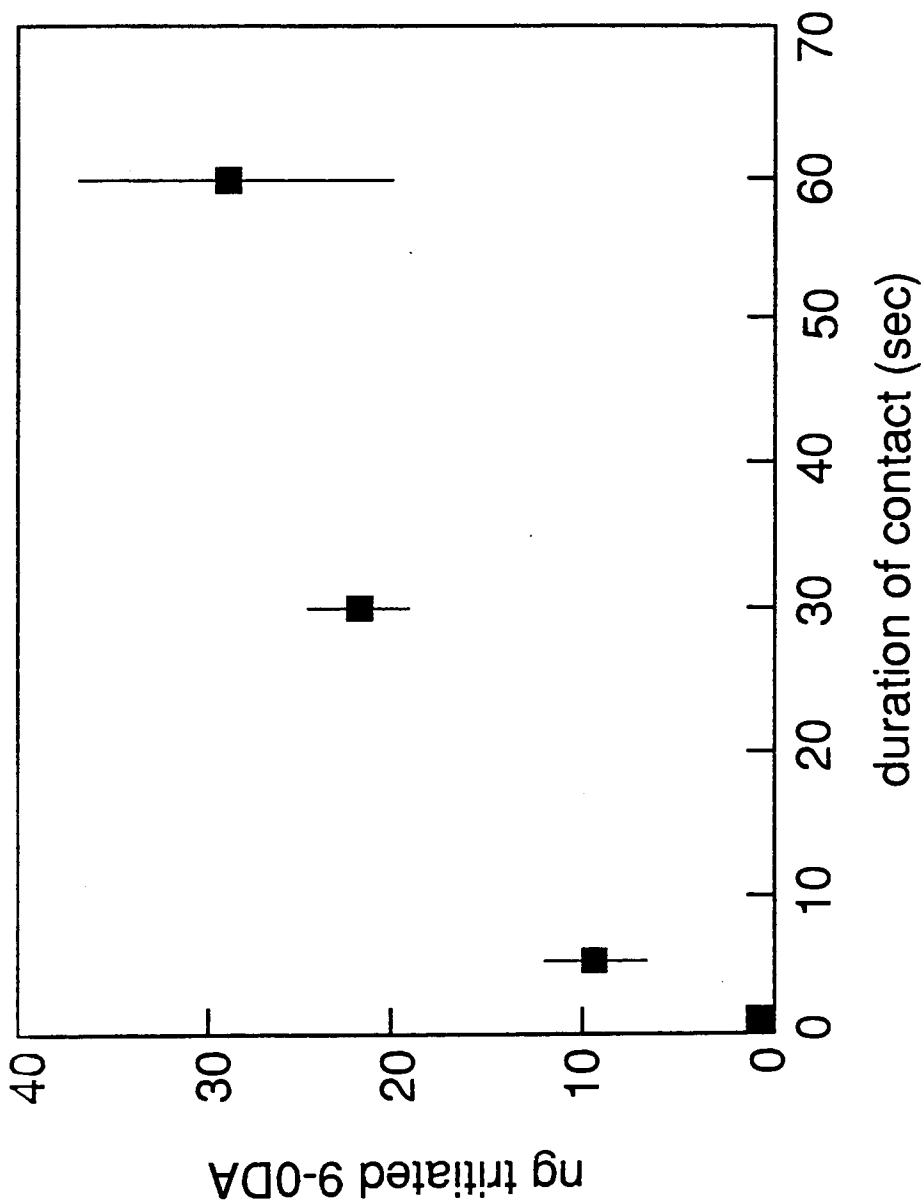
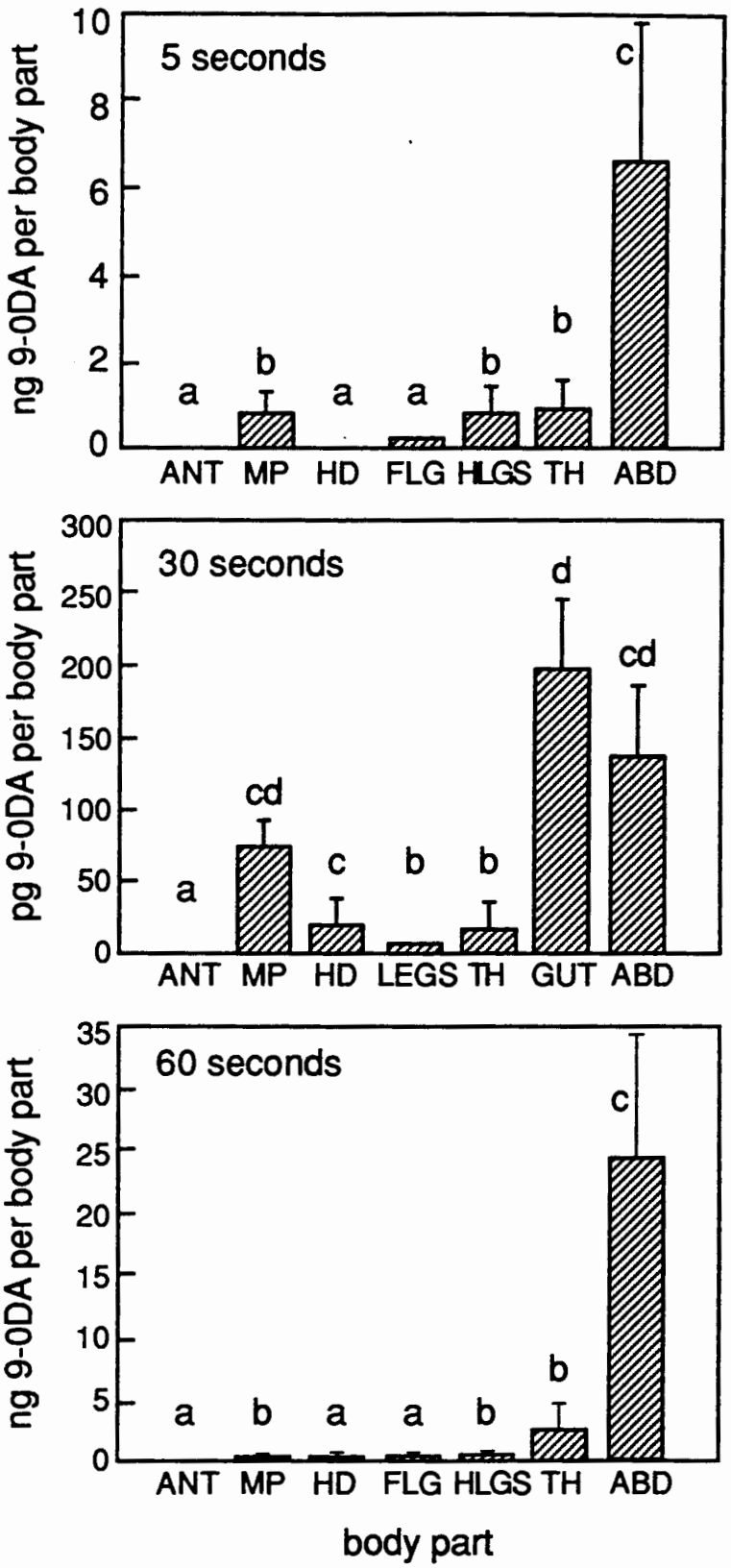

ng tritiated 9-ODA
40
30
20
10
0
0
10
20
30
40
50
60
70
duration of contact (sec)

Figure 2-6.

Pattern of 9-ODA acquisition by licking retinue bees. Amounts are ng or pg of [^{3}H]-9-ODA found on different body parts of workers after different times of contact with topically treated dead-bee lures. Initial applications contained 250 or 15 ng (for 30 s) of [^{3}H]-9-ODA. ANT, antennae; MP, moutparts; HD, head; FLG, forelegs; HLGS, hindlegs; TH, thorax; GUT, gut; ABD, abdomen. Gut dissections were done only for 30 s contact. Different letters above data columns denote significant differences (Kruskal-Wallis test; Conover nonparametric-comparison test; $p < 0.05$). Sample size = 10 at each contact time.


5 seconds
ng 9-0DA per body part
10
8
6
4
2
0
a
b
a
a
b
b
c
ANT MP HD FLG HLGS TH ABD
30 seconds
pg 9-0DA per body part
300
250
200
150
100
50
0
a
cd
c
b
b
d
cd
ANT MP HD LEGS TH GUT ABD
60 seconds
ng 9-0DA per body part
35
30
25
20
15
10
5
0
a
b
a
a
b
b
c
ANT MP HD FLG HLGS TH ABD
body part

(Kruskal-Wallis, Conover nonparametric test comparison test; $P < 0.05$).

Workers which had only antennated the lures gathered a mean of 1.5 ± 0.5 pg, or 0.02% of the total [^{3}H]-9-ODA available, in 5 s. This was only 0.3% of the mean value gathered in the same length of time by licking workers.

Transfer from Queen to Workers Via the Wax

The amount of [^{3}H]-9-ODA deposited by the queens onto the wax substrates or lids of the Petri dishes increased with time (Fig. 2-7).

Three workers introduced into the dishes, after removal of the queens, acquired amounts of radio-labelled 9-ODA that increased with the lengths of time that the queens had spent on the wax, i.e. with the amounts of [^{3}H]-9-ODA deposited (Fig. 2-8). In other words, the more material the queens left on the wax, the more was found on workers which later travelled on the same wax substrate.

Worker to Worker Pheromone Transfer

Direct contacts between a retinue bee and 15 other workers resulted in 27.2 ± 10.2 pg being passed in 5 min. A disproportionate amount of this latter quantity was gathered by a small number of the workers in each dish, suggesting the formation of 'secondary messengers'. In each of the ten

Figure 2-7.

Deposition of 9-ODA on wax by queens. Values are mean ± SE pg [^{3}H]-9-ODA left by queens after different times of isolation on the wax. Initial application to each queen was 10^{-3} queen equivalents of QMP containing 14 ng [^{3}H]-9-ODA. Different letters denote significant differences (ANOVA; $F = 47.0$, $df = 3, 36$, $p < 0.0001$, Student-Newman-Keuls, $\alpha = 0.05$, log-transformed data). Sample size at each time = 10.

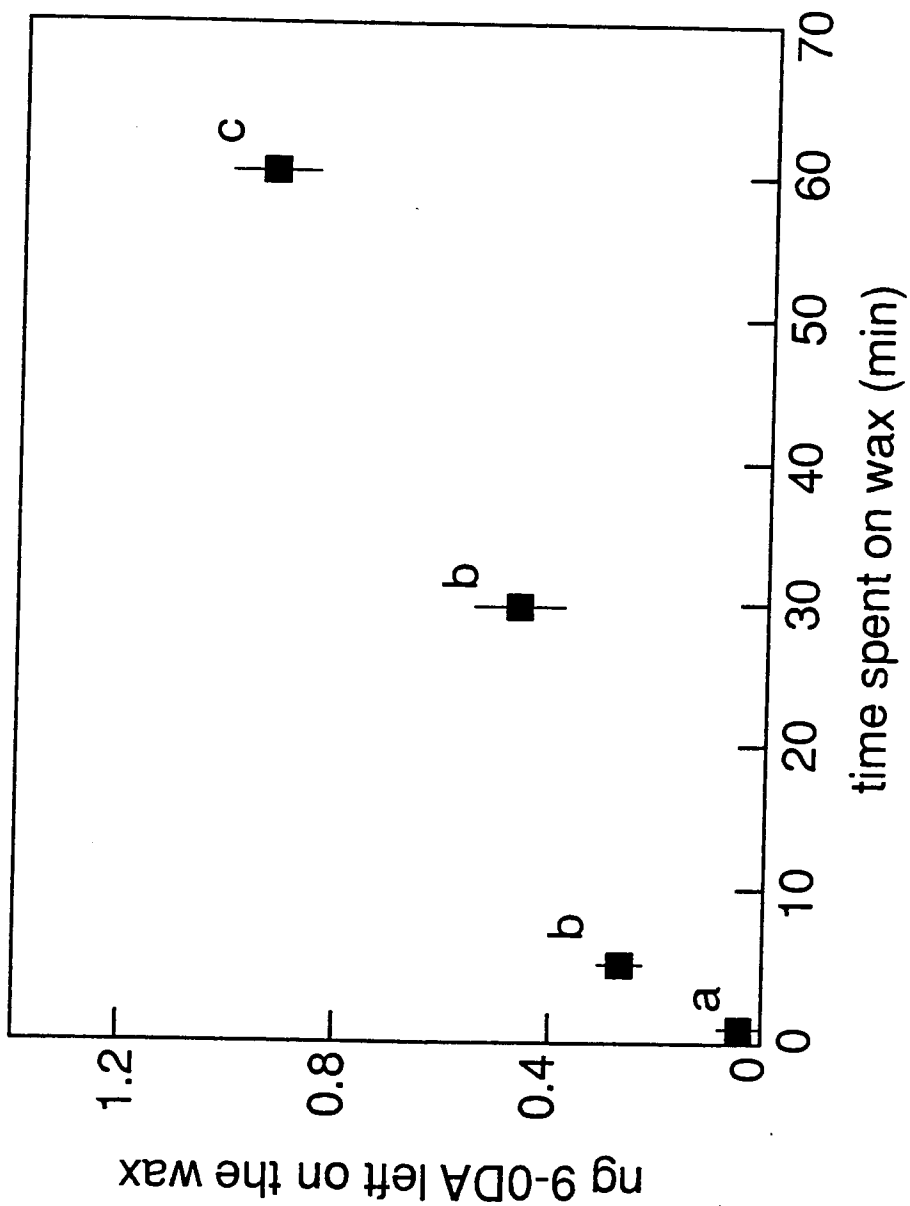
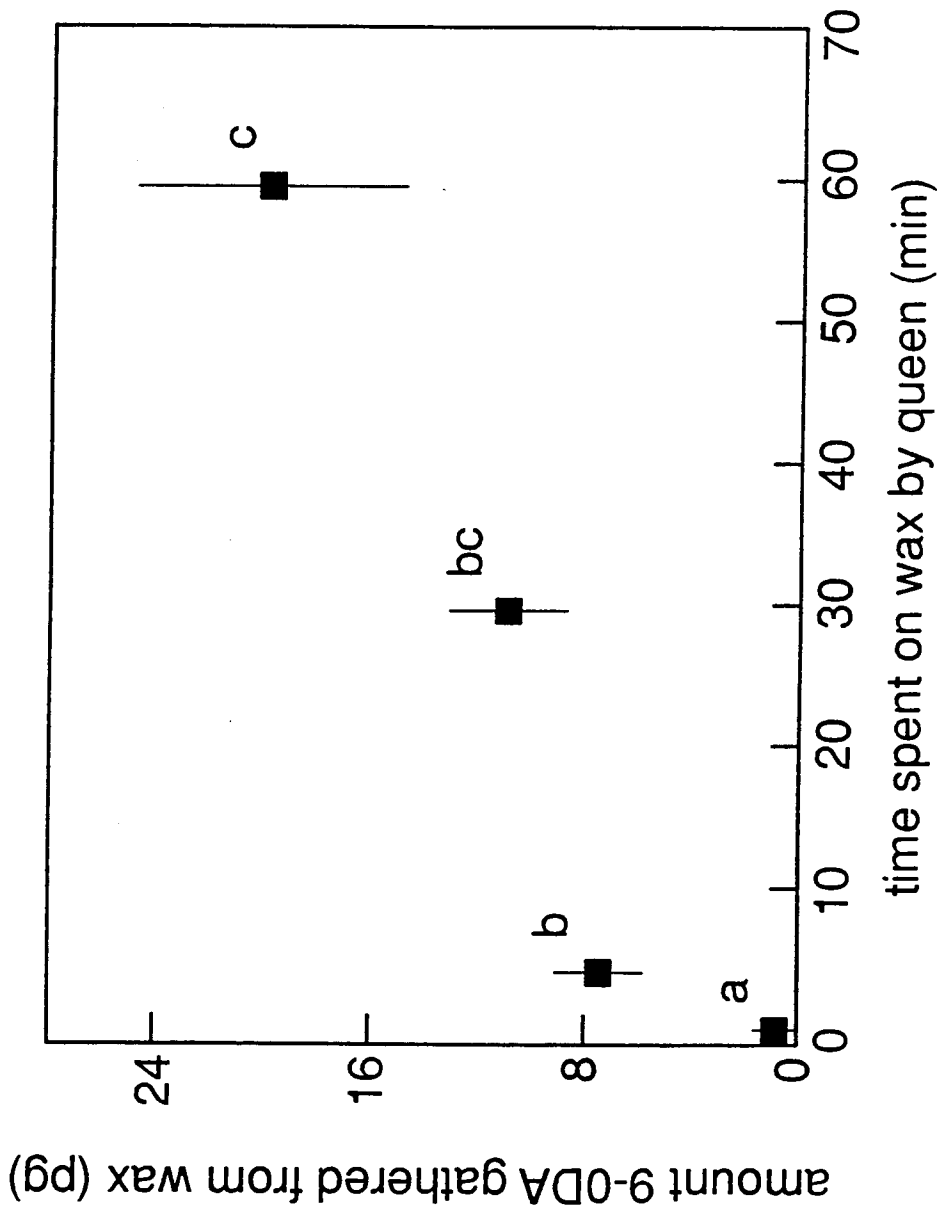

ng 9-ODA left on the wax
1.2
0.8
0.4
0
a
b
b
c
0
10
20
30
40
50
60
70
time spent on wax (min)

Figure 2-8.

Mean (± SE) amounts of [^{3}H]-9-ODA acquired from wax by individual workers following deposition by queens (see Figure 2-7). Contact time with the wax was 5 min. Different letters denote significant differences (ANOVA, F = 31.5, df = 3, 36, p < 0.0001, Student-Newman-Keuls, α = 0.05, log-transformed data). Sample size at each time = 10.


amount 9-ODA gathered from wax (pg)
24
16
8
0
a
b
bc
c
0
10
20
30
40
50
60
70
time spent on wax by queen (min)

replicates, the single individuals gathering the greatest amount of 9-ODA carried an average of 36 ± 7 % of the total carried by all 15 workers. An average of 12.8 ± 0.6 bees carried less than 1% of this total.

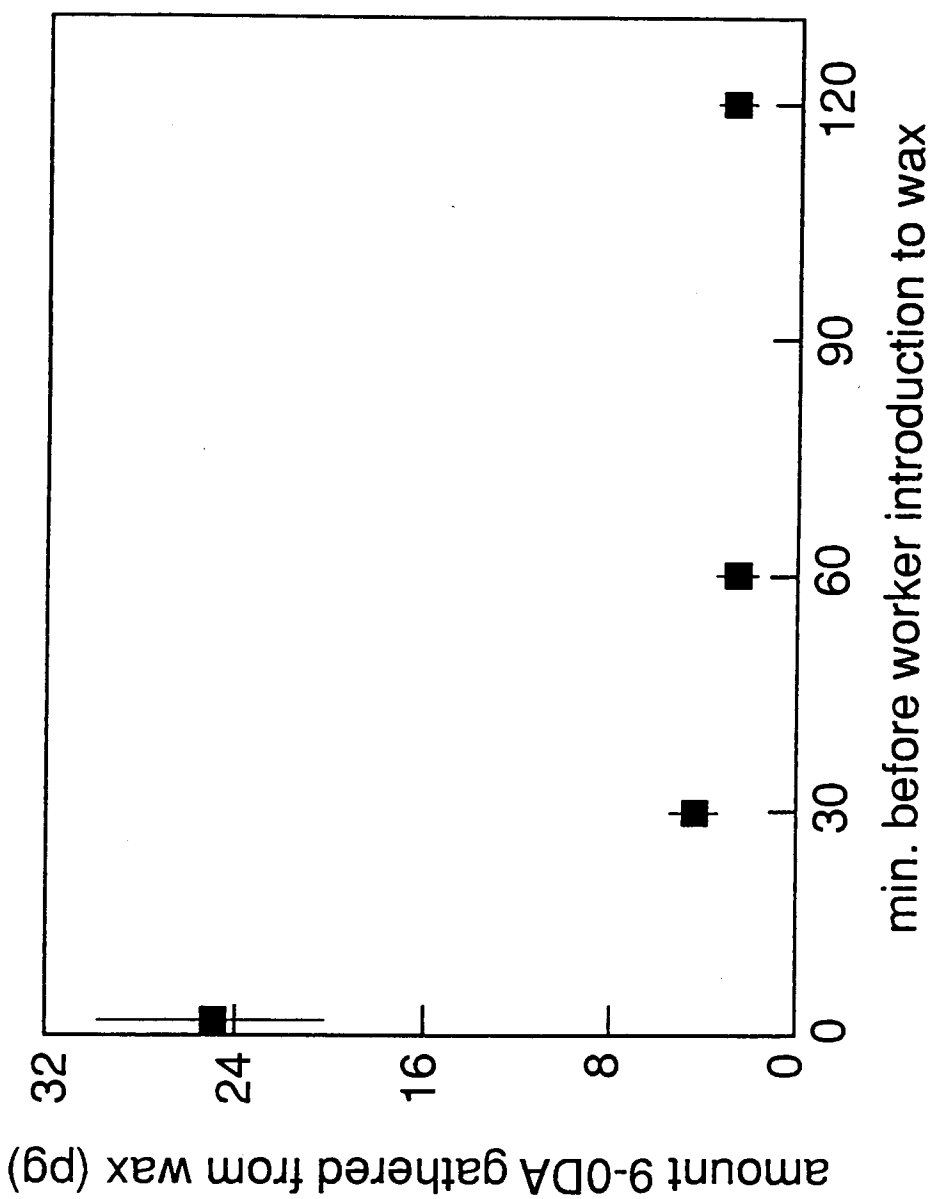
Workers also left [^{3}H]-9-ODA on wax surfaces, following lure contacts, some of which could be gathered by other workers. In 5 min, the retinue workers left 130 ± 9 pg, or 4.5% of the total carried by the messenger bees ($\bar{X}$ = 3.3 ng), on the wax. Fifteen workers, subsequently introduced to the dishes, each picked-up 2.4 ± 0.9 pg, or 2% of the total that had been left on the wax. This was 10 times greater than the mean gathered from blank controls (0.1 ± 0.01 pg), and significantly less than the amount transferred via direct contact (Kruskal Wallis test; X^2 = 23.6; d.f. = 2; $p < 0.001$; Conover nonparametric comparison test).

The Further Role of Wax

The amount of [^{3}H]-9-ODA that two workers picked-up from wax treated with synthetic QMP containing [^{3}H]-9-ODA decreased sharply with the time that the treated wax was allowed to sit before introduction of the workers (Fig. 2-9). The half life of biological availability on the wax foundation was approximately 12 min.

Figure 2-9.

Availability to workers of 9-ODA applied onto wax. Initial applications of QMP containing 14 ng of [^{3}H]-9-ODA were followed by different times before worker introduction. Two workers were then allowed 5 min contact with the wax. Values are means ± SEs for single bees per dish.


amount 9-0DA gathered from wax (pg)
32
24
16
8
0
0
30
60
90
120
min. before worker introduction to wax

Internalization of Synthetic QMP by Workers.

Workers showed a pattern of [^{3}H]-9-ODA internalization similar to that of the queens. [^{3}H]-9-ODA levels in the guts or in the rest of the corpses increased with time while the amounts that could be washed from the cuticle decreased. The half-life of 9-ODA on the cuticle suggested by the negatively sloped curve in Fig. 2-4b was approximately 13 min.

Dead bees showed a similar internalization of topically applied [^{3}H]-9-ODA with time, and a similar half-life on the surface (17 min).

Determination of Rate Constants (summarized in Fig. 2-10)

If the assumptions are made that the rates of pheromone transfer are constant and the amount of pheromone transferred is directly proportional to the quantity present at the source, the amount transferred can be expressed as a "pseudo" first order rate constant, k_n. (In favor of the second assumption, workers acquired approximately 8 times more 9-ODA from lures carrying 250 ng of [^{3}H]-9-ODA than from lures carrying 14 ng, and a strong correlation, r^2 = 0.9, has been found for the relationship between acquisition of another QMP component and the amount available (see data for HVA in Chapter 4).

Amount transferred = k_n * transfer time (t)* initial amount

We refer to these rates as "pseudo" first order rate constants since they are applied to amounts on a bee rather than a solution concentration and are the sum of forward and reverse rate constants, and thus in all likelihood, only approximate first order kinetics. The exponential loss of material at time t by a first order process can be expressed as

$$[conc]_t = [conc]_{t=0} * e^{-kt}.$$

The half-life of the material is

$$t_{1/2} = 0.693/k.$$

It is apparent from Fig. 2-10 that in this model the source of all pheromone in the colony is the amount of 9-ODA present at a given time on the queen's body. The amount of 9-ODA on the queen's body is established by the rate at which her glands produce and secrete the pheromone, by its loss to the wax, by its reabsorption through her own cuticle, and through donation to messenger workers. 9-ODA production was estimated from a least squares regression of Fig. 2-2 to be 2.4 ng/s. In isolation, pick-up by messengers is zero, thus the amount of 9-ODA on the queen's body, [QB], at time t will reach a steady state when the (assumed) linear production is offset by the exponential loss, i.e.

$$2.4 \text{ ng/s} * 1 \text{ s} = [QB]_t - [QB]_t * e^{-(k1+k5)t+1}$$

$$= [QB]_t * (1 - e^{-(k1+k5)})$$

$$e^{-(k1+k5)} = 1 - 2.4/[QB]$$

$$k_1 = -\ln(1 - 2.4/[QB]) - k_5$$

A mean value of 7.7×10^{-5} s^{-1} for k_5 is obtained from Fig. 2-7 and the 2 and 4 h isolated queen data. The equilibrium [QB] value for queens in isolation (Fig. 2-3) is 1091 ng (combining a decay curve with different, potential, constant rates of production/exudation always results in an equilibrium amount of 9-ODA on the surface of the queen being achieved within 2-4 h. Substitution yields a value for k_1 of 2.15×10^{-3} s^{-1} (79 μg/day).

