WORKER RESPONSES TO, AND QUEEN PRODUCTION OF, HONEY BEE (*APIS MELLIFERA* L.) QUEEN MANDIBULAR PHEROMONE

by

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ABSTRACT

Honey bees, like most highly eusocial insects use pheromones to communicate. However, the honey bee is the only social insect for which a primer pheromone has been identified. This pheromone consists of a five-component blend of acids and aromatics produced in the queen's mandibular glands that is involved in both primer and releaser functions. Queen mandibular gland pheromone (QMP) signals queen presence to workers either by direct contact with the pheromone source or indirect serial transmission of QMP from worker to worker. Workers attracted to the pheromone display a releaser response called retinue behavior, characterized by frequent antennations, licking, grooming and feeding activities directed toward the queen or pheromone lure. Removal of the queen from the nest or inhibition of QMP transmission stimulates queen rearing activities, a "primer" effect mediated by QMP. A second primer effect of QMP is the suppression of juvenile hormone III (JH) biosynthesis. Juvenile hormone is an insect development hormone associated with worker honey bee behavioral ontogeny.

Strains of high and low QMP retinue responding workers were used to investigate colony-level QMP-response variation, and releaser-primer relationships. Strain-dependent response was not related to queen production of pheromone, synthetic QMP or gland extract response, or rearing environment. Strain-dependent differences also were maintained over a wide range of dosages. Despite a strong genetic component to the retinue response to QMP, there was no differential strain-dependent queen attendance behavior, suggesting that other cues, such as movement, texture, and perhaps another pheromone singly or in combination

with QMP may be involved in mediating retinue behavior.

High strain workers were significantly more likely to engage in queen cell rearing activities as well as spend more time on these tasks. Thus, QMP retinue response conferred a primer influence of QMP, expressed as worker queen rearing activity. This result suggests a mechanism by which queen rearing behavior is organized in colonies.

High, low, and wild-type QMP-retinue responding strains fostered in queenright colonies with supplemental QMP had significantly lower JH titers and showed a related delay in foraging ontogeny. In addition, forager activity was greater in control colonies. These results provide the first demonstration that a social insect pheromone acts as a modulator of division of labor.

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INTRODUCTION

Honey bees (*Apis mellifera* L.) are highly eusocial insects that maintain perennial colonies containing several thousand facultatively sterile female workers, none to hundreds of drones (males), and a single reproductive female, the queen. Labor is divided such that all tasks are performed concurrently, and individuals change tasks according to internal and external stimuli based on local information (Page and Mitchell, 1990; Huang and Robinson, 1992). A central question in insect sociobiology is how the activities of thousands of individuals are integrated to enable colonies to maintain cohesive units capable of growth and reproduction.

The utilization of communication chemicals is vital to maintaining the cohesiveness of highly eusocial insect colonies. Queen mandibular pheromone (QMP) is a semiochemical used for communication among colony members. Pheromones may be classified according to their various actions as either releaser or primer pheromones. QMP is both a releaser and a primer pheromone. Primer pheromones physiologically alter the endocrine and/or reproductive systems, while a releaser response constitutes a stimulus-response mediated by the nervous system (Hölldobler and Wilson, 1990). The releaser response to QMP known as retinue behavior is characterized by 4-12 workers antennating, licking, feeding and grooming the queen or an artificial QMP source (Seeley, 1979; Slessor *et al.*, 1988; Naumann *et al.*, 1991, 1992). The discovery of the 5-component blend of QMP (Slessor *et al.*, 1988) has permitted an examination of the role of QMP alone as a retinue cue, without any other cues present. The primary function of QMP is to signal queen presence,

accomplished by queen movement (Seeley, 1979), and worker circulation of QMP throughout the nest (Naumann *et al.* 1991, 1992, 1993). QMP elicits retinue behavior (Slessor *et al.*, 1988, 1990; Kaminski *et al.*, 1990), retinue workers remove QMP from the queen (Naumann *et al.*, 1991, 1992), and messenger specialists (Seeley, 1979) circulate QMP throughout the nest (Naumann *et al.*, 1991, 1992). QMP influences several colony-level functions such as, the inhibition of new queen production, the timing of swarming (Winston *et al.*, 1989, 1990, 1991; Pettis *et al.*, 1995a), and the attraction of workers to swarm clusters (Winston *et al.*, 1989).

The reproductive influences of QMP on honey bee colonies constitute a primer effect of QMP, and the releaser and primer functions of QMP with respect to colony reproduction appear to be coupled. Removal of the queen and colony congestion terminates or dilutes the circulation of QMP in the nest (Winston, 1989; Naumann *et al.*, 1993), releasing the inhibitory effects of QMP on queen rearing (Winston *et al.*, 1989, 1990, 1991; Pettis *et al.*, 1995a). One objective of this study was to examine the variability of retinue response to QMP in a laboratory bioassay to determine possible environmental influences on QMP retinue response. Concurrently, a genetic component to the releaser response was also investigated (Chapters 1 and 3) by implementing a breeding program (Chapter 2) to develop high and low QMP retinue responding strains of workers. High and low strain workers were used to determine whether retinue response to QMP in the laboratory bioassay conferred retinue response to a live queen in the nest (Chapter 4). These workers were used to examine the role of QMP retinue response in queen rearing behavior. The purpose of these studies was to uncouple the releaser retinue response and the reproductive primer

influence of QMP in order to examine their relationship (Chapter 5).

As a primer pheromone QMP also may influence the endocrine system of honey bees. Mandibular gland extracts, ODA (9-keto-(*E*)-(2)-decenoic acid), the major component of QMP and the queen inhibit the rate of juvenile hormone III (JH) biosynthesis in worker honey bees (Kaatz *et al.*, 1992). JH is an insect developmental hormone associated with age-related behavioral changes in worker honey bees. Intra-nest activities performed by workers less than 3 weeks old, such as cell cleaning, queen, and larva tending are associated with low JH titers, and extra-nest activities such as guarding, and foraging are associated with high JH titers and performed by workers greater than 3 weeks old (Robinson, 1992). Treatment with JH mimic, JH or a JH analogue will induce precocious foraging (Fluri *et al.*, 1982; Huang *et al.*, 1991, 1994; Jaycox *et al.*, 1974; Robinson 1987a, b; Sasagawa *et al.*, 1989). The results in Kaatz *et al.* (1992) suggest that QMP could induce a JH-based delay in worker foraging ontogeny. In this context, the primer influence of QMP is on the endocrine system. The main objective of Chapter 6 was to examine the endocrinological primer effect of QMP, and to determine the influence of QMP on worker foraging ontogeny and JH titers. The main hypothesis was that workers reared in nest environments with supplemental QMP would display lower JH titers and later foraging ontogeny than untreated colonies. Additionally, a genetic basis to the hypothesis was tested using the high and low QMP-retinue responding workers to determine whether selection based on retinue response conferred a correlated primer response.

CHAPTER 1

VARIATION IN WORKER RESPONSE TO HONEY BEE QUEEN MANDIBULAR PHEROMONE*

ABSTRACT

Genetic and environmental influences on the worker honey bee retinue response to queen mandibular gland pheromone (QMP) were investigated. Worker progeny were reared from queens originating from four sources: Australia, New Zealand, and two locations in British Columbia, Canada (Simon Fraser University and Vancouver Island). Progeny from New Zealand queens responded significantly higher ($P < 0.05$) than progeny from Australia in a QMP retinue bioassay. Retinue response was not related to queen production of pheromone or colony environment, and the strain dependent differences were maintained over a wide range of dosages. Selected high and low responding colonies contacted QMP lures more frequently than low responding colonies ($P < 0.05$) throughout the year except in late summer. We conclude that there is a strong genetic component to QMP response by worker honey bees, as well as a seasonal effect on response.

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* Pankiw, T., Winston, M.L., and Slessor, K.N. 1994. Variation in worker response to honey bee (*Apis mellifera* L.) queen mandibular pheromone (Hymenoptera: Apidae). Journal of Insect Behavior 7(1): 1-15.

INTRODUCTION

The behavioral phenotype of a social insect colony is determined by the intensity and frequency at which individuals respond to stimuli and perform tasks. Physiological, morphological, and/or behavioral phenotypic characteristics are the consequence of inherited potential expressed in varying environmental circumstances (Robinson, 1992). Therefore, behavior is particularly dependent on epigenetic considerations. Behavioral traits are distant from the chemical nature of underlying genes, unlike physiological and morphological traits. Honey bee behavior has been configured by natural selection in a social context, making bee behavior additionally complex. Thus, a bee's behavior is the product of its genetic potential, its ecological and physiological environments, the social conditions of its colony, and various prior and ongoing interactions among these four.

Honey bee (*Apis mellifera* L.) behaviors that have a strong genetic component include defensive behavior (Boch and Rothenbuler, 1974; Breed and Rogers, 1991; Breed *et al.*, 1991; Collins, 1979; Collins *et al.*, 1982, 1984; 1987; 1989; Guzmàn-Novoa and Page, 1993), foraging and pollen hoarding behavior (Calderone and Page, 1988; Danka *et al.*, 1987; Frumhoff and Baker, 1988; Hellmich *et al.*, 1985; Page and Fondrk, 1995; Page and Robinson, 1991; Page *et al.*, 1988; Pesante *et al.*, 1987a, 1987b; Rinderer *et al.*, 1985; Robinson, 1992; Robinson and Page, 1989; Rothenbuler and Page, 1989), hive cleaning behavior (Robinson and Page, 1988; Rothenbuler, 1964a,b), the tendency to swarm (Winston, 1980), grooming (Frumhoff and Baker, 1988: Kolmes, 1989), and temporal caste ontogeny (Guzmán-Novoa *et al.*, 1994; Winston and Katz, 1981).

In honey bees the only pheromone-mediated behavior known to have some genetic basis involves response to the alarm pheromone isopentyl acetate, controlled by two or three loci (Collins, 1979). Heritability estimates for the production of ten alarm pheromones range from high to moderate, suggesting a genetic basis for alarm pheromone production (Collins *et al.*, 1987), and subspecific differences in defensive behavior between Africanized and European bees also support a genetically based alarm pheromone response (Guzmán-Novoa and Page, 1993). Responses to pheromone also have been shown to be heritable in several non-social insects. Sex pheromone perception by males of the European cornborer, *Ostrinia nubilalis*, is inherited as a single autosomal gene with two alleles (Roelofs *et al.*, 1987), and there is no linkage between the genes controlling female pheromone production and male response (Löfstedt *et al.*, 1989). Heritable isomer specificity has been observed in males of the pink bollworm, *Pectinophora gossypiella* (Saunders) (Collins and Cardé, 1989), and male house flies, *Musca domestica* L. inherit responsiveness to female sex pheromone (Cowan and Rogoff, 1968). The southern pine beetle, *Dendroctonus frontalis* (Zimmermann) exhibits geographical variation in response to aggregation pheromones (Berisford *et al.*, 1990), and populations of bark beetle, *Ips pini*, exhibit geographical differences in sex pheromone production and reception (Lanier *et al.*, 1972; Miller *et al.*, 1989). Anosmia (smell deficiency) in *Drosophila melanogaster* is an X-linked mutation with four variants. Two mutants are deficient in responsiveness to aldehydes, one to acetates, and a fourth has multiple defects (Venard and Pichon, 1984).

In this study we examine the genetic basis of the pheromone-mediated retinue response in the honey bee. The presence of the natural or synthetic 5-component queen

mandibular gland pheromone (QMP), induces retinue behavior in worker bees (reviewed by Winston and Slessor, 1992). Workers engaged in retinue behavior antennate and lick the pheromone source while assembled in a fluctuating elliptical congregation (Seeley, 1979; Slessor *et al.*, 1988, 1990), and then disperse the pheromone to other colony workers (Naumann *et al.*, 1991, 1992, 1993). Worker bees display the highest (but not statistically different) response to QMP at seven days after emergence (Kaminski *et al.*, 1990), while workers kept in isolation after emergence are most responsive at ages less than five days old (Pham-Delègue *et al.*, 1991), and are 20-30% less responsive than workers reared in colonies (Masson and Arnold, 1984).

The honey bee queen mandibular pheromone is known to influence several colony activities, including direct reproductive activities such as the inhibition of new queen production (Butler, 1954; Butler and Simpson, 1958; Butler *et al.*, 1961; Winston *et al.*, 1989,

1990, 1991), swarming suppression (Winston *et al.*, 1991), the attraction of workers during swarming (Butler and Simpson, 1967; Velthuis and Van Es, 1964), and drone attraction (Gary, 1962; Butler and Fairey, 1964). Queen pheromone also attracts workers in the colony to the queen (Gary 1961; Zmarlicki and Morse, 1964), and may induce pollen and nectar foraging (Free and Williams, 1974; Higo *et al.*, 1992), comb building and brood rearing (Free, 1987), and the release of Nasonov pheromone at the colony entrance, important for orientation (Fergusson and Free, 1981). The genetic basis for QMP-induced retinue response by workers was investigated by selecting and rearing queens from high and low responding colonies. Environmental influences on retinue response were studied by

monitoring the retinue response of high and low responding colonies under varying environmental conditions including time of year, colony environment, and amount of QMP presented to workers in the laboratory and produced by the queen.

MATERIALS AND METHODS

Bioassay

Responsiveness of non-narcotized workers to QMP was quantified in a retinue bioassay (Kaminski *et al.*, 1990). Each replicate of the bioassay consisted of fifteen workers randomly selected from the brood nest area of the colony. Workers were placed in a plastic Petri dish bioassay arena, and exposed to a glass lure spotted with 10 μl of 10^{-3} queen equivalents (Qeq) of QMP. One queen equivalent is the average amount of pheromone found in a pair of queen mandibular glands; the synthetic blend contained a mixture of 250 μg 9-keto-2-(E)-decenoic acid (ODA), 150 μg 9-hydroxy-2-(E)-decenoic acid (88% R-(-) and 12% S-(+)) (9-HDA), 20 μg methyl p-hydroxybenzoate (HOB), and 2 μg 4-hydroxy-3-methoxyphenylethanol (HVA) (Slessor *et al.*, 1988). The quantity of 10^{-3} Qeq was chosen because it equals the average amount of QMP found on the body of a mated queen (Naumann *et al.*, 1991). Bioassay responsiveness was quantified by summing the number of worker lure contacts at 30 second intervals over a five minute period. An accompanying control bioassay quantified retinue response to a glass lure spotted with 10 of solvent (methanol or 2-propanol). Mean lure contacts per colony represent ten replicates of the retinue bioassay.

Honey bee sources

The colonies were derived from queens (most similar to *Apis mellifera ligustica* L.) originating from four different sources, NZ (Whiteline Queens, New Zealand), SFU (Simon Fraser University stock), VAN (Babe's Honey, Victoria, B.C., Vancouver Island), and AUST (Barden Apiaries, New South Wales, Australia). The queens (first generation) were instrumentally inseminated (Harbo 1985) with the semen from one drone. All drones originated from the same colony of an open mated queen. A single first generation mother queen from each source was selected (retinue bioassay selection) for rearing second generation queens. The second generation queens were allowed to mate naturally before overwintering. There were 11 NZ, 7 SFU, 10 VAN, and 14 AUST colonies headed by their respective second generation queens. Queens (second generation) within sources were super-sisters with a genetic relationship of r=0.75. The worker progeny relationship within each colony ranged from super-sister (r=0.75) to half-sister (r=0.25) (Page and Laidlaw, 1988). The highest (N=5) and lowest (N=5) responding colonies were transferred to ten-frame hives, and allowed to grow to fill two standard Langstroth size brood chambers. These selected colonies were bioassayed during May 1991, September 1991, February 1992, April 1992, and August 1992.

Cross-fostered worker bioassay

Newly emerged workers from one high and one low responding first generation colony were marked and cross-fostered. Workers from the high responding colony were

fostered in the low responding colony, and workers from the low responding colony were fostered in the high responding colony. After seven days of fostering the marked bees were removed and bioassayed. Six replications of the bioassay per colony were conducted for cross-fostered workers and ten for maternally fostered workers.

Age-dependent response

Newly emerged workers from the high (N=5) and low (N=5) responding colonies were marked and bioassayed when workers were seven to eleven days old to determine age-dependent responses. Bioassays on days 10 and 11 consisted of a mixture of randomly selected and marked bees from the brood nest area because an insufficient number of marked bees remained in the colonies for these days. Bioassays were conducted from 13 to 17 May, 1991.

Dose-dependent response of high and low responding colonies

To determine retinue behavior of high (N=5) and low (N=5) responding colonies to different quantities of QMP, workers were exposed to 0, 10^{-4}, 10^{-3}, 10^{-2}, and 10^{-1} Qeq of QMP/ 10 μl in the bioassay arena. Ten replicates per dose per colony were conducted.

Queen Mandibular Gland Components

Mandibular gland components from 8 N.Z., 5 S.F.U., 8 VAN, and 11 AUST queens were analyzed (Slessor *et al.*, 1990). The queens were 321-322 days old, and laying normally when removed from the colony. Queens were placed on dry ice, in a labeled polypropylene Eppendorf Safe-Lock Microcentrifuge Tube (1.5 m), and stored in a -70° C freezer until dissection.

Univariate analysis was used to test for normality among colonies headed by queens from different sources. Tukey's multiple means comparison was used to analyse both retinue response and QMP content differences between colonies headed by queens from different sources (Snedecor and Cochran, 1980). Wilcoxon rank-sum test was used to determine differences between selected high and low retinue responding colonies (Conover, 1980). Correlation analysis was used to determine the relationship between worker retinue response and queen pheromone production (Snedecor and Cochran, 1980; SAS Inst. Inc. Release 6.04, 1988, North Carolina, USA).

RESULTS

Retinue response of workers within sources

Colonies headed by queens from different sources represented a normal distribution for lure contacts in the retinue bioassay ($P < 0.94$, Univariate). The highest responding colony (33.7 ± 16.2, NZ line) was 17.7 times more responsive than the lowest responding

colony (1.9 ± 1.0, AUST line). Workers from colonies headed by queens from NZ contacted the lure 2.5 times more than those from the AUST colonies ($P < 0.05$, Tukey's, Fig. 1). Lure contacts by colonies headed by NZ, VAN, and SFU queens were statistically similar ($P > 0.05$, Tukey's). Response of AUST colonies to QMP and the solvent control was not significantly different ($P > 0.05$, Tukey's).

Cross-fostered worker bioassay

Worker response did not change as a result of cross-fostering (Table 1). The lack of statistical significance between high and low response workers in cross-fostering environments was due to insufficient replication. High response workers fostered in a low response colony remained almost four times more responsive than low response workers fostered in a high response colony environment.

Table 1. High and low worker response fostered in two colony environments; maternal colony and cross-fostered environments. Cross-fostered environments refer to high workers in a low colony and low workers in a high colony.

QMP-Response	Maternal	Cross-Fostered
High	18.7 ± 4.3a*	17.5 ± 10.8ab
Low	3.1 ± 0.7c	4.6 ± 2.2bc

* Means followed by different letters are significantly different ($P<0.05$, Wilcoxon Rank-Sum Test).

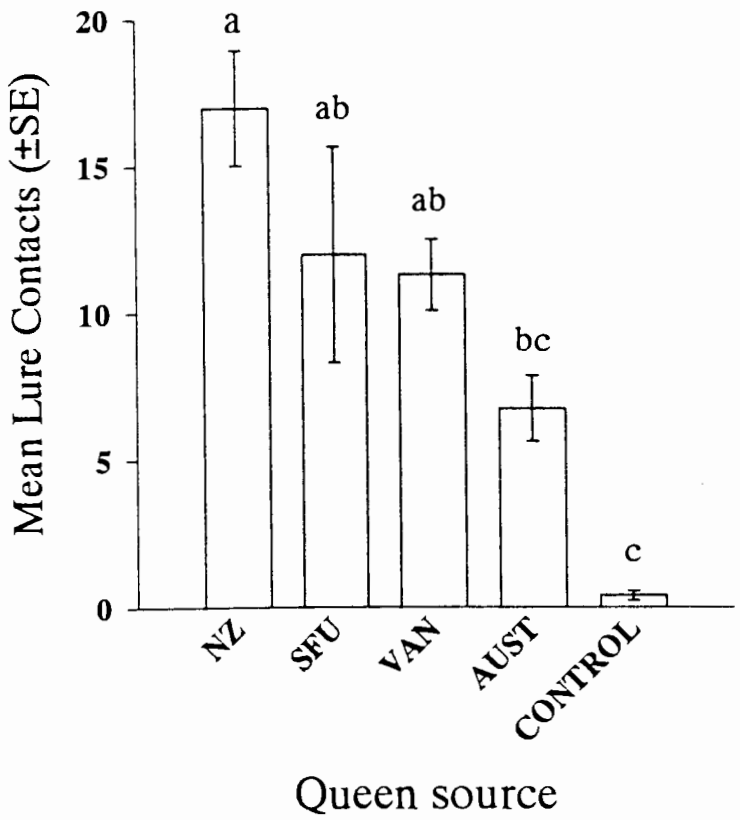


Figure 1. Worker bee responses from colonies headed by queens from different sources: NZ (N=11), VAN (N=10), SFU (N=7), AUST (N=14), and Control (N=1) colonies. Bars with different letters were significantly different (Tukey, $P < 0.05$).

Age-dependent response

There were no age-dependent differences in response within high and low responding colonies ($P > 0.05$, Wilcoxon Rank-Sum, Fig. 2). High response colonies contacted the lure more often than low response colonies in every age category ($P < 0.05$, Wilcoxon Rank-Sum).

Dose response of high and low responding colonies

At all QMP dose levels, high response colonies contacted the lure more than low response colonies ($P < 0.05$, Wilcoxon Rank-Sum, Fig. 3). The latter contacted QMP doses of 10^{-4} and 10^{-1} Qeq as frequently as solvent controls ($P > 0.05$, Wilcoxon Rank-Sum).

Mandibular gland pheromone components of queens from different sources

Quantities of mandibular gland components were not different among queens from different sources ($P > 0.05$, Tukey's, Fig. 4). There were no correlations between any pheromone component and worker response ($P > 0.05$, Correlation Analysis).

Annual retinue response

High responding colonies contacted QMP lures more frequently than low responding colonies at all dates tested except in September '91, and August '92 when contacts by low and high responding colonies did not differ ($P > 0.05$, Wilcoxon Rank-Sum, Fig. 5).

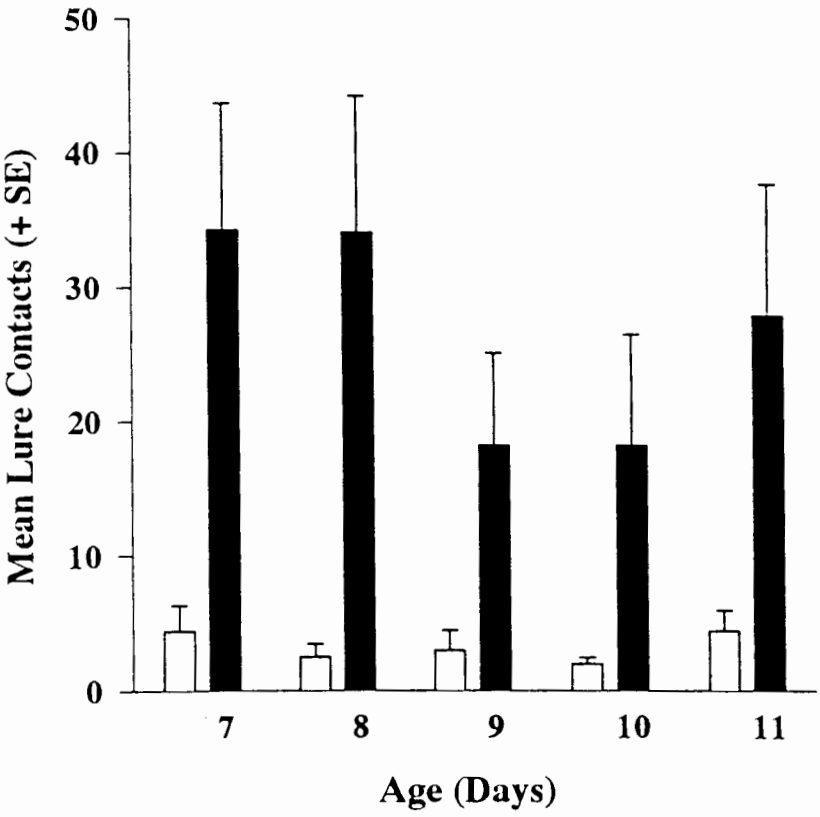


Figure 2. Age-dependent response of ■ high (N=5), and □ low (N=5) responding colonies. The high responding colonies contacted the lure significantly more than the low responding colonies ($P<0.05$).

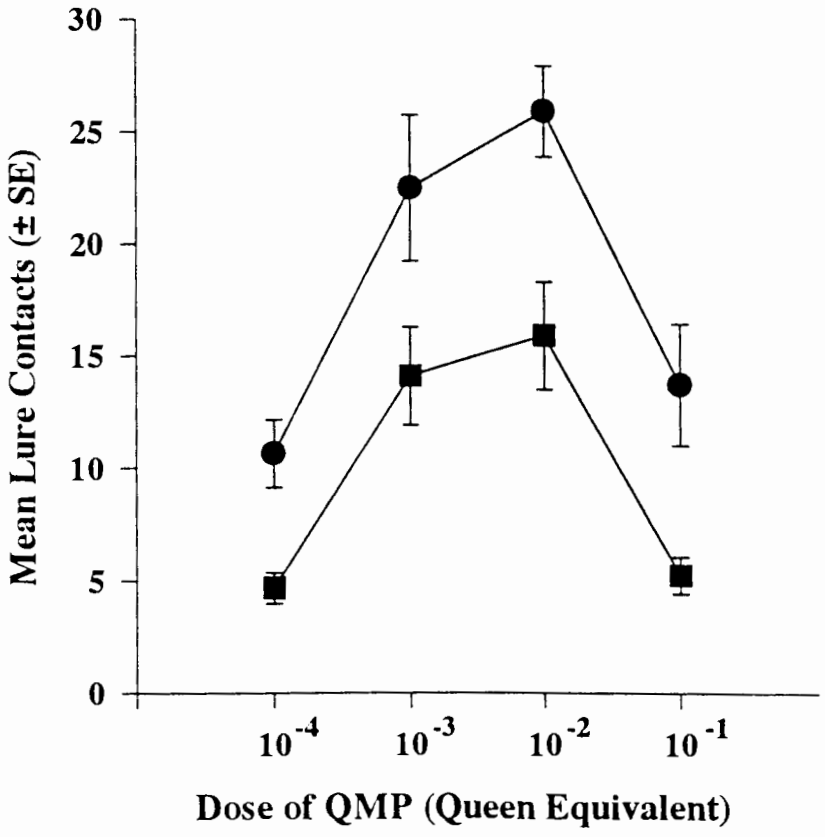


Figure 3. Dose response of —●— high (N=5), and —■— low (N=6) responding colonies to various doses of QMP. High responding colonies contacted the lure significantly more than low responding colonies ($P < 0.05$).

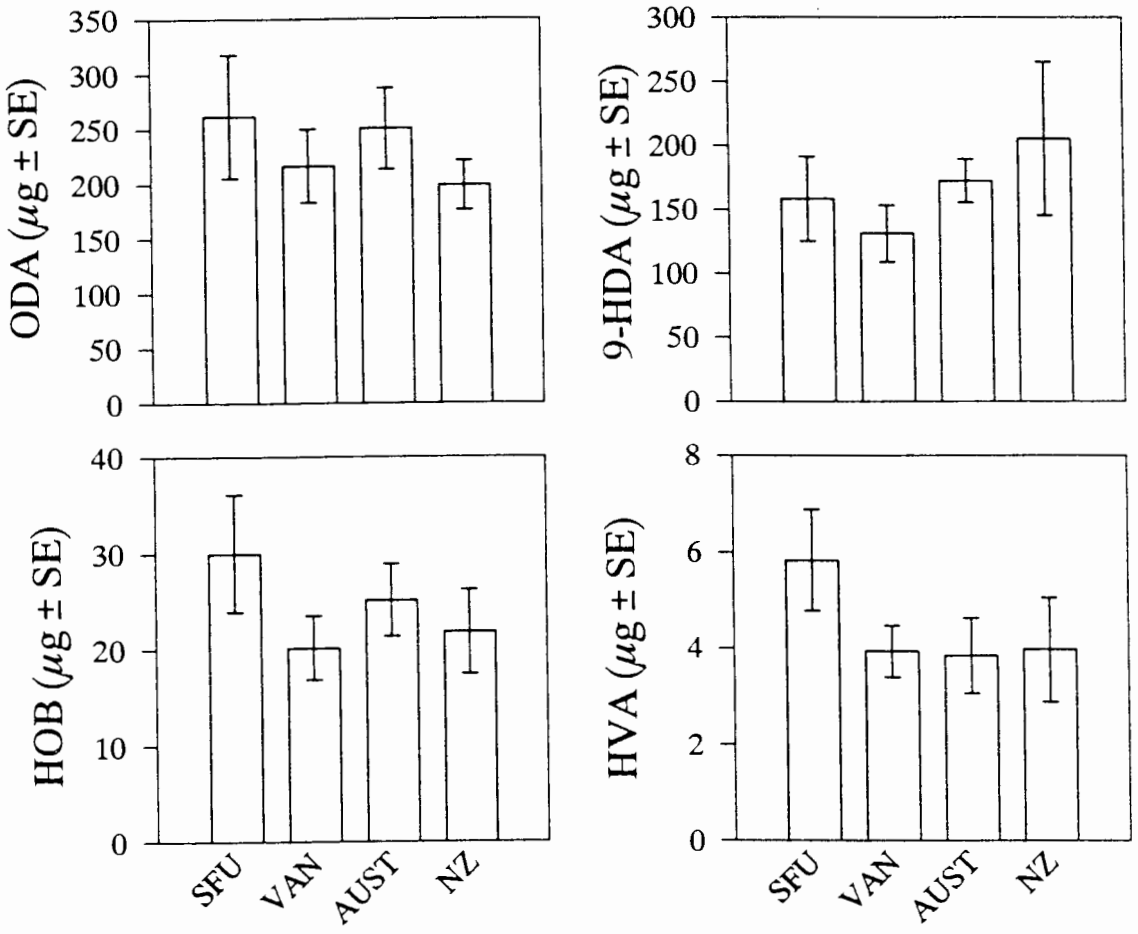


Figure 4. Quantities of mandibular gland pheromone of queens from different sources. Relatedness among queens within sources was 0.75. SFU (N=5), VAN (N=8), AUST (N=11), and NZ (N=8). There were no statistically significant differences between sources for any of the gland components ($P > 0.05$).

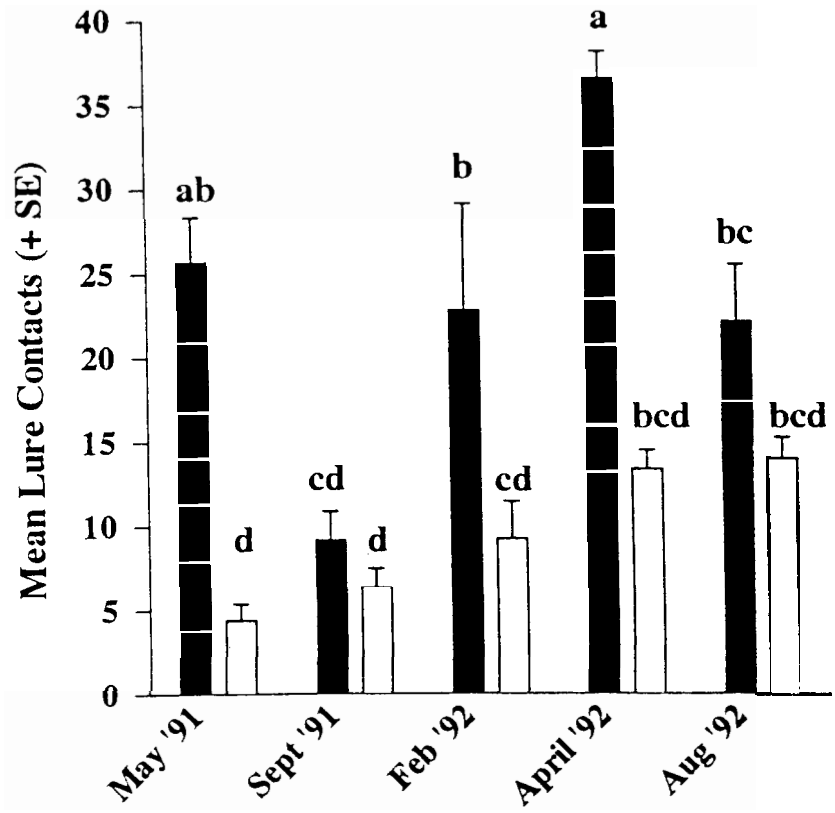


Figure 5. Annual retinue response of ■ high (N=5) and □ low (N=5) responding colonies. Bars with different letters were statistically different ($P < 0.05$).

DISCUSSION

Workers from colonies headed by queens from different sources represented a normal distribution of response to QMP varying from very high to exceptionally low, i.e. not different from the solvent control. The wide range in responses to pheromones by bees within and among colonies needs to be considered when worker bees are being used to determine the most attractive pheromone blend. Colonies headed by AUST queens responded similarly to QMP and solvent, demonstrating that some honey bee stocks may be particularly inferior for use in pheromone bioassays. Statistically significant differences between NZ and AUST queen sources in the retinue bioassay suggest a genetic basis for response. Genetically based differences in pheromone reception or response may have significant consequences for colony functioning.

The cross-fostering experiment indicated that the colony environment of a high or low responding colony did not influence the retinue bioassay response of workers originating from high or low responding colonies, further supporting a strong genetic component to this behavior. Although olfactory conditioning in the critical phase of olfactory development during the first four days after emergence influences response for the rest of the worker's life (Masson and Arnold, 1984), high and low responding workers fostered in opposite environments did not respond differently from workers reared in maternal environments. It may be that worker response is "set" during olfactory neuro-ontogeny as a result of pheromonal environment or some other environmental factor. Antennal sensory bundles develop eight days before emergence, and reach the appearance of adult antennal lobes three days before emergence (Masson and Arnold, 1984). It is not known whether all glomerular

synapses are stabilized morphologically and functionally three days before emergence, or whether olfactory ontogeny and maturation continue four to eight days following emergence, a time when E.A.G. responses are highest in an adult bee's lifetime (Masson and Arnold, 1984). If the pre-emergence environment influences worker response to QMP then response may be "set" before emergence. However, if environment acts to "set" worker response to QMP as high or low after emergence then the cross-fostering experiment is evidence against pheromonal environment influencing worker response, and that genetic factors influence response.

There were no age-dependent differences in QMP response in the 7-11 day old worker bees from high and low responding colonies, which is consistent with the previous finding that the peripheral olfactory system is completely developed four days after emergence (Pham-Delègue *et al.*, 1991). Similarily, worker antennal responses to ODA remained the same in bees 1-60 days after emergence (Allan *et al.* 1987). Topically applied juvenile hormone analogue reduces the response threshold to alarm pheromone, but does not affect the antennal response (Robinson, 1987). Although high or low response in the retinue bioassay has not yet been correlated with colony functioning, any QMP-induced effect should be expressed with greater intensity in high QMP responding colonies.

Throughout the year, except in late summer, high responding colonies contacted QMP lures more frequently than low responding colonies. The low responding colonies were least responsive at all dates tested. Decrease of response to QMP late in the summer by high responding bees is not well understood. Young worker bees perform intra-colony duties such as queen attending and food handling, while older bees are engaged in extra-colony duties such as ventilating, guarding, and finally foraging. Ontogeny of division of labor may be

altered by perturbations in colony age demography and resource availability by adjustments in the proportions of individuals occupied in assorted tasks (Robinson, 1992; Winston, 1987). This plasticity of age-dependent behavior may be regulated on a hormonal and genetic level (Hildebrandt and Kaatz, 1990; Robinson *et al.*, 1989; Robinson *et al.* in press). For example, high titers of juvenile hormone (JH), and topical application of JH to workers are associated with a shift from intra- to extra-colony duties (Robinson, 1987a). It is conceivable that low responses in late summer delay foraging, thereby increasing the number of workers in colonies prior to winter.

Differences in QMP lure contacts by high and low responding colonies was apparent at all doses tested (Fig. 3). Levels of QMP production and worker response were not correlated (Fig. 4), confirming that production and response to QMP are not inter-related. Similar pheromone quantity in queens from different sources suggests that pheromone production is not strongly influenced by genotype. Although, the amount of ODA in glands (Naumann *et al.*, 1991; Slessor *et al.*, 1990), and on the cuticle (Pain and Roger, 1978), can vary greatly between queens, these results suggest that this variation probably does not have a strong genetic basis. Crewe (1982) reported differences in the proportion of mandibular gland components between *Apis mellifera adansonii*, *A. m. mellifera*, and *A. m. capensis*. However, discriminant analysis of *Apis m. mellifera* mandibular gland components indicated that these were not dependable predictor variables for grouping inbred lines. No statistically significant differences have been found in the amount of mandibular components between Africanized, European, and selected high and low pollen hoarding strains of mated queens (Chapter 6). Thus, workers may be more important than the queen in creating the colony's pheromone environment. Indeed workers remove pheromone from the queen's body and

distribute it throughout the nest, thereby determining the amount of pheromone held by the queen and circulated through the colony (Naumann, *et al.*, 1991; 1992; 1993).

The implications of high and low responding workers for colony functioning remain obscure. In an observation hive experiment containing same age cohorts of high and low responding workers, neither high nor low responding cohorts attended the queen more frequently, or differently, suggesting that QMP alone is not responsible for inducing retinue behavior (Chapter 3). Queen mandibular gland pheromone is only one of at least two pheromones (the second as yet unidentified, Slessor and Foster, unpublished observations) that attract workers to the queen, and it may be that QMP plus other queen pheromones are necessary to elicit retinue behavior in low responding workers. The laboratory QMP retinue bioassay exposes workers to a single pheromonal stimulus, and thus reveals the importance of QMP alone for attracting high QMP responding workers. Although low responding workers are not as attracted to QMP as high responding workers, low responding workers may be attracted to another pheromone and subsequently gain as much exposure to QMP as high QMP responding workers. Attraction to QMP alone may or may not confer expression of pheromone-mediated behaviors such as foraging, queen cell building, swarming, and attraction to swarm clusters.

Perhaps low QMP responding workers are demonstrating a type of queen-worker conflict. Honey bee queens show almost no physical dominance over worker bees, but instead have evolved a pheromone-based dominance system. Workers may have evolved mechanisms to bypass queen pheromone control, and this is countered by queens evolving multi-component or additional attractive pheromones to maintain pheromonal control. The full laboratory retinue response is elicited by a blend of 5 compounds found in the

mandibular glands of mated queens. Even the most minor compounds are necessary to attract workers, but worker phenotypes successful at ignoring QMP may be drawn to the queen by other queen odors, thereby exposing the worker to all of the queen pheromones.

CHAPTER 2

COLONY-LEVEL SELECTION FOR WORKER HONEY BEE (*Apis mellifera* L.) HIGH AND LOW RETINUE RESPONSE TO QUEEN MANDIBULAR PHEROMONE.*

ABSTRACT

Colony-level retinue response to queen mandibular gland pheromone (QMP), in which worker bees attend their queen, was normally distributed in a population of 43 domestic colonies. Two-way selection for worker honey bee response to QMP resulted in the production of high and low QMP responding strains. Strains differed in response to QMP after one generation of selection, and by the third generation worker response from high strain colonies was 18.5 times higher than the low strain colonies. Low strain retinue responses to QMP were not significantly different ($P > 0.05$) than responses to solvent controls. Strain response was not related to queen pheromone production, rearing environment, worker age (Chapter 1), or an artifact of the synthetic blend of the pheromone.

* Pankiw, T., and M.K. Fondrk. Colony-level selection for worker honey bee (*Apis mellifera* L.) high and low retinue response to queen mandibular pheromone. (to be submitted)

INTRODUCTION

The behavioral phenotype of a honey bee (*Apis mellifera* L.) colony is determined by the intensity and frequency at which individuals respond to stimuli and perform tasks. Honey bee behavior is the product of genetic potential, ecological and physiological environments, the social conditions of the colony and various prior and ongoing interactions among these four. Additional complexity is introduced because honey bee behavior has been configured by natural selection in a social context, where sterile female workers do not directly transmit their genes to the next generation (reviewed by Winston, 1987).

Many behaviors have been shown to have a genetic component (reviewed in Chapter 1). Pheromone-mediated behaviors known to have a genetic basis in honey bees include alarm response to isopentyl acetate (reviewed in Chapter 1). Responses to pheromone also have been shown to be heritable among several non-social insects (reviewed in Chapter 1). Colony-level variability for QMP retinue response suggested that this trait may be heritable (Kaminski *et al.*, 1990; Chapter 1). The purpose of this study was to conduct a two-way selection program to produce workers with high and low retinue responses to QMP. These workers were used for subsequent studies to determine the relationship between a quantifiable releaser response to QMP, live queen retinue response, and two important QMP-based primer responses, foraging ontogeny and queen rearing behavior.

MATERIALS AND METHODS

Retinue response to QMP was quantified as described in Chapter 1. Controls consisted of the same protocol except the lure was spotted with 10 μl of methanol or 2-propanol.

A population of 43 Simon Fraser University (SFU) colonies were assayed in June 1990 for QMP retinue response to determine response variability. These colonies were headed by naturally mated queens derived from a variety of commercial bee breeders from British Columbia, Canada. The colonies had been managed for honey production and were not part of any other experimental program.

Additional queens were purchased from four different sources to provide us with a diverse sample of genotypes to initiate our two-way selection program; 1) Babe's Honey, Victoria B.C., Canada, (N=20) 2) Aussie Apiaries, New South Wales, Australia, (N=20), 3) Barden Apiaries, New South Wales, Australia, (N=20), and 4) Whiteline Queens, New Zealand (N=20). SFU stock queens (N=20) also were reared for inclusion in the initial evaluations.

Initial selection was established by colony-level performance in the QMP retinue bioassay based upon 5 bioassay replicates per colony. The highest and lowest performing colonies from each of the four sources were selected to provide a genetically diverse population to initiate the breeding program. Colony-level response based on 10 bioassay replicates was determined immediately prior to rearing virgin queens and drones. The Barden subline was eliminated from the selection program due to difficulties in maintaining

fecund queens, so the two-way selection was reduced to 3 sublines per strain. Six selected high and six low QMP-retinue responding colonies were designated H1-H6, and L1-L6 (Fig 6a). Virgin queens were raised using standard methods (Laidlaw, 1979), and instrumentally inseminated with the semen from one drone, a single drone for each queen (Laidlaw, 1977).

Three maternal sublines were maintained within the high and low strain populations throughout the selection program. Sixteen to 18 daughter queens were produced from each subline, each generation. The highest and lowest performing daughter colony of each subline was selected to produce virgin queens and drones for the next generation (Fig. 6b). From each generation 63-79 surviving colonies were bioassayed. Crosses were made between sublines to minimize inbreeding, and between- subline crosses were rotated each generation (Fig. 6b).

Ten high and 10 low strain colonies were tested for response to the whole gland extract, synthetic QMP, and control (methanol or 2-propanol) to determine whether our selection was based on an artifact of the synthetic blend or retinue response to queen mandibular gland pheromone. Whole gland extracts were prepared from excised glands of 3 laying queens. The glands were macerated and extracted twice in HPLC-grade methanol to give a combined total extract of 100 μl per queen. A portion of this extract was diluted to yield a 10^{-3} Qeq in 10 μl.

Selective breeding affects the population distribution of the trait selected, and the retinue response data did not meet assumptions of a normally distributed population. Therefore, the nonparametric Wilcoxon Rank-Sum test was used for statistical analyses (Snedecor and Cochran, 1980). A univariate procedure was used to determine whether the

Figure 6a). Initial crosses of high strain sublines producing the first generation. The low strain crosses were identical, using selected low responding sources.

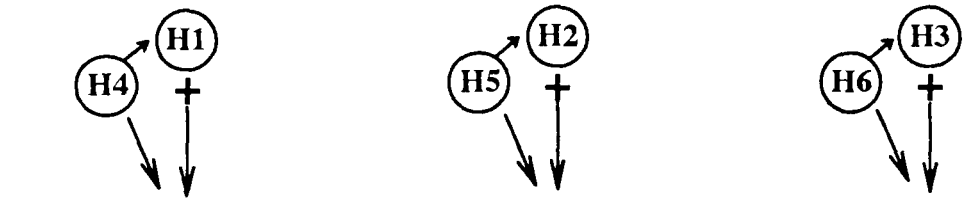
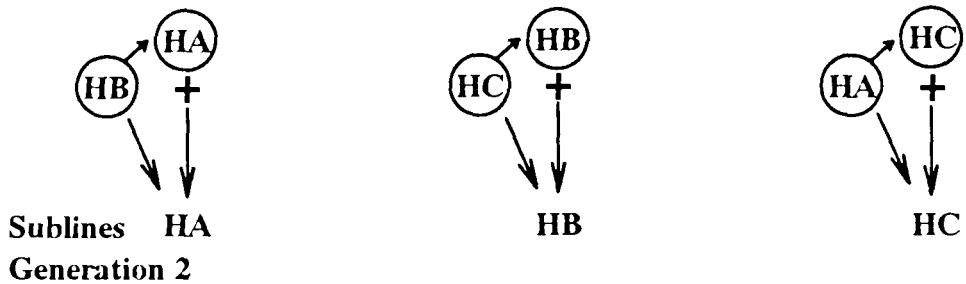


Figure 6b). Mating rotation of the high strain with three sublines. The rotation scheme was the same for the low strain.



QMP retinue responses of the 43 SFU stock colonies were normally distributed (Snedecor and Cochran, 1980).

RESULTS

The 43 SFU stock colonies selected for evaluation had an average retinue response to QMP of $X = 11 \pm 10.3$ (standard error). Responses were normally distributed (Univariate, $P > 0.8$, Fig. 7) (Snedecor and Cochran, 1980). QMP retinue response in the laboratory bioassay was significantly different in each generation ($P<0.05$) (Fig. 8). High strain colonies by generation were; 1) 8.25, 2) 17.5, and 3) 18.5 times as responsive as low strain colonies (Fig. 8). Retinue bioassay responses to control solvents methanol or 2-propanol by generation were; 1) 3.3 ± 0.7, 2) 3.7 ± 0.5, and 3) 3.1 ± 0.3. Generation 1-3 control and low strain responses were not significantly different ($P > 0.05$).

Within-strain responses to synthetic QMP and gland extracts were not significantly different ($P > 0.05$) although the high strains were significantly different from low strain and control colonies ($P < 0.05$, Fig. 9).