Within a colony, a similar steady state exists where the removal of 9-ODA by messenger bees (overall rate of k_3) is occurring.

$$2.4 \text{ ng/s} * 1 \text{ sec} = [QB]_t - ([QB]_t * e^{-(k1+k3+k5)t+1})$$

Fig. 2-3 indicates that queens analyzed immediately after removal from their colony have an average of 423 ng of 9-ODA on the outside of their bodies. Substitution provides a value of 3.62×10^{-3} s^{-1} for k_3.

The rate at which workers internalize pheromone can be obtained from the half-life of the pheromone on the cuticle of a dead worker.

$$k_2 = 0.693/(17 * 60)$$
$$= 6.8 \times 10^{-4} \text{ } s^{-1}.$$

The amount of pheromone acquired by workers from the comb is obtained from the initial slope data in Fig. 2-8. In 5

min., an average bee picked-up 0.025 ng from a wax surface carrying 14 ng.

$$[14\ ng] * k_4 = 0.025/(5 * 60)$$
$$k_4 = 5.9x10^{-6}\ s^{-1}.$$

The rate of deposition of pheromone on the wax by a worker was calculated with data from the experiment which examined messenger to worker transfers. The mean total carried by the messenger bees was 3.3 ng, and 0.13 ng of this was deposited on the wax in 5 min.

$$[3.3\ ng] * k_6 = 0.13/(5 * 60)$$
$$k_6 = 1.31x10^{-4}\ s^{-1}$$

The rate of disappearance of pheromone into the wax can be ascertained from Fig. 2-9, since the half-life is shown to be 12 min.

$$k_7 = 0.693/(12 * 60)$$
$$k_7 = 1.0x10^{-3}\ s^{-1}.$$

The antennating messenger acquisition data indicated a 1.5 pg mean pick-up for a 5 s contact with a lure containing 8.5 ng of [^{3}H]-9-ODA.

$$k_8 = 1.5x10^{-3}\ ng/(5s * [8.5\ ng])$$
$$= 3.53x10^{-5}\ s^{-1}.$$

An average licking messenger acquired 23 ng from a lure carrying 250 ng of [^{3}H]-9-ODA in 30 s, thus

$$[250\ ng] * k_9 = 23\ ng/\ 30\ s.$$

$$k_9 = 3.07x10^{-3}\ s^{-1}.$$

Estimating Pheromone Transfer

Total Calculated 9-ODA production by the queen in the colony

$$= [QB] * (k_3 + k_5 + k_1)$$

$$= 2.47\ ng/s = 214\ \mu g/day.$$

The amount available to other colony members

$$= [QB] * (k_3 + k_5)$$

$$= 1.56\ ng/s = 135\ \mu g/day$$

(a) The transfer of 9-ODA by the queen to the wax can now be evaluated

$$= [\text{amount on the queen in the nest}] * k_5$$

$$= 423\ ng * 7.7x10^{-5}\ s^{-1}$$

$$= 0.032\ ng/s$$

$$= 2.8\ \mu g/day$$

(b) Queen to messengers in the retinue.

To establish the transfer of pheromone in the retinue we must define the ratio of antennating (A) to licking (L) workers. The retinue will consist of 1 licking and 10 antennating workers (see discussion for rationale). Maximal uptake of pheromone by the retinue must be

$$= k_9 + (R*k_8)$$

$= 3.07 \times 10^{-3}\ s^{-1} + (10 * 3.53 \times 10^{-5}\ s^{-1})$

$= 3.42 \times 10^{-3}$ ng s^{-1}, and with 423 ng on the queen

= 1.45 ng/min.

The retinue size, or alternately the frequency with which the full retinue contacts the queen, can be altered until retinue uptake is balanced with queen production.

effective ret. size = ret. * queen production / ret. uptake

=(1 + 10) * 1.56 ng/s / 1.45 ng/s

= 11.8 bees in the average retinue

The daily transfer of 9-ODA from queen to licking messengers can now be obtained

= effective ret. size * fraction of lickers in the ret. * k_9 * [amount on queen] * s/day

$= 11.8 * 1/11 * 3.07 \times 10^{-3}\ s^{-1} * 423 * 86{,}400$

= 121 μg/day to a maximum of 3110 30-s licking contacts

Similarly, transfer to antennating messengers

$= 11.8 * 10/11 * 3.53 \times 10^{-5}\ s^{-1} * 423$ ng * 86,400 s/day

= 13.9 μg/day to a maximum of 31100 5-s antennators

(c)Transfers from messengers to other workers

Secondary transfers from the 2 types of messengers can be considered to be restrained by a retinue of identical size and composition to that of the queen, but are considered to last for only 5 s. Unlike a queen, the concentration of pheromone on a messenger is time dependent and decreases exponentially from the time she receives her "dose". Licking messengers also swallow about half of the pheromone received, therefore, after 30 s contact with a queen, the concentration on the body of a licking messenger would be

$$[LM_1]_t = k_9 * [QB] * \text{contact time} * 0.5$$
$$= 19.5 \text{ ng}$$

The concentration is decreasing exponentially with time, therefore at time t, t sec after contact with the queen, the pheromone on the surface of the first messenger would be

$$[LM_1]_t = [LM_1]_{t=0} * e^{-(k2+k6+(f*k9)+(A*f*k8)t}$$

decreasing due to loss to absorption, wax, licking workers, and antennating workers respectively. But, at time t′, where t′ = retinue contact time/f another LM_1 gets a dose from the queen, the total amount of pheromone present on all the licking messengers will be the sum of a series,

$$[P]_{LM1} = [LM_1] * e^{-kt'} + [LM_2] * e^{-k2t'} +$$
$$[LM_3] * e^{-k3t'} + \text{etc.}$$

This can be shown to equal

$$= [LM_1] * e^{-kt}/(e^{kt} - 1)$$

Numerically, this yields a sawtooth function with maxima at the time that a licking messenger just leaves the queen,

decreasing exponentially to just prior to the next licker leaving the queen at time t'. An average value of 133 ng is used as a sum for calculation of the transfer from licking messengers to the wax

$$= k6 * [LM_1]_{sum}$$
$$= 1.07x10^{-2} \text{ ng/s}$$
$$= 1.5 \ \mu g/day$$

(d)Other workers, and the wax comb

Licking workers

It follows that the maximum amount passed onto the surface of a licking worker, i.e. one that contacted a licking messenger, is maximal when the messenger has just left the queen, and equal to

$$[LW_2] = k_9 * [\text{amount on } LM_1] * \text{contact time} * 0.5$$
$$= 0.15 \text{ ng}$$
$$= 9.3 \ \mu g/day \text{ to } 31100 \ LW_2\text{'s}$$

Transfer of 9-ODA from these workers to the wax is

$$= 0.1 \ \mu g/day$$

Antennating workers

We saw previously that the maximum transfer from a queen to a 5 s antennating worker was

$$= k_8 * [\text{amount on queen}] * \text{contact time}$$
$$= 0.07 \text{ ng each}$$

A summation calculation of the total amount on all of the antennating messengers indicates a mean of 0.15 ng. Transfer to the wax by these antennating messengers is very small

$$= k_6 * [AM]_{sum}$$
$$= 0.2 \text{ ng/day}$$

No evaluation of the recipient antennating workers transfer to the wax is given as the concentrations involved are miniscule.

The wax.

Deposition of 9-ODA on wax can now be evaluated as a contribution from the queen, licking messengers, licking workers, and antennating messengers

$$= 0.051 \text{ ng/s}$$
$$= 4.4 \ \mu\text{g/day}.$$

In a steady state, this deposition will be equalled by the pick-up by broodnest workers and by absorption into the wax. Making the assumption that we have 3,000 broodnest workers,

$$0.051 \text{ ng/s} = [\text{wax}] * (k_4 * 3{,}000 + k_7)$$

Solving for [wax] yields a value of 2.7 ng for the total steady state concentration of pheromone on the surface of the wax. This permits an estimate of the rate of aborption into the wax,

$$= [\text{wax}] * k_7$$
$$= 2.7 \times 10^{-3} \text{ ng/s}$$
$$= 0.24 \ \mu\text{g/day}$$

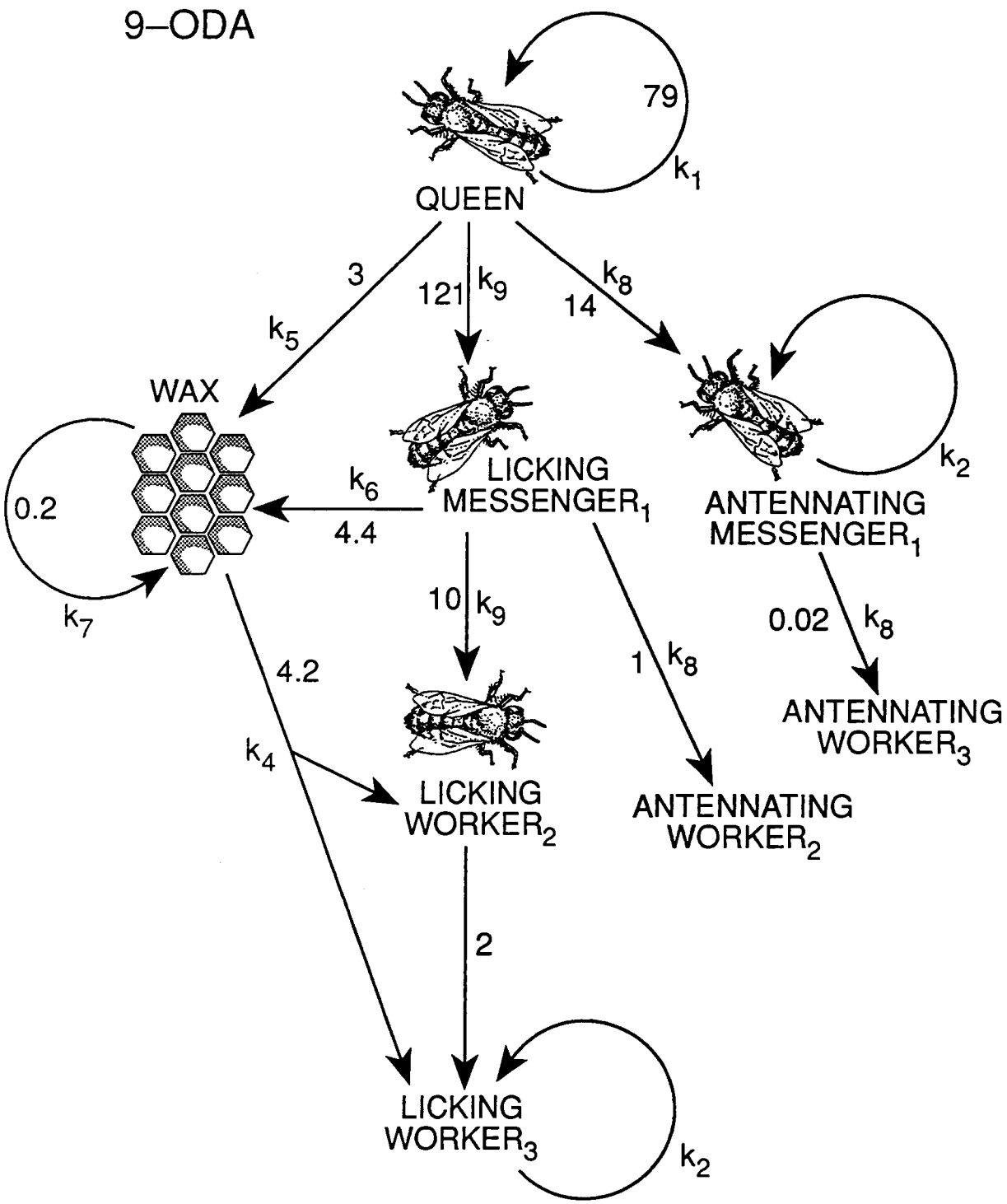
and the pick-up from the wax by broodnest workers,

= [wax] * k_4

= 1.4 ng/day for each broodnest worker, or a total of 4.2 μg/day for all the 3,000 broodnest workers.

Figure 2-10.

Model for 9-ODA transmission within the colony. A messenger is defined as a worker receiving pheromone directly from the queen; a bee receiving pheromone from a messenger is termed a licking or an antennating messenger, depending on the type of contact. Values are μg of 9-ODA movement per day. Assumptions include an equilibrium amount of 423 ng of 9-ODA on the queen's cuticle, 30 s licking and 5 s antennating contacts with the queen, 3000 broodnest workers, and 5 s messenger - worker contacts. Rate constants for each stage of transmission are defined as: k_1, rate at which cuticular 9-ODA is absorbed by queen; k_2, rate at which cuticular 9-ODA is absorbed by workers; k_3, rate at which queen transfers 9-ODA to workers ($k_8 + k_9$); k_4, rate at which workers pick-up 9-ODA from the wax; k_5, rate at which queen deposits 9-ODA onto wax; k_6, rate at which mesengers and workers deposit 9-ODA; k_7, rate at which 9-ODA is absorbed into wax; k_8, rate at which antennating messengers receive 9-ODA; k_9, rate at which licking messengers recieve 9-ODA.


9–ODA
79
k_1
QUEEN
3
k_5
121
k_9
k_8
14
WAX
LICKING
MESSENGER$_1$
ANTENNATING
MESSENGER$_1$
k_2
0.2
k_6
4.4
k_7
10
k_9
0.02
k_8
4.2
1
k_8
ANTENNATING
WORKER$_3$
k_4
LICKING
WORKER$_2$
ANTENNATING
WORKER$_2$
2
LICKING
WORKER$_3$
k_2

Attraction

If the limit of pheromone perception is considered to be in the range of 0.025 ng of 9-ODA, or 10^{-7} Qeq (Slessor et al., 1988), the following estimates can be made of the time necessary for the following consitituents to lose their attractiveness in normal colony conditions.

$$t = \ln([\text{conc. at start}]/[0.025\ \text{ng}])/\ k's$$

	peak value (ng)	attractiveness lost (min)	half-life (min)
wax ($k=k_4+k_7$)	>5.1	75	12
licking messenger	19.5	25	3
ant. messenger	0.07	45	11
licking $worker_2$	0.9	7	3
licking $worker_3$	0.005	-	3
ant. $worker_2$	<0.0001	-	11
ant. $worker_3$	<0.0001	-	11

Summary of Model

The model is very robust in that drastic changes have to be made in the constants before major changes in pheromone flux occur. For example changing k_2 from $6.8\text{x}10^{-4}$ to $2.00\text{x}10^{-4}$ s^{-1} causes only a minor change in the amount of 9-ODA deposited on the wax; changing k_9 from $3.0\text{x}10^{-3}$ to $7.44\text{x}10^{-3}$ s^{-1} causes transfer from the queen to LM1's to

change by approximately 6 μg/day, and an increase in transfer from LM_1 to LW_2 of 13 μg/day; Altering the rate of 9-ODA build-up in the mandibular glands fom 2.4 to 1.4 μg/hr, causes an 30% decrease in the rate of 9-ODA transfer from queen to LM_1, and a 5μg/day decrease of movement from LM1's to LW2's. The quantity moved by the antennating messengers is also reduced, from 13 to 4 μg/day; notable in itself, but a small part of the total pheromone flux. Qualitatively, the scheme is little affected. The model is linear with respect to the amount of 9-ODA on the queen's body, doubling of this value doubles each of the final numbers in Fig. 2-10.

DISCUSSION

In this group of experiments, we have, for the first time, succeeded in quantifying the production, secretion, and intra-colony transfer of a primer pheromone in a eusocial insect species. Such pheromones influence many worker activities, including attraction to the queen, inhibition of queen rearing, and possibly foraging. As well as being important for a basic understanding of the system, these results have implications for the proper design and use of pheromones in bee management.

The results obtained using [^{3}H]-9-ODA provide information on the rates by which this QMP component is transmitted from one entity to another within the nest. A summary of these processes is described in Fig. 2-10, with a fuller explanation of the model in the results.

Mated queen honey bees produce between 0.1 and 2 Qeq (20 - 500 μg of 9-ODA) of mandibular gland pheromone per day. Pain and Roger (1978) found a unimodal, diurnal variation in the amount of 9-ODA in the mandibular glands of unmated queens. The production was highest between 11 A.M. and 5 P.M., and the daily rate of production suggested by their results was a minimum of approximately 500 μg (approximately 2 Qeq), with almost all of that gain coming in the 6 midday hours. The diurnal pattern was hypothesized to be related to the sexual activity of drones. Pain and Roger (1978) reported no measure of the variance in their data, but it

appears that their findings are not greatly different from those of this study, given the high levels of variation between individuals which we have observed. Mandibular pheromone production by mated, laying queens might be expected to show a more consistent rate of production due to the role of the pheromone in colony functions. A constant production rate and a constant loss rate from the cuticle, as described earlier, result in equilibrium amounts of 9-ODA on the queens' body. Seeley and Fell (1981) extracted approximately 75 μg of 9-ODA from mated queens which had been frozen within an hour of being removed from their colonies, and analyzed later. These results are of the same order as the mean of 92 μg found in this study in the glands of queens which had just been removed from their colonies.

Retinue pheromone is released onto the queen's body surface, where, probably through grooming, it becomes distributed mainly on the head and feet (Slessor et al. 1990). The amounts on the cuticle are likely to vary greatly between individuals, and possibly between different races. We found large (order of magnitude) variation in the amounts of 9-ODA carried by different queens, and Kaminski et al. (1990) found differences in the dose response for retinue behavior of workers from different geographical regions.

A portion of the exuded pheromone is re-internalized by the queen. The nature of this internalization is unknown, but the rapid appearance of radio-label in the gut suggests

that some material is swallowed. However, the greatest amount of the material which could not be washed from the body surface was not associated with the gut or the haemolymph, but with the rest of the corpse. This material may have been held on the cuticle or been in the process of moving through it. The radio-label detected in the haemolymph might have reached there via the gut, through the cuticle, or both. Given the position of tritium labelling, at C-3, there would be essentially no non-specific tritium exchange from the ß position of the α,ß-unsaturated acid during the course of the experiments. Butler et al. (1974) demonstrated that 9-ODA tritiated alpha to the 9-keto group was transmitted internally when applied to the thoraces of workers. However, the authors suggested that their result may have been due, at least in part, to tritium exchange with tissue material. The large amount of 9-ODA apparently internalized by the queen is somewhat surprising, but might involve active transport of 9-ODA or a metabolite. The estimated rate of re-internalization may be one of the weakest components of our model, because, unlike workers, the disappearance of radio-label from the body surface of queens did not conform ideally to a first order function (see Fig. 2-4a). The reason for such a difference is unclear.

A second portion of the queen's 9-ODA production is deposited on the comb wax, with a 12 minute half-life of availability to workers. This is less material than is

usually removed from the queen by retinue bees. The effectiveness of queen tracks, which we have shown here contain QMP, has been calculated to decline exponentially with a half life of 20 minutes (Juska, 1978). Small amounts of pheromones in the wax may remain attractive to workers (Juska's bioassay) even though the amounts which are acquired by the workers decline at a faster rate.

The greatest fraction of the 9-ODA on the surface of the queen is removed by workers in the retinue. Relatively few workers in the retinue make close physical contact with the queen or remove significant quantities of 9-ODA; those workers that made prolonged contact (licking) were able to remove pheromone at a rate approaching 0.5 ng/s. Other evidence for the efficiency of honey bees in removing this pheromone comes from Winston et al. (1989) who found that glass slides containing 10 Qeq of synthetic QMP were completely cleaned by colonies of 8,000 - 10,000 workers in less than 24 h (>30 ng/s). Allen (1957) found that, in a colony of 3000 - 7000 bees, fewer bees were engaged in licking than in examining (antennating) the queen. The mean number of workers attending the queen in the summer was 6 while she was moving, 8 when egg-laying, and 10 when stationary. In contrast, only 1 out of 89 attendants licked the queen while she was moving, compared with 1 out of 24 while egg laying, and 1 out of 9 when stationary. Combined with the observation that queens spend 55% of their time stationary, 15% laying, and 30% moving (Seeley, 1979), we

can estimate a mean retinue size of approximately 8 workers, and a licker to antennator ratio of 1:14. Vaitkeviciene and Skirkevicius (1982) reported an antennator to licker ratio of 3:1, and Butler (1954) a ratio closer to 1:1. Thus, most of the workers in the retinue acquire small amounts of QMP (antennators), while only about 10% gather large quantities (lickers). This may explain Seeley's (1979) inability to detect 9-ODA on samples of bees which had just left the retinue. However, even the small amounts gathered are evidently enough to stimulate many retinue bees to a short-lived burst of increased activity and social contacts (Seeley 1979). Using a licking to antennating messenger ratio of 1:10, our model predicted an average retinue size of 11.8 for interactions with a stationary lure.

The mouthparts are far more important for acquiring pheromone than the antennae. We detected very little 9-ODA on the antennae, but considerable amounts on the mouthparts and head, and surprisingly large quantities in the gut almost immediately after worker - lure contacts. These findings are contrary to those of Free and Fergusson (1982) who reported circumstantial evidence that the attractiveness of bees that have recently been in close proximity with their queen is due to the presence of queen pheromone on their antennae. But, Fergusson and Free's study focussed on antennation in worker - queen and worker - worker contacts, and their results do not give evidence against workers carrying QMP on their body surfaces. A large quantity of

pheromone also is quickly translocated backwards on the body surface to the abdomen (see also Butler et al. 1974). A grooming behavior observed in retinue workers, and its relationship to pheromone translocation are described in the following chapter. The backwards movement of pheromone may also be partly passive. 9-ODA is present in queen mandibular glands, and undoubtedly on their bodies, as an oily mixture with other substances, and oils can spread rapidly by passive diffusion over the integument of living, active insects (Lewis, 1962).

Messenger workers, like queens, deposit 9-ODA on the wax comb, and some of this material is transferred to other workers. The greatest amount is transferred to other bees via direct worker to worker contacts, but it is not clear how material is passed between workers. Indirect evidence supporting the involvement of the antennae in worker-worker pheromone transfer has been reported by Free (1978), Seeley (1979), and Fergusson and Free (1980). Workers which have contacted a queen have an increased tendency to make antennal contacts with other workers, and this tendency is reinforced when these workers have licked the queen (Fergusson and Free 1980), suggesting, as we have shown, that workers who lick the queen acquire a stronger pheromone signal than those that only palpate her with their antennae.

Past observational studies have tended to conclude that trophallaxis plays a minor or nonexistent role in QMP transfer. In small groups (2-5 workers), less than 5% of

trophallactic interactions resulted in food transfer (Korst and Velthuis, 1982), and, in groups of 20 workers, approximately half of all contacts lasted less than 1 second (Montagner and Galliot, 1982), little time for food transfer. Van Erp (1960) found that the mere ingestion of queen extracts was ineffective at inhibiting queen cell construction.

Labor specialization as dispersers of queen pheromone lasts slightly less than 30 minutes after retinue workers leave the queen (Seeley 1979; Juska et al. 1981). Our model predicts attractiveness of 25 minutes for messengers contacted by workers. Contacts with some retinue bees appear to create secondary messengers, i.e. workers which accept a large fraction of the messengers retinue bee's load. Their subsequent behaviors have not been studied, but they might act like messenger bees, i.e. show a period of increased travel throughout the nest and increased contact frequency with other workers.

The role of the wax in the transfer of retinue pheromone has been previously underestimated. It appears to act as both a medium for pheromone movement through the nest, and as a sink. The combination of the low volatility of the decenoic acid components of the pheromone, and the ability of these compounds to diffuse into wax may provide a partial explanation for the attractiveness of old comb to bees. Our model gives an estimate of 75 minutes for the total loss of attractiveness of wax from the neighborhood of the queen

(due to 9-ODA alone). Breed et al. (1988) reported that the comb also absorbs and re-releases odors associated with incoming food, and they suggested that such odors are acquired by colony members and are important in nestmate recognition.

Most pheromone is probably made unavailable by worker internalization, both by being swallowed and via the cuticle. The rate at which 9-ODA is removed from the body surface of a worker multiplied by the thousands of individuals in a colony suggests that this internalization provides a reasonable mechanism to explain the rapidity with which the queen's signal is lost after her disappearance. Juska et al. (1981) proposed that retinue workers lose their dispersal function exponentially due mainly to volatilization of queen pheromone carried on their bodies, and suggested that the elimination of those airborne pheromones while ventilating the nest is balanced by the queen's pheromone release. The extremely low volatility of the decenoic acids at nest temperatures makes such a model unlikely. Juska et al. (1981) also considered that QMP may be removed from circulation by enzymatic degradation inside of workers. The level of the queen's signal in the nest may be maintained by a balance between the queen's production, reabsorption by the queen, and internalization and metabolic breakdown by the many workers. As will be discussed further in Chapter 4, the cleansing of queen pheromone from workers, i.e the loss of the queen's signal, is not likely due to the

dissociation of the different components of the full pheromone blend as it is moved around the nest.

CHAPTER 3

WORKER SELF-GROOMING AND THE TRANSLOCATION OF QMP

Self-grooming, a behavior common to all insects, can function to manipulate pollen, remove irritant particulate matter from the body, clean and straighten wings, remove ectoparasites, and spread pheromones of low volatility about the body (reviewed by Walker and Archer, 1988). Evidence of pheromone translocation by grooming has been reported for the house fly, *Musca domestica* L. (Dillwith and Blomquist, 1982) and the tsetse fly, *Glossina morsitans morsitans* Westwood (Langley and Carlson, 1983). Butler (1954) found that honey bee workers could obtain 'queen substance' (mandibular gland pheromone) from any part of a queen's body, suggesting that it had been moved backwards from its source glands in the head, but the mode of this movement was not investigated.