DISCUSSION

Substantial levels of variability existed in the initial population from which the strains were derived, contributing to the rapid response to colony-level selection. The statistically similar within-strain responses to synthetic QMP and gland extract demonstrated that

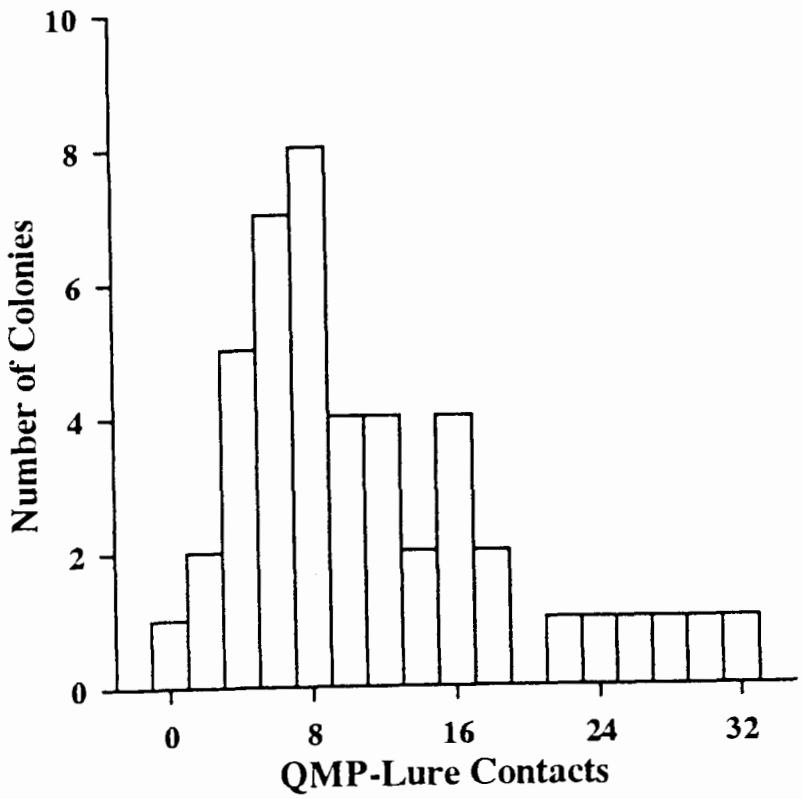


Figure 7. Frequency distribution of retinue responses of 43 SFU colonies.

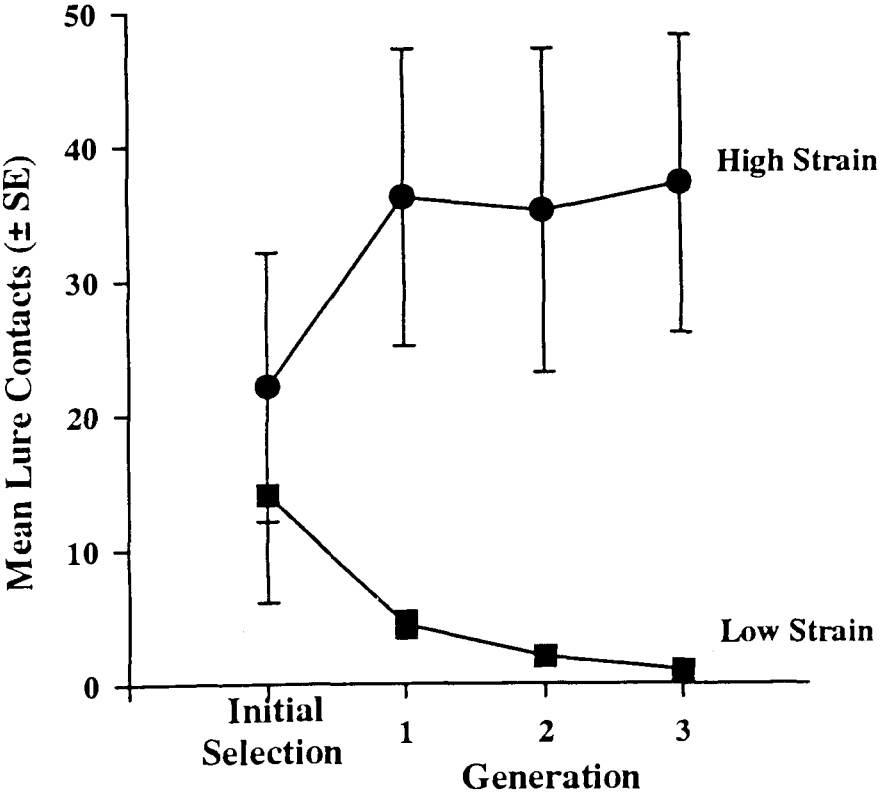


Figure 8. Colony level selection for worker retinue response to QMP from the initial selection to the third generation. Initial selection high (N=8), and low (N=8) colonies. Generation 1: High N=30, Low N=33; Generation 2: High N=38, Low N=24; Generation 3: High N=40, Low N=35. Invisible error bars are less than the symbol size.

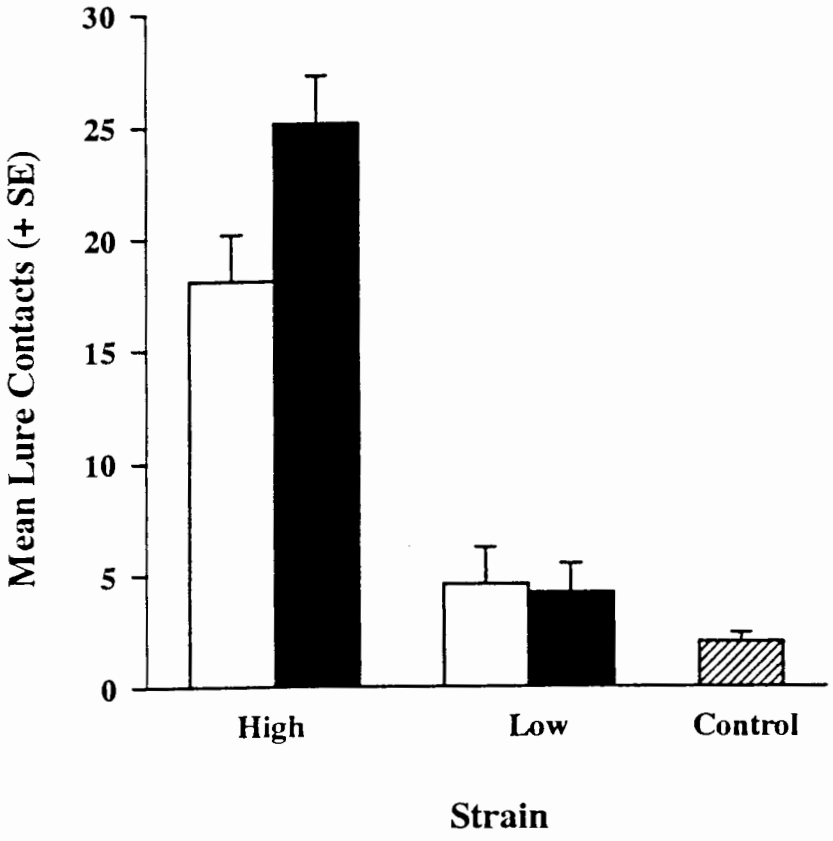


Figure 9. High- (N=10) and low-strain (N=10) responses to 10^{-3} Qeq of ■ QMP, and □ gland extract. There was no significant ($P > 0.05$) within strain difference in in response to either pheromone source. High- and low-strain differences were maintained ($P < 0.05$). Low-strain and ▨ control (N=10) responses were not significantly different ($P > 0.05$).

selection response was not an artifact of the synthetic blend. We did not vary the synthetic blend to test the possibility of differential response by high and low strains to particular components. However, it is unlikely that the differential response between high and low strains would change with different component blend proportions, because queens produce a wide range of component quantities (Chapter 3). Gland component comparisons between European and Africanized mated queens revealed differences in quantities and proportions of QMP components in these two races (Chapter 3), but queens of either race may head colonies composed of workers of both races (Winston, 1992). Thus, it appears that workers will accept racially different queen signals. Further, a European-like synthetic blend of QMP is equally effective at preventing queen rearing in colonies of either race (Pettis *et al.*, 1995a). This, coupled with the maintenance of strain-dependent differences over a wide range of QMP dosages (Chapter 1), suggests that strain-dependent differences over a range of blends are likely.

It is possible, although unlikely, that differences in QMP retinue response occurred between strains due to chance alone. The retinue responses in the laboratory bioassay could have occurred as a result of random fixation of a genetically-variable trait in the small breeding population (3 sublines per strain), within each strain (Falconer, 1981). Fixation is the attainment of homozygosity in a population which thereby becomes monomorphic with respect to a given allele, or phenotypic effect (Lincoln *et al.*, 1990). It would be necessary to repeat the selection program using a larger breeding population with more than three sublines per strain to exclude this possibility.

Retinue behavior is strictly a social behavior. A lone worker with a queen or QMP

will not engage in retinue behavior (personal observation), in contrast to other worker bee behaviors having a strong genetic component. For example, defensive behavior (Boch and Rothenbuler, 1974; Breed and Rogers, 1991; Collins, 1979; Collins *et al.*, 1982, 1984, 1987, 1988; Guzmán-Nova and Page, 1993), high and low foraging and pollen hoarding (Calderone and Page, 1988; Danka *et al,*. 1987; Frumhoff and Baker, 1988; Hellmich *et al,*. 1985; Page and Robinson, 1991; Pesante *et al.*, 1987a, b; Rinderer *et al.*, 1985; Robinson, 1992; Robinson and Page, 1989), and hive cleaning (Robinson and Page, 1988; Rothenbuler, 1964a, b) phenotypes are determined by the intensity and frequency at which individuals perform these tasks. The only genetically based colony-level behaviors that are performed in a strictly social context (meaning that solitary individuals don't perform these behaviors) include the tendency to swarm (Winston, 1980) and retinue response to QMP. Queen rearing was the only strain-dependent behavior observed based on high and low responses (Chapter 5). Since QMP influences the number of queen cells reared and the likelihood of swarming (Winston *et al.*, 1989, 1990, 1991; Pettis *et al.*, 1995), these two colony-level behaviors may be influenced by a strain-dependent effect, which would be an interesting subject for further study.

The high and low strain populations responded rapidly to colony-level selection, expressing the wide range of genetic variability in the initial population from which the strains originated. The adaptive significance and factors maintaining variability for this trait are unknown. Genetic variability is essential for evolution by natural selection. The ecological success of honey bees is often attributed to its behavioral flexibility (Robinson, 1992), maintained by polyandry which maximizes worker genotypes within colonies (Crozier

and Page, 1985). Although the colony-level functioning of high and low strain workers has not been fully elucidated, it appears that the maintenance of variation in pheromone responses may be an important aspect of colony fitness, and deserves additional study.

CHAPTER 3

MANDIBULAR GLAND COMPONENTS OF EUROPEAN AND AFRICANIZED HONEY BEE QUEENS*

ABSTRACT

Composition of the five-component honey bee queen mandibular gland pheromone of mated European honey bee queens was compared to those of virgin and drone laying European queens and Africanized mated queens. QMP of mated European queens showed significantly greater quantities of individual components than all queen types compared, except for a significantly greater quantity of 9-HDA found in Africanized queens. Glands of European drone-laying queens contained quantities intermediate between virgin and mated queens, reflecting their intermediate reproductive state and age. QMP ontogeny shifted from a high proportion of ODA in young unmated queens to roughly equal proportions of ODA and 9-HDA in mated queens. A biosynthetic shift occurs after mating that results in a greater proportion of 9-HDA, HOB, and HVA production, accompanied by a decreased proportion of ODA. Africanized QMP proportions of ODA and 9-HDA were significantly different from European queens. QMP components from the body surfaces of European and Africanized mated and virgin queens were highly variable and not correlated to quantities found in individual glands. Body surface QMP was approximately 10^{-3} of that found in glands. A quantitative definition of a "queen equivalent" of QMP was proposed for the various queen types, and a standard queen equivalent for mated European honey bee queen mandibular gland pheromone is suggested as 200 μg ODA, 80 μg 9-HDA, 20 μg HOB, and 2 μg HVA.

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* Pankiw, T., Plettner, E., Pettis, J.S, Winston, M.L, Slessor, K.N., and Taylor, O.R. *in press.* Mandibular gland components of European and Africanized honey bee queens (*Apis mellifera* L.). Journal of Chemical Ecology. (**Note:** Bodywash data are unpublished.)

INTRODUCTION

One of the most interesting aspects of honey bee biology is the variability found within and between races of *Apis mellifera*. Bees vary in behavioral, morphological and physiological characteristics. Much of the world-wide success of this species is attributed to the variation among colony members, providing adaptive advantages in different habitats (Seeley, 1985; Winston, 1987, 1992). A great deal is known about the phenotypic and genotypic variability among worker bees within colonies, but the variability among queen honey bees is not as widely studied. Here we present a detailed characterization and comparison of inter- and intra-racial variation of queen mandibular gland pheromone.

QMP is a blend of the three abundant aliphatic acids, and two aromatic compounds (Slessor *et al.* 1988). As a primer pheromone, QMP has been shown to delay swarming (Winston *et al.*, 1991), suppress queen rearing (Pettis *et al.*, 1995; Winston *et al.*, 1989, 1990), inhibit worker juvenile hormone (JH) biosynthesis in the laboratory (Gast, 1967; Hildebrandt and Kaatz, 1990; Kaatz *et al.*, 1992), and regulate foraging age of adult worker bees and suppress JH titers in colonies (Chapter 6). QMP also has some releaser pheromone functions, including retinue formation around the queen (Slessor *et al.*, 1988; Kaminski *et al.*, 1990), attraction of workers to swarms (Winston *et al.*, 1989), stimulation of pollen foraging (Higo *et al.*, 1992), calming of queenless workers (Naumann *et al.*, 1990), and attraction of foragers to crops (Currie *et al.*, 1992a, b).

In this study we extracted QMP from the glands of various European and Africanized queen types and compared the quantities and ratios of individual components. The ontogenetic development of the QMP signal also is presented, and a definition of one queen

equivalent is proposed that reflects racial and reproductive differences, while allowing for the natural variation found among individuals.

The only previous descriptions of the 5-component blend of QMP in *Apis mellifera* L. have been done by Slessor *et al.*, (1988, 1990). The study presented here analyses a greater number and range of queen types, and permits a more complete description of QMP in *Apis mellifera*. The queen types examined here include European mated, virgin, and drone-laying queens, representing various female honey bee reproductive types, and mated Africanized queens. Africanized bees are derived from South African bees imported to South America. The rapid expansion of Africanized honey bees throughout South and Central America can be attributed to their propensity to higher swarming rates with smaller swarm sizes (Winston *et al.*, 1983). Since QMP is important to colony cohesion and plays a role in the timing of swarming both in European and Africanized honey bees (Pettis *et al.*, 1995b; Winston *et al.*, 1991), QMP differences may influence variation in swarming behavior between these races.

MATERIALS AND METHODS

Queen Collection and Mandibular Gland Component Analysis

Queens were immediately placed on dry ice upon collection, then into labelled Eppendorf® tubes, and stored at -70°C prior to dissection, except where stated otherwise.

Mandibular glands were extirpated, then extracted in 2 X 50 μl of methanol containing 10-undecenoic acid (0.22 mg/ml) as an internal standard. Two μl aliquots of the

extracts were derivatized with BSTFA (Slessor *et al.,* 1990), diluted with hexane and analysed by splitless capillary gas chromatography on an HP5890 gas chromatograph equipped with a flame ionization detector (FID), and a 30 m DB-1 fused-silica capillary column. The FID was calibrated with standards of known concentration, and these FID responses were used to calculate the amount of each component in the mandibular gland extracts.

Subsamples of Africanized, mated and virgin European queens were washed with 10 portions of 100 μl of methanol to determine the amount of QMP on their bodies.

European Mated Queens

Mandibular gland extract data from 125 one and two year old mated queens of Simon Fraser University stock most similar to *Apis mellifera ligustica* were combined to establish a baseline mated queen mandibular gland component profile. Data from 10 queens have not been reported previously, and the remainder are from Slessor *et al.,* 1990, Pettis *et al.,* 1995b, and Chapter 1.

Virgin Queens

Ten virgin queens of Simon Fraser University stock most similar to *Apis mellifera ligustica* were reared until two weeks of age then collected in July and August, 1992. Mandibular gland component analysis results from these queens were combined with 7

queens from Slessor *et al.,* 1990 for a total of 17 queens.

European Drone-Laying Queens

Twenty-two queens of mixed stock most similar to *Apis mellifera ligustica* were narcotized two times with CO_2 7 days after emergence, and inseminated with insect saline solution to induce haploid male egg laying, then collected from 5-frame nucleus colonies at eight weeks of age on 21 and 22 August, 1991, as above. Queens were dissected from 20 May to 6 June, 1992.

Africanized Mated Queens

Twenty mated Africanized queens were obtained from Mexico in 1992. Morphometric (Daly and Balling, 1978) analysis of the progeny confirmed the racial status of these queens. Queens were transported on dry ice by overnight courier from Mexico and received 3 March, 1992. The queens were stored at -70 °C prior to dissection from 22 to 29 June, 1992.

Proportions and Ratios of QMP

To develop an ontological profile of queen mandibular gland components, the results reported in Slessor *et al.,* 1990, Pettis *et al.,* 1995a, Chapter 1, and this study were combined.

Ratios of HVA, HOB, and HVA to ODA were calculated to develop a definitive formula of a QMP queen equivalent.

Statistical Analysis

Analysis for statistical differences between European mated and all other queen types were performed using one-tailed T-test, the Satterthwaite method for unequal variances (Snedecor and Cochran, 1980). Analysis for statistical differences between European mated and all other queen types for proportion and ratio data were by the Kruskal-Wallis test (Conover, 1980). SAS® was used for all statistical analyses (SAS Institute Inc. Release 6.04, 1988). The level of significance throughout was at the 0.05 level, unless stated otherwise.

RESULTS

Quantities

Glands of European mated queens contained statistically greater quantities of all QMP components compared to virgin, drone-laying and Africanized queens (Fig. 10), except for a statistically similar quantity of 9-HDA in Africanized and European mated queens (Fig. 10).

Body wash results of HVA were below detectable levels and therefore not available. ODA quantities washed from the bodies of mated European and Africanized queens were statistically similar ($P > 0.22$) (Fig 11a). Significantly lower quantities were washed from

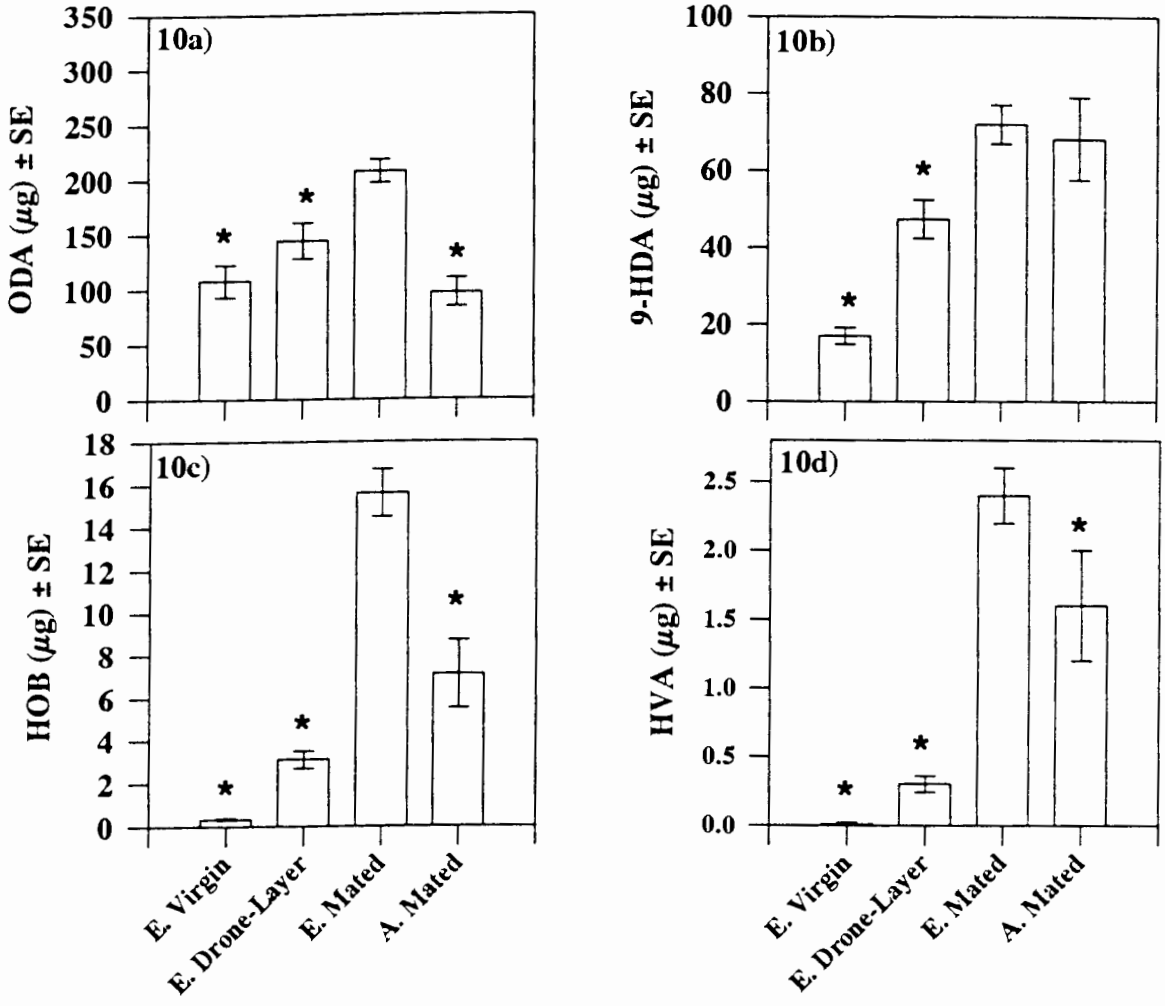


Figure 10. Whole gland quantities of individual queen mandibular pheromone components. A * indicates statistically significant differences from the mated European (**E**) queen mean at the 0.05 level. **A** signifies Africanized queens.

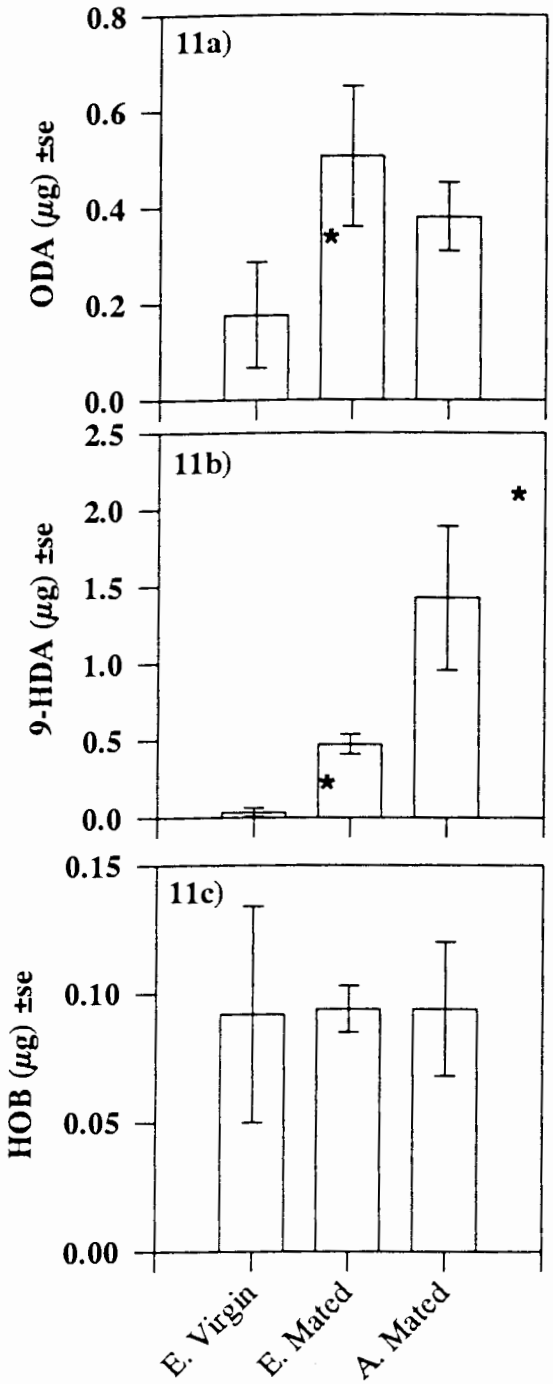


Figure 11. Quantities of QMP extracted from the body surfaces of queens. An 'E' indicates European queens, an 'A' Africanized queens, and a * indicates significant differences from the mated European queen mean at the 0.05 level.

virgin queen bodies than European mated queens ($P < 0.05$). Significantly greater ($P < 0.03$) quantities of HDA were washed from Africanized queens compared to mated European queens, and significantly lower ($P < 0.0001$) quantities from virgin queens (Fig 11b). The quantities of HOB washed from all queens were similar (Fig 11c).