The most abundant QMP component, 9-keto-2(E)-decenoic acid, or 9-ODA, is rapidly translocated, in substantial amounts, to the legs, thorax, and abdomen of retinue workers after contacting a pseudo-queen lure, despite the fact that this pheromone is gathered primarily with the mouthparts (Chapter 2). The backwards movement may be due to grooming, passive diffusion, or both. Transfer of QMP to the feet and abdomen of retinue workers may increase the rate at which pheromone can be passed on to other workers, directly

through contact, or indirectly via the comb wax. Grooming behaviors of the honey bee have been described, in part, by Jander (1976), and I have observed a common pattern of grooming associated with queen attendance and the removal of synthetic QMP from lures. In this Chapter, I describe retinue worker grooming and experiments designed to investigate the importance of such grooming in the translocation of QMP on workers.

MATERIALS AND METHODS

The Grooming Behavior of Retinue Bees

Artificial-queen lures were created by topically applying, to freshly killed workers, 11 μl of methanol, MeOH, containing 10^{-3} Qeq of synthetic QMP (250 ng 9-ODA, 150 ng 9-hydroxy-2(E)-decenoic acid (71% R-(-), 29% S-(+)), 13 ng methyl p-hydroxybenzoate, and 1.5 ng 4-hydroxy-3-methoxyphenylethanol), and 8 ng of [^{3}H]-9-ODA (Webster and Prestwich, 1988) (activity = 450,000 disintegrations/min). This quantity of QMP is approximately the mean amount found on the body surface of mated queens (Chapter 2) and approximately 10^{-3} the mean amount found in the mandibular glands of a mated queen (Slessor et al., 1990). Dead workers were used because they present a surface for pheromone acquisition that is most like that of a queen. Queens themselves were not used for lures because foreign workers may greet them with aggression and behaviors not normally associated with the retinue. After 2-3 min, these lures were placed into Petri dishes with 15 workers which had been removed from their host colony at least 1 h previously. This number of workers is sufficient to yield retinue behavior. Interactions between workers and lures, and subsequent worker behaviors, including self-grooming, were videotaped using a Panasonic VW-CD110 color camera, an AG-1950 video cassette recorder, and a CT-1330MC color video

monitor (Matsushita Electric Industrial Co. Inc., Fujisawa, Kanagawa, Japan). Recordings were analyzed for time between the beginning of lure contact and first occurrence of grooming behaviors, and/or probability and frequency of occurrence of grooming behaviors in the next 60 - 90 s. To test for whether or not observed grooming behaviors were exclusive to pheromone transfer, and to control for the effect of MeOH on worker behaviors, workers from the same host colony were also observed after contacts with dead bees treated with MeOH, or with untreated bees.

Grooming Behaviors and Pheromone Translocation

Lures treated with synthetic QMP containing [^{3}H]-9-ODA were placed into Petri dishes containing 15 workers. After making sustained (>5 s) contacts with the lure, individuals which had also subsequently or concurrently groomed their mouthparts, thorax, and abdomen, for any length of time, were removed. An equal number (n = 13) of lure-contacting, workers were pulled from the dishes before any grooming had occurred. Removed workers were quickly dissected to yield mouthparts, head, legs, thorax, abdominal integument, and abdominal contents. The thorax and abdomen were first frozen on dry ice to allow complete removal of the alimentary tract and its contents from the abdomen. To remove contaminating radio-label, all dissecting implements were twice washed with MeOH between operations. The body

parts were then immersed in 10 ml of BCS biodegradable counting scintillant (Amersham Canada Ltd, Oakville, Ont.). After 24 hr, the samples were separately analyzed for radio-label content with a Beckmann LS 5801 liquid scintillation counter (Irvine, CA).

Interfering with Grooming

In order to interfere with grooming patterns, all legs of 2 workers were secured to the bottom of plastic Petri dishes with Duco^{R} cement (Devcon Corp., Wood Dale, Illinois), 13 nestmates were then introduced, and the dishes of bees left undisturbed, with a source of sugar syrup, for at least 1 h. Lures treated with synthetic QMP containing [^{3}H]-9-ODA were then introduced to the dishes by presenting them directly to the restrained workers. The unrestrained workers were free to make contact with the lures. After 2 - 4 min, the glued bees and several of the unrestrained workers which had made contact with the lure were removed, killed by freezing with dry ice, and dissected to yield mouthparts, head, front legs, middle legs, hind legs, thorax, and abdomen. Antennae were also dissected from some individuals. The abdomens were washed with 6 consecutive rinses of 100 μl of MeOH to collect the 9-ODA from the surface. All of the body parts and the MeOH wash were then analyzed for [^{3}H]-9-ODA content as described above. Fourteen glued bees and 10 free running bees were analyzed.

Passive movement of 9-ODA was investigated by topically applying 1 μl of the synthetic pheromone solution to the heads of freshly-killed workers. After 2 min, head, thorax, and abdomen of each worker (n = 25) were removed and individually analyzed for radio-label content by scintillation counter.

Data were analyzed by t-tests using Minitab^{R} data analysis software (State College, PA). Proportional data were arcsin-square root transformed. The analyses are only for workers which had gathered amounts of radio-label at least 2 standard deviations greater than average background levels; workers containing only background counts can show false and ambiguous patterns of radio-label distribution.

RESULTS

Grooming Behavior of Retinue Bees:

The grooming behaviors most commonly observed for workers in, or just leaving, the retinue were as follows: **Antennal cleaning.** This was the most frequent grooming behavior of the retinue bees. They typically scraped one antenna at a time by stroking it distad with the ipsilateral foreleg cleaner. **Head cleaning.** The rest of the head also was occasionally swiped by downward movements of a foreleg. **Mouthpart grooming.** The mouthparts were frequently cleaned by individuals that had made contact with a lure or queen with their proboscis. Usually, both foretarsi would simultaneously scrape the unfolded proboscis, in a direction away from the head, often 3-4 times consecutively. **Thorax and abdomen grooming.** Less frequently, retinue workers would step back from the lure to groom legs, thorax, and abdomen, i.e. forelegs rubbed middle legs and thorax, middle legs contacted hind legs, thorax, and/or abdomen. Some individuals stroked the hind legs several times along the sides of the abdomen. Stroking of the legs against each other, the thorax, or the abdomen occurred more commonly when individuals had contacted a lure with their legs, or after they had scraped their mouthparts several times with their forelegs. The workers which had visited the synthetic

QMP-treated lures had all made mouthpart (licking) contact with a lure. In Chapter 2 it was shown that such workers gather considerably more QMP than workers making only antennal contact.

There was a greater tendency for bees that visited lures containing synthetic pheromone or MeOH to perform grooming behaviors (percentage performing each behavior) than those visiting the blank controls (Table 3-1). However, there was little difference in the types of grooming behaviors observed between individuals visiting lures, i.e. once an individual was grooming, frequencies and times to initiation of individual movements were similar, regardless of the type of lure contacted. An exception was contact with a synthetic QMP source, which released a more rapid initiation of mouthpart grooming, than contact with either type of control lure.

Grooming Behaviors and Pheromone Translocation

Grooming and non-grooming workers gathered similar total amounts of radio-label (40.2 ± 16.1 pg and 37.8 ± 12.1 pg respectively; t-test, $t = -0.11$, $df = 24$, $p = 0.91$), and carried similar amounts externally (21.9 ± 9.4 vs 26.5 ± 8.7 pg; $t = 0.37$, $df = 24$, $p = 0.72$). However, the proportion of the externally-carried total that was found on the mouthparts was significantly greater for the non-grooming workers (Fig. 3-1; $t = 4.22$, $df = 24$, $p = 0.0006$), and that

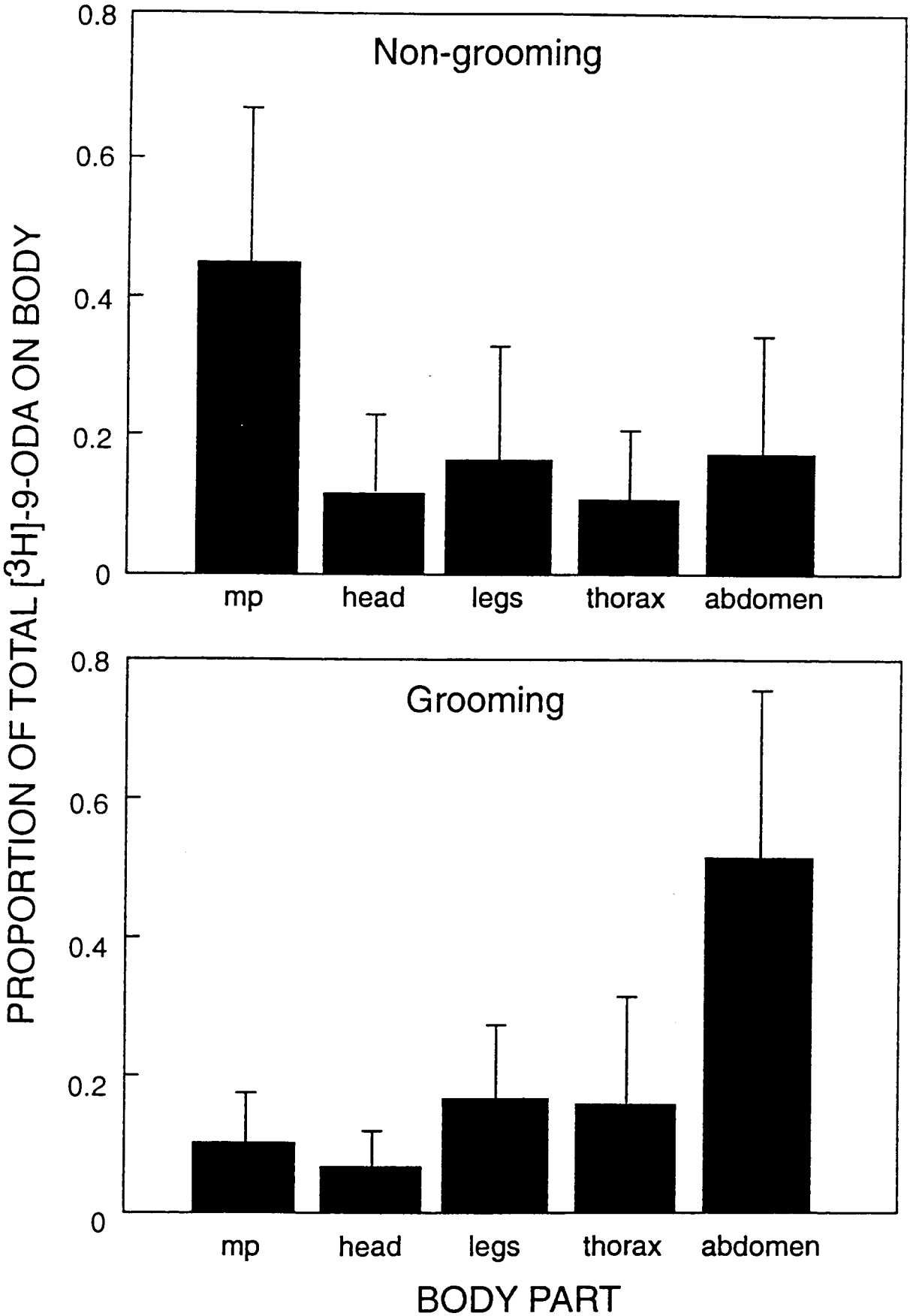
Table 3-1. Grooming characteristics of workers visiting lures containing synthetic QMP, MeOH, or blanks.

	duration of contact (sec)	time to first ant. groom	time between ant. groomings	time to first m.p. groom	time between m.p. groomings	time between f.l. groomings	time between m.l. groomings	time between r.l. - abd. groomings
QMP	38.4 ± 23.3a	4.4 ± 2.2a	14.8 ± 10.3	7.9 ± 5.5	20.4 ± 18.0	42.2 ± 42.8	27.9 ± 25.4	21.9 ± 12.2
percentage of bees performing each behavior		(92)	(92)	(100)a	(100)	(92)	(75)	(50)
MeOH	4.1 ± 2.0b	6.8 ± 6.0a	17.8 ± 13.8	21.3 ± 29.1	20.0 ± 10.0	17.1 ± 8.5	20.8 ± 7.4	15.8 ± 7.4
percentage of bees performing each behavior		(88)	(88)	(19)b	(19)	(75)	(38)	(38)
Blank	1.5 ± 0.5c	20.0 ± 12.0b	18.3 ± 6.9	16 ± 0	6.7 ± 0	13.2 ± 5.7	17.5 ± 11.4	22.9 ± 12.1
percentage of bees performing each behavior		(60)	(60)	(10)b	(10)	(80)	(30)	(30)

Values are means ± standard dev. Sample sizes were QMP 12, MeOH 16, and blank 12. Times are seconds. Ant., antennae; m.p., mouthparts; f.l.,fore legs; m.l., middle legs; r.l., rear legs; abd., abdomen. Within a column, values followed by different letters are significantly different. Only significantly different groupings areso marked, (duration of contact, ANOVA; time to antennal groom, ANOVA, $F=11.4$, $d.f.=2.28$, $p<0.05$, Student Newman Keuls, $\alpha=0.05$; percentage involved in grooming, binomial tests, $p<0.001$), hypothesis QMP > MeOH = blank).

Figure 3-1.

Mean (± SD) relative proportions of acquired [^{3}H]-9-ODA on different body parts of self-groomed (mouthparts, thorax, and abdomen) and non-groomed workers, after conatct with a pheromone lure. Mp, mouthparts.


Non-grooming
Grooming
0.8
0.6
0.4
0.2
0
mp
head
legs
thorax
abdomen
PROPORTION OF TOTAL [3H]-9-ODA ON BODY
BODY PART

on the abdomen was significantly less ($t = -3.98$, $df = 24$, $p = 0.0008$). There were no significant differences for the relative amounts on other body parts, however the non-grooming workers showed a trend towards carrying relatively more material on their heads ($t = 2.06$, $df = 24$, $p = 0.051$).

Interfering with Grooming:

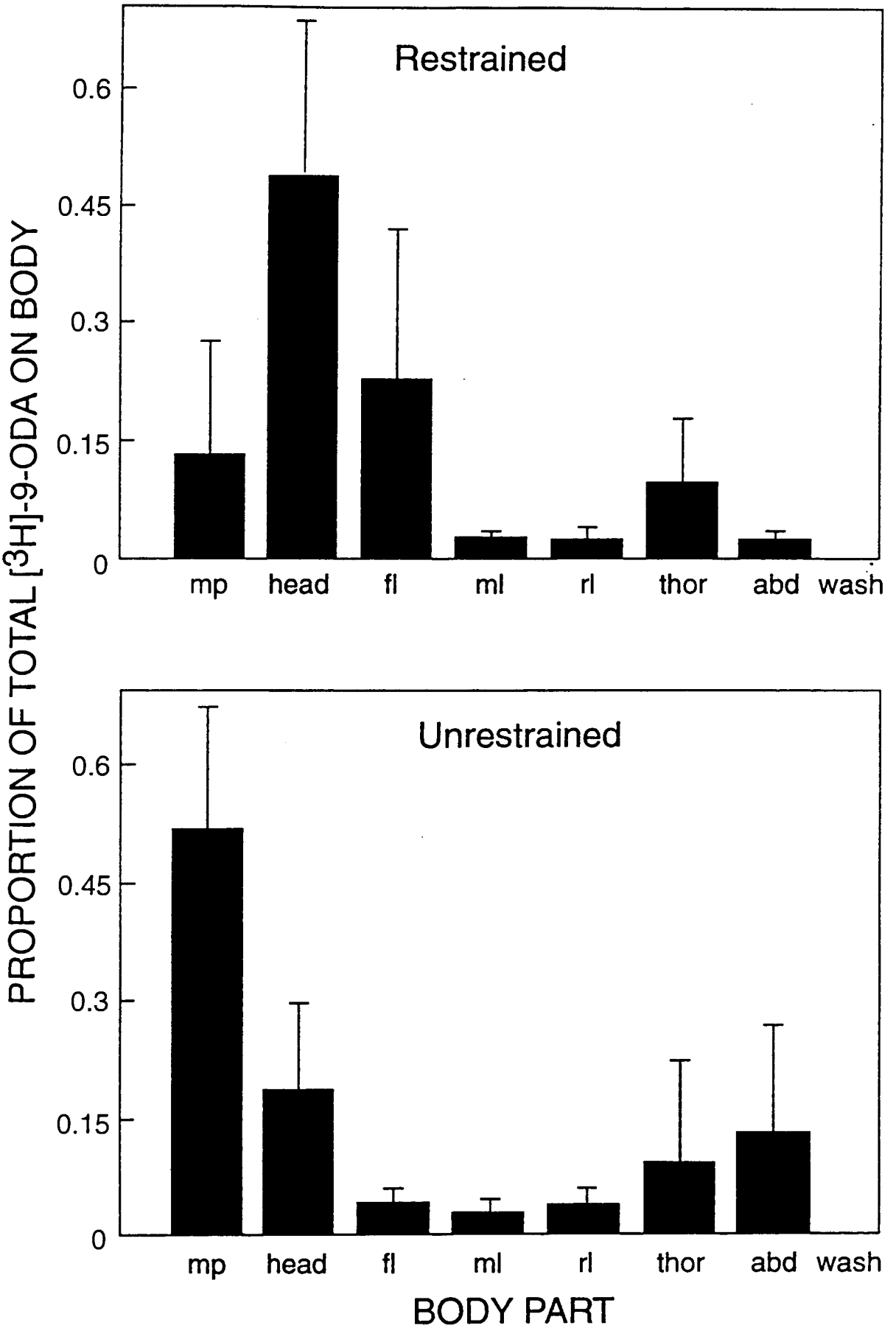
Workers with legs fixed to the substrate took up tritiated pheromone from a dead bee lure. However, their contacts were not as prolonged as those of free running bees, and usually did not involve as much proboscis contact. As a result, free running bees gathered greater amounts of [^{3}H]-9-ODA (203 ± 63 pg vs 44 ± 10 pg ($\bar{X}$ ± S.E.); t-test, $t = 2.51$, $p = 0.033$)(untreated controls yielded a background value of 0.2 pg), and swallowed substantially more (abdomen counts; 148 ± 54 pg vs 3 ± 1 pg). If only the amounts on the body surface are considered, then both types of bees gathered similar amounts (65 ± 16 pg vs 41 ± 10 pg; $t = 1.27$, $p = 0.22$), but the pattern of [^{3}H]-9-ODA distribution on the body was different (Fig. 3-2). The proportion of the total recovered quantity of externally carried [^{3}H]-9-ODA that was found on the head was significantly less for the free running workers ($t = 4.0$, $p = 0.0007$). The proportion of the total found on the forelegs of the free running workers was also significantly less ($t = 2.91$, $p = 0.012$), but the proportion of the total that was on the abdomen,

ie., in the MeOH wash, was significantly higher ($t = -2.69$, $p = 0.025$). Restrained workers had significantly greater mean amounts of tritiated 9-ODA on their antennae than free running bees (2.5 ± 0.9 pg vs 0.35 ± 0.05 pg; $n = 11, 4$, $t = -2.43$, $p = 0.036$).

There was no detectable movement of [^{3}H]-9-ODA from the head to the abdomen of dead workers in 2 min; 100% of the recovered [^{3}H]-9-ODA being detected on the heads.

Figure 3-2.

Mean (± SD) relative proportions of acquired [^{3}H]-9-ODA on different body parts of workers that had legs fastened to substrate (restrained), or workers free to move. Radio-label was gathered by the workers from a lure. mp, mouthparts; fl, front legs; ml, middle legs; rl, rear legs; thor, thorax; abd wash, methanol wash of the abdomen.


Restrained
0.6
0.45
0.3
0.15
0
mp
head
fl
ml
rl
thor
abd
wash
Unrestrained
0.6
0.45
0.3
0.15
0
mp
head
fl
ml
rl
thor
abd
wash
PROPORTION OF TOTAL [3H]-9-ODA ON BODY
BODY PART

DISCUSSION

Backwards translocation of queen mandibular gland pheromone on worker honey bees occurred, at least in part, as a result of grooming. Butler et al. (1974) reported that tritiated 9-ODA applied to the thoraces of workers was translocated to the head and abdomen. However, as was mentioned earlier, their 9-ODA was tritiated α to the 9-keto carbon, and they suggested that the translocation that they observed may have been due to tritium that had exchanged with tissue material. Also, their results could not be used to deduce whether the route of translocation was internal or external. Individuals that lick a pheromone source swallow some of the material, thus moving it rapidly backwards.

The grooming movements observed in this study do not appear to be unique to pheromone transfer. Movements involving the forelegs were the same as those in Jander's (1976) description of self cleaning and pollen manipulation, and behaviors of workers visiting pheromone-lures were not qualitatively different from those visiting MeOH-treated ones. The spreading of pheromone over the body as a result of grooming is somewhat unusual in that grooming is usually undertaken to remove materials from the body. The persistent cleaning of the antennae by retinue bees, which removes 9-ODA, probably serves to keep chemo-receptor sites clear, and grooming of the proboscis may similarly remove materials from an area with chemo-sensory cells. The free

running workers in the second part of this study, and those of Chapter 2, showed almost no 9-ODA on their antennae, suggesting that normally the antennae are rapidly and effectively cleaned after contact with a 9-ODA source. Contact with MeOH lures also led rapidly to antennal grooming, and even some workers contacting blank dead bee lures frequently cleaned their antennae. However, workers that had visited pheromone treated lures were more likely to lick the lures and to clean their mouthparts afterwards. Similarly there was no evidence that leg - abdomen contacts were used specifically to translocate pheromone since only some lure-visiting individuals performed such activities. The grooming of the legs, thorax, and abdomen may simply be the self cleaning behaviors of individuals that find themselves carrying an oily substance; even such incidental grooming can facilitate the spread of 9-ODA over the bodies of workers.

Little of the pheromone spread over workers can be attributed to a tendency for 9-ODA to spread passively over the body, although Lewis (1962) demonstrated that oils can, in this way, spread rapidly over the integument of living, active insects. However, occasional bumping by the free running bees, together with some passive translocation on the immobilized but struggling bees, may have deposited some of the [^{3}H]-9-ODA which was recovered from the abdominal surfaces of the bound workers.

The backwards spread of pheromone on the body may aid in transfer to other individuals by increasing the effective body surface area from which other workers can gather QMP, or via comb contact with a greater area of cuticle contaminated with pheromone. Retinue workers leave 9-ODA on the wax of the comb, and other workers can collect it from there (Chapter 2). The dispersal of 9-ODA over the body surface of a bee also may serve to increase the rate at which it is internalized and removed from circulation in the nest. The two processes acting in combination could operate to ensure that the queen's signal is both rapidly transferred through the nest, and non-persistent.

This study has provided the first evidence in a social insect species for a mechanism of contact pheromone movement on an individual. Other eusocial species may utilize similar pheromone dispersal systems. As such, grooming behaviors which serve to translocate queen-produced pheromones may play a significant role in the queen's communication with her workers.

CHAPTER 4

INTRA-NEST TRANSFER OF THE AROMATIC COMPONENTS OF QMP: TRANSPORT OF THE COMPLEX AS A UNIT

Most insect semiochemicals have proven to be complex mixtures (Hölldobler and Carlin, 1987; Silverstein and Young, 1976), and the honey bee queen mandibular gland pheromone complex (QMP) is no exception (Slessor et al., 1988). Different components within a blend may potentially act together as a whole, or the various constituents may occasionally be involved in separate functions. Different components also may be transported differentially, especially in the case of contact pheromones, resulting in the loss of the chemical signal provided by the whole blend.

The purpose of the research reported here was to investigate the intra-nest transfer of the 2 aromatic components of the queen mandibular gland pheromone complex, HVA (4-hydroxy-3-methoxyphenylethanol) and HOB (methyl p-hydroxybenzoate). These 2 compounds are chemically different from 9-ODA as well as being much less abundant in the full blend (0.6% as abundant for HVA and 5.2% for HOB). They have the potential therefore to be transferred differently, or in different relative amounts than 9-ODA, resulting in the break-up of the queen's pheromone signal at some point after its exudation by the queen. Evidence is presented however, that these 2 components, and 9-ODA, are transferred between different entitites within the nest at

similar rates, suggesting that the pheromone complex is received by workers throughout the nest as a full-blend complex.

MATERIALS AND METHODS

Pheromone Production and Exudation

Estimates for the production rates and equilibrium amounts of 9-ODA on the body surface of queens were earlier determined by GC and GC-MS (Chapter 2). The sensitivity of the methodology used, however, did not allow accurate determinations of HVA or HOB production levels. The assumption is made, therefore, that production and exudation rates of 9-ODA, HVA, and HOB rates are in the same proportions as are the mean amounts of those 3 compounds in the mandibular glands of queens. It is unlikely that different QMP components are excreted in ratios different from those at which they are found in the glands, because of mixing in the lumena of the glands.

Pheromone Internalization by Queens and Workers and Deposition on Wax by Queens

To determine the rate of absorption and/or binding of HVA and HOB by the cuticle of queens, 10^{-3} Qeq of synthetic QMP, containing 1.2 ng of either $[^3H_2]$-HVA or $[^3H_2]$-HOB (2 atoms of tritium per molecule), was topically applied, in 8 μl of methanol, to the heads of 40 mated queens. Slessor et al. (1988) found the greatest proportion of externally

carried QMP could be found on a queen's head and on the feet, presumably as a result of grooming. The queens were isolated in 60 x 15 mm plastic Petri dishes, on a thin layer of wax, for 0, 5, 30, or 60 min (10 replicates each). They were then anesthetized with CO_2, and washed by dribbling 10 100 μl portions of methanol over their bodies. Two μl of haemolymph were removed, and for HVA, their alimentary canals excised.

After the queens were removed from the isolation dishes, 3 workers were admitted for 5 min each to determine if workers can pick up pheromone left on the wax by queens. The sides and tops of the Petri dishes were then washed with methanol. The queen body washes, alimentary tracts, haemolymph samples, wax substrates, dish washes, remainders of the corpses, and the workers were individually analyzed for amounts of [$^3H_2$]-HVA or [$^3H_2$]-HOB by liquid scintillation counting (L.C). The samples first were placed into plastic scintillation vials and topped with BCS biodegradable scintillation cocktail (Amersham Canada Ltd., Oakville, Ont.). Scintillations were measured, in this and the other experiments which follow, for 1 min with a Beckman LS 5801 liquid scintillation counter (Irvine, CA). Recovery efficiency of radio-label was generally greater than 90%.

The above experiments were repeated with workers. The protocol was the same, with the exception that no workers were later introduced into the dishes, and the wax and dish washes were not analyzed. The body wash represented

pheromone still on the cuticle after the isolation time, the corpse count indicated how much had passed into the body or was bound in the cuticle. Ten replicates of 1 worker each were used per treatment, including a control group of workers which were untreated but otherwise subjected to the same protocol.

Removal from a Lure: Pick-up in the Retinue

For all experiments involving the transfer of pheromone, test workers were collected in groups of 15 from a queenright colony, placed into 150x25 mm plastic tissue culture dishes, and given a source of 1:1 sugar syrup (Kaminski et al. 1990). They were then left undisturbed for a minimum of 1 hr before taking part in any test. Unless otherwise stated, ten replicates of each treatment were completed for all experiments.

The nature and amounts of pheromone removal by retinue workers (messengers) were determined by allowing workers to contact a pheromone-treated lure placed into each dish of 15 bees. Lures consisted of sister workers, killed on dry ice, and treated topically with 10^{-3} Qeq of synthetic QMP containing either 0.8 ng of supplemental $[^3H_2]$-HVA or 1.0 ng of $[^3H_2]$-HOB, on the dorsal surface. Workers showed a typical retinue response to such a lure. Individuals were allowed to make either 5, 30, or 60 s licking contact or 5 s of antennal contact with a lure. Lures for the licking

messengers carried 0.8 ng of [$^3H_2$]-HVA or 1.0 ng of [$^3H_2$]-HOB, and lures for the antennating messengers carried 2.5 ng of [$^3H_2$]-HVA or 1.0 ng of [$^3H_2$]-HOB. The licking messengers were dissected to yield antennae, mouthparts (mandibles, maxillae, and labium), head, legs, thorax, crop and gut, and abdomen. Antennal messengers were analyzed whole. Untreated workers were used as blank controls.

A further experiment was carried out to test the assumption that the amount of pheromone gathered from a source is proportional to the amount at the source. Dead bee lures were treated with 10 μl of MeOH and synthetic QMP containing radio-labelled HVA yielding either 270, 420, 1200, or 2720 dpm. Lures were individually placed into Petri dishes containing 15 workers. Lure-visiting workers were removed after 30 s of contact, and analyzed by L.C.

Transfer From Messengers to Other Workers Via Comb and Direct Contact

Dead-bee lures treated with 10^{-3} Qeq of synthetic QMP (containing a supplementary 2.5 ng [$^3H_2$]-HVA (1,950,000 dpm), or 3.5 ng of [$^3H_2$]-HOB (2,750,000 dpm)) were placed into dishes containing 15 sister-workers. When one of these workers had contacted the lure for 30 s, she was removed to either a 100 x 15 mm plastic Petri dish with a floor of wax foundation and 15 sister workers, or a similar dish with no workers. After 5 min, the bees and the wax from the first

group of dishes were analyzed by L.C. For the second group, the lone bees were removed for counting and replaced by 15 workers. These bees and the wax were counted after a further 5 min. Untreated workers and wax were used as blank controls. The wax-only treatment allowed pheromone transfer only via the wax; the second treatment allowed transfer via both the wax and direct contacts.

Movement Through Beeswax.

Synthetic QMP (10^{-3} Qeq) containing 0.8 ng of [$^3H_2$]-HVA or 1.0 ng of [$^3H_2$]-HOB, in 8 μl of methanol, was spread over 8.1 cm^3 discs of beeswax on the bottom of 30 x 10 mm plastic Petri dishes. These were left undisturbed at 32° C (nest temperature) for 0, 30, 60, or 120 min. At that time, 2 workers were introduced to the dishes for 5 min. Each was quantitatively analyzed for radio-label content by L.C. Untreated workers on untreated wax were used as controls.

Data Analysis

Individuals in all experiments were assigned randomly to experiments, and, except where indicated, 10 replicates of each treatment were performed. Data, occasionally transformed, were analyzed by correlation or one way analysis of variance (ANOVA) with a completely randomized design, and, where significance was found, by a Student

Newman Keuls (snk) multiple comparison test for differences between treatments (Zar, 1984). Data are presented as means ± standard errors. Analyses were performed using SAS statistical software (Cary, NC).

Determination of Rate Constants

Rate constants for the transfer of HVA and HOB were determined in the same way as those for 9-ODA (see results section of Chapter 2).

RESULTS

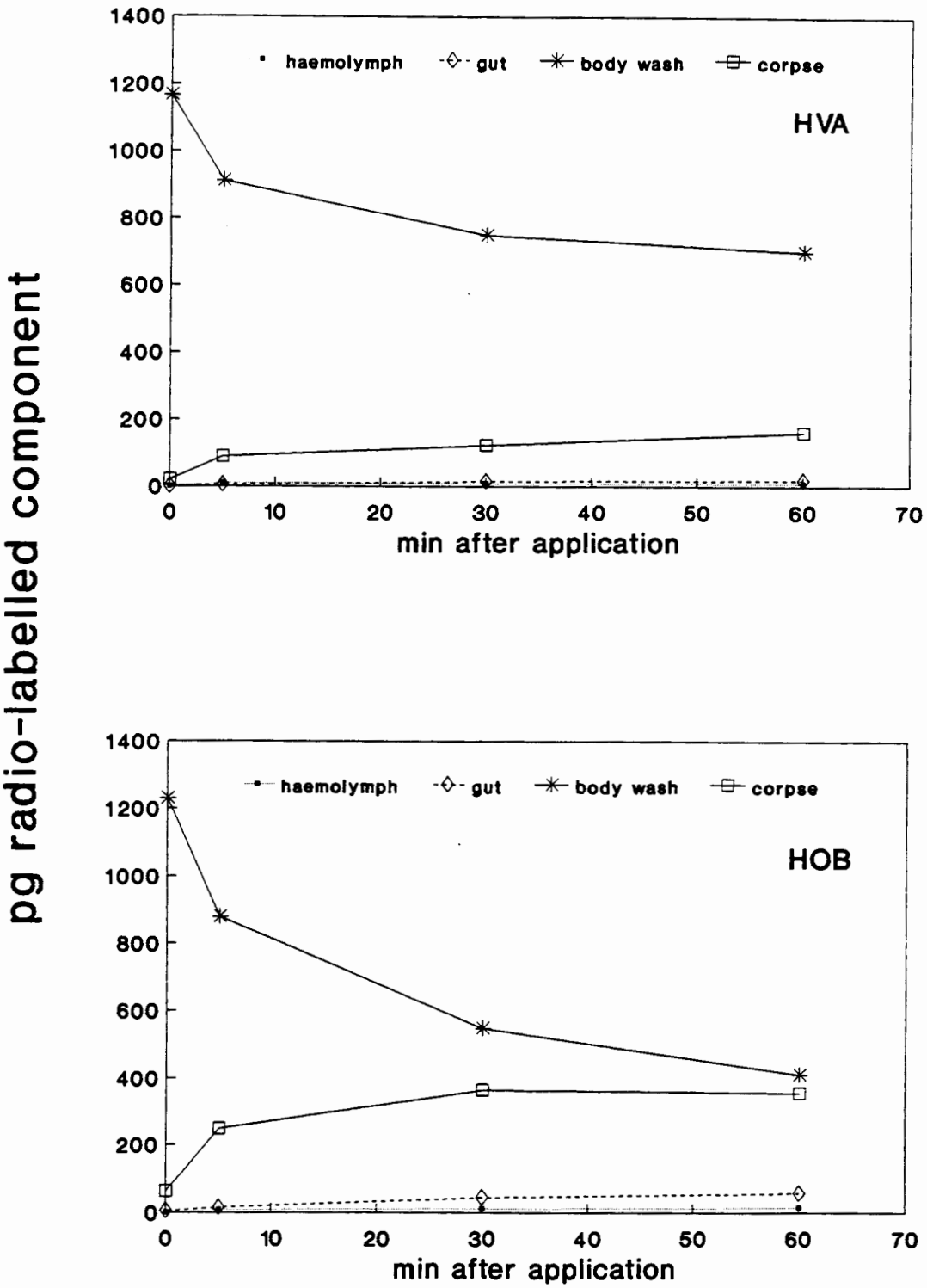
Rates of Production

Assuming that quantities of QMP components on the body surface of a queen are in the same ratios as found in the glands (Slessor et al., 1990; Chapter 2), it can be estimated that 5.1 ng of HVA and 30.5 ng of HOB can be found on the body of a mated queen at equilibrium.

Fig. 4-1 shows the pattern of radio-label internalization from the cuticular surfaces of live queens. From the body surface, some material was apparently swallowed, a lesser amount entered the haemolymph, and other material became associated with the remainder of the corpse. By 5 min after application, the quantity of radio-label detected in the haemolymph was significantly greater than control values for both HVA (t-test; $t = 5.6$; d.f. = 9; $p < 0.001$) and HOB ($t = 3.0$; d.f. = 9; $p = 0.01$), as were values for the gut (HVA, $t = 6.0$, d.f. = 9, $p < 0.001$; HOB, $t = 3.9$, d.f. = 9, $p = 0.001$). The increases in the amounts of radio-label detected with time were significant for all body parts, and for both components (correlations, p's <0.001), with the exception of the amounts of [$^3H_2$]-HVA in the haemolymph ($r^2 = 0.24$, $p = 0.13$), where the increase did not continue significantly beyond 5 min. The amounts of radio-label that could be washed from the queens decreased with the time spent in isolation ($p < 0.001$).

Figure 4-1.

Fate of HVA and HOB on the body surface of queens. Queens were isolated for different lengths of time after topical applications of synthetic QMP containing 1.2 ng of [$^3H_2$]-HVA or [$^3H_2$]-HOB. Sample size at each time = 10.


pg radio-labelled component
haemolymph
gut
body wash
corpse
HVA
1400
1200
1000
800
600
400
200
0
0
10
20
30
40
50
60
70
min after application
haemolymph
gut
body wash
corpse
HOB
1400
1200
1000
800
600
400
200
0
0
10
20
30
40
50
60
70
min after application

Pheromone Acquisition by Worker Bees from a Lure

For both compounds, the quantities detected on workers making licking contact with synthetic QMP-treated lures increased with the duration of contact (Fig. 4-2). Five seconds of licking contact gathered 35 ± 15 pg of HVA, or 3.5% of what was available on the lure. The values for HOB were 12 ± 6 pg, or 1.2% of the total on the lure. For 30 s of contact, the amounts were 54 ± 24 pg (5.4 % of the total available) for HVA, and 25 ± 12 pg (2.5 %) for HOB. The pattern of pick-up (Fig. 4-3) was consistent for both compounds and for the duration of contact, and was qualitatively the same as that which has been observed for 9-ODA. The amount of radio-label gathered was proportional to the amount available on the lure (Fig. 4-4; $r^2 = 0.64$, $p = 0.0001$, log transformed data). For antennating bees, a mean of 0.5 ± 0.1 pg of [$^3H_2$]-HVA and 0.6 ± 0.1 pg of [$^3H_2$]-HOB were gathered in 5 s antennal contacts with a radio-label treated lure carrying 2500 pg of [$^3H_2$]-HVA or 1000 pg of [$^3H_2$]-HOB. These represent 0.02% and 0.06% respectively of the totals available. The percentages gathered were significantly less than those for 5 second licking contacts (t-tests; HVA, $t = -3.14$, d.f. = 13, $p = 0.01$; HOB, $t = -3.00$, d.f. = 11, $p = 0.015$, square root transformed data).

Figure 4-2.

Acquisition of HVA and HOB by licking retinue bees. Values are pg of [$^3H_2$]-HVA and [$^3H_2$]-HOB gathered from dead workers to which synthetic QMP containing 0.8 ng of supplemental [$^3H_2$]-HVA or 1.0 ng of [$^3H_2$]-HOB had been topically applied. Values are means ± SEs.

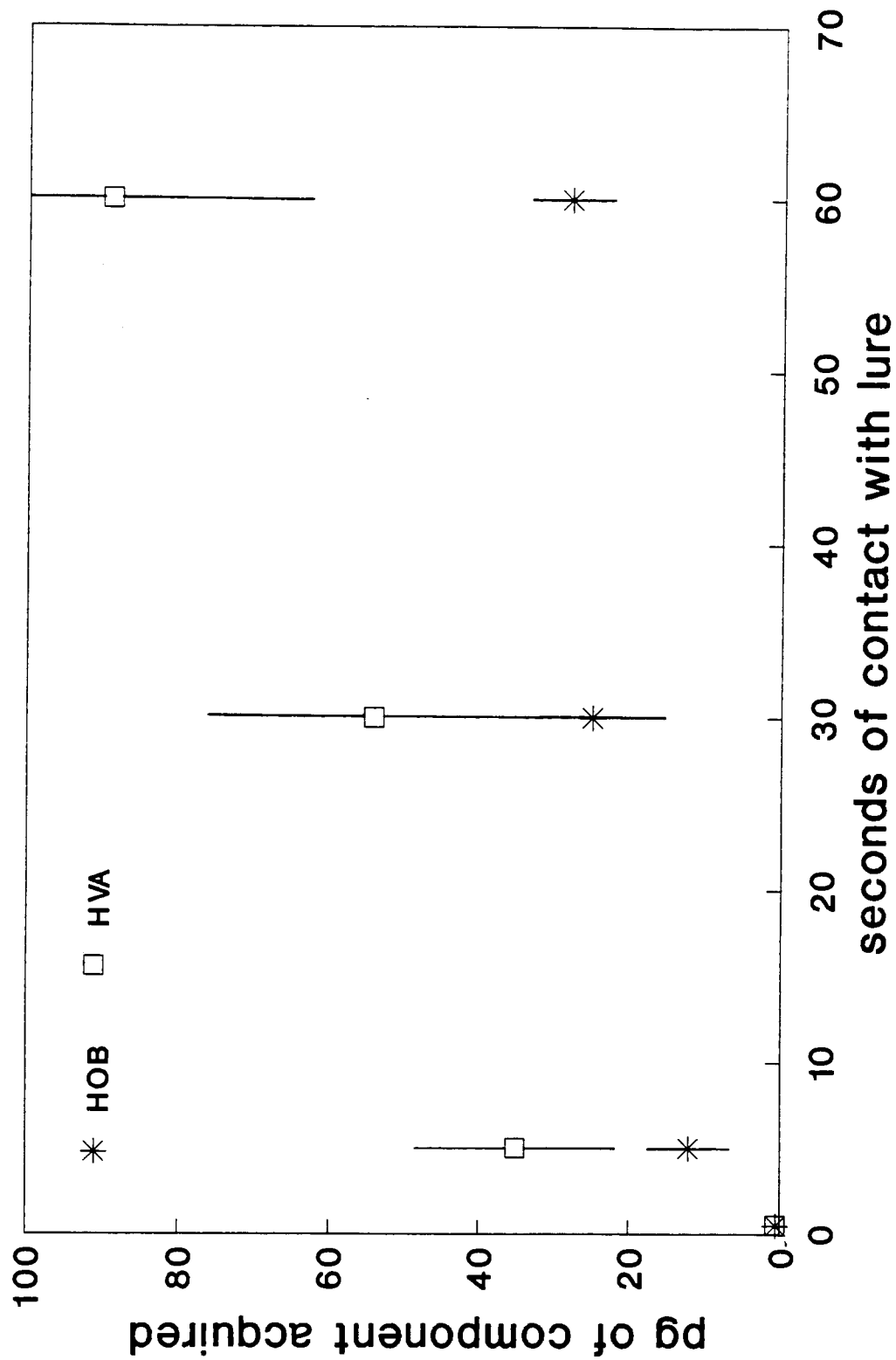
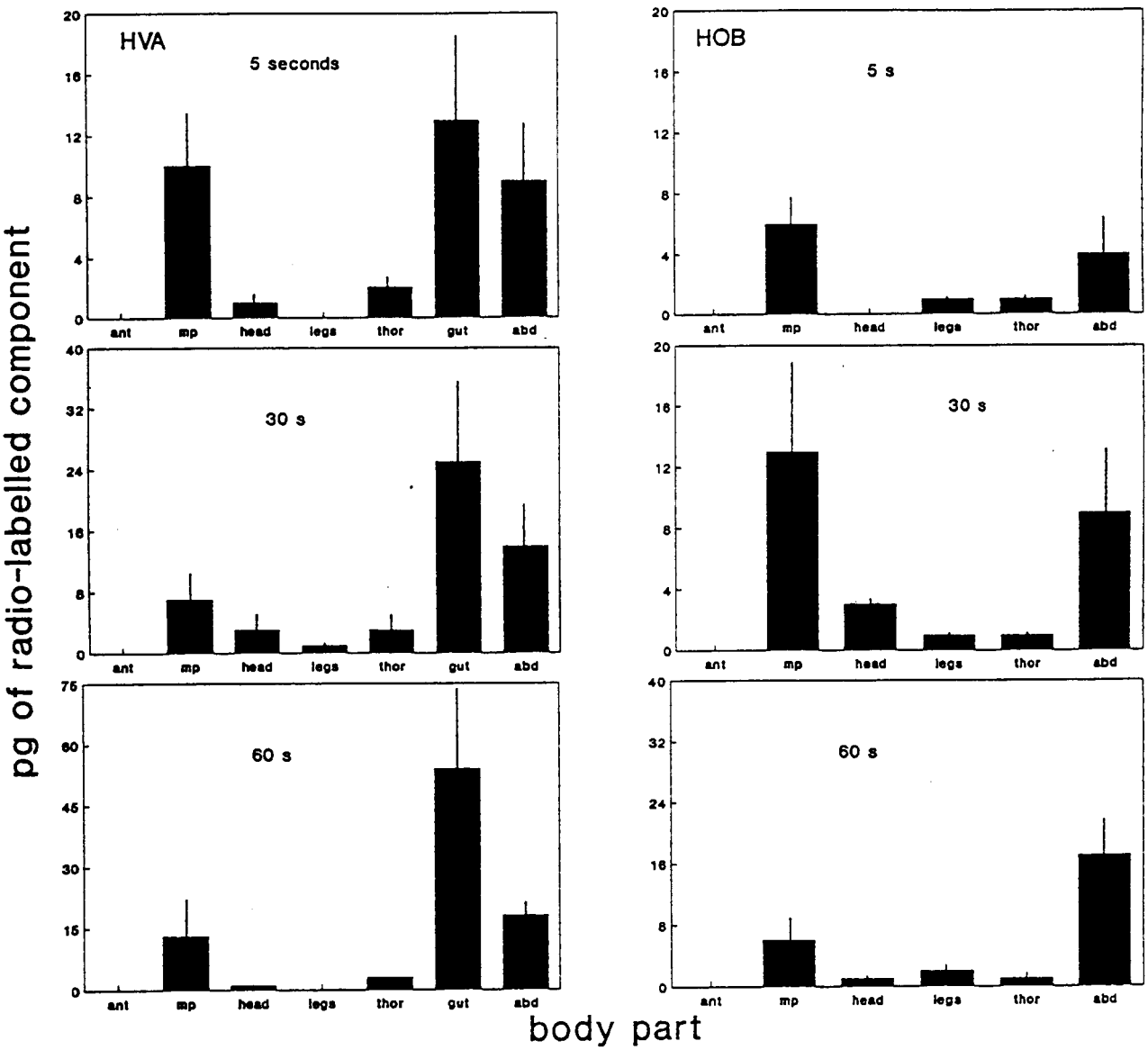

pg of component acquired
100
80
60
40
20
0
0
10
20
30
40
50
60
70
seconds of contact with lure
HOB
HVA

Figure 4-3.

Pattern of HVA and HOB acquisition by licking retinue bees. Amounts are mean ± SE pg of $[^3H_2]$-HVA or $[^3H_2]$-HOB found on different body parts of workers after different times of contact with dead bee lures topically treated with 10^{-3} queen equivalents of synthetic QMP with a supplemental 0.8 ng of $[^3H_2]$-HVA or 1.0 ng of $[^3H_2]$-HOB. Abbreviations: ant, antennae; mp, mouthparts; thor, thorax; gut, alimentary tract; abd, abdomen. Note that there are no gut data for HOB.


HVA
5 seconds
HOB
5 s
30 s
30 s
60 s
60 s
ant
mp
head
legs
thor
gut
abd
pg of radio-labelled component
body part

Transfer from Queens to Workers via the Wax

The amounts of [$^3H_2$]-HVA and [$^3H_2$]-HOB deposited by the queens onto a wax substrate (Fig. 4-5), and later acquired by workers from the wax (Fig. 4-6), both increased with the length of time spent on the wax by the queens (correlations of amounts vs time, p's < 0.01).

Worker to Worker Pheromone Transfer

In this experiment, retinue bees, i.e. those that had licked a lure, gathered 32 ± 10 pg of HVA and 42 ± 13 pg of HOB. Five min of direct individual to individual contacts between groups of 15 workers and a retinue bee resulted in 2 ± 0.6 pg of [$^3H_2$]-HVA (or 2.2% of the total available) and 1.4 ± 0.7 pg of [$^3H_2$]-HOB (2.0%) being transferred to each of the 15 bees. The retinue bees left 3.0 ± 1.1 pg of [$^3H_2$]-HVA and 1.2 ± 0.3 pg of [$^3H_2$]-HOB on a wax surface in the same length of time, and 15 workers subsequently introduced onto the wax each gathered 0.1 ± 0.05 pg of [$^3H_2$]-HVA (i.e. 0.5% of what the average retinue bee carried) and 0.1 ± 0.01 pg of [$^3H_2$]-HOB (0.3%) in 5 min. The latter quantities were not significantly different than the mean gathered from blank controls (0.03 ± 0.001 pg), but were significantly less than the amounts of each compound transferred via direct contacts (ANOVA; HVA, F = 16.4, d.f.

Figure 4-4.

Mean (± SE) pg of [$^3H_2$]-HVA gathered by workers making 30 s licking contact with dead bee lures topically treated with different amounts of synthetic QMP containing [$^3H_2$]-HVA.

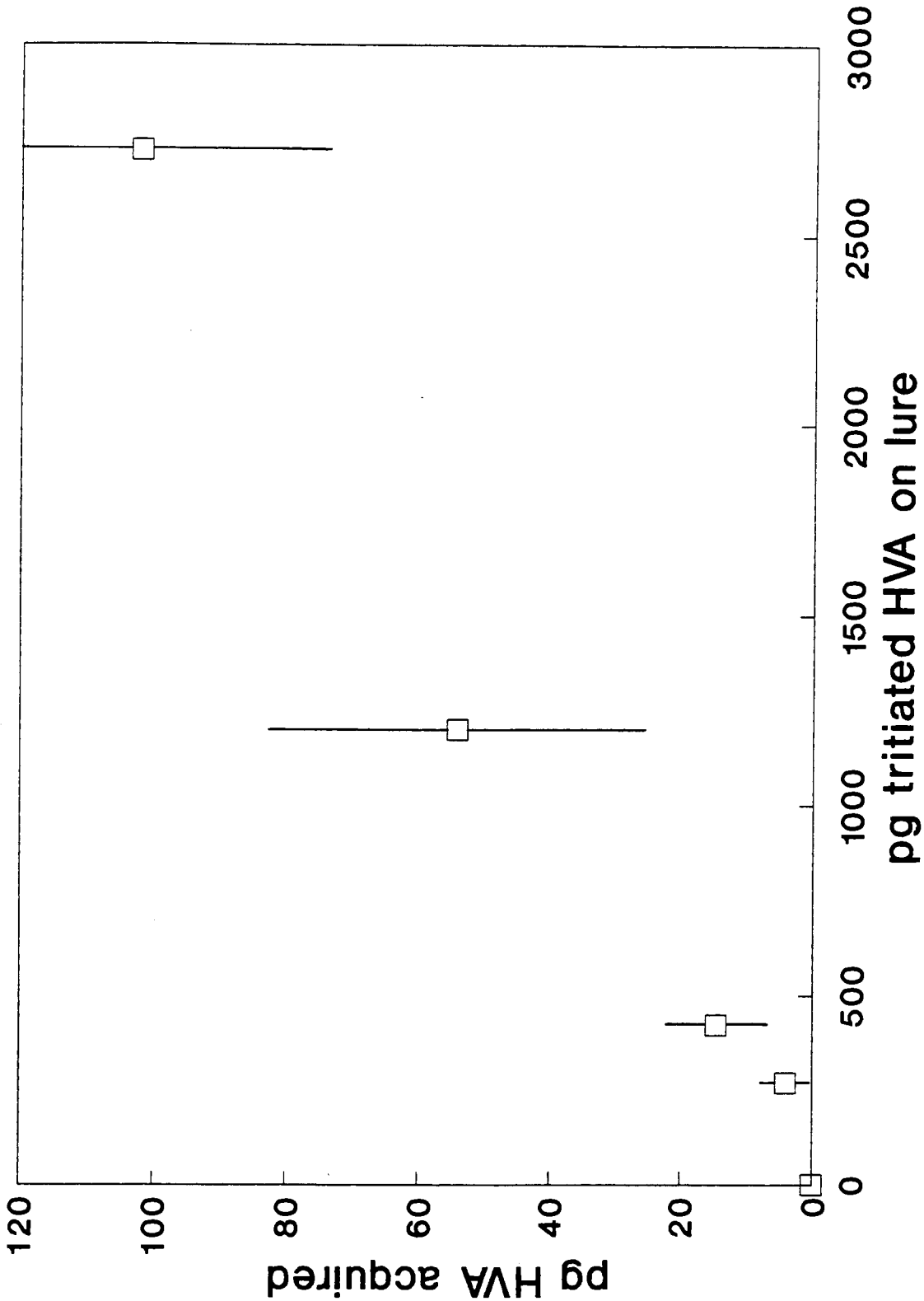

pg HVA acquired
120
100
80
60
40
20
0
0
500
1000
1500
2000
2500
3000
pg tritiated HVA on lure

Figure 4-5.