Proportions and Ratios of QMP

Queen mandibular gland biosynthetic capabilities appears to be ontogenetic (Fig. 12); an examination of individual QMP component proportions to the total amount of QMP reveals a reproductive shift in the pheromonal "bouquet" (Fig. 12). Virgin queen glands contained high proportions of ODA, and lower 9-HDA proportions. Drone-laying queen ODA and 9-HDA proportions were intermediate between virgin and mated queens. Mated queen glands contained significantly greater proportions of 9-HDA compared to virgin queens (Fig. 12). Africanized queen ODA proportions were significantly lower, and 9-HDA higher, compared to mated European queens.

The aromatic component (HOB and HVA) proportions changed significantly with reproductive status (Fig. 12). Virgin and drone-laying queen glands contained significantly lower proportions of the aromatic components. Africanized queen gland aromatic proportions were similar to mated European queens (Fig. 12).

The ratios of 9-HDA, HOB, and HVA to ODA in the glands and bodywashes for the various queens are summarized in Table 2. The gland ratio of 9-HDA/ODA was significantly higher in Africanized than European mated queens, and significantly lower in

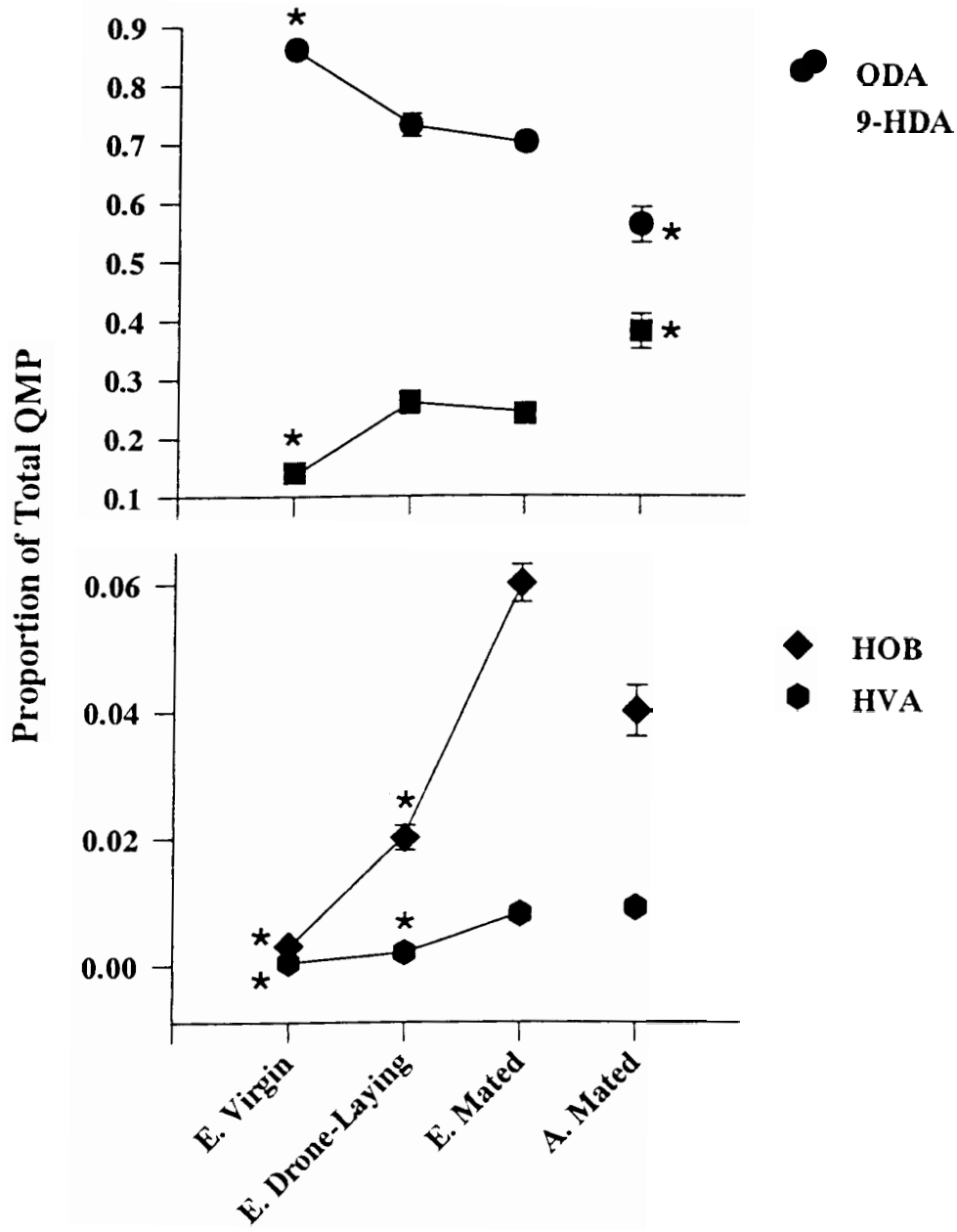


Figure 12. Ontogenetic and reproductive profile of QMP extracted from glands. A * indicates a statistically significant difference from the mated European queen at the 0.05 level. An **E** indicates European queens, and an **A** Africanized queens. Invisible standard error bars are less than the size of the symbol.

the virgin queens. The virgin and drone-laying queen glands contained significantly lower ratios of HOB/ODA, and HVA/ODA compared to the European queens, whereas Africanized queen glands contained similar ratios. In every case the bodywash ratio is significantly greater than that found in the gland (Table 2), except for 9-HDA/ODA in the virgin European queens.

Table 3 demonstrates examples of calculated queen equivalents of glandular QMP based on the mean ODA values from Fig. 10a.

Table 2. Mean ratios of QMP components to ODA for various queen types.

Queen Type	Source	Ratios		
		9-HDA/ODA	HOB/ODA	HVA/ODA
European Mated	Gland	0.40	0.09	0.01
	Bodywash	1.80*	0.40*	-
Africanized Mated	Gland	0.75¤	0.07	0.02
	Bodywash	4.70*	0.30*	-
European Drone-Laying	Gland	0.40	0.03¤	0.002¤
European Virgin	Gland	0.20¤	0.004¤	0.0004¤
	Bodywash	0.10¤	1.30*	-

¤ Indicates significant (P<0.0002) difference from mated European queen gland or bodywash.
* Indicates significant (P<0.0002) difference from gland and bodywash ration for individual queen type.

Table 3. Calculated Values for One Queen Equivalent of QMP.

Queen Type	Queen Equivalent QMP Components (μg)*			
	Mean ODA	Calculated 9-HDA	Calculated HOB	Calculated HVA
European Mated	208 (200)	83 (80)	19 (20)	2 (2)
Africanized Mated	97 (100)	39 (40)	7 (10)	2 (2)
European Drone-Layer	145 (145)	58 (60)	4 (5)	0.4 (1)
European Virgin	108 (100)	22 (20)	0.8 (1)	0.7 (1)

* Values in parentheses are proposed "standard" queen equivalent values.

Frequency Distributions

Frequency distributions of QMP component quantities and proportions of the mated European queens (Figs. 13 and 14) indicated a wide range of variability in the composition of this pheromone.

DISCUSSION

We define one queen equivalent (Qeq) based on the mean ratios of 9-HDA, HOB, and HVA quantities to ODA for the various queen types (Tables 2 and 3). A standard queen equivalent for the mated European queens, derived by rounding-off from values in Table 3 is: 200 μg ODA, 80 μg 9-HDA, 20 μg HOB, and 2 μg HVA. Proportions of the total amount of QMP also may be used to achieve similar queen equivalent values. The primary purpose for proposing a queen equivalent definition is to reflect racial and reproductive differences

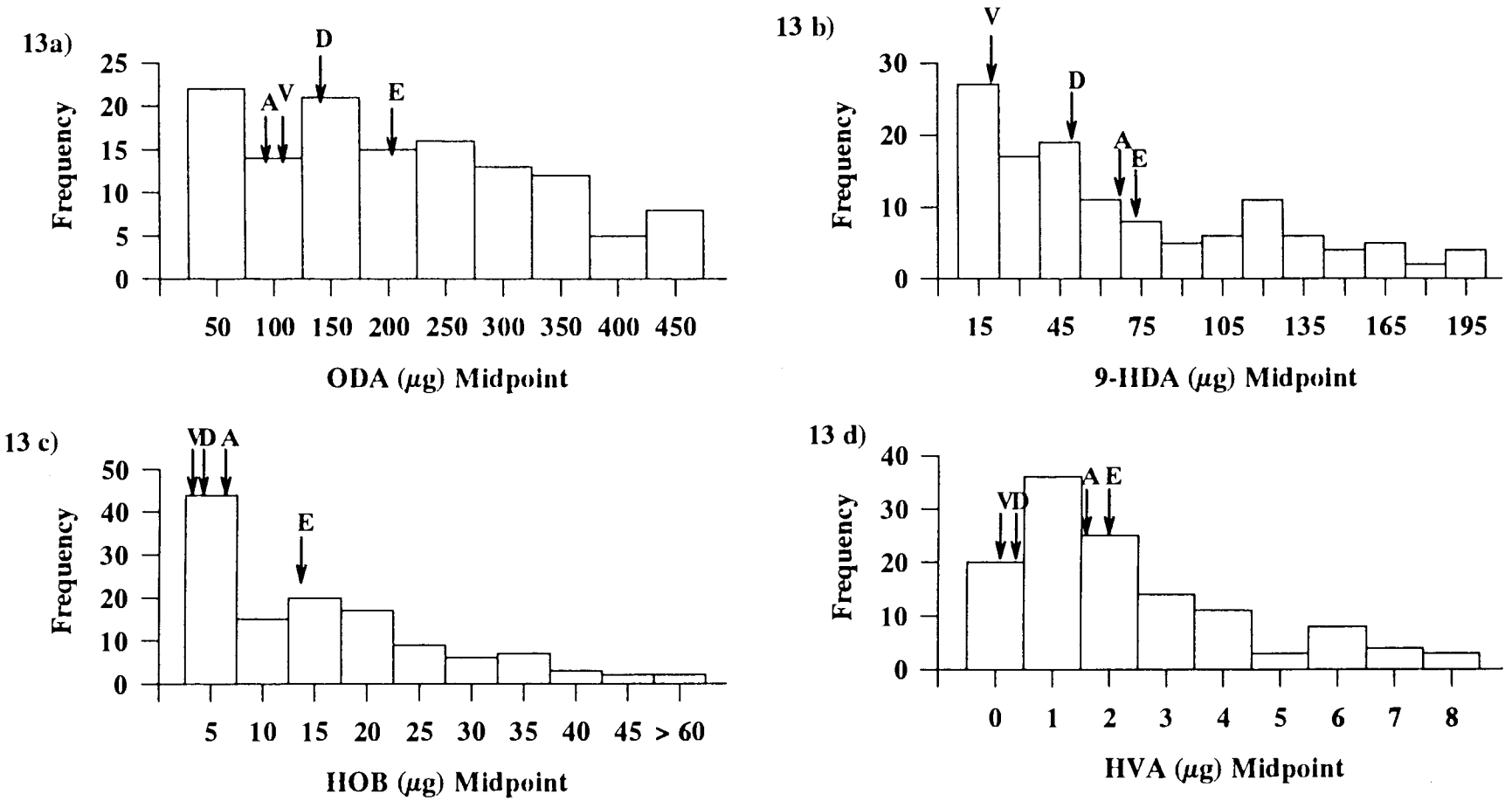


Figure 13. Frequency distribution histograms of QMP component quantities of 125 mated European queens. Mean quantities for the queens are indicated by arrows and; **A** = Africanized mated queens; **D** = European drone-laying queens; **E** = European mated queens; **V** = European virgin queens.

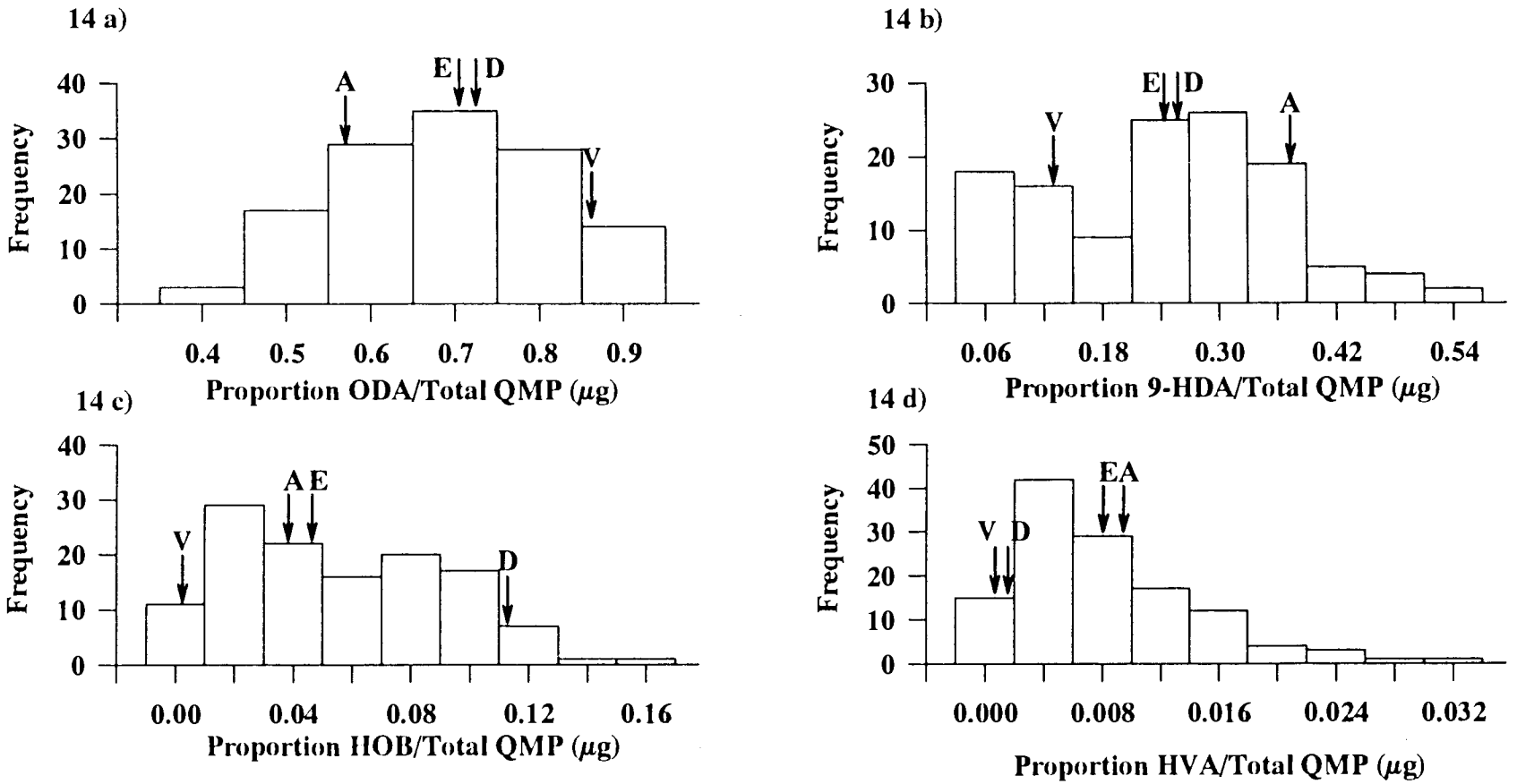


Figure 14. Frequency distribution histograms for proportions of QMP components for 125 mated European queens. Mean proportions for the queens are indicated by arrows and; **A** = Africanized mated queens; **D** = European drone-laying queens; **E** = European mated queens; **V** = European virgin queens.

while incorporating the variation found in nature. Previously proposed definitions of one queen equivalent used for synthetic formulations were: 1) 150 μg ODA, 55 μg 9-HDA, 13 μg HOB, and 1.5 μg HVA (Slessor *et al.,* 1988), and 2) 250 μg ODA, 150 μg 9-HDA, 20 μg HOB, and 2 μg HVA (Slessor *et al.*, 1990). These two queen equivalent values were formulated based on the most current knowledge of mean queen equivalent QMP component values at the time, and fit well within the distributions reported here (Fig. 13). The first queen equivalent definition was more reflective of mean ratios than the second (Table 2).

The comparisons between European and Africanized mated queens reveal some differences in the quantities and proportions of QMP components in these two honey bee races. However, this trait would not be reliable for classifying individual queens into a race category due to the high variability between individual queens (Figs. 13 and 14). For all variables, Africanized means fall within high frequency categories of European means, except perhaps the quantity of 9-HDA. Crewe (1982) reported differences in the proportion of mandibular gland components among *A. m. adansonii, A. m. mellifera,* and *A. m. capensis,* but discriminant analysis indicated that gland components were not dependable predictor variables for grouping inbred lines. Methodological differences between previously published African queen mandibular gland component quantities or percentages (Crewe and Velthuis, 1980; Crewe, 1982; Allsopp, 1988), and results reported in this study make comparisons unrealistic, but comparative studies would be of interest in the future.

Queens of either race may head colonies composed of workers of both races (Winston, 1992), and thus it appears that workers will accept racially different queen signals. Further, a European-like synthetic blend of QMP is equally effective at preventing queen

rearing in colonies of either European or Africanized bees (Pettis *et al.,* 1995b). Nevertheless, the lower total quantity of QMP found in Africanized queens may contribute to the tendency for Africanized colonies to swarm with smaller parent colony sizes. The higher proportion of 9-HDA in Africanized queen glands, and greater quantities found on the body (Figs. 10 and 11) may reflect an adaptation to greater swarming activity in this race, because 9-HDA is an important component for swarm clustering behavior (Winston *et al.*, 1982).

Unmated virgin queens not only produce significantly lower quantities of QMP components, but the proportion of components are significantly different from mated queens (Fig. 10 and Table 2). The older (8 wks) drone-laying queen glands contained ODA and 9-HDA proportions similar to the European mated queens, but the younger (6-14 days) virgin queen proportions were significantly different from the mated queens. The older drone-laying queen ODA and 9-HDA proportions may reflect ontogenetic development and enzymatic capabilities of the gland. Conversely, the significantly lower proportions of the aromatic components represent a non-reproductive status (Fig. 11) (Slessor *et al.,* 1990). Virgin queens were producing a significantly different signal from the mated queens, suggesting an important biosynthetic change occurs with mating that cannot be explained by age alone, as seen by the intermediate signal produced in drone-laying queens. The essence of the different signals are a combination of quantity and proportion.

The ratios of surface to gland components calculated confirm that 10^{-3} glandular Qeq's is representative of QMP surface concentration (Slessor *et al.*, 1988). Gland component and bodywash quantities were not correlated, and quantities found on the body were not in the same ratios as those found in the glands (Table 2), except for 9-HDA/ODA

in virgin queens. A comparatively high ratio of 9-HDA/ODA was found on the bodies of European virgin queens compared to mated queens. European mated queen glands contain approximately 46 times more HOB than virgin queens, yet have similar amounts of HOB on the body. The glandular "bouquet" of virgin queens (Fig. 12) has HOB as a relatively minor component in the total blend, but body QMP shows HOB as a proportionately greater component. The bodywash results are enigmatic, and suggest that QMP is not consistent as a signal, and not correlated to the QMP exuded from the gland. Many factors may affect quantities of QMP that can be extracted from the body including varied absorption into the cuticle, removal by workers, and queen re-internalization (Naumann *et al.*, 1992). Those factors related to ontogeny, such as cuticle and queen behavior, may be relevant to the ontogeny of the signal, and warrant further investigation.

The wide variation in the quantity of QMP components suggests a generic signal indicating the individual is a queen, but there are important ontogenetic and reproductive differences in the signal as well. More will need to be known about biological and behavioral effects of individual QMP components before any inferences can be made concerning the meaning of total or relative presence of any component in the blend.

CHAPTER 4

Queen Attendance Behavior of Worker Honey Bees that are High and Low Responding to Queen Mandibular Pheromone*

ABSTRACT

Queen attendance behavior of workers from selected honey bee colonies with high and low worker retinue response to QMP was investigated. Antennating, licking, grooming and feeding of the queen by workers from high and low responding colonies were examined. High and low QMP responding workers did not attend the queen differently. However, workers originating from different colonies antennated and licked the queen more frequently than others, suggesting there may be a genetic basis for queen attendance behavior not necessarily associated with response to QMP. The median age of queen attendance was independent of strain. Other cues may be important in retinue behavior such as movement, texture, and other odors associated with a live queen.

* Pankiw, T., Winston, M.L., and Slessor, K.N. *in press.* Queen attendance behavior of worker honey bees (*Apis mellifera* L.) that are high and low responding to queen mandibular pheromone. Insectes Sociaux

INTRODUCTION

The court or group of worker honey bees surrounding a queen has been a captivating subject of interest for many years (Allen, 1957; van der Blom, 1992; Butler, 1954; Free *et al.,* 1992; Seeley, 1979), but only recently has the role of retinue attending bees in transmitting queen pheromone throughout the nest been demonstrated and modeled (Naumann *et al.,* 1991, 1992). Worker bees engaged in retinue behavior antennate, lick, groom, and feed the queen while surrounding her in a fluctuating elliptical grouping (Seeley, 1979). Social cohesiveness is maintained in colonies by the subsequent transmission of queen pheromones throughout the nest by workers that have contacted the queen, thereby substituting a semiochemical form of queen dominance for the physical dominance used by the more primitive forms of social insects.

A blend of five compounds known as queen mandibular pheromone (QMP) elicits retinue behavior (Slessor *et al.*, 1988), and a number of other bee behaviors (reviewed in Chapter 1).

The present study provides the first examination of queen attendance behavior by workers of selected QMP responding phenotypes. Previous studies have concentrated on queen attendance by individual workers or individuals within age cohorts (Seeley, 1979; Free *et al.,* 1992; van der Blom, 1992). Queen attendance appears to be random, with workers engaging in retinue behavior or retreating from the queen when within the queen's retinue radius, a distance of one to two cm from the queen (Seeley, 1979; van der Blom, 1992). The frequency with which workers from different sources might attend the queen is a measure of

the tendency of those workers to attend or retreat from the queen.

The objectives of this study were to determine whether retinue response to QMP alone in the retinue bioassay could predict the likelihood of retinue participation in the colony. The selected strains of high and low QMP-responding workers were used to determine the role of QMP-induced behaviors at the colony level. Presumably, any QMP-induced effects should be expressed with greater intensity in high QMP-responding workers and colonies. High QMP rather than low QMP responding workers would be expected to be observed performing queen attendance behaviors more frequently if 1) QMP is the primary retinue-inducing pheromone for all worker phenotypes, 2) response to synthetic QMP is associated with queen attendance behavior, and/or 3) queen attendance behavior has a genetic basis associated with QMP response. Differences between high and low QMP responding workers could further include the frequency in which they engage in queen attending behaviors, such as antennating, licking, grooming, or feeding the queen (Seeley, 1979).