Deposition of HVA and HOB on wax by queens. Values are mean (± SE) pg of [$^3H_2$]-HVA or [$^3H_2$]-HOB left by queens after different times of isolation on wax discs. Queens were initially given topical applications of synthetic QMP containing 1.2 ng of [$^3H_2$]-HVA or [$^3H_2$]-HOB. Sample size at each time = 10.

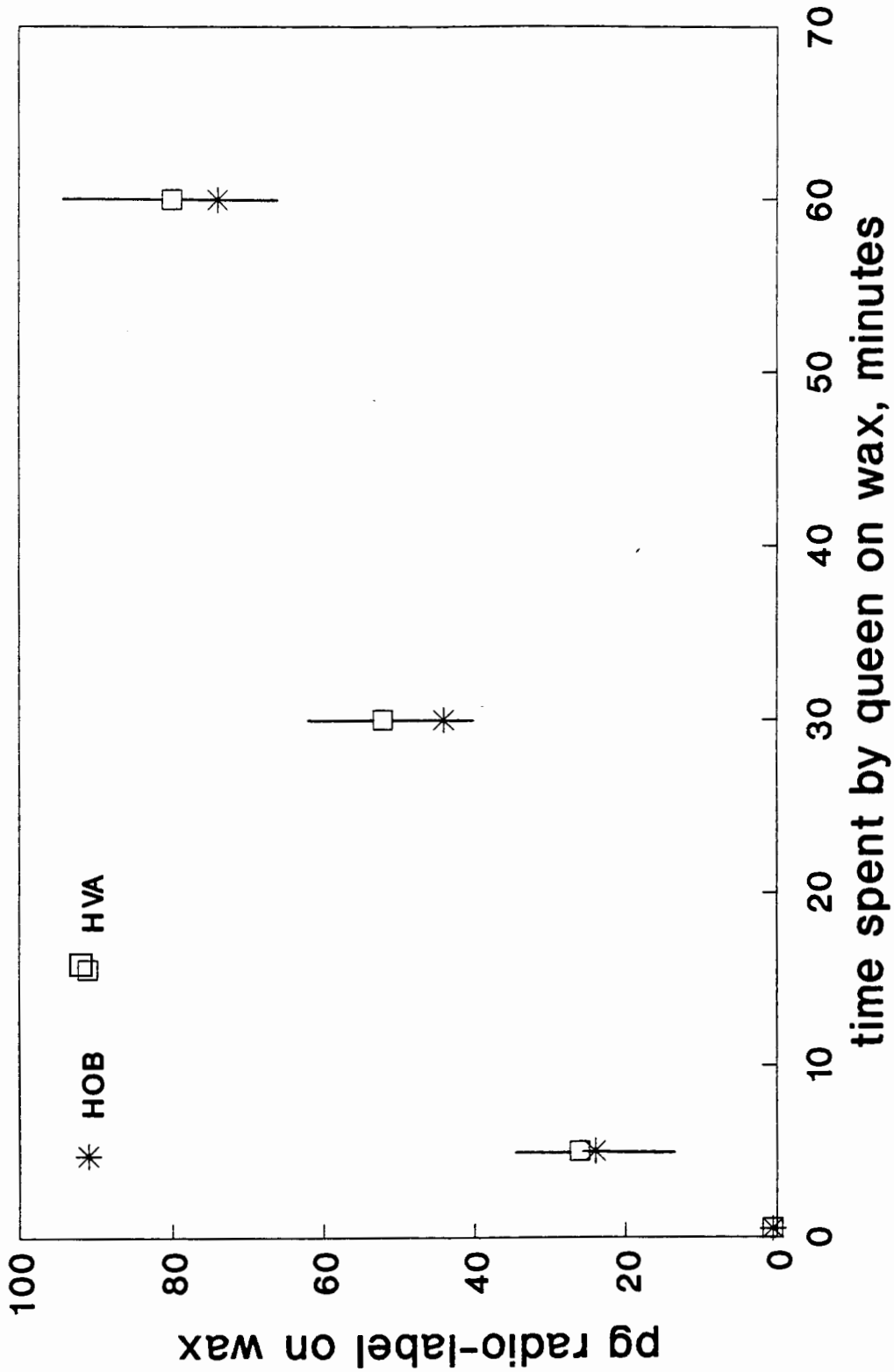
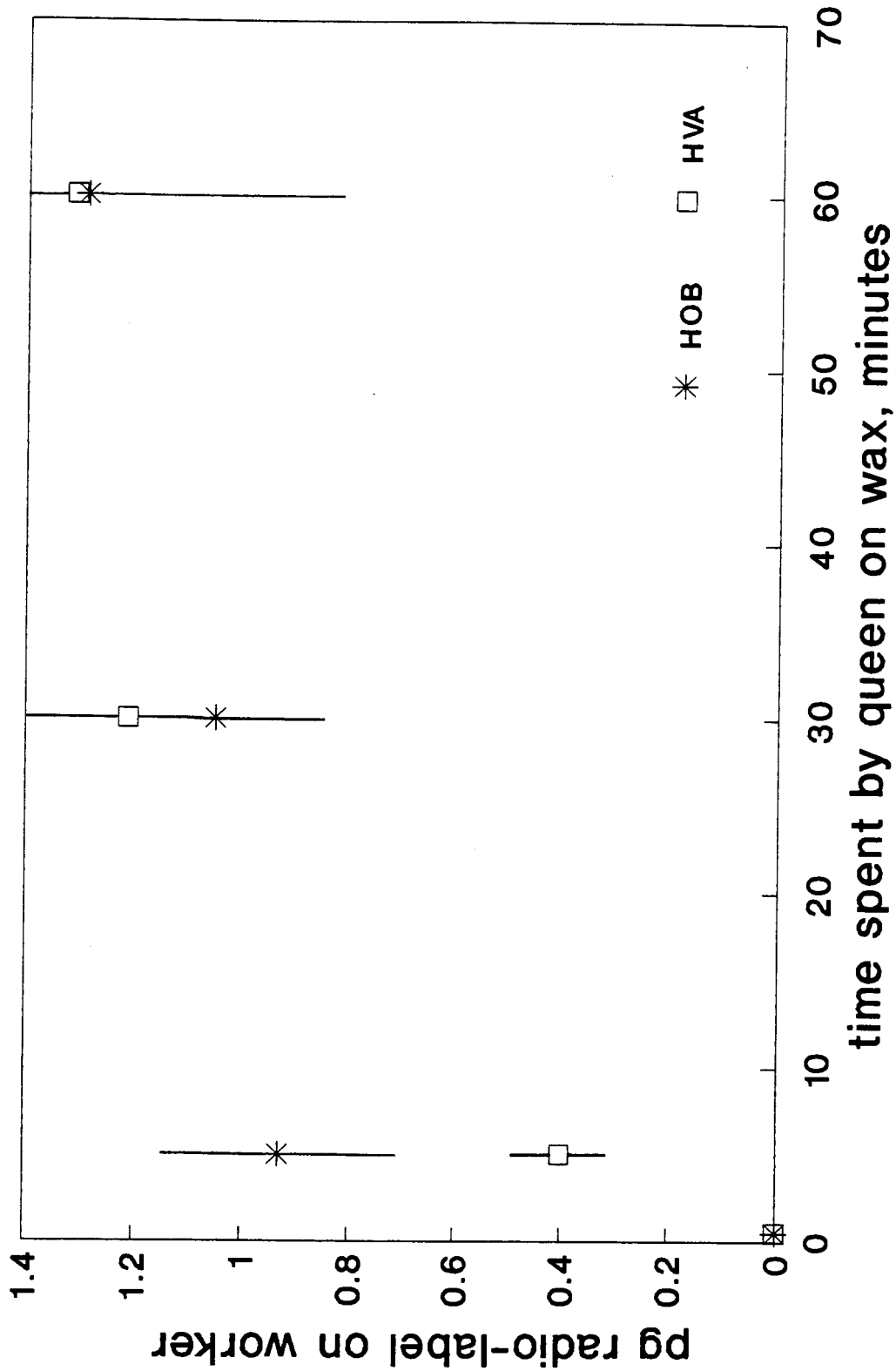

pg radio-label on wax
100
80
60
40
20
0
0
10
20
30
40
50
60
70
time spent by queen on wax, minutes
HOB
HVA

Figure 4-6.

Mean (± SE) amounts of tritiated HVA or HOB acquired from wax discs by individual workers following deposition there by queens (see Figure 4-5). Sample size at each time = 10.


pg radio-label on worker
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
60
70
time spent by queen on wax, minutes
HOB
HVA

= 2, 27, $p < 0.0001$, log transformed data; HOB, $F = 17.4$, d.f. =2, 27, $p = 0.0001$, log transformed; snk comparison test, $\alpha = 0.05$).

The Further Role of Wax

The amounts of [$^3H_2$]-HVA and [$^3H_2$]-HOB which 2 workers acquired from wax treated with synthetic QMP and either of the tritiated aromatics decreased with the time that the treated wax was left to sit before worker introduction (Fig. 4-7)(correlations of amounts vs time, p's < 0.001).

Internalization of Aromatic Components by Workers

Workers showed a pattern of radio-label internalization similar to that of the queens; levels in the guts and the rest of the corpses increased with time, while the amounts that could be washed from the cuticle decreased (Fig. 4-8)(correlations of amounts vs time, p's < 0.01).

Rates of Pheromone Component Translocation

Rate constants (k_n) for HVA and HOB were derived from the data, and from data for 9-ODA (from Chapter 2), as follows.

The amount of a QMP component on a queen's body is established by the rate at which her glands produce and

Figure 4-7.

Availability to workers of [$^{3}H_2$]-HVA and [$^{3}H^2$]-HOB applied to wax. Initial application was 10^{-3} queen equivalents of synthetic QMP containing 0.8 ng of [$^{3}H_2$]-HVA or 1.0 ng of [$^{3}H_2$]-HOB. Values are mean (± SE) pg of tritiated pheromone component acquired after different time intervals before workers were introduced to the wax. Two workers were then allowed 5 min contact with the wax, and the amount acquired averaged. Sample size at each time = 10.

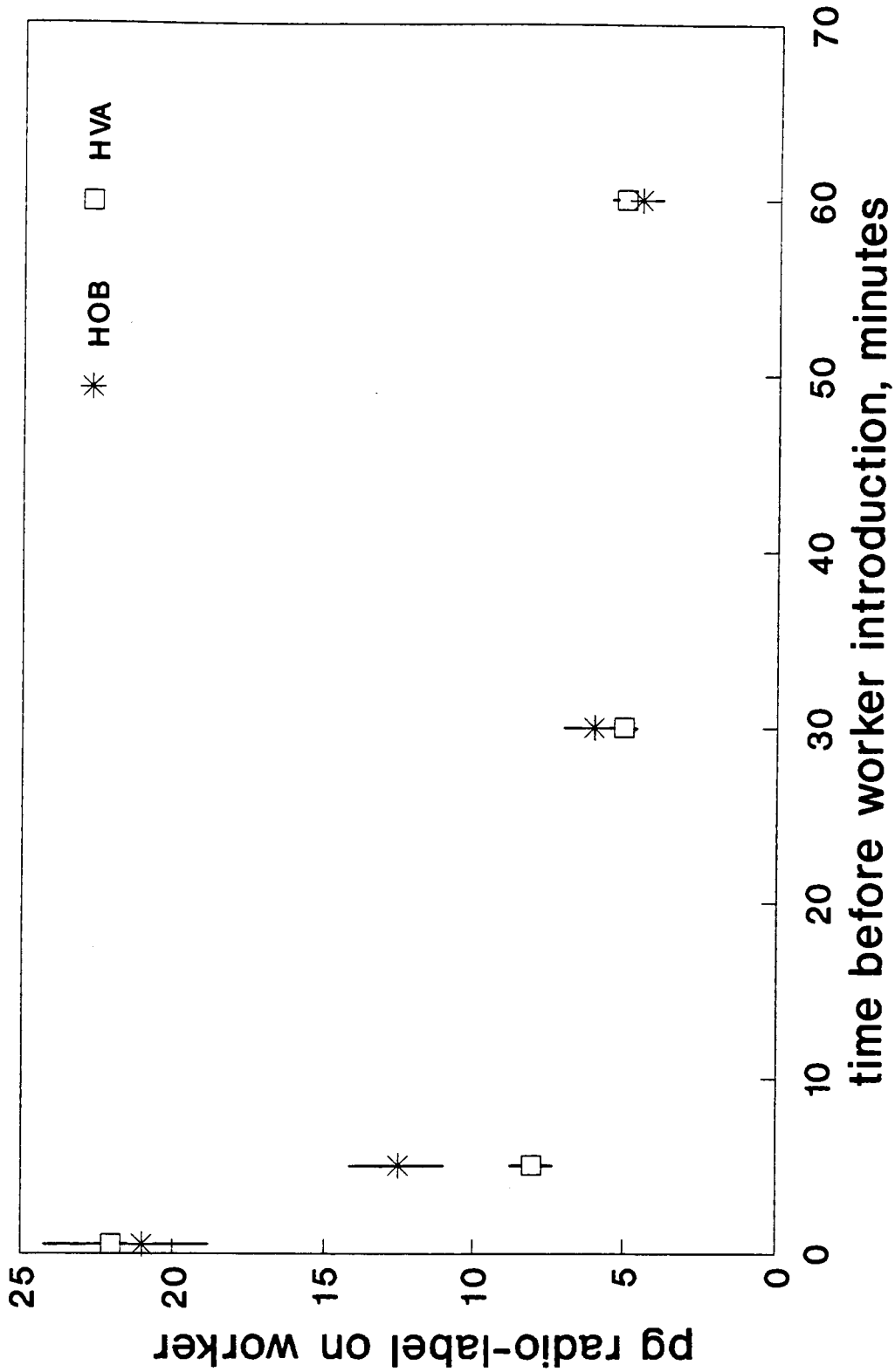
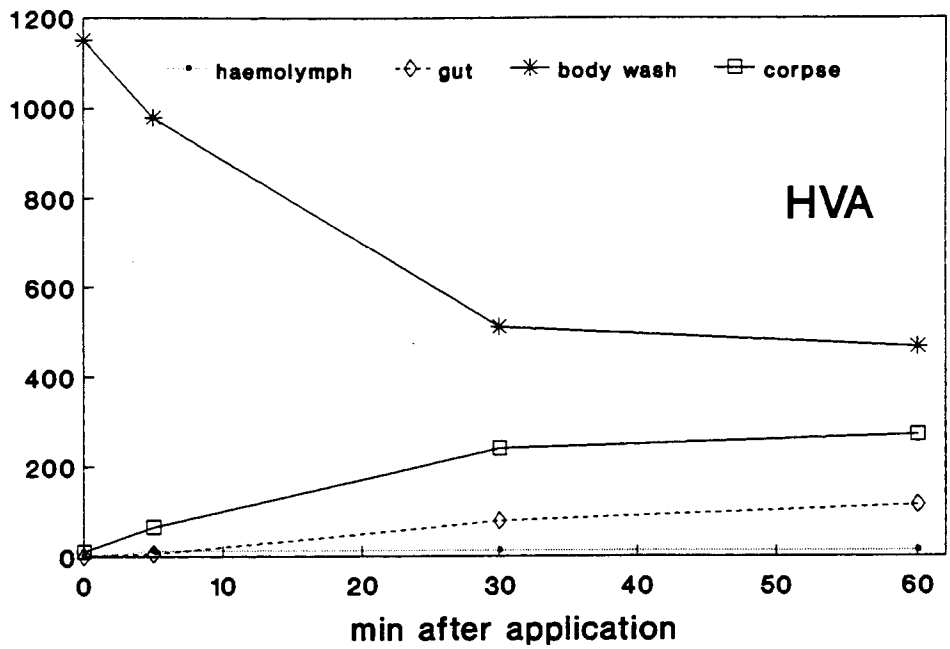
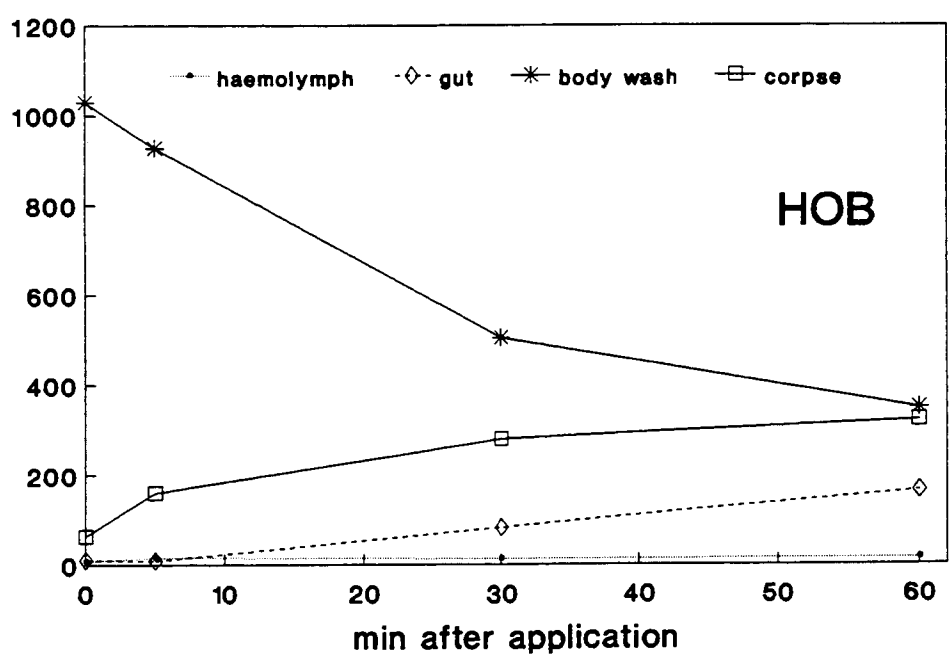

pg radio-label on worker
25
20
15
10
5
0
0
10
20
30
40
50
60
70
time before worker introduction, minutes
HOB
HVA

Figure 4-8.

Fate of HVA and HOB on the body surface of workers. Workers were isolated for different lengths of time after topical application of synthetic QMP containing 1.2 ng of [$^3H_2$]-HVA or [$^3H_2$]-HOB. Sample size at each time = 10.


1200
1000
800
600
400
200
0
haemolymph
gut
body wash
corpse
HVA
0
10
20
30
40
50
60
min after application


1200
1000
800
600
400
200
0
haemolymph
gut
body wash
corpse
HOB
0
10
20
30
40
50
60
min after application

secrete that component, by its loss to the wax, by its reabsorption through her own cuticle, and through donation to messenger workers. As mentioned earlier, HVA and HOB production were estimated using the 9-ODA data of Chapter 2. In isolation from workers, acquisition by messenger bees is zero, thus the amount of a component on the queen's body, [QB], at time t, will reach a steady state when the production is offset by the exponential loss, i.e. for HVA,

production/s = $[QB]_t - [QB]_t * e^{-(k1+k5)t+1}$

$-k_5$ = rate of deposition by queen onto wax

= for HVA, 26 pg out of a possible 1,200 pg, in 5 min (see Fig. 4-5)

= (26 pg)/(5 min)(60 s/min)(1200 pg)

= 7.2×10^{-5} s^{-1}

k_1 = estimated rate of internalization by queens

= $-\ln(1 - 0.029 \text{ pg/s}/[QB]) - k_5$

(The equilibrium value for isolated queens extrapolated from the 9-ODA data of Chapter 2

= 0.029 ng/s).

= 2.1×10^{-3} s^{-1}.

Within a colony, a similar steady state exists where the removal of pheromone by messenger bees (overall rate of k_3, where $k_3 = k_8 + k_9$) is occurring.

0.029 ng/s = $[QB]_t - ([QB]_t * e^{-(k1+k3+k5)t+1}$

$k_3 = 3.5 \times 10^{-3}$.

k_2 = rate of internalization by workers, obtained from half-life of HVA on the cuticle of a worker (Fig. 4-8).

= 0.693/(26 min*60 s/min) = 4.44×10^{-4} s^{-1}.

k_4 = rate of acquisition by workers, from the comb

= 1.3 pg from wax which carried 81 pg, in 5 min (after queens on wax for 60 min).

= 1.3 pg/(5 min * 60 s/min)(81 pg) = 5.3×10^{-5} s^{-1}.

k_6 = rate of deposition by workers on wax = 5 pg out of 31 pg carried, in 5 min.

= 3 pg/(32 pg)(5 min * 60 s/min) = 3.1×10^{-4} s^{-1}.

k_7 = rate of diffusion into wax (from Fig. 4-7)

= 0.693/8 min * 60 s/min = 1.4×10^{-3} s^{-1}.

k_8 = rate of acquisition by antennating messengers

= 0.504 pg/2500 pg * 5 s = 4.0×10^{-5} s^{-1}.

k_9 = rate of acquisition by licking messengers (Fig. 4-2).

= 54 pg/1000 pg * 30 s = 1.8×10^{-3} s^{-1}.

Values of k_n for HOB were calculated in the same way. The derived rate constants for HVA and HOB are compared with those for the major QMP component, 9-ODA, in Table 4-1. Note that the standard errors presented in that table are

based upon the standard errors of the parameters used to estimate the various rate constants. Direct statistical comparisons between the corresponding values for different QMP comonents would be imprudent because the movements of the different components were not studied simultaneously, i.e. they were not part of a single experiment.

Amounts of HVA and HOB transferred daily in a model colony are shown in Figures 4-9 and 4-10. Amounts were calculated using the rate constants derived in this study, and the model developed in Chapter 2.

Table 4-1. Comparison of rate constants for the intra-nest transfer of 3 components of honey bee queen mandibular gland pheromone: 9-ODA*, HVA, and HOB. Values are rate constants ± standard errors. The standard error values are based on the standard errors of the parameters used to calculate the rate constants. All units are s^{-1}

Rate constant	9-ODA	HVA	HOB
k_1	$2.2\times10^{-3} \pm 0.2\times10^{-3}$	$2.1\times10^{-3} \pm 0.4\times10^{-3}$	$1.9\times10^{-3} \pm 0.2\times10^{-3}$
k_2	$6.8\times10^{-4} \pm 0.7\times10^{-4}$	$4.4\times10^{-4} \pm 0.7\times10^{-4}$	$5.8\times10^{-4} \pm 0.9\times10^{-4}$
k_4	$5.9\times10^{-6} \pm 0.9\times10-6$	$5.3\times10^{-5} \pm 0.5\times10-5$	$5.9\times10^{-5} \pm 0.6\times10-5$
k_5	$7.7\times10^{-5} \pm 0.8\times10^{-5}$	$7.2\times10^{-5} \pm 1.8\times10^{-5}$	$8.0\times10^{-5} \pm 2.0\times10^{-5}$
k_6	$1.3\times10^{-4} \pm 0.1\times10^{-4}$	$3.1\times10^{-4} \pm 0.8\times10^{-4}$	$1.1\times10^{-4} \pm 1.1\times10^{-4}$
k_7	$1.0\times10^{-3} \pm 0.1\times10-3$	$1.4\times10^{-3} \pm 0.1\times10-3$	$1.2\times10^{-3} \pm 0.4\times10^{-3}$
k_8	$3.5\times10^{-5} \pm 1.1\times10^{-5}$	$4.0\times10^{-5} \pm 0.8\times10^{-5}$	$1.2\times10^{-4} \pm 0.2\times10^{-4}$
k_9	$3.1\times10^{-3} \pm 0.3\times10^{-3}$	$1.8\times10^{-3} \pm 0.5\times10^{-3}$	$8.3\times10^{-4} \pm 2.5\times10^{-4}$

k_1 = absorption into queen
k_2 = absorption into worker
k_3 = total transfer from queen to workers (k_8 + k_9)
k_4 = transfer from wax to workers
k_5 = queen deposition on wax
k_6 = transfer from workers to wax
k_7 = absorption into wax
k_8 = transfer from queen to antennating messenger
k_9 = transfer from queen to licking messenger
*data for 9-ODA from Chapter 2

Figure 4-9.

Estimated quantities of HVA transferred in 24 hr. Amounts are ng. A messenger is defined as a worker receiving pheromone directly from a queen; a bee receiving pheromone from a messenger is termed a licking or antennating worker, depending on the type of contact. Derivation of amounts is described in the results section of this chapter and assumptions of the model are outlined in Chapter 2.

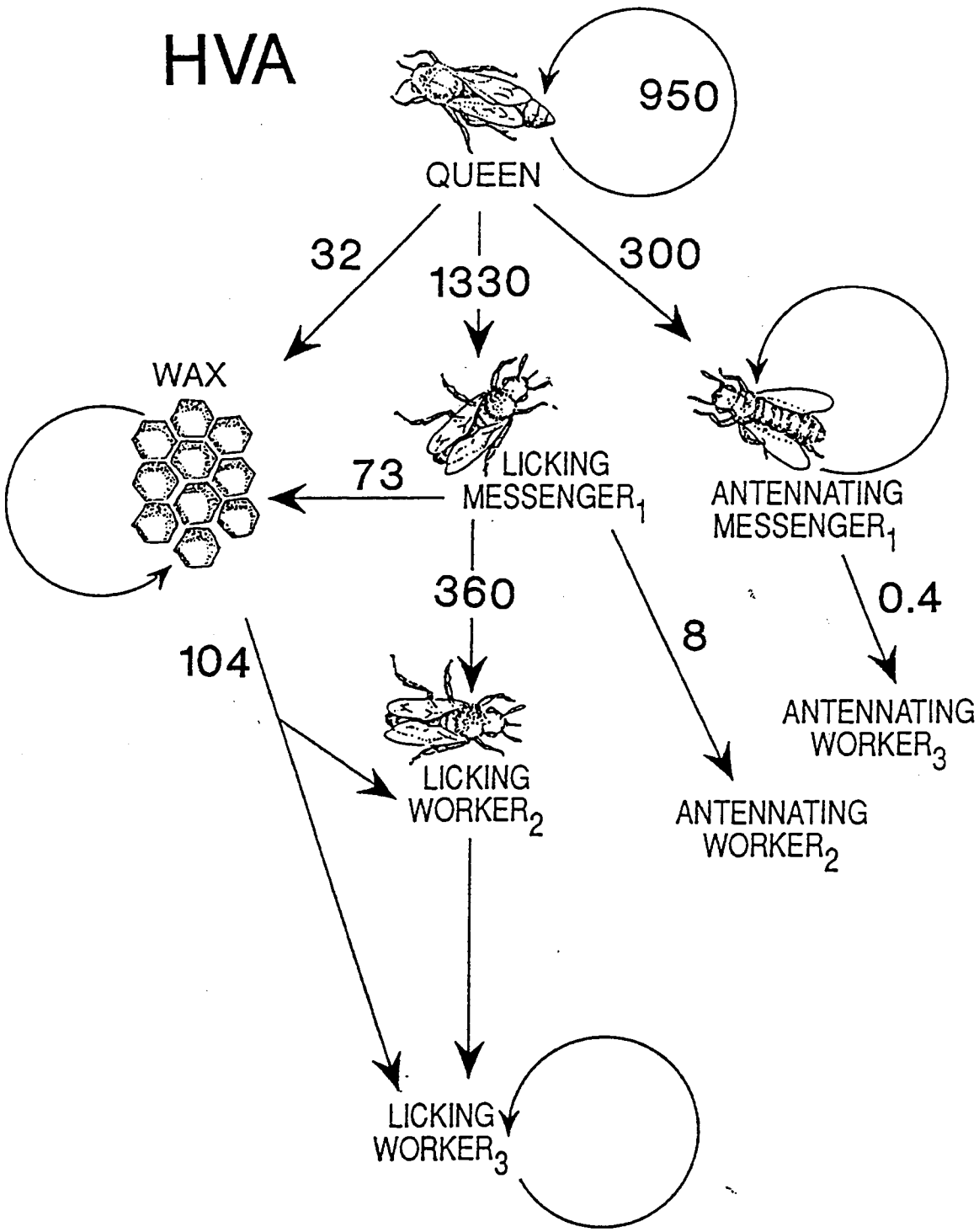
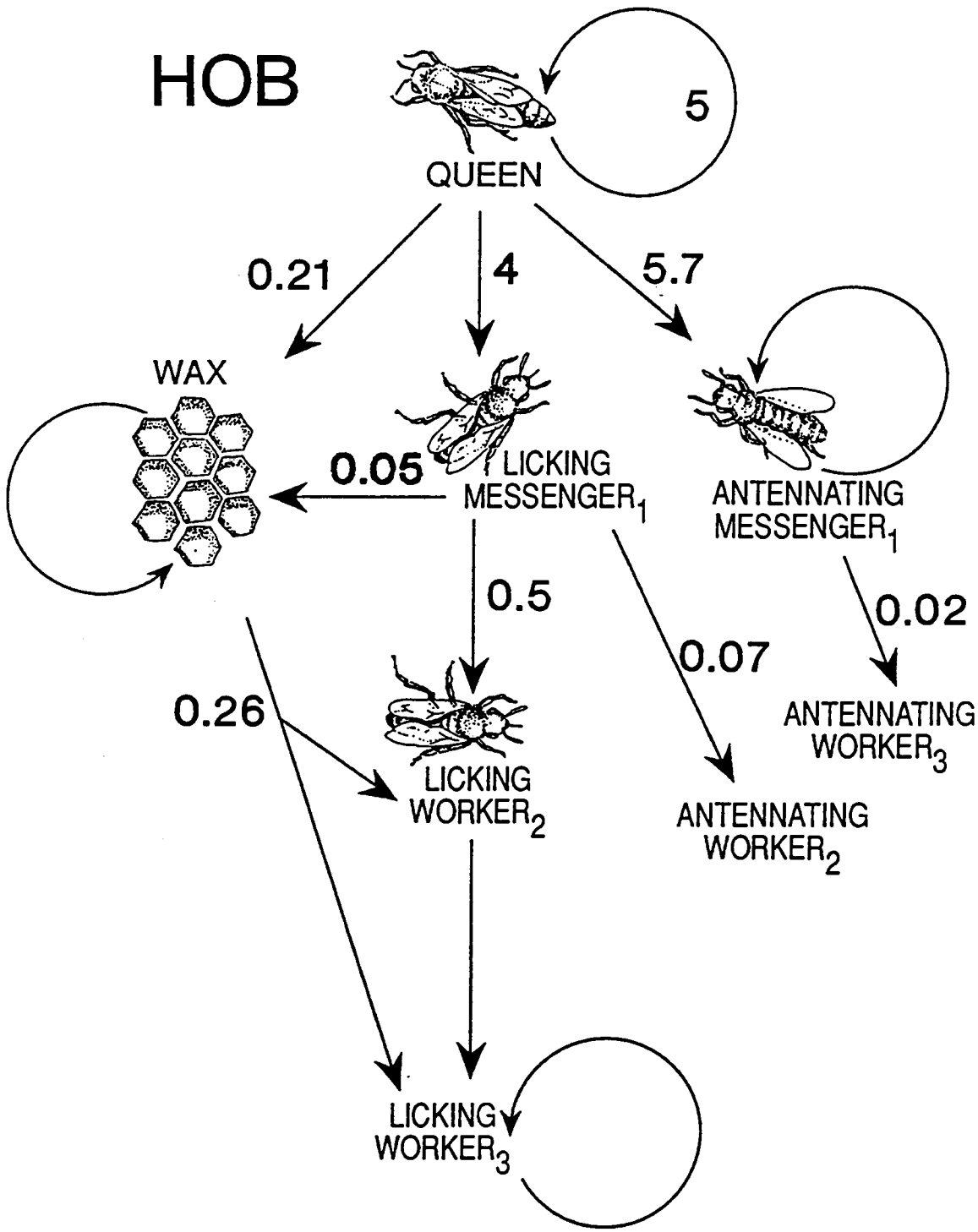

HVA
950
QUEEN
32
1330
300
WAX
73
LICKING
MESSENGER$_1$
ANTENNATING
MESSENGER$_1$
360
0.4
104
8
ANTENNATING
WORKER$_3$
LICKING
WORKER$_2$
ANTENNATING
WORKER$_2$
LICKING
WORKER$_3$

Figure 4-10.

Estimated quantities of HOB transferred in 24 hr. Amounts are μg. A messenger is defined as a worker receiving pheromone directly from a queen; a bee receiving pheromone from a messenger is termed a licking or antennating worker, depending on the type of contact. Derivation of amounts is described in the results section of this chapter and assumptions of the model are outlined in Chapter 2.


HOB
5
QUEEN
0.21
4
5.7
WAX
0.05
LICKING
MESSENGER$_1$
ANTENNATING
MESSENGER$_1$
0.5
0.02
0.07
0.26
ANTENNATING
WORKER$_3$
LICKING
WORKER$_2$
ANTENNATING
WORKER$_2$
LICKING
WORKER$_3$

DISCUSSION

The intra-nest transfer of the aromatic components of QMP occurs, qualitatively and quantitatively, in a similar fashion to that of the primary constituent, 9-ODA. After exudation onto the queen's body surface, HVA and HOB are either re-internalized by the queen, gathered by retinue bees, or left as footprints on the wax comb. Licking retinue bees, although less abundant than those making only antennal contact, are more important individually in gathering QMP components from the queen. However, the results of these studies with the aromatics, especially HOB, suggest that the role of antennating messengers may be more important in overall pheromone acquisition from the queen that was indicated by the 9-ODA study. In the case of HOB, the model estimates that antennating messengers actually gather more material daily from the queen than licking messengers. While it is possible that HOB differs from the other components at this stage of transfer, it is more likely that the different results reflect natural variance. The rate constants for the three compounds do not differ greatly, and it is possible that the average rate of the three would best predict what actually occurs in a colony. Worker - worker contacts, beginning with retinue bees, result in the serial transfer of pheromone to areas of the nest removed from the queen. Non-retinue workers can also come into contact with QMP components that have been

deposited on the wax by the queen and other workers. The rate constants for transfer from the wax to workers differ between the aromatics and 9-ODA, however this difference results in a relatively minor affect on the net flux through a colony. The rate constants derived for the individual stages of transfer are remarkably similar for 9-ODA, HVA, and HOB, especially considering the high degree of variation which can occur in individual behaviors, and the fact that the 3 components were not investigated simultaneously. They were, however, studied at the same time of the year.

Several authors (Crewe, 1988; Boch and Lensky, 1976) have suggested that QMP may be partitioned in some way after production by the queen, with the result that it supplies both a short-term and a long-term signal. If so, a relatively ephemeral (more volatile) signal from the mandibular glands might regulate activities such as queen rearing and worker responses to their own queen, while the C_{10} fatty acids give a more persistent signal. However, the aromatics in QMP are relatively non-volatile, and results in this chapter suggest that all QMP components move through the colony at a similar rate. We conclude that the elements of QMP are not partitioned, but are transferred as a unit. This is not to say that there is not some distortion of the queen's original secretion. Slight differences in movement between the QMP components would result in workers far from the queen receiving a slightly altered chemical message. Velthuis (1990) speculated that only those workers attending

the queen (i.e. those in an age-based task group which results in frequent contacts with the queen) receive an individual queen's 'true' signal. Workers that are farther removed may receive a more 'generic' queen message, but one which still acts to affect behaviors.

In the Lepidoptera, where sex attractant pheromones are also multicomponent, evidence is accumulating that males respond to the complete mixture of components (see Linn and Roelofs, 1989). The implication is that, even though minor components may be present in low amounts, their presence is still necessary to affect male response at distances far downwind of the female. An analogous situation may occur with the relatively non-volatile sociochemicals of the eusocial Hymenoptera, with the complication that queen-produced pheromones may act to both release behaviors and to prime physiological functions. Initial attraction to the compounds on the queen's body is the result of a distinctive blend of compounds acting at short distances (cm's) within the nest. Once the complex is transferred to retinue bees or the wax substrate, it is disseminated as a unit throughout the nest.

The question remains, what is the adaptive significance of messages that consist of several compounds? Linn and Roelofs (1989) suggested 3 hypotheses to explain the role of minor components in Lepidopteran sex attractants: i) minor components are by-products of some process and are neutral, i.e. they do not deter the important effects of the major

components, ii) minor components may substitute for each other, i.e. they have common receptor sites, and iii) minor components may act as behavioral antagonists between closely related species. The first hypothesis is unlikely to be correct for honey bee QMP because the presence of minor components is required to elicit the full mandibular-based retinue response (Slessor et al., 1988). Similarly, the minor components are not interchangeable, and therefore do not likely share common receptors. Not enough is known about the minor components of QMP in other *Apis* species to suggest if they play a role in reproductive isolation. However, males of *A. dorsata*, *A. cerana*, and *A. florea* are all attracted to *A. mellifera* queens and to synthetic 9-ODA alone (reviewed in Free, 1987), suggesting that QMP may not be used as a specific discriminator by drones. It is possible that subtle differences in the ratio of components plays a role in the recognition of individual queens, and/or in the formation of nestmate recognition signals. Redundancy of the chemical message may also act as a mechanism to separate the queen's signal from "background noise".

An understanding of the significance of the chemical complexity of such socio-chemicals may lie in better knowledge of the modes of perception and action of individual pheromone components. Currently, little is known about the mechanisms of perception of multiple component pheromones, and the cascade of events that occurs after

perception. For example, are individual compounds truly perceived by specific receptors? If not, how are parallel nervous messages from different pheromone component receptors organized so as to give an insect information about component ratios? These interesting questions remain for future research.

CHAPTER 5

QMP MOVEMENT IN POPULOUS AND UNPOPULOUS COLONIES

Reproductive swarming represents the greatest change in worker organization that occurs during the annual cycle of honey bee (*Apis mellifera* L.) colonies. In a process that spans several weeks, the workers raise several queens, and over half of the work force subsequently leaves the nest with the old queen and founds a new colony. At the original nest, smaller afterswarms may later issue with recently emerged virgin queens, leaving the remaining queens to fight for reproductive control of the nest. Queen rearing associated with the onset of swarming is initiated by a complex combination of demographic factors and resource abundance, so as to produce swarms at a time of year when survival is likely to be high. Primary stimuli for queen rearing include colony size, brood nest congestion, worker age distribution, and possibly reduced availability of queen pheromone (reviewed by Winston, 1987).

Workers are inhibited from producing new queens by the queen mandibular gland pheromone complex (QMP) (Butler, 1954; Butler and Simpson, 1958; Winston et al., 1989, 1990, 1991). Hypotheses linking the role of QMP and reproductive swarming have proposed that queen pheromones have less inhibitory effect in colonies prior to swarming, either because 1) queen pheromone production declines, 2) worker

responses to given amounts of QMP decline when conditions favoring swarming arise, or 3) colony crowding interferes with the movement of these substances through the nest. Seeley and Fell (1981) found that queens from crowded colonies that were rearing queens in preparation for swarming contained similar amounts of one QMP component, 9-ODA, as similarly-aged queens from colonies not preparing to swarm. Simpson (1973) reported that colonies that have relatively small population sizes, but are crowded, will rear queens, whereas populous but uncrowded colonies will tend not to. These two results are inconsistent with the hypotheses that changes in queen output of pheromone are important in initiating colony reproduction. It remains possible however that worker responses to given amounts of QMP decline when swarming conditions arise, i.e. crowding and abundant brood. Recently, Winston et al. (1991) found that supplemental applications of synthetic QMP on glass slides delayed swarming in large, uncongested colonies, but not in smaller, congested ones. Use of an aerosol spray that better dispersed the pheromone was more effective in the crowded colonies than pheromone delivered on glass slides. These results are consistent with the hypothesis that pheromone transmission is slowed as colonies grow and become congested, resulting in diminished inhibition by the queen of worker behaviors that lead to queen rearing.

The purpose of the research reported here was to investigate and compare the transmission of QMP more

directly in populous, more congested colonies compared to less populous, uncrowded colonies. Radio-labelled 9-keto-2(E)-decenoic acid was used to follow the intra-nest movement of queen pheromone.

MATERIALS AND METHODS

In May, 1991, 20 test colonies were initiated by shaking worker bees from several source colonies into 4-frame (approximately 17 L) cardboard nucleus boxes. In the populous, more congested treatment (called Populous), 10 colonies received 2 kg of bees each (mean population size = 12,400 ± 900 bees), and the unpopulous, uncongested 10 colonies received 0.9 kg each (population size = 4,100 ± 300; Unpopulous). All shaking was done with standard equipment, consisting of a large metal funnel, wire screen storage container, and a portable scale. Each colony was supplied with a queen, 1 frame of comb that was half-occupied with eggs and larvae, 1 frame containing pollen and honey, and 2 empty frames of drawn-out comb. All frames were marked so as to divide the faces into 10 sample areas of equal size. The colonies were left undisturbed for 5 days.

Pheromone transfer was examined using radio-labelled pheromone. Untreated control samples (n=160) were gathered periodically through the course of the experiment by sampling workers from test colonies before pheromone application. To initiate the experiment, queens were removed from each colony, killed on dry ice, and treated by the topical application of 10 μl of MeOH containing 10^{-3} Qeq of synthetic QMP (1 Qeq is defined as the mean amounts of

the pheromone components that are found in the mandibular glands of mated queens), with 250 ng of [^{3}H]-9-keto-2(E)-decenoic acid ([^{3}H]-9-ODA) (activity = 15,000,000 disintegration/min (dpm)), 150 ng 9-hydroxy-2(e)-decenoic acid (9-HDA) (66% R-(-)), 12 ng methyl p-hydroxybenzoate (HOB), and 2 ng of 4-hydroxy-3-methoxyphenylethanol (HVA). This blend of compounds was found by Slessor et al. (1988) to be indistinguishable from mandibular gland extracts in eliciting the retinue response. The amounts applied represent the approximate mean amounts found on the body surface of a mated queen in a colony (Slessor et al, 1988; Chapter 2), and are equivalent to 1/1000 of the mean amounts of the components that are found in the mandibular glands of such queens. After allowing the solvent to evaporate, the queens were affixed to the center of one of the center-most frames so that they were accessible to the workers.

Samples of 2 workers were taken from each sampling area on both sides of each frame at 15 min. and 1, 2, 4, 6, and 24 h after the treated queens were introduced. At 24 h, the queens were collected. Only 8 colonies of each treatment were sampled at 4 and 6 h, and, due to vandalism, only 8 of the Populous colonies could be sampled at 24 h.

All samples were placed into 20 ml plastic scintillation vials immediately upon collection, and covered with 10 ml of BCS biodegradable scintillation cocktail (Amersham Canada Ltd, Oakville, Ont.). Samples were allowed to soak for 48

h, and then were analyzed for radio-label content with a LS 5801 liquid scintillation counter (Irvine, CA).

The following comparisons were made between treatments at each sampling time:

a) the total number of samples that were found to be carrying radio-label (only samples yielding greater than 2 standard deviations above the mean background (control) levels (i.e. > 1.4 pg = approximately $5x10^{-8}$ Qeq) were considered to have acquired pheromone),

b) the mean amounts of radio-label per sample, and the distribution of radio-label on different frame sides,

c) the numbers of bee samples that had acquired [^{3}H]-9-ODA on different frame sides, and

d) the total numbers and relative abundance of those bees carrying large amounts (>10,000 dpm = 165 pg) of [^{3}H]-9-ODA, and likely to be especially influential in disseminating pheromone through the nest. (The value of 10,000 dpm represents approximately the 1% of bees gathering the most radio-label).

Statistical analyses consisted of one-way analyses of variance (ANOVAs) with a split plot design. Such a design allowed for analysis of treatment effects, time effects, and the interaction of any treatment affects with time. Proportional data (eg. the number of tritium-carrying samples (out of some maximum possible number)) were first arcsine square-root transformed. Other data were

transformed to better meet the assumptions of the ANOVA model. Transformation functions were derived from regressions of log variance vs. log means (Taylor's power law (Southwood, 1978)). Analyses were performed using SAS (SAS Institute, Cary, NC) statistical software.

Results

There was no difference in the spatial pattern of radio-label movement between the Populous and Unpopulous colonies (Fig. 5-1). Workers that had acquired radio-label could be collected from the outermost frame sides, i.e. at the farthest point from the lure, only 15 min after lure introduction in colonies of both treatments. Thereafter, there was no difference between treatments, at any time, in the relative proportions of all of the radioactive samples in each colony that were collected from the outer frame sides (split plot ANOVA; arcsine squareroot transformed data; $F = 2.51$, d.f. = 1, 98, $p = 0.13$), although, the relative proportion of radioactive samples on the outer frame sides increased with time in both treatments (same ANOVA as above; $F = 3.52$, d.f. = 5, 80, $p = 0.006$). There was, however, a trend towards a difference between treatments in the absolute amounts of [^{3}H]-9-ODA on workers from this outer region (Fig. 5-2) (split plot ANOVA; transformed data = data$^{-0.33}$; $F = 3.5$, d.f. = 1, 86, $p = 0.08$), and in the proportion of the total dpm's sampled from each colony that were contributed by the workers from the outer frame sides (split plot ANOVA; $F = 2.99$, d.f = 1,98, $p = 0.10$). In both cases, workers in the Populous colonies tended to carry less radio-label.

The degree of congestion had no significant effect on the amount of [^{3}H]-9-ODA on individual workers that had

Figure 5-1.

Distribution in the nest of workers that acquired detectable quantities of radio-label. Values are percentages of 2-bee samples from each frame side that carried radio-label, at different times after introduction of a lure carrying QMP with [^{3}H]-9-ODA. Lures were placed in the center of each colony, on frame side 4. Number of colonies in each treatment = 10.

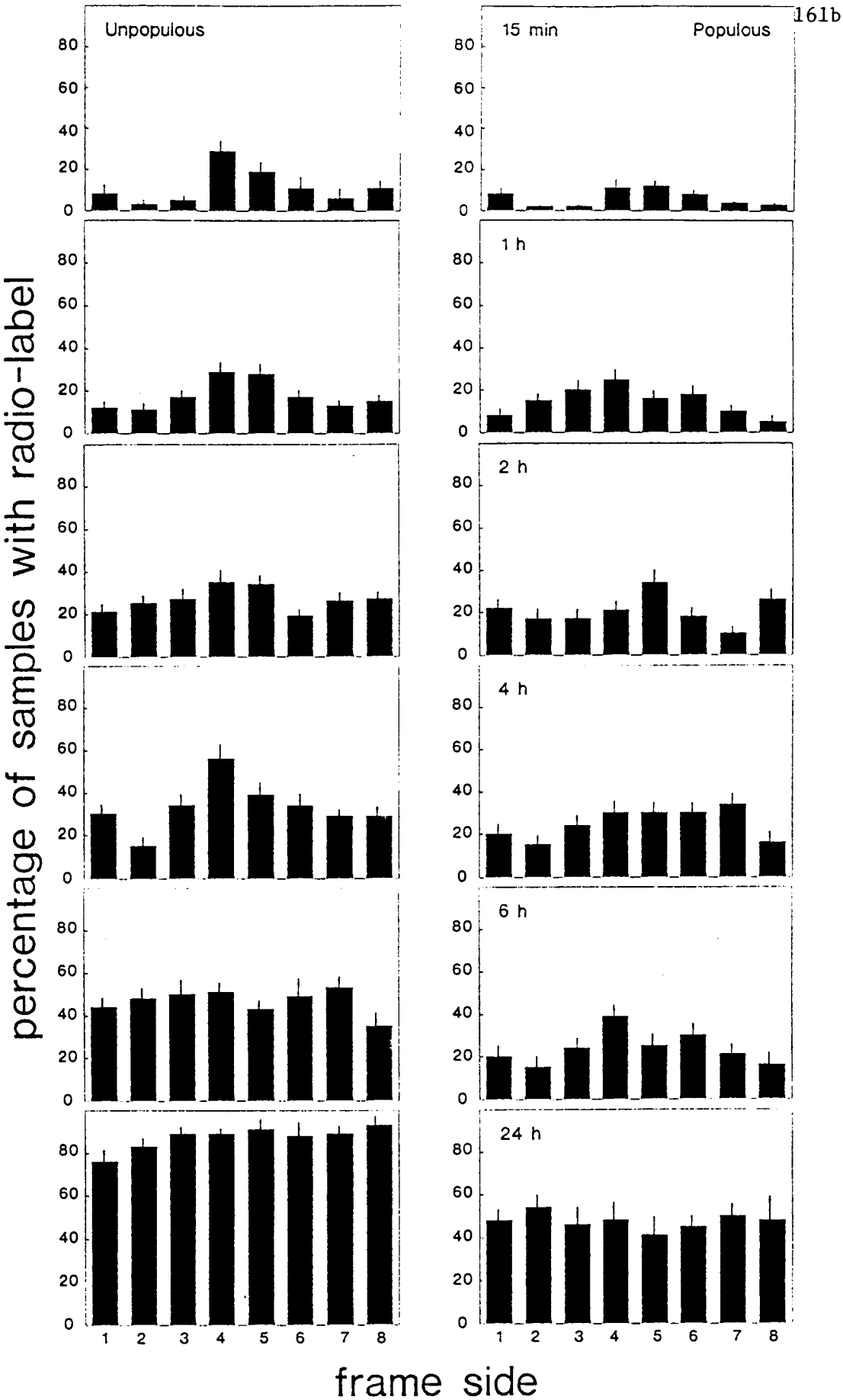
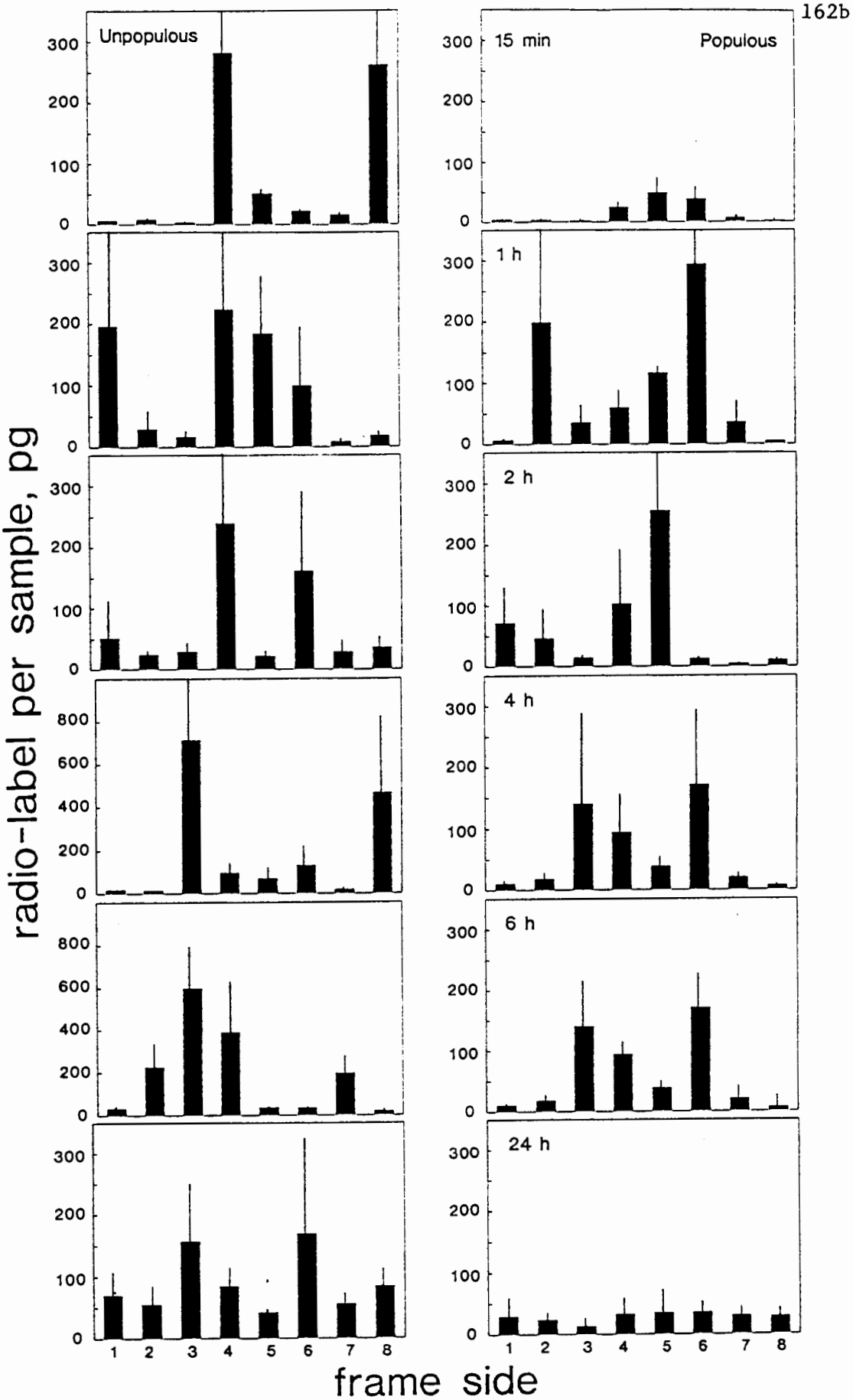

Unpopulous
15 min
Populous
1 h
2 h
4 h
6 h
24 h
percentage of samples with radio-label
frame side
0
20
40
60
80
1
2
3
4
5
6
7
8

Figure 5-2.