Materials and Methods

Workers originated from colonies headed by second generation queens from a closed circular mating breeding program for the selection of high and low QMP responding workers (Laidlaw and Page, 1986). The queens were open mated; progeny relatedness ranged from super-sisters ($r=0.75$) to half-sisters ($r=0.25$) (Page and Laidlaw, 1988). The retinue response of 4 high and 4 low QMP-responding colonies was monitored periodically for one year, and was determined to be consistently high or low responding in the laboratory retinue

bioassay (Chapter 1). The average QMP bioassay lure contact response was 23.4 ± 3.1, and 7.6 ± 1.9 for high and low responding colonies, respectively.

A four-frame (Langstroth deep frame, with 2 brood and 2 food frames) observation hive with about 4,000 workers (most similar to *Apis mellifera ligustica* L.), and a naturally mated one year old queen (Simon Fraser University stock) was initiated on 13 July, 1992. Seven days (20 July 1992) later, 500 newly emerged workers with colony-specific paint marks on the thorax, from each of four high and four low QMP responding colonies were introduced. Workers will be referred to as belonging to one of these eight cohorts. The observation hive contained about 8,000 workers when introductions were completed over the course of two days. The following retinue behaviors were recorded beginning the next day; 1) antennating (antennae palpating the queen), 2) licking (proboscis licking any part of the queen), 3) grooming (mandibles gently scouring any part of the queen), and 4) trophallaxis (feeding the queen). Workers observed in the retinue for greater than 9 seconds were considered retinue attendants. The retinue was observed for one minute intervals while tape recording the cohort identity of the workers engaged in the various retinue behaviors. Five recording periods were separated by 5-10 minute intervals. Seeley (1979) found that workers spend an average of 116.8 s (standard deviation = 112.3) in the retinue. A 5-10 minute interval was chosen to minimize the possibility of counting the same worker more than once. The observer adjusted the interval depending on the queen's activities. When the queen was stationary the between-count interval was 10 minutes or longer, and if moving the interval was 5 minutes. Counts were conducted each morning and afternoon for a total of 10 periods per day. The workers were observed until they were thirty-two days old for a total of 320

observations.

The number of surviving marked workers was determined by counting marked workers in ten randomly selected sections of an 18 X 19 ($5cm^2$) grid on both sides of the observation hive on days fifteen and thirty-two.

Comparisons of retinue behavior among cohorts were analysed using Wilcoxon Rank Sum statistic (Conover, 1980). This non-parametric test was used because the honey bee phenotypes were specifically as opposed to randomly selected.

RESULTS

The number of marked workers in 20 randomly selected grids (10 on each side of the hive) on days 15 (estimated total number of workers per cohort 456.5 ± 18.8 std. dev.), and 32 (36.4 ± 9.3 std. dev.) was not significantly different between any of the cohorts ($P > 0.05$). Comparisons were made only between the selected cohorts, so the relative equality of their respective numbers were important to a balanced analysis. Because there were no significant differences in cohort numbers on days 15 and 32 it was assumed for the purposes of analysis the numbers were statistically equal on all other days. There were no differences between the number of high and low QMP retinue responding workers antennating, and licking the queen ($P > 0.05$, Figure 15a), but there were significant differences in the number of antennating and licking workers between cohorts ($P < 0.05$, Figures 15a and 15b). There were no differences between strains in grooming, and feeding the queen ($P > 0.05$, Figure 16). Grooming and trophallaxis events were too infrequent for

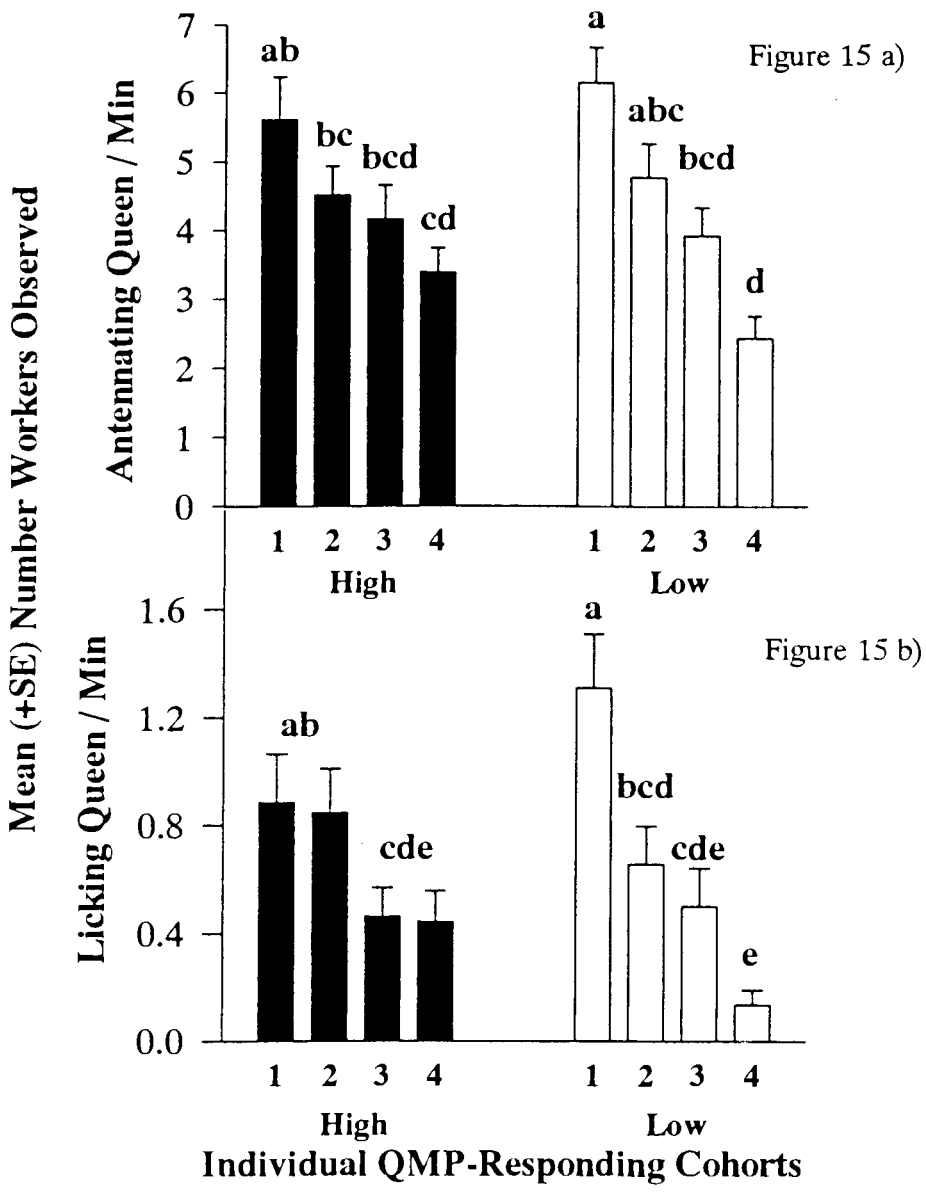


Figure 15. **a)** Mean number of workers from individual ■ high and □ low QMP-responding cohorts antennating the queen. **b)** Mean number of workers from individual ■ high and □ low QMP-responding cohorts licking the queen. Means followed by different letters were significantly different ($P < 0.05$).

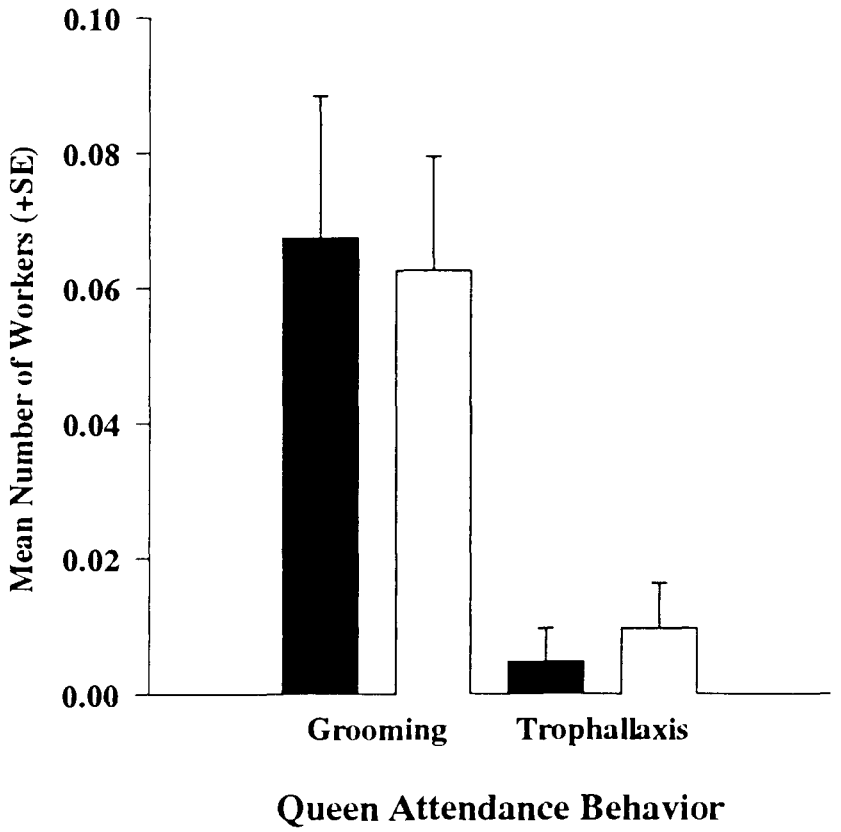


Figure 16. Mean number of workers grooming and feeding the queen. Worker-queen encounters by ■ high (N=4), and □ low (N=4) QMP pheromone-responding phenotypes. Differences were not statistically significant ($P > 0.05$).

statistical analysis on an individual cohort level. The overall mean age for queen attendance was 11.3 ± 1.5 days (±se). There were no strain differences in the mean age of queen attendance ($P > 0.05$, Figure 17).

DISCUSSION

The number of workers engaged in the various retinue behaviors in this study were most similar to those reported by Butler (1954) because comparable counting methods were used. Butler (1954) used a snap-shot method recording 12 counts separated by 5 minute intervals, and observed on average 2.5 workers licking the queen and 2.2 workers "examining" the queen, of approximately 1500 unmarked workers in an observation hive. Allen (1957) observed the retinue for one hour periods, and saw 8 to 10 workers attend the queen out of 100-110 marked in a three-frame observation hive containing about 4,500 workers. Seeley (1979) demonstrated that the average number of workers engaged in retinue behavior at any one time depends on queen activity. Retinue size was 4.2 workers when the queen is travelling, 10.9 when laying, and 17.2 when the queen was stationary (Seeley, 1979). Seeley (1979) also found that longer observation periods showed greater percentages of marked individuals engaged in retinue behavior. For example, after 5 hrs of continuous observation 21.3% of the total number of marked individuals were observed, after 10 hrs 35.6%, and after 15 hrs 48.6%, in a four-frame observation hive with approximately 17,500 workers. Van der Blom (1992) observed the retinue behavior of 200 marked individuals in four, two-frame observation hives of approximately 1500 workers for continuous periods of 2

to 4 hours. The total number of workers in the retinue during that time ranged from 182 to 268 (van der Blom, 1992). This brief review of methods reveals that the number of marked workers observed in retinue behavior depends on the ratio of marked workers to total colony size, and observation time. Overall, our results are not methodologically comparable to others except Butler (1954), but the retinue activity in all of these studies appears to be in a similar range.

Surprisingly, the QMP bioassay response was not correlated to any queen attendance behavior in the hive by high and low responding workers. Attraction to QMP as a single stimulus evidently does not confer expression of queen attendance behavior. Another queen pheromone may be involved in mediating the extent of retinue behavior, singly or in combination with QMP. Thus, those cohorts that were low QMP responders in the bioassay but high queen attenders in the colony could be responding to other queen odors, alone or in combination with QMP. Tactile cues and queen movement also may be important in the hierarchy of cues attracting workers to the queen. QMP is not highly volatile, so workers must be within 1 to 2 cm of the queen to recognize her presence (Seeley, 1979). A glass lure spotted with QMP placed in the brood nest area of a colony, or in a petri dish with 15 workers is highly attractive to workers (Slessor *et al.*, 1988), but not all workers are drawn to the lure. A glass lure lacks movement, texture, and other odors associated with a live queen that may be significant factors in queen recognition for some workers.

There are several explanations for our results. Firstly, significant differences in queen attendance behavior by some cohorts may indicate some genetic component to queen attendance behavior, albeit not based on QMP response. This finding is consistent with

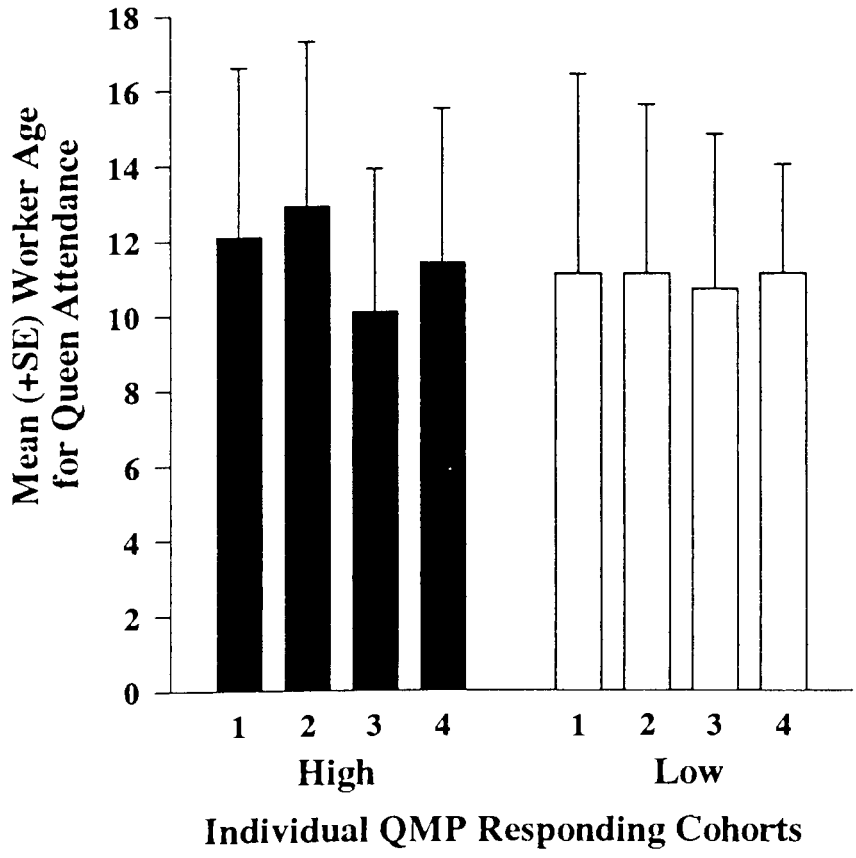


Figure 17. Mean age of queen attendance (P > 0.05) including antennation licking, grooming, and trophallaxis behaviors by individual cohorts.

many other genetically based behavioral differences in honey bees (reviewed by Page and Robinson, 1991; Robinson, 1992). Our data indicate a greater tendency for queen attendance at the phenotypic level, similar to that found for high and low pollen hoarding strains (Page *et al.*, 1988). Individuals from the different cohorts may attend the queen with the same frequency (i.e. individuals' frequency of queen attendance is stochastic), but cohort-specifically more or fewer workers may be engaged in the activity, as for high and low pollen hoarding behavior (Page *et al.*, 1989). Secondly, there may be genetically determined differences in cohort spatial distribution due to local preferences within the nest that may affect the probability of encountering the queen. We don't believe this was the case for two reasons. The queen travelled extensively throughout the nest, and the cohorts didn't appear to be clumped in any areas within the nest. Thirdly, there may be an environmental effect on queen attendance. If so, then evidently high queen attending cohorts displayed a lower threshold of response than low queen attending cohorts, which may point to a genetic component to queen attendance.

In summary, we have shown that queen attendance in honey bees may have a genetically variable component, but genetic differences between cohorts are not based on QMP response as measured in our bioassay. Future work might examine other queen odors to determine whether they play a role in queen attendance. Low QMP responding workers may be particularly suited for use in exploring additional queen attraction odors because some of these workers are equally attracted to the queen as some high QMP responding workers. Also, genetic differences in the frequency of queen attendance could have profound effects on colony pheromone distribution and pheromone-influences on worker behaviors, but

these topics are almost completely unexplored.

CHAPTER 5

PHENOTYPIC DIFFERENCES IN QUEEN REARING BY HIGH AND LOW QUEEN MANDIBULAR PHEROMONE RESPONDING WORKER HONEY BEES.*

ABSTRACT

The behavioral response to queen mandibular pheromone (QMP) known as retinue behavior has been shown to have a genetic basis (Chapters 1 & 2). In this study we demonstrate that the responses to QMP are related to another effect of QMP, queen rearing activity. Worker honey bees selected on the basis of high retinue response to QMP in a laboratory bioassay were significantly more likely to be engaged in queen rearing activities than were workers with a low retinue response to QMP. High response workers spent proportionately more time working on and in queen cells than low response workers and there were significant age by response effects for time spent rearing queen cells. No interindividual differences were detected among the response phenotypes in the tendency to rear queens. Results from this experiment suggest that QMP response may be a mechanism upon which colony-level selection acts for cooperative queen rearing behavior.

*Pankiw, T. Phenotypic differences in queen rearing by high and low queen mandibular pheromone responding worker honey bees. (*to be submitted*)

INTRODUCTION

Honey bee (*Apis mellifera* L.) queens produce a signal known as queen mandibular gland pheromone (QMP) (Slessor *et al.*, 1988). QMP signals queen presence to workers and elicits worker retinue behavior distinguished by frequent anntenations, licking, grooming and feeding activities directed toward the queen. This behavior is a releaser response, comprising a stimulus-response mediated by the nervous system (Hölldobler and Wilson, 1990). Queen presence is made known throughout the nest by serial transmissions of QMP from worker to worker (Naumann *et al.*, 1991, 1992). Removal of the queen from the nest or inhibition of QMP transmission as a result of high adult numbers (a dilution effect) results in queen rearing, normally inhibited by QMP (Winston *et al.*, 1989, 1990, 1991; Pettis *et al.*, 1995). The queen's pheromonal inhibition of new queen rearing by workers is a primer effect of QMP, in which the endocrine or reproductive systems are altered physiologically (Hölldobler and Wilson, 1990). This study asks whether workers selected on the basis of their releaser response to QMP show a differential response to the primer effect of QMP inhibiting queen rearing.

This experiment focuses on investigating QMP queen rearing behavior by high and low strain workers in the absence of QMP. Female larvae three days old or younger may develop as workers or queens depending on diet. Larvae chosen to be queens are fed an enriched diet containing secretions mainly from mandibular glands of brood tending workers, and larvae destined to be sterile workers are fed a combination of mainly hypopharyngeal and some mandibular gland secretions (reviewed in Winston, 1987). I

hypothesized that workers showing a high response to the pheromone that signals queen presence (QMP) will have a low threshold of response in the absence of QMP, expressed as greater queen rearing activity, than workers with a low retinue response to QMP.

MATERIALS AND METHODS

Experimental Observation Hives

Four-frame Langstroth deep observation hives, with glass sides containing 2 brood and 2 food frames, 6-7000 workers, and a naturally mated queen were set-up three days before focal bees were introduced. The experiment was divided into three rounds, 2 observation hives in round 1, and 3 hives each in rounds 2 and 3, for a total of 8 observation hives.

Focal Bees

High and low QMP retinue responding workers were selected from colonies headed by naturally mated queens (Chapters 1 and 2). QMP retinue responses were determined by the number of worker contacts with a glass bulb spotted with QMP (Chapter 1). Responses of focal bee colony sources for each replicate were as follows: 1) X_{high}=57, X_{low}=5; 2) X_{high}=36, X_{low}=4; 3) X_{high}=58, X_{low}=5; 4) X_{high}=47, X_{low}=4; 5) X_{high}=51, X_{low}=7; 6) X_{high}=38, X_{low}=3; 7) X_{high}=45, X_{low}=4; 8) X_{high}=49, X_{low}=3.

Bees of known age were obtained by removing combs containing developing pupae from parent colonies 24 hours before adult emergence. The combs were placed in an incubator maintained at 33°C. The newly emerged adults were marked with colored numbered tags and paint marks. The numbered tags were glued to the thorax and the paint mark applied to the abdomen. A corresponding paint mark was dabbed on to the numbered tag as a back-up reference to the abdominal paint mark. Focal bees were introduced to the observation hives on five days separated by 24 hour periods to create age cohorts of 1, 3, 5, 7, and 9 days after emergence at the beginning of the experiment. Each day bees were introduced, 200 high and 200 low QMP responding workers were prepared for a total of 1000 high and 1000 low workers per observation hive.

Experimental Procedures

The queen and all larvae were removed from the colony. First to fourth instar female larvae fed a relatively rich diet develop into queens and those fed a poorer diet develop into sterile workers (reviewed in Winston, 1987). Twelve hours later, workers were presented with 10 larvae chosen randomly from unrelated sources (unrelated to focal bees and observation hive bees) and transferred into dry artificial queen cells arranged on one face of an empty comb. The cells were spaced at least 8 cm apart and affixed using gentle heat on the area to which the cell was positioned. Average acceptance of larvae for queen rearing was 70%. All cells were inspected daily for evidence of royal jelly (primarily mandibular gland secretions fed to developing queens). Abandoned cells were eliminated from the

experiment.

Behavioral Observations

24 Hour Continuous Observation

Detailed observations were made on a single queen cell for a 24 hour period to detect any periodicity to queen rearing activity. The cell was illuminated with a glass-fiber optic light, and videotape recordings were later transcribed. Behavioral observations were divided into 2 categories: 1) workers observed ON queen cells which included any activities where workers made physical contact with the outside of the queen cell, and 2) workers observed IN cells for any amount of time. Workers entering the queen cell were likely feeding the larvae, because most queen larva feeding bouts last less than 50 seconds (Browers *et al.*, 1987), although a visual confirmation was not possible. Workers ON the cell were engaged in cell building by shaping and elongating the wax body with their mandibles, and were presumably gauging their activities by frequent antennations (reviewed by Winston, 1987). A third category, TEND the queen cell was generated for some statistical analysis purposes by combining the ON and IN cell categories. Data collected from this 24 hr period were used to test for interindividual differences in the tendency to specialize in rearing queens. Observations of the 24 hour recording were used to determine the frequency with which each bee was observed ON or IN the queen cell. Frequency distributions were calculated by summing all bees for each behavioral category (ON, IN, and TEND the queen cell). The

distributions were tested against expected frequencies generated by Poisson distributions. Specialists would appear as bees with unexpectedly high frequencies (Sokal and Rohlf, 1981).

5 Minute Scans

Scan samples were conducted on 8 colonies as follows. Each queen cell was observed for a 5 min period while recording the activity of focal bees (5 min scans). Activities of focal bees working queen cells were scanned twice daily, from 11 a.m. to 2 p.m., and from 3 to 5 p.m. Only two behaviors were monitored and classified as workers ON and IN queen cells, as described above. Observations continued for 4 or 5 days until the cells were capped over.

30 Minutes Continuous Observation

To determine whether there were phenotypic biases in the relative likelihood of time spent rearing queens, all of the cells (40) in six observation hives were videotaped for 30 minutes (30 minute observations) daily for 4-5 days until the larvae pupated. Cells were illuminated with a glass-fiber optic light. The tapes were reviewed for durations of ON and IN cell activities. Rearing bout categories represent the following time ranges; 10 sec ($\leq$ 10 sec), 30 sec (10<sec<30), 60 sec (30<sec<60), 120 sec (60<sec<120), 180 sec (120<sec<180), 240 sec (180<sec<240), 300 sec (240<sec<300), and 600 (> 300 sec).