Distribution of radio-label in the nest. Values are mean (± SE) amounts of radio-label (as pg of [^{3}H]-9-ODA) on 2-bee samples from each frame side, at different times after lure introduction. Lures containing QMP with [^{3}H]-9-ODA were placed on frame side 4.


Unpopulous
15 min
Populous
1 h
2 h
4 h
6 h
24 h
radio-label per sample, pg
frame side
0
100
200
300
400
600
800
1
2
3
4
5
6
7
8

acquired radio-label (Fig. 5-3) (split plot ANOVA; transformed data = data$^{-0.28}$; $F = 0.43$, d.f. = 1, 2570, $p = 0.52$). In other words, workers in both colony types acquired and carried similar amounts of the radio-label. There was, however, a significant decrease in the mean amounts per sample with time (same ANOVA as above; $F = 8.31$, d.f. = 5, 2552, $p = 0.0001$).

The greatest difference in radio-label distribution between the Populous and Unpopulous colonies was in the proportion of the sampled workers that had acquired detectable amounts of radio-label (Fig's. 5-4 and 5-1). The numbers were lower for the Populous colonies throughout the experiment (split plot ANOVA; arcsine squareroot transformed data; $F = 24.4$, d.f. = 1, 98, $p < 0.0001$). After 24 h, 88% of the sampled workers in the Unpopulous colonies and 47% of those in the Populous colonies had acquired detectable radio-label. The difference between treatments also increased with the time after lure introduction (same ANOVA; treatment*time interaction; $F = 15.2$; d.f. = 5, 80, $p < 0.0001$), although the number of radioactive samples per colony increased significantly with time in both groups ($F = 128.8$, d.f. = 5, 80, $p < 0.0001$).

The Populous colonies also yielded fewer samples with especially large amounts (>10,000 dpm = 165 pg) of radio-label, at all times after lure introduction, although there was no difference in the relative proportion of such individuals to all the radioactive samples from a colony

Figure 5-3.

Amounts of radio-label acquired by individual workers. Values are mean (± SE) pg of [^{3}H]-9-ODA per radioactive 2-worker sample, i.e. only of those samples wherein at least one worker had acquired detectable radio-label.

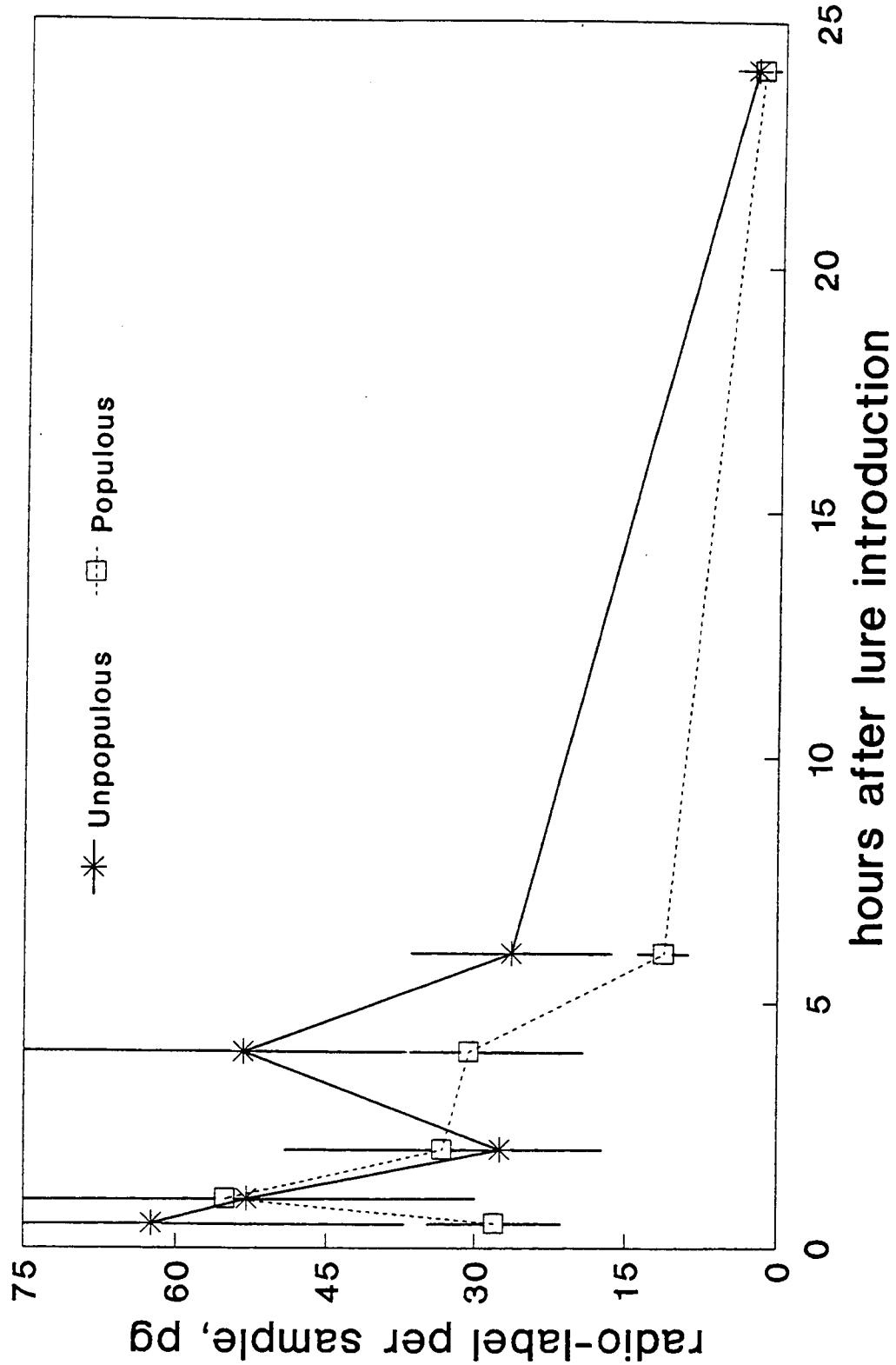
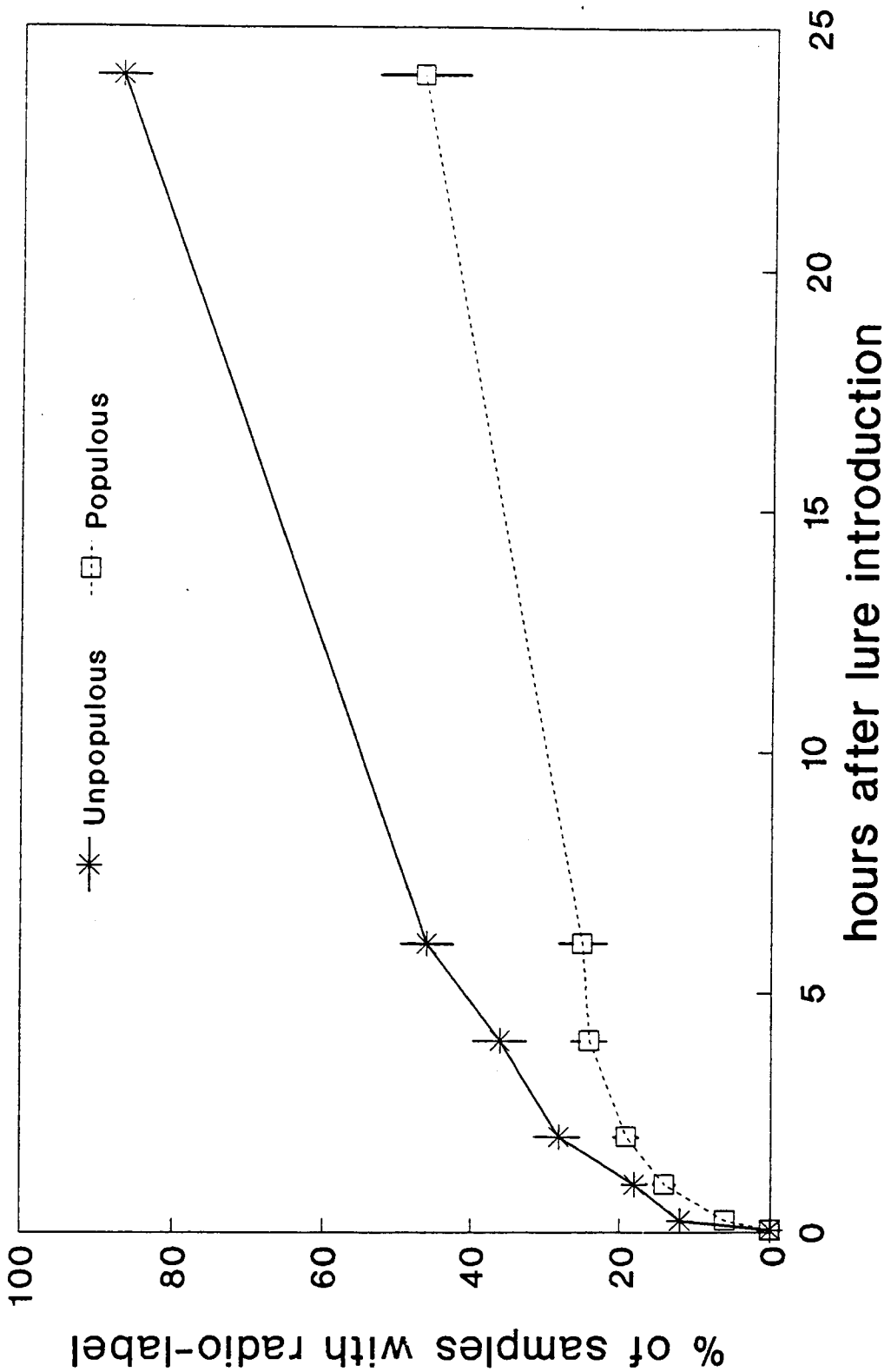

radio-label per sample, pg
75
60
45
30
15
0
0
5
10
15
20
25
hours after lure introduction
Unpopulous
Populous

Figure 5-4.

Proportion of all sampled workers with radio-label. Values are percentages of samples per colony that contained detectable radio-label at different times after lure introduction.


% of samples with radio-label
100
80
60
40
20
0
0
5
10
15
20
25
hours after lure introduction
Unpopulous
Populous

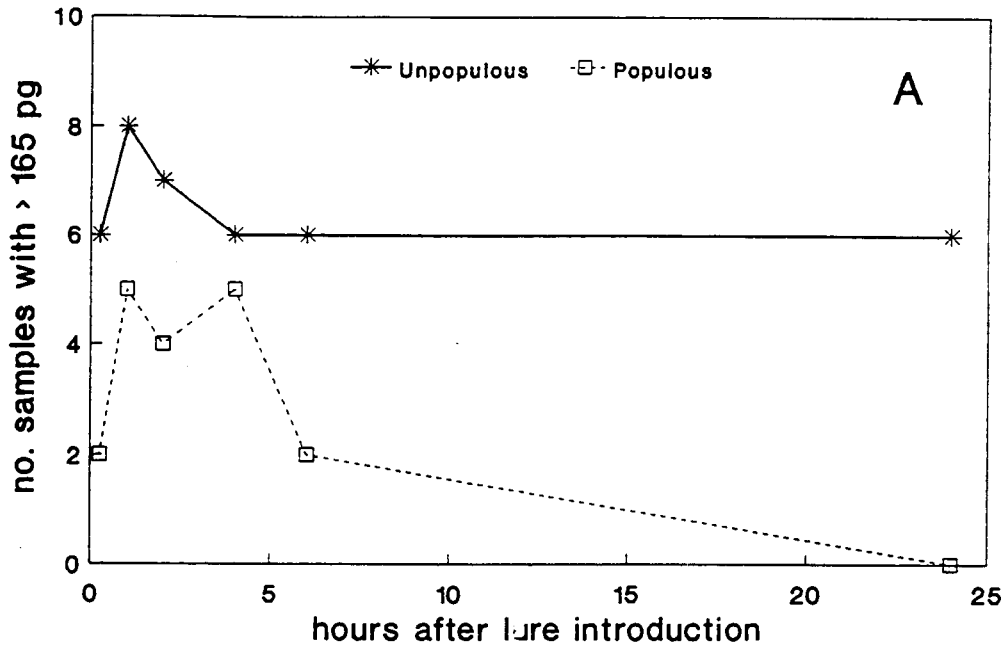
(Fig. 5-5) (due to small numbers of such individuals, the data were pooled for all replicates, and no statistical analyses were performed).

Colony congestion had no significant effect on the estimated amounts of [^{3}H]-9-ODA distributed throughout the whole nest at any time (Fig. 5-6) (calculated as mean amount of radio-label/bee in each colony * no. of bees/colony) (split plot ANOVA; transformed data = data$^{-0.38}$; $F = 0.02$, d.f. = 1, 86, $p = 0.90$). In both treatments, the amount of [^{3}H]-9-ODA on or in the workers appeared to increase until 4 h after lure introduction, after which, this value declined. The time effect was significant (same ANOVA as above; $F = 2.85$, d.f. = 5, 70, $p = 0.02$). After 24 h, an average worker in an Unpopulous colony received 10.2 ± 2.4 pg, and in a Populous colony 5.7 ± 0.9 pg from a one-time source of 250 ng of 9-ODA.

The mean amounts of [^{3}H]-9-ODA remaining on the queens after 24 hrs were 17.5 ± 3.1 ng for the Populous and 16.6 ± 3.1 ng for the Unpopulous colonies (t-test; $t = 0.22$, d.f. = 15, $p = 0.83$).

Figure 5-5.

A) numbers and B) proportions of all the radioactive samples (data pooled for all colonies within a treatment) represented by those samples carrying relatively large amounts of radio-label (equivalent to >165 pg of [^{3}H]-9-ODA). Such bees were likely to be especially important in disseminating pheromone.


Unpopulous
Populous
A
no. samples with › 165 pg
hours after lure introduction
0
2
4
6
8
10
0
5
10
15
20
25

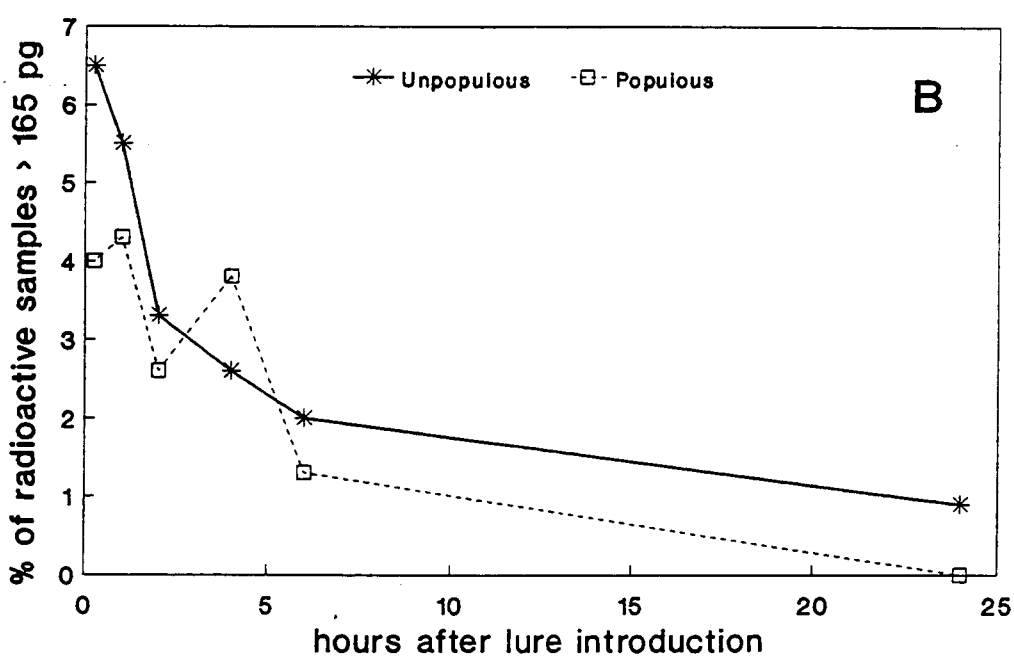
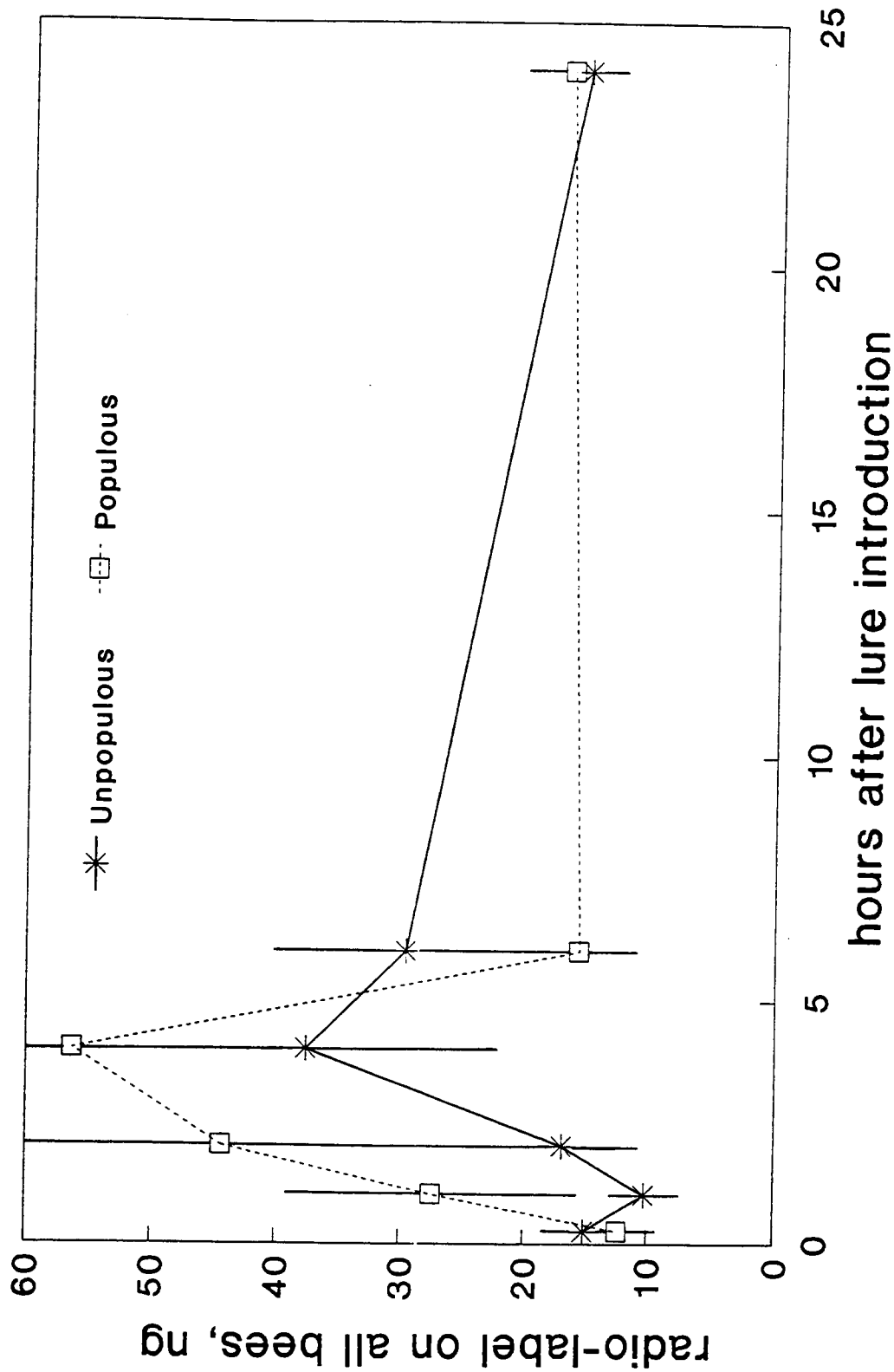

Unpopulous
Populous
B
% of radioactive samples › 165 pg
hours after lure introduction
0
1
2
3
4
5
6
7
0
5
10
15
20
25

Figure 5-6.

Estimated total amounts of radio-label (as mean ± SE ng of [^{3}H]-9-ODA) carried by all the workers of the nest. Each lure initially held 250 ng of [^{3}H]-9-ODA.


radio-label on all bees, ng
60
50
40
30
20
10
0
0
5
10
15
20
25
hours after lure introduction
Unpopulous
Populous

DISCUSSION

The results presented here have demonstrated that individual workers in the populous, mildly congested colonies received, on average, lower levels of queen pheromone than those in unpopulous, uncongested colonies. However, this reduction in queen pheromone can be largely explained by increased population size (the ratio of populations bewteen the 2 colony types was 3.0 and the ratio of bees with QMP after 24 h was 2.0); the limited amount of congestion used in this experiment evidently had little or no effects on the pheromone transmission mechanisms. There were no significant effects of crowding on the relative rate of movement from the center to the periphery of the nest, the amounts of radio-label on individual bees, the amounts distributed among the workers of the colony as a whole, or on the relative frequency, among those workers that had acquired radio-label, of individuals that were carrying especially large amounts. There was a tendency, however, for workers at the periphery of the congested colonies to carry smaller amounts of radio-label than equivalent workers in the uncrowded nests, although this effect was not statistically significant. The greatest difference between treatments was that the Populous colonies showed a much smaller proportion of workers receiving detectable amounts (>1.4 pg) of [^{3}H]-9-ODA, and fewer workers carried large quantities. This sort of result is predicted by the model

of Chapter 2 which suggested that the number of retinue bees that lick the queen (these bees gather the most QMP and are the most important in transferring it to nestmates), is limited. Such bees should therefore be less frequently encountered in the sampling of a more populous colony. In other words, regardless of colony size, only a fraction of the bees in a colony can acquire abundant quantities of queen pheromone.

The results also provide evidence that queen pheromone is disseminated by queen-worker and worker-worker contacts, confirming the behavioral evidence of Seeley (1979) and the laboratory-based studies of Chapters 2 to 4. The rapid appearance of radio-label at the periphery of the nest, the increase in the numbers of radio-label carrying workers with time, and the decreasing mean load of radio-label per bee are all consistent with pheromone acquisition from the queen, followed by dissemination among the workers.

It is important to note that chemical breakdown of [^{3}H]-9-ODA is likely to have occurred at some time after application to the queens. Although the time interval is unknown, it is likely that the radio-label detected after several hours was no longer part of the pheromone component. As such, quantitative conversions of tritium to 9-ODA become increasingly tentative with increasing time after pheromone application. It would therefore be imprudent to make too many conclusions about the estimated quantities of pheromone on individual bees, especially at the later times. The

actual amounts of QMP that are required to influence the behaviors of individual workers have now been determined for several activities. Winston et al. (1989, 1990, 1991) found that approximately 1-10 Qeq of synthetic QMP per day (150-2500 μg/day of 9-ODA) are sufficient to inhibit queen rearing in queenless and queenright colonies. Slessor et al. (1988) found that elicitation of the retinue response required a source containing at least 10^{-7} Qeq of QMP, equivalent to approximately 2 pg of 9-ODA; this functional attractive dose is well above the lower detection limit of 1.4 pg in this experiment. Thus, bees for which no radio-label was detected, did not come into contact with biologically meaningful amounts of pheromone. However, for bees carrying radio-label, the amounts of pheromone that they received cannot be accurately calculated.

Differences in population size were the most important factor contributing to the observed differences in radio-label movement between treatments. The presence of more bees in the Populous colonies resulted in fewer individuals receiving pheromone. However, there was dilution effect due to difference in population size, since those workers in the populous, congested colonies that had acquired radio-label carried, on average, the same amounts as those in the uncongested colonies. If pheromone were simply being diluted equally among the workers, an average worker in the more populous colonies should have carried less than one from the less populous nests. Also, movement of radio-label

to peripheral areas of the Populous colonies should have been delayed, or of a lesser magnitude, compared to the Unpopulous colonies, but spatial patterns of radio-label distribution were similar in both treatments. Therefore, I conclude that individual queen - worker and worker - worker transfers were similar in Populous and Unpopulous colonies, but the high population size in the Populous colonies resulted in reduced transfers relative to the overall population. Because population size per se has been shown not to be directly related to the onset of swarming, the effects seen in this study are probably not of primary importance in causing reproductive swarming; much more populous colonies than those used here can be prevented from swarming by providing adequate space for expansion, thereby relieving congestion.