Censuses of Focal Bees

Colony censuses were conducted the day before focal bees were introduced to determine adult bee numbers, and whether possible frequency differences between colonies were based on differential access to queen cells. Censuses were conducted on the final day of the observation period to determine whether possible phenotypic differences were based on differential mortality among the focal bees. Censuses were conducted by scanning each face of each observation hive that was divided into grids for orientation purposes. The identification of focal bees, and the total number of workers per grid were recorded on an audio recorder.

Statistical Analyses

All results from 5 minute scan were based on the first observation for each bee in each observation period. Thus, the observations of individuals within a 5 minute scan period were independent. Observations of individuals within a 30 minute observation period also were independent, but the duration of repeated visitations of individuals were treated independently as well as summed to determine the overall effect of repeated visitations. Durations of ON and IN categories were analysed as proportions of total time spent engaged in the respective activities to determine whether there were any phenotypic biases in the relative likelihood of bout duration.

Colony mean proportions were pooled because high and low proportions between

colonies were homogeneous, respectively (Sokal and Rohlf, 1981). The effects of strain, worker age, and strain by worker age on queen rearing were analysed by two- and three-way *G*-tests (Sokal and Rohlf, 1981).

RESULTS

24 Hour Continuous Observation

There was no periodic activity observed ON the queen cell in a 24 hour observation period (Fig. 18a). Workers observed IN the cell appeared to be more frequent from 1100 h to 1900 h, interspersed with a periodicity of 2-3 hours (Fig. 18b). The frequency of individuals' tendency to work ON (χ^2=0.57, df=2, P>0.50), IN (χ^2=1.3, df=2, P>0.25), or TEND a queen cell (χ^2=0.44, df=2, P>0.50, Fig. 19) were distributed in a manner indistinguishable from a Poisson distribution.

5 Minute Scans

Colony mean proportions were pooled for statistical analysis (Sokal and Rohlf, 1981). High strain proportions were homogeneous between colonies, as were low strain proportions: 5 minute scans: G_{High}=8.7, df=8, *P*>20.150, G_{Low}=10.6, df=8, *P*>0.100 (Fig. 20); 30 minute observations: G_{High}=7.87, df=6, *P*>0.25, G_{Low}=5.37, df=6, *P*>0.50) (Sokal and Rohlf, 1981). There was a significantly greater probability for high QMP responding workers to be

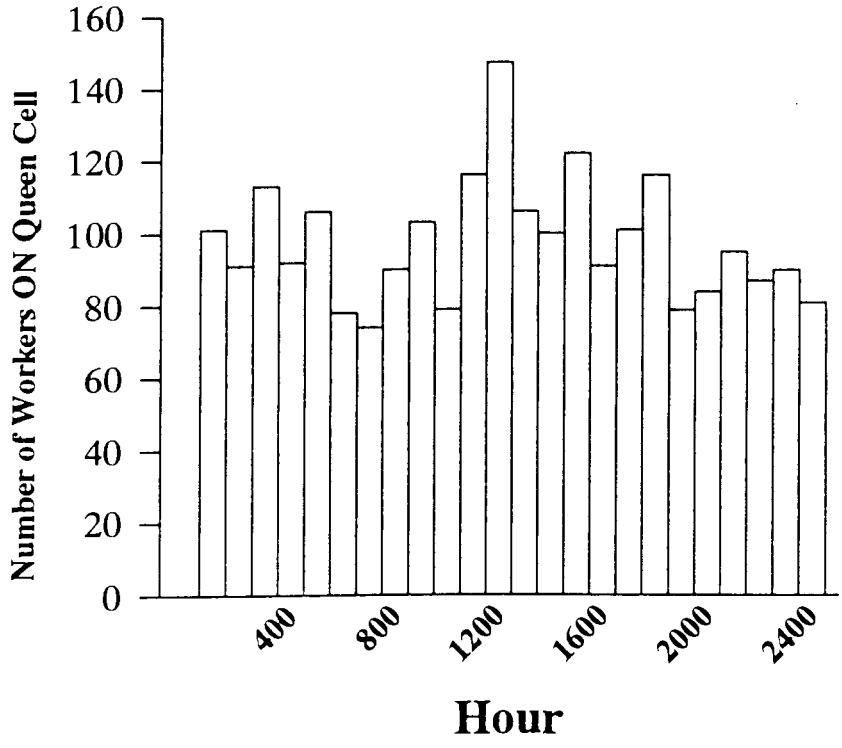


Figure 18a. Periodic worker activity ON a queen cell observed continuously for 24 h.

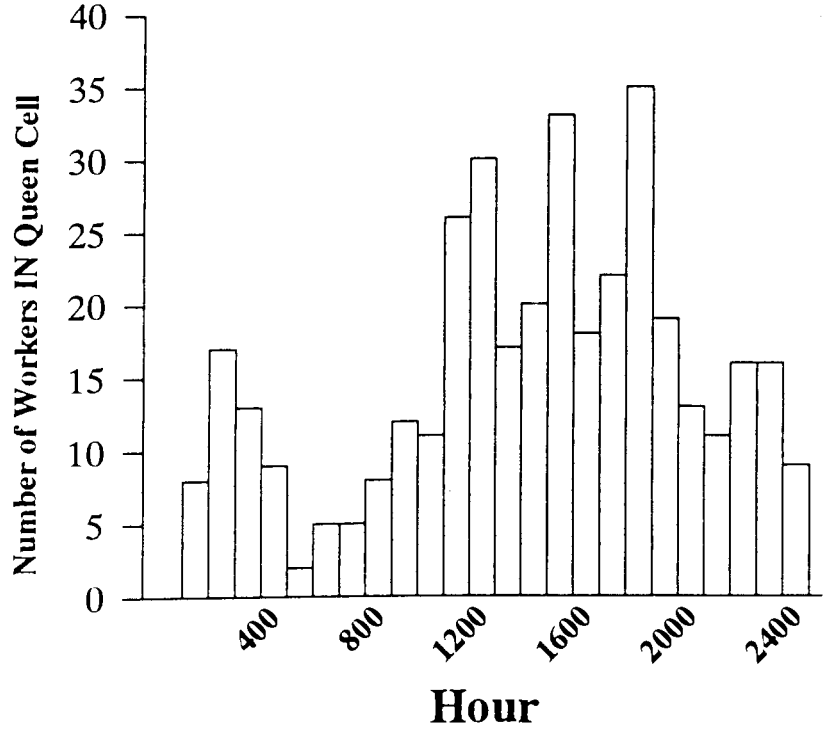


Figure 18b. Periodic worker activity IN a queen cell observed continuously for 24 h.

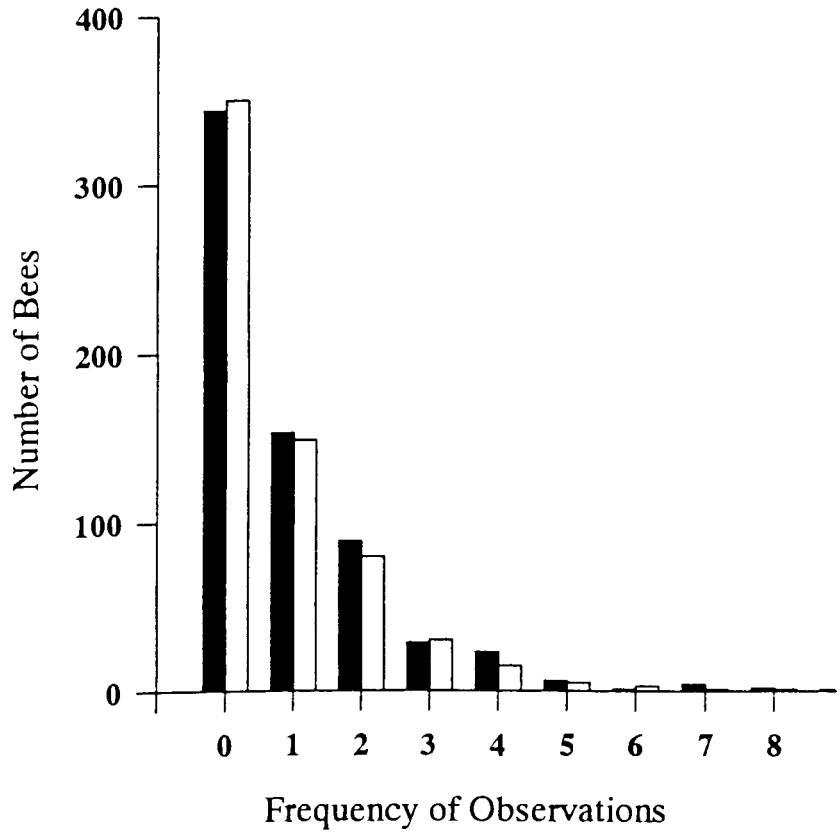


Figure 19. Observed ■ and expected □ probability distribution of queen cell TENDing frequencies. The probability that the observed distribution deviates from the Poisson distribution by chance alone is 0.05 (χ^2=0.44, df=2).

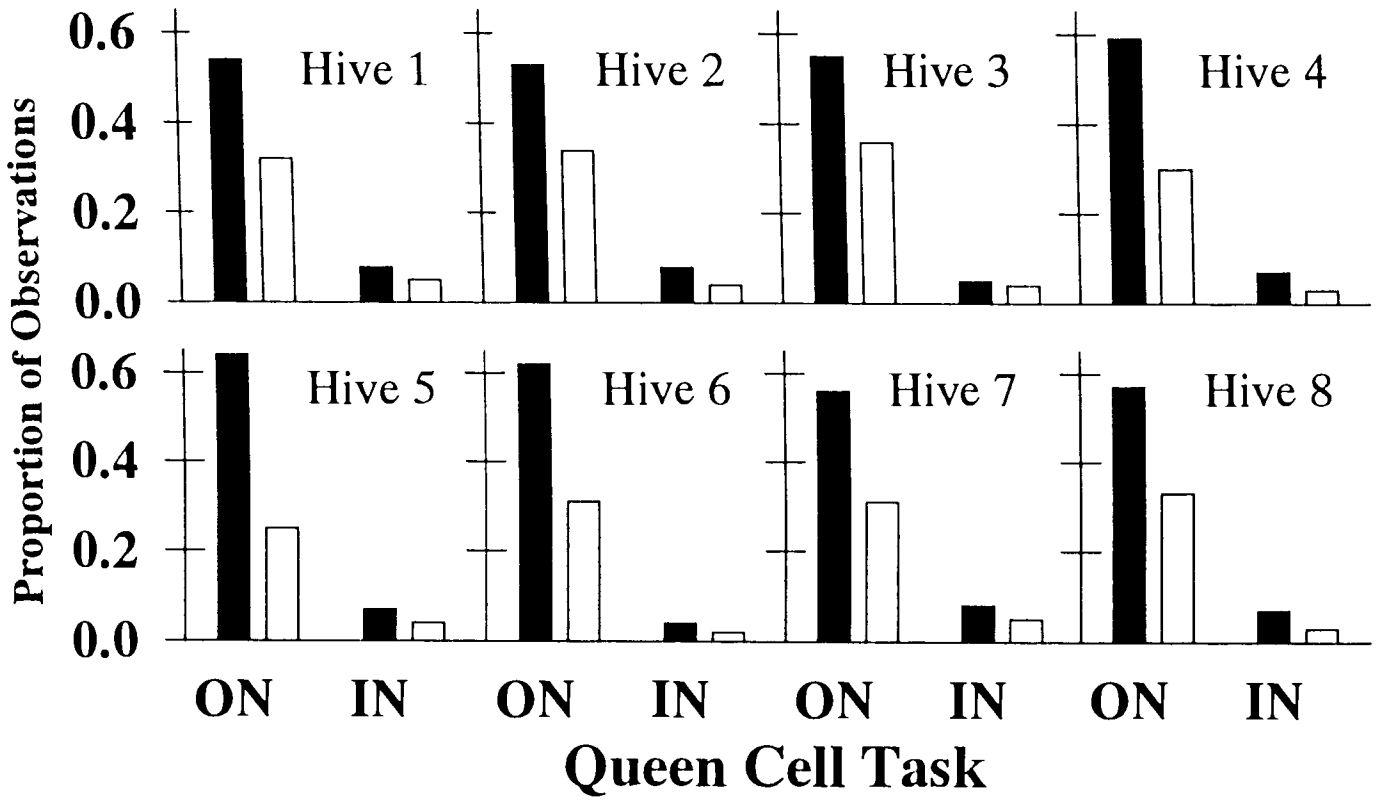


Figure 20. Effect of QMP retinue response on the likelihood of being observed ON or IN queen cells. ■ High workers were significantly more likely to work IN and ON queen cells than □ low workers, see text for G-Test probabilities and values.

observed ON and IN queen cells than low QMP responding workers (G= 7.3, df=2 $P<0.02$). There was no significant effect of age on the likelihood of workers to TEND queen cells (G=4.5, df=5, $P>0.5$), but there were significant age by strain effects. High strain workers were more likely to TEND queen cells at 3, 7, and 9 days; Age 1, G=4.7, df=2; Age 3, G =9.6, df=2; Age 5, G=6.3,df=2; Age 7,G=8.3, df=2; Age 9, G=10.5, df=2.

30 Minute Continuous Observations

High strain workers spent significantly more time TENDing queen cells than low strain workers (ON cells G=13.7, df=3, $P < 0.001$; IN cells G=12.7, df=2, $P < 0.001$) (Fig. 21). There also were significant age by strain effects in the proportion of time spent ON (Fig. 22a) and IN cells (Fig. 22b). High strain workers spent proportionately more time ON queen cells than low strain workers for all ages (Day 1: G=6.7, df=2, $P < 0.03$; Day 3: G=6.4, df=2, $P< 0.02$; Day 5: G=7.5, df=2, $P<0.02$; Day 7: G=7.5, df=2, $P<0.02$; Day 9: G=5.9, df=2, $P<0.05$). High strain workers aged 5, 7, and 9 days spent significantly more time IN queen cells than low strain workers (Day 1: G=1.3, df=2, $P > 0.05$: Day 3: G=4.5, df=2, $P>0.05$; Day 5: G=6.1, df=2, $P<0.05$; Day 7: G=6.3, df=2, $P<0.05$; Day 9: G=7.1, df=2, $P<0.03$). These results reflect the cumulative effects of individuals' activities in a 30 minute period. High responding workers were significantly more likely to be ON cells in bouts lasting 10 seconds or less (Fig. 23a), but not for any other durations. High responding workers were significantly more likely to be observed IN cells for bouts lasting 10, 30 and

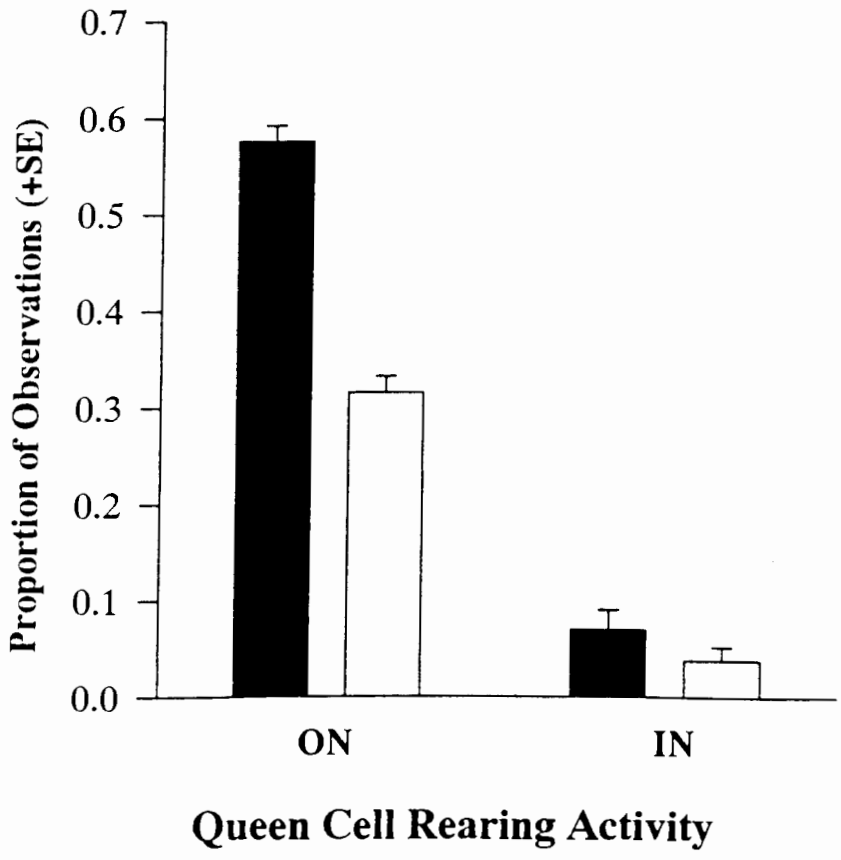


Figure 21. ■ High strain workers were significantly more likely to be observed IN (G-Test = 13.7, df = 2, P < 0.001), and ON (G-Test = 12.7, df=2, P < 0.001) queen cells than □ low strain workers.

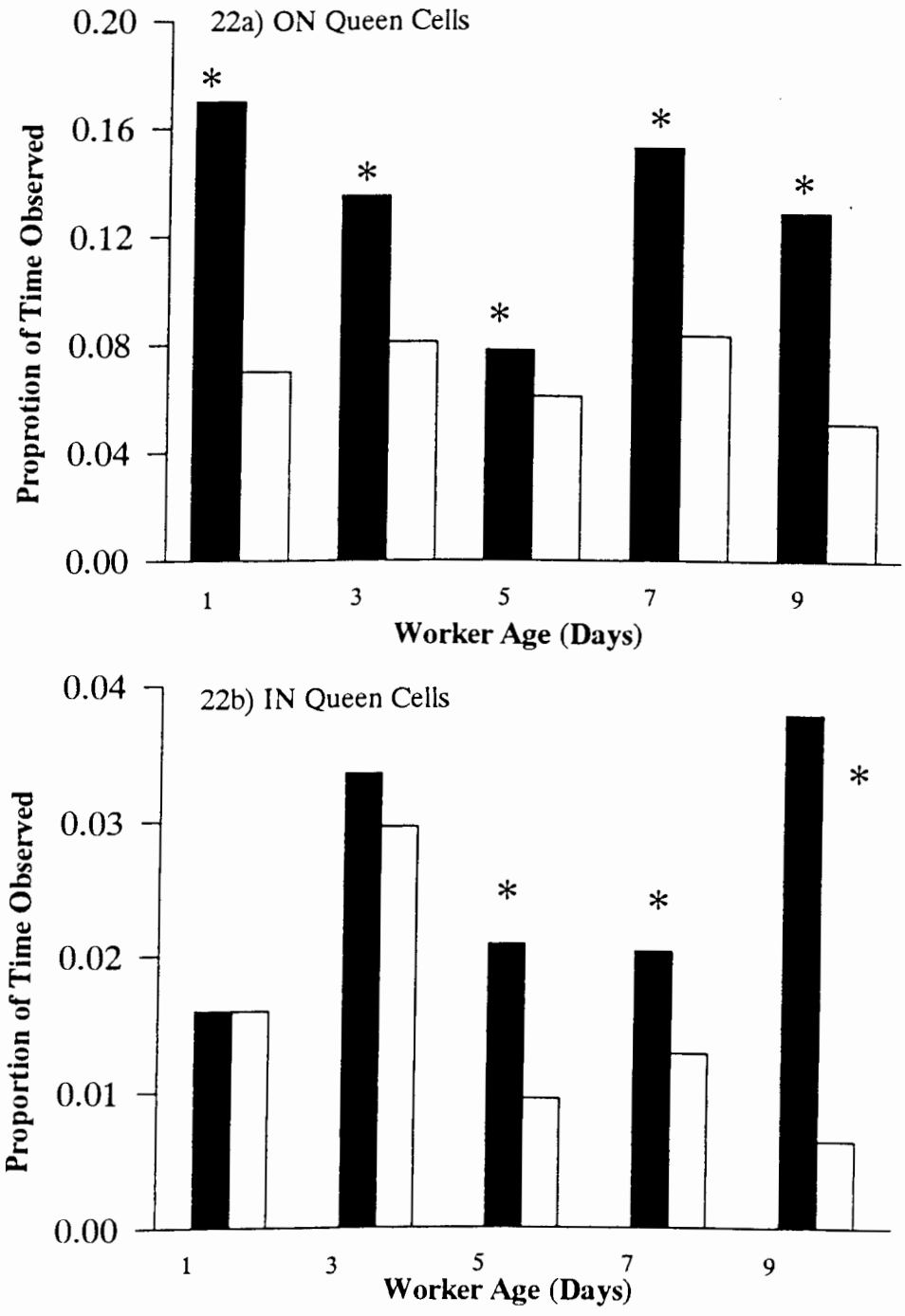


Figure 22. Effect of QMP response and worker age on the proportion of time observed **a)** ON or **b)** IN queen cells. ■ High strain workers spent proportionately more time ON in or IN queen cells than □ low strain workers where and a * indicates a significant difference by at most $P < 0.05$.

Figure 23 a)

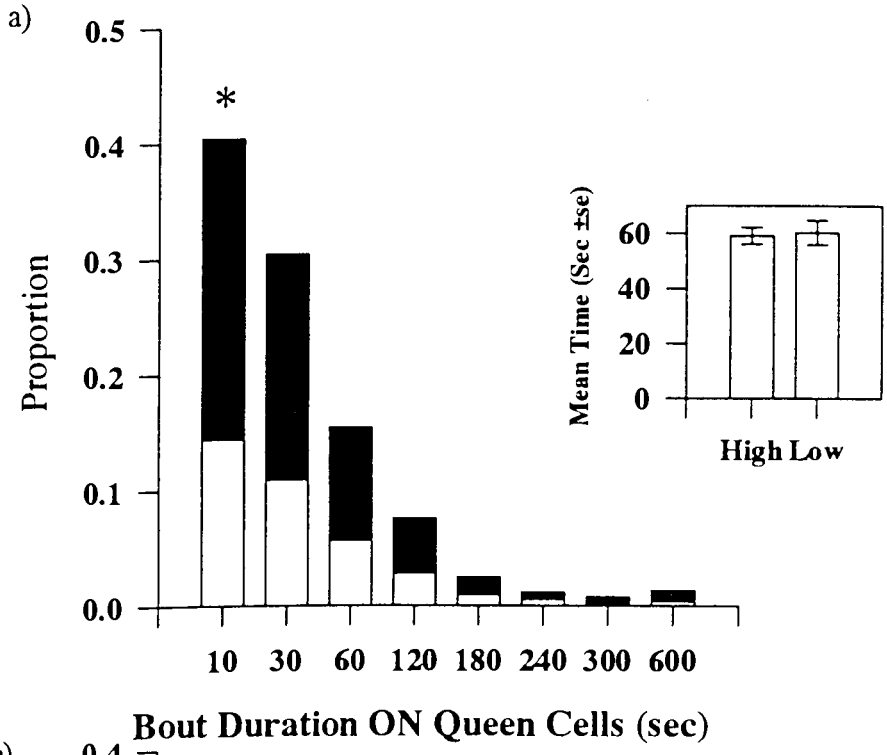


Figure 23 b)

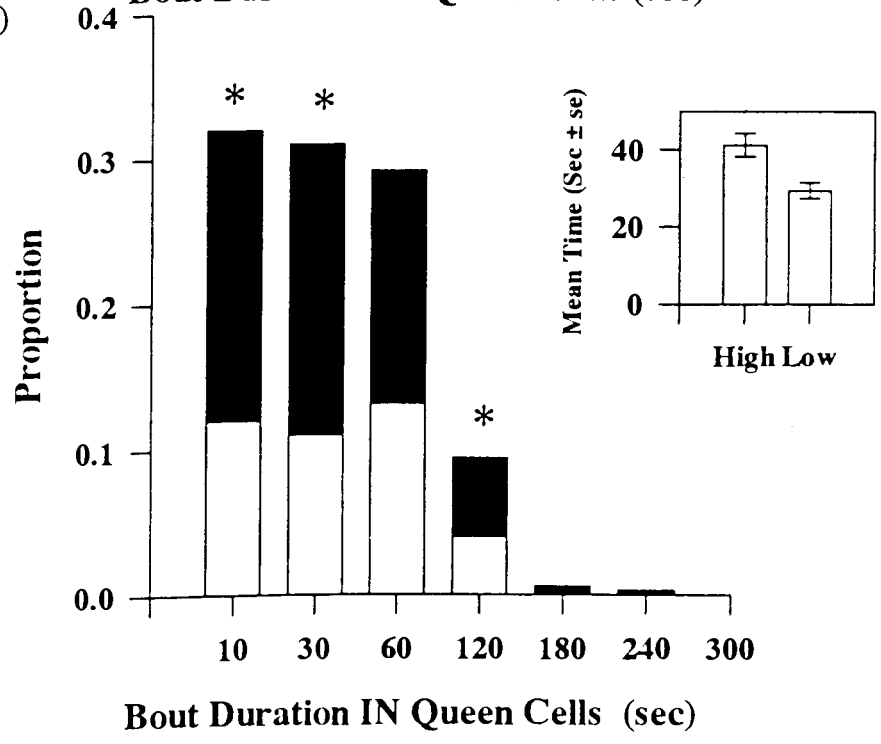


Figure 23. Effect of QMP response and bouts durations **a)** ON and **b)** IN queen cells. A * indicates significant ($P<0.05$) difference between ■ high and □ low QMP responding phenotypes. Insets show the mean time workers spent **a)** ON or **b)** IN queen cells.