The lack of any apparent affects of colony congestion on pheromone transport, and therefore on the stimulation of reproductive swarming, is at odds with the theory that disruption in pheromone transport due to crowding leads to queen rearing. However, the effects of crowding on pheromone transfer would be more pronounced in colonies of greater physical dimensions and populations than were used in this study. Colonies of 10 frames or more might have shown more dramatic differences between treatments in the outwards rate of pheromone movement. The tendency of workers that were sampled from the periphery of the crowded colonies to be carrying smaller quantities of radio-label

than corresponding workers in the uncrowded colonies, although not statistically significant, suggests that this may be the case. Greater congestion in the nest might also have greater impact upon queen pheromone transmission. The congested colonies used in this study were still in the ergonomic stage of development, a period of rapid growth that precedes the reproductive, or swarming, stage. Congestion could become more intense as colonies enter the reproductive phase, and interference with pheromone transfer could increase correspondingly. Thus, crowding and population size may interact in larger colonies to affect the distribution of queen mandibular gland pheromone in the nest. The onset of queen rearing may also not be due to changes in queen pheromone transport due to crowding. Workers may be able to assess crowding or other colony factors and to respond by raising the threshold of inibitory response to queen pheromone. Most experiments that have investigated the role of colony crowding and/or the effect of supplemental applications of QMP (see Winston et al., 1991) have yielded results that are also consistent with this latter theory.

Overall access to the "queen" did not differ between treatments because the total amounts of radio-label that were dispersed among the workers of the 2 colony types, and the amounts remaining on the queens after 24 h, did not differ. In other words, similar amounts of material were gathered from the queens in both treatments. In an

undisturbed colony, the queen can increase the dispersal of her own pheromones by moving to different areas of the nest. This allows access to different workers and spreads pheromones via the queen's footprints (Chapters 2, 4). Seeley (1979) showed that the queen may come into direct contact with as many as a third of the nurse bees in her colony frequently enough to inhibit them from constructing queen cells. In congested colonies, she may be able to move over less comb area than in uncongested nests, thereby accentuating the influence of colony population size and crowding on pheromone transfer.

This study has focussed only on the intra-nest movement of one QMP component, 9-ODA. The results presented in Chapter 4 suggest that the rates of transfer between queens, workers, and the comb wax are similar for 9-ODA and the two aromatic components of the pheromone complex, HVA and HOB, suggesting that the results and conclusions of this study can be extended beyond 9-ODA, to QMP as a whole.

In general, the methods by which queen pheromones reach the queen's nestmates are as poorly understood as the mechanism(s) by which these compounds affect behavior and physiology. Aside from honey bee QMP, no queen pheromones with a primer function have been identified for any eusocial species. It is therefore difficult to extrapolate what is known about honey bees to other species. Fletcher (1986) and Fletcher and Blum (1983) suggested that the maintenance of monogyny in the fire ant, *Solenopsis invicta* (Buren)

involves the maintenance of levels of queen inhibitory pheromones within an optimal range. By this hypothesis, if the queen dies, or the colony becomes populous enough to hinder pheromone dispersal, the levels fall, and workers are stimulated to produce, or accept, a new queen. If the levels are too high due to supernumerary queens, some queens are executed. As in *A. mellifera*, queen pheromones in the fire ant may be transported by physical contacts. Sorensen et al. (1985) demonstrated, using radio-labelled protein, that physical contact and trophallaxis could both be efficient modes of transmission of inhibitory substance in that species. Similar patterns may be found in other species. Thus, in at least two well-studied eusocial hymenopteran species, it appears that the maintenance of the queen's unique position in the nest is the result of either a maintained level of inhibitory compounds in the nest or of changes in workers thresholds of inhibition. The balance can be under the influence of pheromone production levels, the rates of absorption and breakdown, the rate and efficiency of transport, and/or the ability of the workers themselves to alter their thresholds of inhibition in response to demographic conditions in the colony. Future experiments should seek to uncouple the different roles of pheromonal transport and workers abilities to alter thresholds of pheromonal inhibition in the context of the queen's influence on colony-level reproduction.

CHAPTER 6

THE EFFECT OF SYNTHETIC QMP ON WORKERS IN PACKAGES

The recent resurgence in research on honey bee (*Apis mellifera* L.) pheromones has resulted in the application of several synthetic compounds in commercial beekeeping. For example, synthetic Nasonov pheromones are now being marketed as attractants for both pollinators (Mayer et al., 1989), and swarms (Schmidt & Thoenes, 1987a,b). In this chapter, I report on experiments to assess the use of synthetic honey bee queen mandibular gland pheromone to substitute for queens during the shipment of package bees.

Packages containing 0.9 or 1.4 kg (2 and 3 lbs) of workers are produced commercially from overwintered colonies and sold to initiate new colonies or to replace colonies that have been lost or killed. Packages almost invariably contain a mated queen when shipped because her presence prevents the workers from becoming agitated and overheated while in transit, thereby reducing worker mortality and improving the ease of establishing colonies. Because a queen is always necessary once a package is shaken into a hive, there are circumstances where shipping packages without queens might be desirable. For example, there are not always convenient or reliable sources of queens for package producers, and some beekeepers prefer to produce their own queens and purchase only bulk worker bees. These

situations have increased in frequency in recent years due to restrictions on bee movement in North America. Currently, the movement of all honey bees from the United States to Canada has been eliminated in an effort to prevent the entry of the parasitic mites *Acarapis woodii* Rennie and *Varroa jacobsoni* Oudemans into Canada. Such restrictions can be expected to continue since Africanized bees have entered the southern United States. This quarantine has halted the traditional northward flow of packaged bees and queens, and, although this has contributed to the development of a package and queen rearing industry in Canada (Winston, 1986; Winston et al., 1985), it also has increased the circumstances in which the shipment of queenless packages is desirable, particularly in the spring, before queens can be reared in Canada.

One potential application of synthetic queen mandibular gland pheromone is the utilization of 'pseudo-queen' lures for queenless package transport. Such pheromone lures might prove useful for keeping queenless, packaged workers clustered and calm, thereby making them easier to handle, decreasing in-transit mortality, and leading to improved early colony development. Also, queenless packages could be sold at lower cost per package to buyers who can produce their own queens, and would allow producers to create and sell packages when no queens are locally available. In this study we compared the behavior of packaged workers with

queens, without queens, and with two different doses of synthetic queen mandibular pheromone.

MATERIALS AND METHODS

In April, the usual time for package production and shipment in southern British Columbia, 32 0.9 kg (2 lb.) packages were created from a melange of workers shaken from several overwintered colonies. The packages were randomly assigned to four different treatments: 1) queenright, with a mated queen shipped from Australia in a small cage, 2) queenless, 3) 3 queen-equivalents (Qeq) of synthetic QMP, and 4) 10 Qeq of synthetic QMP. The pheromone was applied on lures consisting of 2.5 cm lengths of cotton wicking with pheromone dissolved in methanol injected into the center. Lures were suspended into each package by wire, in a way which allowed workers to form a cluster around them, and each package was fitted with a can containing 0.8 liters of 1:1 sugar syrup medicated with Oxytet-25-S^{R} (Medivet Pharmaceuticals Ltd, High River, AB) and Fumagilin B^{R} (Medivet Pharmaceuticals). The packages without queens or pheromone lures were given methanol-treated 'blank' lures so that, with the exception of the queenright group, observers could not distinguish between treatments. The morning after packaging, the bees were transported for 24 h in a van, returning finally to the study site where they remained in storage for 2 more days. During this entire interval the bees were kept cool (15 - 20^{o} C), dark, and were sprayed several times daily with a mist of water. After 4.5 days, each package was shaken into hives of standard Langstroth

equipment and the bees supplied with 5 l of medicated sugar syrup and 0.5 kg of a pollen supplement to stimulate colony development. At that time, mated queens also were introduced, in cages, into the blank and pheromone-treated colonies. All queens were released 48 h later. Thus, the packages were made up and handled in the same manner as they would be by commercial package sellers and buyers.

Worker responses to the treatments were characterized by determining tightness of the cluster, sound production, the rate of syrup consumption, the general degree of agitation, and worker mortality. All of these measures reflect the tendency for queenless workers to be more agitated and active than those with a queen, and provide an indication of how workers behave in packages with a pheromone lure relative to workers with or without a queen.

(i) Cluster tightness was estimated by determining the density of the cluster (g/l). Five to ten measurements each of length, height, and width were taken across the cluster, and used to calculate the approximate volume. Cluster weight was taken as the total weight of the package and the bees minus the weight of the empty package and feeding can.

(ii) Loudness and frequency distribution of buzzing were used to compare the sound production of bees in the different treatments. The sound produced by each package of bees was tape-recorded using a portable stereo cassette deck (Model CD 1636, JVC Corp, Tokyo, Japan) with the microphone held 5 cm from the center of the cluster. Short (2.4

second) intervals of these sounds were analyzed for range of frequencies and the relative contribution of different frequencies using a Sona-Graph 6061-B audio frequency spectrum analyzer (Kay Electric Co., Pine Brook, N.J.) with the following settings: AGC = 5, input selector = 10,000 Hz, record level = 0, band selector = narrow, and a 500 Hz calibration marker. The recorded sounds were played back in a partially sound-insulated box, and the volume level of the sounds measured (in decibels) with a General Radio Company 1551-C sound-level meter (Concord, Mass.).

(iii) Syrup consumption also was used as a measure of worker activity; calm, clustered workers might utilize syrup at a lower rate than restless, queenless workers. Uptake was determined by measuring the decrease in feed-can weight.

(iv) An observational estimate of worker agitation was made using the following index:

(1) workers 'humming' and hanging together in a cluster

(2) workers walking over the sides of the package

(3) workers flighty, agitated, and buzzy

(v) Worker mortality was measured directly by removing and counting corpses from each package just before hiving. This was accomplished without disrupting the remaining workers by using packages with removable bottoms.

Most measurements of the aforementioned parameters were made on three occasions: the morning after packages were shaken, after transport, and the morning before hiving (approximately after 1, 2, and 4 days). Sound measurements

were made after 1 and 4 days. The temperatures at the sampling times were 18° C, 16° C, and 17° C respectively.

After hiving, lures were collected from all packages and analyzed by capillary gas chromatography to determine the amounts of pheromone remaining.

To gauge the relative development of the package-founded colonies, queen acceptance, the adult population size, and brood area of each colony were determined, by measuring the number of frame sides covered, to the nearest quarter of one side of a frame, 22 days after queen release.

Data for the tests yielding continuous data were analyzed by one-way analysis of variance (ANOVA) and subsequent tests of means (Student-Newman-Keuls)(Zar, 1984). The categorical data for the observational estimates of worker agitation and (due to small sample sizes) the measures of adult and brood population size were analyzed by Kruskal-Wallis and a non-parametric test of comparisons (Conover, 1980).

RESULTS

There were no significant differences between the queenright and pheromone treatments for any of the parameters studied, while the blank treatment usually showed qualitative or statistically significant differences from the other three treatments. The blank treatment always scored the highest in the behavioral index (reflecting greater activity), although differences between the blank packages and the other three treatments were significant only at one day after package creation (Kruskal-Wallis, X^2 = 23.31; d.f. = 3; Conover nonparametric comparison test; $p < 0.05$) (Table 6-1). The blank treatment also showed the loosest clusters, with the workers often clustered away from the blank lures. Cluster densities were significantly lower in the blank treatment at 1 and 2 days after shaking (ANOVA, F = 11.04 and 8.16; d.f. = 31; Student-Newman-Keuls, critical ranges = 0.021 and 0.028; $p < 0.05$). At 4 days after shaking only the blank and queenright treatments were significantly different from each other (ANOVA, F = 5.56; d.f. = 31; Student-Newman-Keuls, critical ranges = 0.029; $p < 0.05$) (Table 6-1).

All treatments showed a consistent pattern of sound production, with harmonics occurring at intervals of approximately 190 Hz. There were no differences in the range of frequencies produced (ANOVA; d.f. = 31; day-1 F = 0.86; p = 0.47 ; day-4 F = 1.62; p = 0.21), or in the

Table 6-1. Effect of queen or lures containing synthetic queen mandibular gland pheromone (QMP) on three mneasures of packaged bee behavior. Values are means ± SE's. n = 8.

Treatment	Days after package formation		
	1	2	4
		Behavioral index[a]	
Blank	2.13 ± 0.13b	1.50 ± 0.19a	1.88 ± 0.13a
3 Qeq QMP[b]	1.13 ± 0.13a	1.00 ± 0a	1.50 ± 0.25a
10 Qeq QMP	1.13 ± 0.13a	1.13 ± 0.13a	1.75 ± 0.27a
Queen	1.00 ± 0a	1.00 ± 0a	1.25 ± 0.16a
		Cluster density[c] (g/litre)	
Blank	180 ± 6.1b	180 ± 15b	190 ± 9.9b
3 Qeq QMP	220 ± 8.1a	220 ± 9.1a	220 ± 7.5ab
10 Qeq QMP	230 ± 7.0a	230 ± 5.4a	230 ± 11ab
Queen	210 ± 7.9a	240 ± 4.9a	250 ± 11a
		Sound amplitude[c] (Db)	
Blank	69.9 ± 1.29b	-	60.5 ± 0.71a
3 Qeq QMP	64.0 ± 1.78a	-	60.4 ± 0.93a
10 Qeq QMP	64.5 ± 1.49a	-	62.1 ± 0.85a
Queen	61.8 ± 0.75a	-	59.6 ± 0.38a

[a]Within a column, values followed by different letters were significantly different (Kruskal-Wallis; Conover nonparametric comparison test; $p < 0.05$).
[b]Qeq, queen equivalents of pheromone per dose.
[c]Within a column, values followed by different letters were significantly different (one-way ANOVA; Student Newman-Keuls test; $p < 0.05$).

relative contributions of the 2 dominant harmonics (approximately 190 Hz for the first and 380 Hz for the second)(ANOVA; d.f. = 31; day 1 $F = 2.75$, $p = 0.06$; day 4 $F = 0.90$, $p = 0.45$). The volume-level of the recorded buzzing by the blank packages was significantly greater than for the other three treatments at 1 day after package formation (ANOVA, $F = 6.23$; d.f. = 31; Student-Newman-Keuls, critical range = 4.00; $p = 0.02$) (Table 6-1). The queenright packages were the quietest. At 4 days, the differences between treatments were not significant (ANOVA, d.f. = 31; $F = 1.99$; $p = 0.14$).

The packages in the blank treatment had significantly greater numbers of dead workers than the queenright or pheromone treatments after 5 days: blank 743 $\pm$ 119, 3 Qeq 203 $\pm$ 19, 10 Qeq 253 $\pm$ 57, and queenright 224 $\pm$ 51 (mean $\pm$ standard error) (ANOVA, $F = 13.24$; d.f. = 31; Student-Newman-Keuls test, critical range = 206; $p < 0.05$).

There was no difference in the amount of syrup consumed between treatments. One day after package creation, the means ($\pm$ standard errors) of the treatments were: queenright 400 $\pm$ 43 ml; 3 Qeq 368 $\pm$ 28 ml; 10 Qeq 386 $\pm$ 15 ml; and blank 446 $\pm$ 21 ml (Kruskal-Wallis, $X^2 = 4.04$; d.f. = 3; $p = 0.26$). No comparisons were made for subsequent sampling dates because many packages from each treatment had consumed all of their syrup supply.

Packages in all treatments were hived with ease, possibly because the temperature was cool (approximately 12^{o}

C), and the bees were relatively inactive. Two days after queen release, two colonies were queenless in both the 10 Qeq and blank treatments, and four were without queens in the 3 Qeq treatment. All of the queens in the queenright group had been accepted and were laying. New queens were introduced into all of the colonies without queens, and after 3 days in cages, all 8 were released and accepted. However, these 8 colonies were excluded from the remainder of the experiment.

Twenty-two days after queen release, a further 3 colonies (one from each of the blank, 10 Qeq, and queenright treatments) had superseded their queens, and were therefore excluded from analysis. There were no significant differences between treatments in either adult population size or brood area (Kruskal-Wallis; $p > 0.05$) (Table 6-2).

Gas chromatographic analysis of methanol washes of the experimental lures showed that only a negligible amount of pheromone had been removed from the cotton wicks after approximately 4.5 days in the 0.9 kg packages. The Qeq amounts calculated to be remaining in the 3 Qeq treatment lures were 3.4 ± 0.3 for 9-ODA, 3.4 ± 0.3 for 9-HDA, and 2.7 ± 02 for HOB (means $\pm$ standard errors; $n = 8$). The lures used in the 10 Qeq treatment yielded 9.9 ± 1.0 Qeq of 9-ODA, 8.0 ± 1.0 of 9-HDA, and 9.1 ± 0.5 Qeq of HOB. Washes of lures treated with 3 Qeq of synthetic QMC, but not used in the experiment, yielded 2.5 ± 0.1 Qeq of 9-ODA, 3.6 ± 0.5 of 9-HDA, and 3.2 ± 0.5 Qeq of HOB. These latter three values

were not significantly greater than the correesponding values from the 3 Qeq treatment lures (t tests; d.f. = 14; $p > 0.05$). There were no mandibular-gland pheromone components detected on the blank lures, and too little HVA was detected on any lures to allow for analysis.

Table 6-2. Adult and brood population sizes in colonies founded from packages of honey bees that had been supplied with either a queen, a lure containing synthetic mandibular gland pheromone (QMP), or a blank lure.

Treatment[a]	Measure	
	Adults	Brood
Blank	4.10 ± 0.45[bc]	3.45 ± 0.23
3 Qeq QMP[d]	4.00 ± 0.35	3.44 ± 0.46
10 Qeq QMP	4.40 ± 0.70	3.80 ± 0.49
Queen	4.71 ± 0.28	4.00 ± 0.38

[a]Sample sizes were blank 5, 3-Qeq 4, 10-Qeq 5, and queen 7.
[b]Values are means ± SEs of the number of frame sides covered, measured to the nearest quarter of one side of one frame.
[c]There were no significant differences between treatments (Kruskal-Wallis; $p < 0.05$).
[d]Qeq, queen equivalents of pheromone.

DISCUSSION

Workers in packages containing lures with 3 or 10 Qeq of synthetic queen mandibular pheromone behaved similarly to workers in queenright packages, and differently from workers in queenless packages, particularly early in the experiment. Statistically significant differences were seen on day 1 in almost all measures of worker agitation, as well as in the worker mortality levels at the end of the package period. Thus, synthetic queen mandibular gland pheromone is a good substitute for queens for the shipment and temporary storage of packaged workers.

The results do not suggest that the presence of a pheromone lure in a package has any detrimental effect on early colony development compared with queenright packages, or any beneficial effect compared with queenless packages. However, the small sample size caused by queen loss makes these results only tentative.

The rate of queen acceptance may have been unusually low in this experiment due to the Australian source of the queens. These queens were unusually prone to flight, a behavior observed in other, non-experimental queens of the same shipment. Poor acceptance of queens from Australia has been noted by Jaycox (1989) who suggested that Australian queens may have difficulty adjusting to the sudden reversal of seasons when they are shipped to North America. He recommended that the queens be kept in their shipment cages

for more than 3 days following package and queen introduction to hives, before allowing their release. In addition, we recommend a 1 or 2 day period with caged queens in the packages if pheromone lures are used to ship bees.

The small amounts of synthetic QMP removed from the lures suggests that minute quantities can quiet workers, and also that release from lures of cotton wicking is slow. Slessor et al. (1988) showed that retinue behavior of workers can be elicited in laboratory bioassays by as little as 10^{-7} Qeq of QMP, yet Winston et al. (1990) have suggested that 1 to 10 Qeq are needed daily to suppress emergency queen rearing. Clearly, the dose of synthetic QMP required for a specific function is highly context-dependent. The need for continued research on slow dispensing lures is apparent. For practical commercial use, lures should deliver pheromone at a constant rate for several days, and different release rates will be needed for different uses; the shipment of queenless packages apparently requires less pheromone than the suppression of queen rearing in established colonies.

Differences between treatments became less significant over the 4 days in which the packages were sampled, but because the queenright packages did not perform better than the synthetic QMP packages on the latter days, there is no evidence that the pheromone effect diminished. Rather, the higher ambient temperature on the first day of sampling may

have accentuated differences in worker agitation between the treatments.

The results demonstrated that synthetic queen mandibular gland pheromone lures can be used as queen substitutes in the shipment of packaged bees, and possibly in other applications. Northern beekeepers could initiate new colonies by matching domestically raised queens with packages of workers shipped from areas such as southern British Columbia, with mild climates. Alternatively, queens from allowable foreign sources, such as Australia or New Zealand, might be shipped directly to the beekeeper for introduction into queenless packages or incipient colonies, provided that problems with queen introduction can be overcome. Pheromone lures could also serve as queen substitutes in situations where logistical problems result in queens being temporarily unavailable. The ability to pacify workers may also make synthetic QMP useful for maintaining units of bees larger than packages. Ultimately, large colonies might be pacified during periods of queenlessness, or made purposely queenless in order to direct segments of the workforce away from brood rearing, and to foraging.

SUMMARY

This study has elucidated the rates of production and the rates and mode of transmission of queen mandibular gland pheromone. These are the first such descriptions for a social insect. Pheromones such as QMP are important in integrating the diverse behaviors of the workers with the result that the colony functions as a single unit. QMP acts as both releaser, arresting nearby workers, and as a primer, inhibiting the production of further queens. As the results of this study show, these two different modes of action are interrelated, since the attraction of nearby workers creates a retinue around the queen and facilitates the transfer of the pheromone to the rest of the workforce.

Pheromone transfer to the workers begins with secretion onto the body surface of the queen. From there, pheromone is removed by workers in the queen's court, reentry into the queen's body, and tracking onto the comb, in order of diminishing quantities. Queen-contacting workers in the court normally show one of two behaviors, licking the queen, or antennal contact. The former behavior results in the acquisition of greater quantities of QMP, in terms of both individual and colony-wide amounts. Upon leaving the retinue, workers act as messengers and transfer some of their acquired pheromone to nestmates via bee to bee contacts. Like the queen, they also leave pheromone

footprints in their path. Non-retinue workers can receive and transfer QMP via the same processes. The sum result is a set of serial transfers of pheromone from the queen to most of the nest residents. The pheromone signal is removed from circulation by an internal movement from the body surface of each worker. Such a system of pheromone transport and removal results in both efficient, rapid transfer through the nest, and a similarly rapid signal of any loss in the supply of QMP, i.e. the failure or death of the queen.

QMP transfer, and removal from circulation, are both accelerated by worker self-grooming, which results in the backwards translocation of pheromone on the body. Individual grooming movements are not specific to pheromone translocation but are likely a typical response to contacting an oily substance.

The speed with which QMP is removed from circulation suggests that some individuals, such as foragers, may experience long intervals without receiving an inhibitory signal from the queen. This raises the interesting possibility that certain individuals, or type of individuals, do not require chemical inhibition to refrain from performing certain tasks. In other words, it may be that only workers in certain age or task windows are the major target of the queen's inhibition. Such workers would be those most likely, or most competent, to construct queen cells, raise young larvae into queens, or begin egg laying

themselves. These tasks are performed by young and intermediately aged adults, bees that are normally found in the area of the brood nest (Seeley, 1982). The critical area of the queen's chemical 'control' may thus be the brood nest.

The method of QMP perception and action is of major importance for a full understanding of the information gleaned from this study. QMP could produce its primer effects via stimulation of antennal receptors, or via specific binding proteins in the hemolymph. If antennal perception proves to be the actual mechanism, then internalization of pheromone by workers is simply a method of removing pheromone from circulation. If, however, binding proteins in the haemolymph are utilized, then internalization has the dual role of limiting further transfer to other individuals, and of bringing pheromone and binding proteins together.

The results of this study suggest that QMP does not dissociate into various components as it is moved through the nest. In this respect, QMP is similar to other multicomponent pheromones in other insect taxa. The reasons for the evolution of such a complex pheromone remain unclear. Winston (1990) has suggested that such complexity may be evidence of an evolutionary arms race, whereby queens have responded to efforts by workers to avoid inhibition with an expanding arsenal of chemical inhibitors. But why do workers possess receptors or binding proteins for such

compounds? The apparent complicity of the workers hints that they are in evolutionary "cooperation" with the queen, in effect, allowing themselves to be affected by the queen's messages. In such a scenario, however, it would be energetically less costly for queens to produce a single compound, and that in small quantities. Perhaps the complexity of the QMP signal evolved as a defence against potential, or past, social parasites that might attempt to usurp the queen's position. Honey bees are currently unaffected by such social parasites although they are common in some other social insect species. Alternatively, the various components of QMP may have specific functions that have not been discovered, or the reason for chemical redundancy may simply be to better separate the queen's signal from background noise.

The results presented in Chapter 5 have demonstrated that a larger proportion of the workers in populous colonies do not receive adequate queen pheromone as compared to less populous, uncrowded colonies. There was however, no difference in the way in which pheromone was moved through the nest in the two treatments. The onset of queen rearing that accompanies the initiation of reproductive swarming is most likely caused by an interaction between the same quantity of pheromone being distributed among a larger population, and possibly decreased transport resulting from increased congestion, and/or from changes in workers' thresholds to queen inhibitory pheromone.

Synthetic QMP may prove useful in a number of commercial applications because of its effects on worker behaviors. The results of Chapter 6 demonstrate that synthetic QMP lures can be used as queen substitutes in the temporary storage and transport of packaged workers. The lures act to attract and pacify the workers. This attractive ability could also be used to lessen the drifting away of workers from small colonies used for queen mating, and to attract foragers to crops for pollination. QMP-treated lures or supplemental applications might also be used to inhibit queen rearing in queenless, or populous, queenright colonies. More research is required to determine when, where, and how much pheromone is optimal for each use.

Many questions concerning queen pheromones and the role of queens in coordinating colony functions remain to be answered. No primer pheromone, excepting QMP, has been identified in any other social insect. In fact, the role of queens in colony organization has been studied in barely a handful of species. As a result, nothing is known about the biosynthetic pathways, the metabolic costs of production, or routes of catabolism of such compounds, and only in the honey bees can estimates be made about pheromone production/secretion and its relationship to colony functions. The physiological mechanisms underlying primer pheromone function and endocrinological functions are all but unknown. Information on how queens and workers have come to interact in different species and ecological

settings may give valuable insights into understanding how eusocial systems have arisen in the Hymenoptera.

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