120 seconds (Fig. 23b).

Censuses of Focal Bees

Colony censuses indicated that strain differences in queen rearing were not due to differential mortality, access to queen cells among the strains, or between-colony differences (Table 4). Colony populations, numbers of high and low QMP responding workers, and numbers of workers per age cohort were similar (Table 4).

Table 4. Frequency of focal bees from high and low strains. One thousand individually marked workers were introduced over a 10 day period on alternate days, 200 workers per strain.

Colony	Population at commencement[1]	Number (%) of focal bees at completion.	
		High[2]	Low[2]
1	6239	751 (75)	728 (73)
2	6912	705 (71)	735 (74)
3	6699	729 (73)	757 (76)
4	6754	748 (75)	719 (72)
5	6587	737 (74)	776 (78)
6	6075	723 (72)	711 (71)
7	6651	718 (72)	708 (70)
8	6876	769 (77)	783 (78)

[1] There were no significant differences among colony sizes (G-Tests, $P>0.05$).
[2] There were no significant differences between high and low QMP-responding workers within and between colonies (G-Tests, $P>0.05$). There were no significant differences among and between age groups (not shown, G-Tests, $P>0.05$).

DISCUSSION

The results in this study support the hypothesis that workers showing high response to the pheromone that signals queen presence (QMP) expressed greater queen rearing activity than workers with a low response to QMP. Evidence for phenotypic variability in queen rearing behavioral traits supports a Darwinian model for the evolution of colony organization and suggests a mechanism for queen rearing division of labor upon which natural selection can act (Page and Robinson, 1991). The high QMP responding phenotype was more likely to be observed ON and IN queen cells, as well as spend proportionately more time engaged in these activities. This suggests that attractiveness to the queen-produced social cohesion pheromone (QMP) is associated with another trait important to colony survival, queen rearing.

There was no strong rythmic effect of activity ON queen cells, although there appeared to be increased IN cell activity during the late morning to early evening hours, perhaps coinciding with a more active period in the hive. However, more replications over a greater range of queen larval ages would be necessary to infer any periodic queen rearing activity. There also were no interindividual differences in the tendency of workers to TEND queen cells (Fig. 19). Thus, workers did not specialize in queen rearing activities (see also Robinson *et al.*, 1994; Oldroyd *et al.*, 1990). The tendency for high QMP responding workers to engage in queen rearing activities was consistent in all 8 of 8 replicates (Fig. 20). High strain workers may have a reduced response threshold to the absence of the queen, but this was not reflected as a high frequency of repeated behaviors by individuals of this

phenotype. High strain workers were more likely to be observed ON and IN queen cells than low strain workers (Fig. 23), spent more time ON cells (Fig 22a), and were significantly more likely to be observed ON cells for bout durations lasting 10 seconds or less (Fig. 23a). For all other bout durations there were no differences between the strains, suggesting that persistent specialization was not a factor for this activity, although a more thorough accounting of time spent on all other tasks would more accurately assess this possibility.

High strain workers aged 5, 7, and 9 days spent more time IN cells than low strain workers (Fig. 22b). Interestingly, in this study high strain workers were significantly more likely to be observed IN cells for durations of 10, 30 , and 120 seconds (Fig. 23b). The greater likelihood of observing high strain workers IN cells (Figs. 20), especially for long bout durations (Fig. 23b), suggests that high strain workers were more likely to have been feeding larvae than low strain workers. To accomplish queen larval feeding the high strain worker would have made a physiological shift to producing queen as opposed to worker food. The food of queen larvae differs from worker food in containing mostly mandibular gland secretions, whereas worker jelly contains a combination of mainly hypopharyngeal and some mandibular gland secretions (reviewed in Winston, 1987). The results from this study suggests that high strain workers were more likely to have well-developed mandibular gland food production than low strain workers, likely induced through an endocrine-based mechanism for the regulation of queen rearing. This is an area warranting further investigation.

The behavioral and physiological mechanisms underlying queen rearing are

unknown. I propose that one mechanism for the differences observed here is the response threshold to the absence of the queen signal QMP by high QMP responding workers. The response sequence to the absence of the queen may occur in the following order: 1) the absence of the queen is perceived, 2) there is an endocrine-based physiological response to the released queen-rearing inhibition (perhaps through a neurohormonal-stimulated increase in mandibular gland food production), and 3) there is a behavioral response expressed as queen rearing. QMP is an important queen presence signal, and there is a genetic basis for worker response to QMP (Chapters 1 and 2). This study has demonstrated that there is a similar genetic basis to worker responses to the absence of QMP, and suggest that response to QMP is a trait involved in the organization of queen rearing.

CHAPTER 6

QUEEN MANDIBULAR GLAND PHEROMONE INFLUENCES WORKER HONEY BEE JUVENILE HORMO TITERS AND FORAGING ONTOGENY.*

ABSTRACT

Synthetic QMP was applied to honey bee colonies to test two hypotheses: 1) QMP acts like a primer pheromone in the regulation of age-related division of labor in honey bee colonies, and 2) the primer effect varies in three strains of workers that show genetically-based differences in their retinue attraction response to QMP. High, low and "wild-type" QMP retinue responding bees were fostered in queenright colonies with or without supplemental QMP. Effects of QMP on juvenile hormone III (JH) blood titers and the ontogeny of foraging in worker honey bees were measured. Although there were no differences in foraging ontogeny and JH titers among the three strains, bees in the QMP-supplemented colony treatment had significantly lower JH titers and showed a related delay in foraging ontogeny. In addition, forager activity was greater in control colonies. We conclude that 1) QMP can delay the ontogeny of foraging by inhibiting JH production, 2) this QMP primer response is independent of the retinue releaser response, and 3) QMP can play an important role in regulating division of labor.

* Pankiw, T., M.L. Winston, Z-.Y. Huang, and G.E. Robinson. Queen mandibular gland pheromone influences worker honey bee (*Apis mellifera* L.) juvenile hormone titers and foragning ontogeny. (*to be submitted*).

INTRODUCTION

The dynamic organization of honey bee (*Apis mellifera* L.) division of labor is an important attribute of this and other highly eusocial insects. A primary advantage of eusocial over solitary life is that all labor may be performed concurrently instead of sequentially, and the maintenance and ordering of division of labor has been a major concern in social insect studies. Workers are differentiated into behavioral groups, an effect of age-related change known as temporal polyethism. Temporal polyethism refers to the age-related behavioral repertory found among all of the ants, many social wasps and bees (Hölldobler and Wilson, 1990; Jeanne, 1991). The adult workers of most social insects change roles as they age usually progressing from tasks performed within to outside the nest.

The honey bee behavioral catalogue is lengthy and not all individuals will perform every possible task (Robinson, 1992; Winston, 1987), but rather the labor schedule reflects a compromise between task performance and location efficiencies (Seeley, 1982). Tasks are generally divided among four basic categories; 1) cell cleaning and capping, 2) brood and queen tending, 3) comb building, cleaning, and food handling, and 4) outside tasks such as ventilating, guarding, and foraging (Seeley, 1982). The nearly universal characteristic of progression from within- to outside-nest tasks is believed to be adaptive because the most hazardous tasks are delegated to the latter part of a worker's life. Workers undergo physiological changes with age in such a way that their response threshold to various environmental stimuli change. These behavioral changes are accompanied and induced in part by shifts in endocrine gland activity.

As honey bee workers age juvenile hormone (JH) titers increase, and treatment with JH mimic, JH, or JH analog will induce precocious foraging (Fluri *et al.*, 1982; Huang *et al.*, 1991, 1994; Jaycox, 1976; Jaycox *et a.*, 1974; Robinson, 1985, 1987a, b; Robinson and Ratnieks, 1987; Sasagawa *et al.*, 1989). Individual behavioral development may be delayed, accelerated or reversed in response to changing internal and external colony environment correlated with JH titers (Robinson *et al.*, 1989; Huang and Robinson, 1992, 1995). Accelerated behavioral development (precocious foraging) may be induced as a response to a lack of older forager age bees, retarded behavioral (overage nurse bees) development may be a response to a lack of young bees, and behavioral reversion (from foraging to nursing behavior) may be induced in response to a deficit of young bees (Page *et al,*. 1989; Robinson *et al.*, 1992; Huang and Robinson, 1992; Robinson, 1992). Accelerated development is associated with a precocious rise in JH titers, retarded development with an extended duration of low JH titers, and behavioral reversion with a decrease in JH titers (Robinson *et al.*, 1992).

The regulation of age-related division of labor is enigmatic because in large colony species like the honey bee it is unlikely that an individual worker is capable of integrating all of the information on internal and external colony environments and the activities of all other workers and then adjusting her behavior accordingly. An "activator-inhibitor" model has been proposed suggesting that workers develop in response to localized stimuli within the colony, and worker development is mediated by interactions with the environment and other workers (Huang and Robinson, 1992). According to this model, there is an interaction between JH, designated as an inherent "activator" that promotes behavioral development, and

an "inhibitor", an unidentified factor(s) transferred among workers that suppresses development (Huang and Robinson, 1992). The activator-inhibitor model predicts that the presence of older forager-age workers will suppress JH titers and promote delayed behavioral development in young workers. The absence of older workers removes the "inhibitor", allowing biosynthesis of the JH "activator" which promotes precocious behavioral development.

Although the activator-inhibitor model hypothesizes that age at first foraging is modulated by a worker-produced inhibitor in the colony, other non-worker produced inhibitors may exist. For example, extracts of queen mandibular gland pheromone (QMP), and it's major component ODA inhibit the rate of JH biosynthesis (Kaatz *et al.*, 1992), that could induce a JH-based delay in worker foraging ontogeny. To date demonstrated queen effects on division of labor have been limited to a genetic contribution (Page *et al.*, 1989), despite known primer pheromone effects of queen mandibular gland pheromone on workers, such as the suppression of reproductive queen rearing (Pettis *et al.*, 1995; Winston and Slessor, 1992; Kaatz *et al.*, 1992). QMP also elicits a releaser-type behavioral response called retinue behavior, characterized by workers surrounding the queen or a QMP-dosed lure in an elliptical formation, antennating and removing QMP from the surface with their antennae, proboscides, and forelegs (Slessor *et al.*, 1988; Kaminski *et al.*, 1990). There is a genetic component to this worker retinue response to QMP, and strains of high and low QMP retinue responding strains have been reared for the purpose of examining the role of QMP in colony-level functioning and individual bee behavior (Chapters 1 and 2).

The main objective of our study was to examine the influence of QMP on worker foraging

ontogeny and JH titers. Our primary hypothesis was that workers reared in nest environments with supplemental QMP would display lower JH titers and later foraging ontogeny than in untreated colonies. In addition, we tested for a genetic basis to this hypothesized influence of QMP on JH and foraging. High and low QMP-retinue responding workers were used as "probes" to determine whether selection based on the retinue response to QMP conferred a correlated response to the proposed influence of QMP on foraging ontogeny and JH titers.

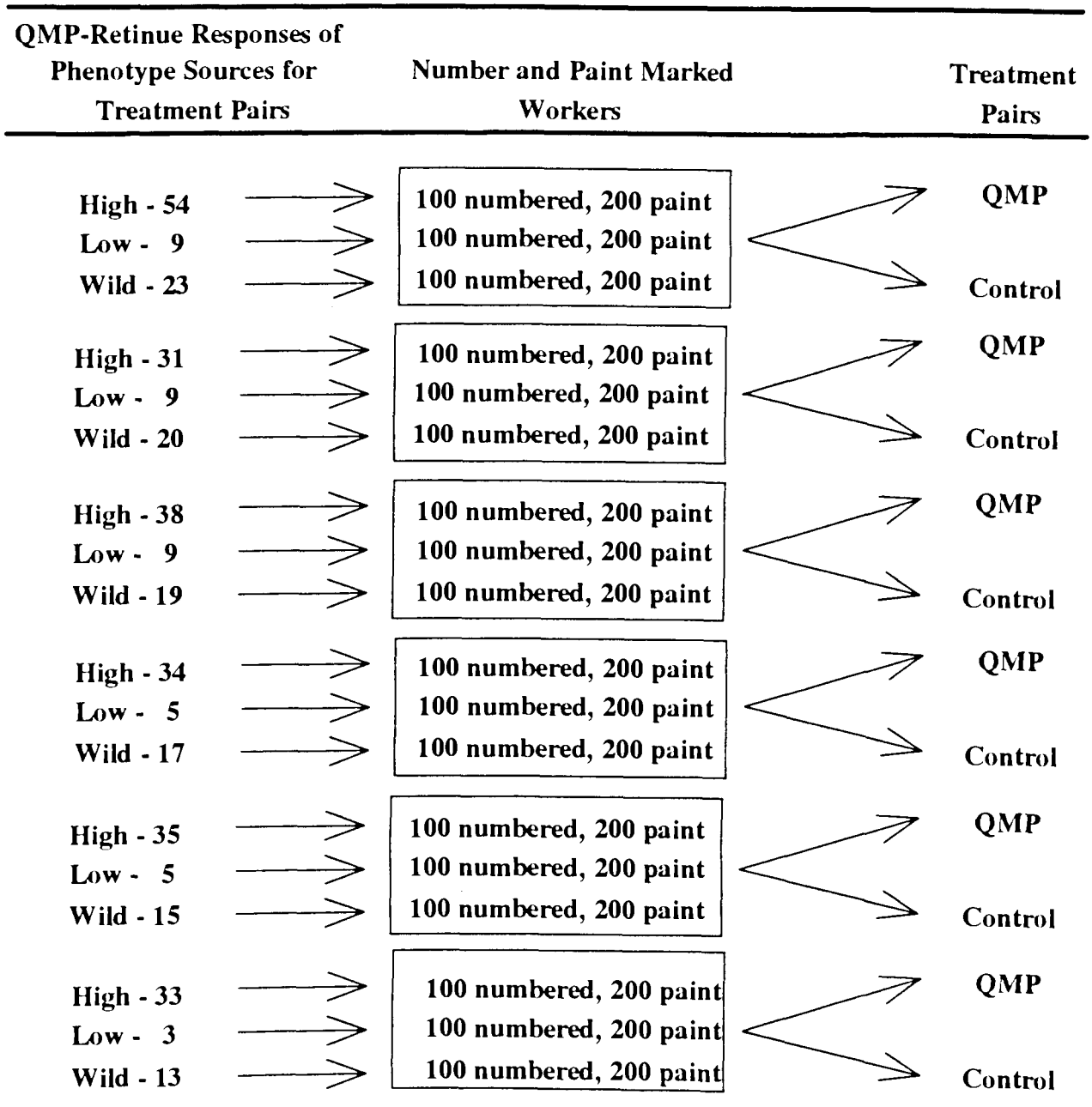
Materials and Methods

Bees

Worker honey bees were obtained from the third generation of "high"- and "low"-QMP retinue responding strains from stocks originally selected in 1990 (Chapter 2). "Wild-type" refers to Simon Fraser University stock colonies headed by open mated queens, therefore worker relatedness was variable. Colony-level QMP-retinue response for all phenotype sources was measured two days prior to introducing workers to experimental treatments (Fig. 24). Means and standard errors of QMP-retinue responses were as follows: X_{High}=37.5 ± 3.4, X_{Low}=6.6 ± 1.1, X_{Wild}=17.8 ± 1.5.

Bees of known age were obtained by removing combs containing developing pupae from pre-selected single-patriline high- and low-QMP responding phenotypes, and "wild-type" colonies (Fig. 24). The combs were placed in an incubator maintained at 33°C for a

Figure 24. Experimental design. Known-age cohorts of number tagged and paint-marked workers were introduced to either QMP treated or control colonies. QMP and control colonies were paired to share phenotype sources. A total of 18 colonies representing high, low or wild-type QMP-retinue responding phenotypes were the sources of introduced bees. High and low QMP-retinue responding sources were obtained from the third generation of selected strains, and worker relatedness within colonies averaged 0.75. Wild-type workers were from open mated SFU stock colonies with variable relatedness.



24 hr emergence period. The newly emerging adults (N=100 per phenotype per colony: grand total of 3,600) were marked with colored and numbered tags, a different color for each of the 3 phenotypes. An additional 200 per phenotype per colony (N=7,200) of newly emerged workers were paint-marked with different colors to designate phenotype (Fig. 24). Sampling without replacement would reduce the number tagged workers being observed for foraging ontogeny, therefore two marking methods were used, number tagging for foraging ontogeny, and paint marking for JH titer measurement.

All marked workers were introduced to QMP treated and control 4-frame colonies equalized for adult numbers (approx. 10,000), brood area (2 frames), and food stores (2 frames). Treatments were replicated 6 times with different worker phenotype sources for each pairing (Fig. 24). Number tagging and introduction of newly emerged workers into respective treatment and control colonies were staggered equally over 3 days from 8 to 10 July, 1993. The experiment was terminated when all number tagged workers had foraged or could no longer be found in the colony.

Treatments

QMP-treatment colonies received a glass microscope slide daily loaded with one queen equivalent of QMP (Slessor *et al.*, 1990) in 10 μL 2-propanol, and centrally placed on the wax comb frame tops of each colony. The amount of QMP used in this study was within the median range of the distribution of QMP extracted from the mandibular glands of mated queens. The amount of ODA may range from 0.2 to 1.8 times the queen equivalent

used here, so the additional amount of QMP that was added was well within what could be expected to be naturally available to colonies. Control-treatment colonies received glass microscope slides daily loaded with 10 μL of 2-propanol.

Sampling Scheme

One worker from each phenotype (high, low, and wild-type) was collected randomly from each frame face of the four frames in the hive. A total of 8 workers per phenotype per colony per treatment was collected and placed in a Ziploc® plastic bag, and stored temporarily in a container of ice. In the laboratory, five workers per phenotype per colony per treatment were used to obtain blood (hemolymph) samples. The additional 3 workers served as back-up workers in the event of a blood collection mishap. The sampling regime, 0, 7, 14, 21, and 28 days after emergence was chosen to follow temporal polyethic progression from nursing to older forager tasks, as established in previous studies (reviewed in Robinson, 1992). Collections took place prior to 10 a.m. to ensure against excluding the collection of any workers that may be outside the nest. During the course of this experiment no foraging took place before 10 a.m.

Measurement of JH titer

After collection, bees were immobilized on ice for 20 min - 1 h until hemolymph was taken. Hemolymph was removed from each bee using a 5 μL Drummond Wiretrol® #5-00-

1005 microcapillary tube, measured to the nearest 0.5mm, and stored in 500 μl acetonitrile. Individual hemolymph samples were stored at -70°C.

A chiral-specific radioimmunoassay (RIA) (Hunnicutt *et al.*, 1989) was used to measure the JH III titer in the sampled hemolymph. JH III is the only JH homolog found in honey bees (Hagenguth and Rembold, 1978). This assay has been validated for adult worker honey bees by Huang *et al.* (1994). Previous results (Huang *et al.*, 1994; Huang and Robinson, 1995) indicate that values from this RIA agree with values obtained with both the Strambi and Goodman JH RIA's, both of which have been validated with gas chromatography/mass spectroscopy (de Kort *et al.*, 1985; Goodman *et al.*, 1990).

Hemolymph may become contaminated with sucrose if the midgut is punctured during hemolymph collection. Sucrose contamination would be reflected as a greater than actual volume of hemolymph resulting in JH titer determination errors. Therefore, sucrose contamination was determined using techniques described by Woodring *et al.* (1993). Seven randomly selected samples of hemolymph were run with standards for 4 sugars (fructose, glucose, trehalose, and sucrose), and no sucrose was detected.

All solvents used were HPLC grade, obtained from either EM Science, Fisher Scientific, or J.T. Baxter Chemical Co. Glassware was baked at 500°C for 3.5 h prior to use to minimized JH absorption (Strambi *et al.*, 1981).

Random vs Selected Bee Collection for Measurement of JH Titer

This experiment was performed because levels of JH in this study were lower than in

other studies which used the same JH RIA (Huang *et al.,* 1994; Huang and Robinson, 1995). We wanted to determine whether JH titer and hemolymph volumes could be biased due to within or outside nest collection among same age bees.

Into four 4-frame colonies (approx. 10,000 workers) 100 paint-marked, newly emerged workers were introduced (as above). Twenty-eight days later 10 workers per colony were collected randomly from within the colony (as above) in the morning before foraging began, and another 10 per colony were collected as returning foragers at the colony entrances (as per Huang *et al.,* 1994). Hemolymph collection and JH analysis were performed as above.

Behavioral Measurements

To measure number tagged forager activity, entrance counts were conducted. Hive entrances were partially blocked with wide mesh screen to slow forager entry into the colony, allowing the observer to distinguish the identity and type of forager. Measurements were taken for 5-minute intervals, four times daily, twice in the morning and twice in the afternoon, beginning 5 days after worker introductions. The number tag worker identities were called out and tape recorded or immediately recorded by another person. Observers were not informed of the treatment or marked worker codes nor were they aware of hypothesized predictions.

Colony entrances were blocked completely twice daily, once in the morning and once in the afternoon for 15 minutes after entrance counts were completed. The identity of the

number tagged workers at blocked entrances was recorded, as well as forager type. Type of forager was determined by the presence or absence of pollen on corbiculae. The date of the first foraging trip was considered to be the date that a marked worker was first seen outside the hive during entrance observations. While a small number of these first flights may have been orientation rather than foraging flights, previous studies have shown that error because of this factor is insignificant (Winston and Katz, 1982; Winston and Punnett, 1982).

Census data were collected to correct for differential mortality for the purposes of statistical analysis. Censuses were conducted on the 1st,15th, and 35th day after worker introductions to determine whether possible treatment or phenotypic differences were based on differential mortality among the focal bees. Censuses were conducted by scanning each face of each frame, as well as the interior of the hive body. The identification of number tagged workers was recorded.

Statistical Analyses

All results are presented as means and standard errors.

Foraging Ontogeny - Chi-square analysis was performed on percentage data adjusted for mortality among phenotypes within colonies to determine differences among phenotype and treatment classifications (Sokal and Rohlf, 1981).

Foraging Activity - Data were log transformed prior to an analysis of variance test (Steel and Torrie, 1980).

JH Titer Measurements - To increase precision for comparing the average effects

of treatments on phenotypes and interactions, the data were analysed based on an split plot design with two error terms based on the following degrees of freedom sources 1) pairs X treatments, and 2) pairs[(phenotypes)(treatments X phenotypes)] (Steel and Torrie, 1980). Differences in JH titer among randomly in-hive selected and behaviorally selected workers was determined by analysis of variance (Steel and Torrie, 1980).

RESULTS

Foraging Ontogeny

Exposing workers to colony environments with supplemental QMP, which we predicted would delay foraging ontogeny, did result in delayed foraging (Fig. 25a). Workers in control colonies were significantly precocious foragers compared to QMP treatment, foraging an average of 2.7 days earlier (χ^2=4.3, df=1, $P < 0.05$) (Fig. 25a). There were no differences (χ^2=1.3, df=2, $P > 0.05$) between the QMP-responding phenotypes within treatments (Fig. 25b), so data within treatments were pooled for analysis (Fig. 25a).

Foraging Activity

The number of workers returning from foraging flights was significantly greater (F=5.3, df=1, $P < 0.05$) in control colonies, 2.0 ± 0.09/ 5 min, than QMP treated colonies 1.4 ± 0.014/ 5 min. There were no differences within treatments between high, low or wild-type

Figure 25 a)

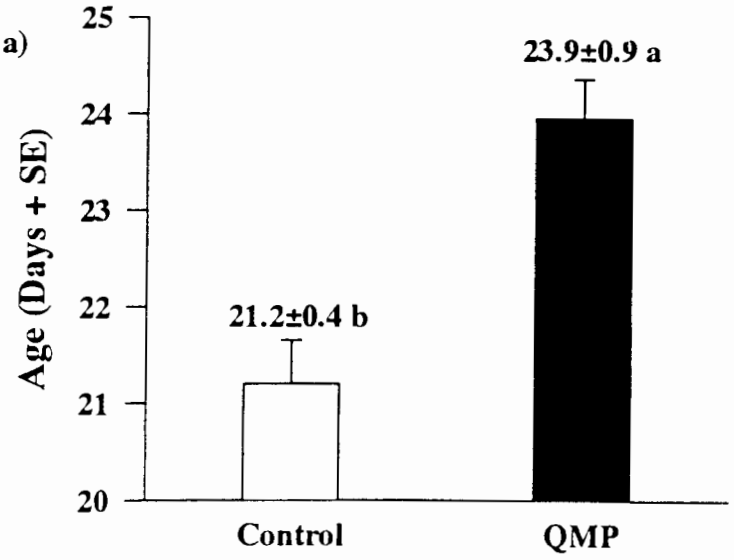


Figure 25 b)

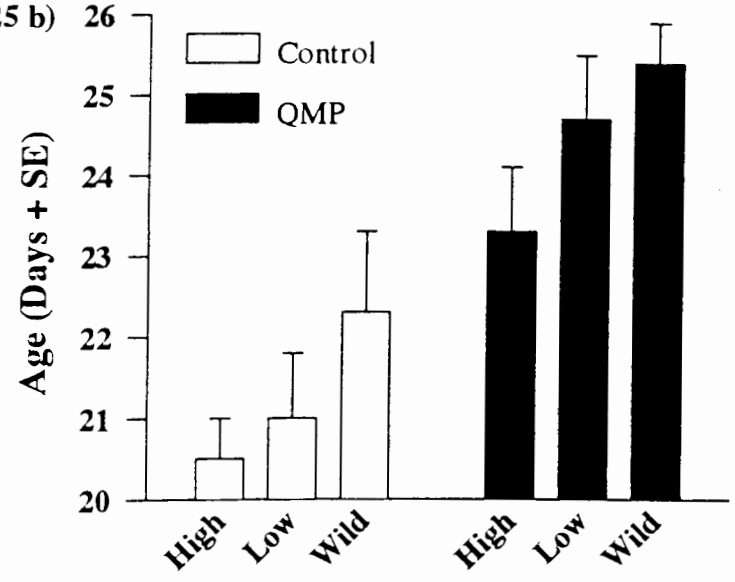


Figure 25. a) Pooled mean foraging age of control and QMP-treated colonies. Control workers (N=6 colonies) began foraging at significantly younger ages than QMP-treated (N=6 colonies, $P < 0.05$). **b)** Mean foraging age of number-tagged workers in control and treatment colonies. The bars represent mean ages of high, low, and "wild-type" QMP-responding phenotypes (N=6 colonies). There was no significant difference among phenotypes within treatments ($P>0.05$).

QMP-responding phenotypes (F=3.1, df=2, P>0.05).

JH Titers

Split plot analysis of variance indicated there were significant differences among treatments (F=6.21, df=1, P<0.01), no differences among phenotypes (F=1.44, df=2, P>0.24), and no treatment x phenotype interaction (F=2.19, df=2, P>0.11) (Fig. 26a, b).

Further analysis of variance using means of colony-level means (i.e. treatment N=6 colonies), pooling phenotypes (because there was no phenotype effect from split plot analysis of variance), indicated an overall treatment effect (F=6.40, df=1, P<0.01). T-test analysis of variance to examine treatment differences on days 0, 7, 14, 21, and 28 individually on pooled means (i.e. means of colony means, N=6), indicated that day 14 contributed the most to treatment differences (T=74, df=1, P>0.0001) (Fig. 26c).

Random vs Selected Bee Collection Method

There were no differences in JH titers between 28-day old workers collected inside the colony (148.8 ± 15.9 ng/ml), and returning foragers (157.0 ± 124.4) (F=0.08, df=1, P>0.78). There also were no differences in blood volume collected between workers collected in the hive (3.8 ± 0.2 μl), and returning foragers (3.9 ± 0.3 μl) (F=0.09, df=1, P>0.76). These results indicate that there is no blood volume - worker location relationship. Although JH titer is slightly higher among returning foragers, this factor was insignificant,

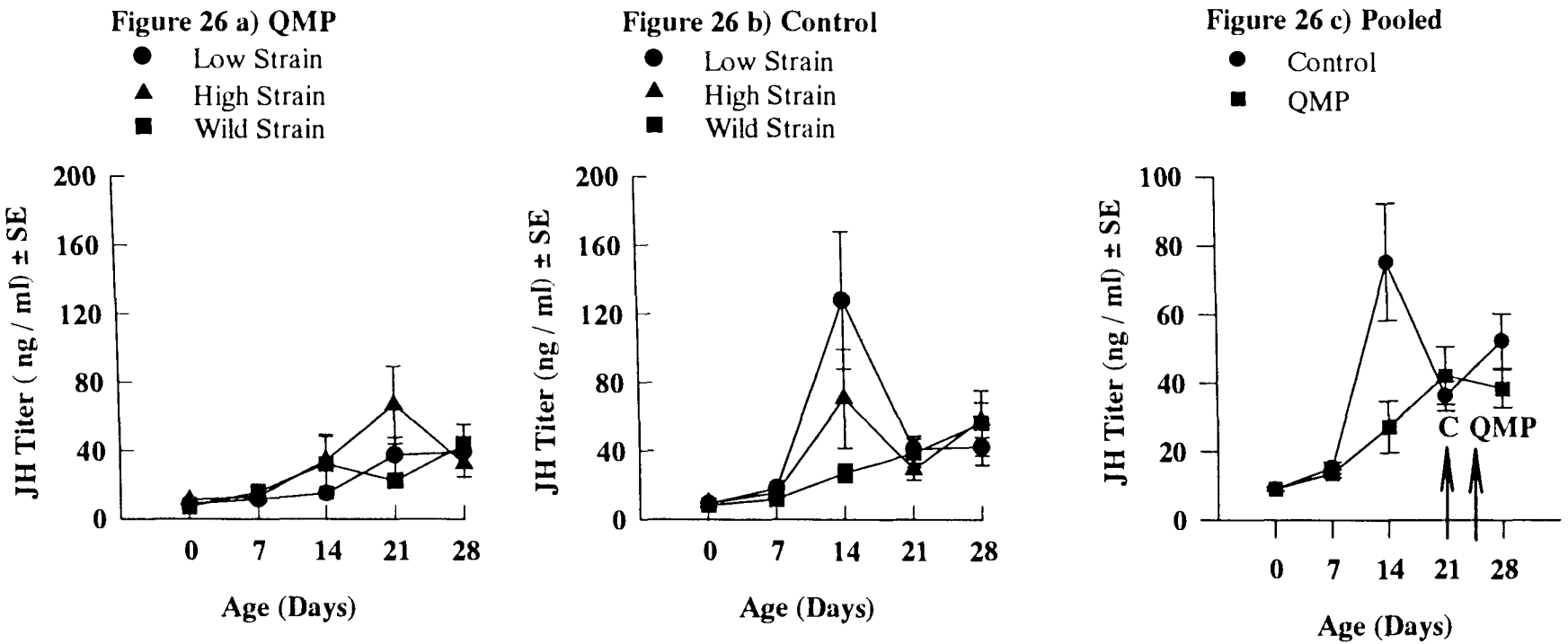


Figure 26. Worker honey bee juvenile hormone (JH) titers. **a)** Mean JH titers of high, low and "wild-type" QMP-responding worker phenotypes (N=5bees/phenotype/day) in QMP-treated colonies (N=6). There were no significant differences among phenotypes (P>0.05). **b)** Mean JH titers of high, low and "wild-type" QMP-responding phenotypes (N=5 bees/phenotype/day) in control colonies (N=6). There were no differences among phenotypes. (P>0.05). **c)** Pooled JH titers for worker honey bees in control (N=6) and QMP-treated (N=6) colonies. Control JH titers were significantly greater than (P<0.01) than QMP-treated titers. Day 14 contributed most (P<0.0001) to the overall difference. Arrows with **C** and **QMP** indicated mean foraging age for control and QMP-treated workers respectively.

validating the in-hive random collection methodology used in this study.

DISCUSSION

These results provide the first demonstration that a social insect queen pheromone, in this case honey bee QMP, acts as a modulator for division of labor. Evidently, QMP can influence foraging ontogeny by inhibiting JH titers. QMP delayed the age at which foraging began, and associated with this delayed foraging ontogeny were lower JH titers among QMP-treated colonies compared to control colonies. The higher mean foraging rate by control workers that resulted from this lower mean foraging age produced a greater accumulation of foragers over time than in QMP colonies (Fig. 27, see discussion below). Finally, we found no genetic basis to QMP inhibition of JH titers and foraging ontogeny.

These findings expand the activator-inhibitor model of division of labor (Huang and Robinson, 1992) to include queen effects in addition to worker factors. This model, and the experimental results that support it, suggest that forager-age workers suppress JH levels and temporal caste development of young workers. The removal of old workers removes this "inhibitor", allowing the biosynthesis of the "activator" JH and prompting precocious behavioral development (Huang and Robinson, 1992). However, QMP also inhibits JH levels and behavioral development, indicating that caste ontogeny is mediated by at least two parallel systems, queen pheromone and feedback from colony demographic factors.

QMP functions to moderate the rate at which workers progress from within to outside nest tasks. A modulator such as QMP may impose a labor schedule that promotes efficiency

Figure 27 a) Control

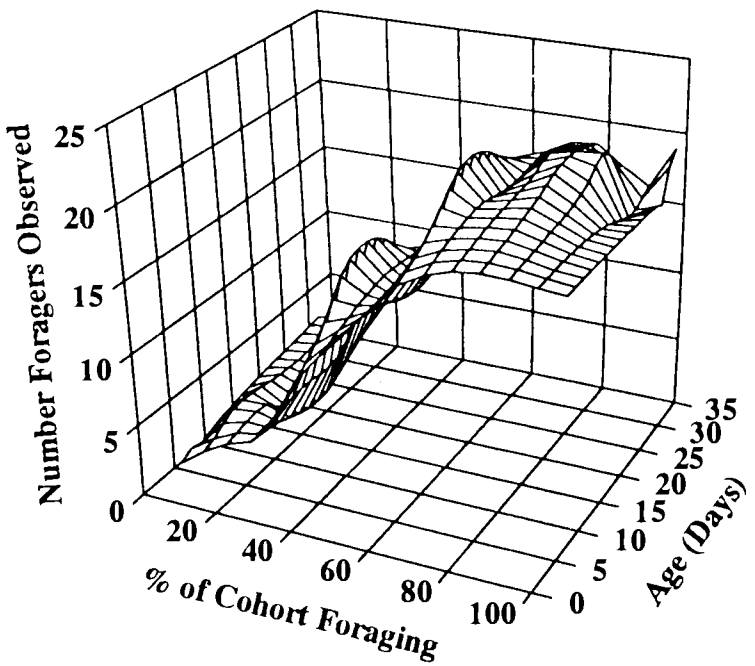


Figure 27 b) QMP-Treated

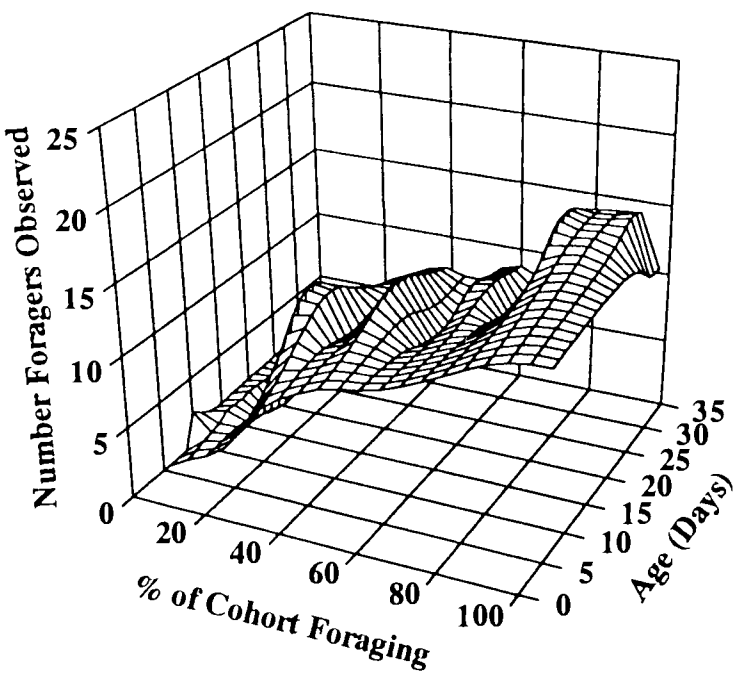


Figure 27. Foraging activity of control (**a**), and (**b**) QMP-treated high, low, and "wild-type" QMP-responding worker phenotypes. The mean number of foragers observed represents the total number of number-tagged foragers on days foraging took place. An individual was counted only once, and any multiple observations of a single individual were eliminated.

in task performance by physiologically delaying a shift to the next age caste. QMP also may lengthen transition phases from age caste to age caste, resulting in worker response thresholds that overlap castes, thereby producing a worker generalist capable of behavioral plasticity. In addition, the presence of this secondary queen-based inhibitor would prevent a colony's young workers from developing too rapidly towards foraging tasks in the event of a sudden loss of older workers due to predation, rapid weather changes or other factors.

QMP may be most influential in suppressing JH titers and foraging in small rather than large colonies because the source (queen) is more accessible. QMP is most effective in inhibiting queen rearing activities among unpopulous versus populous colonies (Winston *et al.*, 1991), and QMP is detectable in a smaller proportion of workers in populous versus unpopulous colonies (Naumann *et al.* 1993). Although there is no link between the two known QMP primer effects, swarming suppression and delayed foraging ontogeny, it seems reasonable to assume that QMP transmission mechanisms would be similar for both. Thus, the QMP effects we found here may diminish as colonies become more populous, and the worker-based inhibitory factors might become more significant. Studies of QMP and worker inhibitory factors in colonies of different population and age structures would be useful to further explore this subject.

We examined how control and QMP-treatments affected the ergonomic structure of the number-tagged cohorts by plotting three variables, worker age, percent of cohort foraging, and the number of foragers observed (Fig. 27). Control workers (Fig. 27a) began foraging at significantly younger ages (*X*-axis) than QMP-treated workers (see also Fig 25a). As a result the control cohort began to shift earlier toward foraging tasks, expressed as

percent of the cohort foraging (*Y*-axis). As a greater percentage of the cohort foraged, the mean number of foragers observed increases (*Z*-axis). This plot demonstrates that a small change in the task performance of a cohort can induce a large shift in ergonomic structure. QMP-treated workers began foraging at older ages, the cohort did not shift to foraging tasks as quickly as the control workers, and the number of foragers observed was lower compared with controls until a greater percentage of the cohort began foraging (Fig. 27b), thereby indicating how a QMP-induced shift in age of first foraging can have profound implications for colony foraging structure.

The JH titers in this experiment were low compared with other studies using bees of similar age and behavioral development (Huang *et al.*, 1991; Huang *et al.*, 1994). These low values do not appear to be an artifact of our sampling procedure, because in-hive and forager samples were similar. Also, the drop in JH levels following the single JH peak observed on day 14 was unusual. This peak JH titer on day 14 (Fig. 26c) may have "set" control workers to a physiological foraging state predisposing relatively earlier foraging (Huang and Robinson, 1995). In contrast, a comparable JH peak was not observed among QMP treated workers, but rather we observed a slower, continuous increase (Fig 26c).

Cool (average night temp. 8°C, average A.M. temp. 15°C, average P.M. temp. 19°C), rainy conditions interspersed with brief warm sunny weather prevailed throughout the duration of this experiment and may have contributed significantly to the low JH titers observed in this experiment. During seasonally cool and cold periods, or experimentally induced cold in which foragers are less or inactive, JH titers decline to pre-foraging levels (Huang and Robinson, 1995). Decreases in JH in the fall, and increases in JH in the spring

may be attributed to seasonal decreases and increases in temperature as suggested by Bühler *et al.* (1983). Characteristically, winter foragers have low JH titers (Fluri *et al.*, 1977), associated with prolonged longevity. Despite low JH titers and extended longevity, winter foragers generally don't survive to the spring (Huang and Robinson, 1995). However, summer foragers presented with adverse climatic conditions must be capable of taking advantage of brief periods of favorable weather, and then possibly waiting long periods for another foraging opportunity. The functional significance of low JH titers and low metabolic rates (Huang *et al.,* 1994; Harrison, 1986) in summer foragers may induce greater longevity and lower consumption rates of limited resources. This behavioral and physiological plasticity in JH levels and foraging ages may be principal factors in honey bee ecological success.

QMP retinue response phenotypes (high, low, and wild-type) did not respond significantly differently to QMP primer influences on ontogeny of foraging and JH titers. Evidently the QMP-induced releaser retinue response does not confer a correlated response to this primer influence, and the primer does not appear to have a similar genetically variable nature, at least in the strains we tested. No genetic component was found in QMP-based suppression of queen rearing between Africanized and European bees (Pettis *et al.*, 1995), suggesting that QMP primer effects may generally be quantitatively similar between honey bee races or populations.

In summary, QMP delays foraging ontogeny in worker honey bees, associated with lower JH titers. QMP should be included with worker genotype (Page and Robinson, 1991; Giray and Robinson, 1994), and environment (Robinson, 1992) as a factor that influences

temporal polyethism. The integration of QMP (and perhaps other honey bee pheromones such as worker or brood pheromones), genetics, and environment into the activator-inhibitor model for the plasticity of division of labor will undoubtedly lead to a refinement in our understanding of the mechanisms regulating temporal polyethism.

SUMMARY

Releaser and primer influences of queen mandibular pheromone on workers were examined in this study. Variation in QMP retinue response was due primarily to genotype. Strain dependent differences were maintained over a wide range of dosages, were not an artifact of the synthetic blend, worker age or rearing environment, and there was no correlation between queen pheromone production and worker response. There was a seasonal component to response observed in high QMP-responding workers distinguished by a low response in the autumn. Similar pheromone quantities were extracted from queens originating from different sources, suggesting that mandibular production is not strongly influenced by genotype. Comparisons between European and Africanized queens revealed some racial differences, but, these differences would not be reliable for classifying individual queens according to race. The wide variation in the quantity of QMP components suggests a generic signal indicating the queen is present. Much of the observed variability can be attributed to gland substrate uptake, enzyme levels, and reaction volumes in individual queen glands (Plettner, 1995).

More needs to be known about the biological and behavioral influences of the individual components before suggestions can be made concerning the relevance of the total or relative presence of any component in the blend. However, ontogenetic and reproductive states are apparent through component proportion shifts. Virgin queen glands produce QMP with a high proportion of ODA, mated queens produce QMP with approximately equal proportions of ODA and 9-HDA, and drone-laying queens produce proportions that are

intermediate, reflecting their intermediate reproductive status. A quantitative definition of a "queen equivalent" of QMP was proposed for the various queen types, and a standard queen equivalent for mated European honey bee queen mandibular gland pheromone is to be 200 μg 9-ODA, 80 μg 9-HDA, 20 μg HOB, and 2 μg HVA.

QMP components from the body surfaces of European and Africanized mated and virgin queens were highly variable and not correlated to quantities found in individual glands, Body surface QMP was approximately 10^{-3} found in glands.

Strain-dependent QMP retinue response in the laboratory did not confer retinue response to a live queen in the hive. Nevertheless, differential queen attendance among worker sources suggested that there may be a genetic basis for queen attendance. Other cues may be involved in retinue response such as queen movement, texture, and queen odors alone or in combination with QMP. Phenotypic differences in queen attendance behavior is puzzling. Presumably phenotypes with a greater tendency to attend the queen are more likely to be exposed to QMP. Whether these phenotypes are differentially removing, circulating, and/or affected by QMP, is unknown. A more thorough examination of behaviors and fate of workers following queen attendance might elucidate the significance of retinue behavior.

Response to QMP is related to another QMP effect, queen rearing activity. Worker honey bees selected on the basis of high retinue response to QMP in the laboratory bioassay were significantly more likely to be engaged in queen rearing activities than were workers with a low retinue response to QMP. High response workers spent proportionately more time working on and in queen cells than low response workers, and there were significant

age by response effects for time spent rearing queen cells. No interindividual differences were detected among the QMP-response phenotypes in the tendency to rear queens meaning that phenotypic differences in queen rearing were not a result of individual specialization, but rather demonstrated a phenotype-based differential tendency to rear queens. Results from this experiment suggest that QMP response may be a mechanism upon which colony-level selection acts for cooperative division of queen rearing labor. For high-responding strains, releaser effect of QMP retinue response is coupled with the primer function queen rearing. The response sequence to the absence of the queen may occur in the following order: 1) the absence of the queen is perceived, 2) there is an endocrine-based physiological response to the released queen-rearing inhibition, possibly by a neurohormonal-stimulated increase in gland food production, and 3) there is a behavioral response expressed as queen rearing activities.

This study also provided the first demonstration that a social insect pheromone (QMP) acts to modulate division of labor. QMP influences worker foraging ontogeny by inhibiting JH titers thereby, delaying the age at which workers begin foraging. The overall effect on the ergonomic structure of the colony is indicative of how small changes in worker physiology can rapidly shift a cohort from intra- to extra-hive tasks. High and low retinue response phenotypes did not respond differently from each other or from wild-type phenotypes to this primer influence of QMP. Thus, QMP-induced releaser response does not confer a correlated response to foraging ontogeny and JH titers, at least in the strains tested.

The releaser-primer relationship of QMP has functional advantages with respect to

queen presence and rearing. Those phenotypes most responsive to QMP have a low threshold of response for the absence of QMP, expressed as greater queen rearing activity. This relationship appears to be directly related to continuously monitoring queen presence, and to act quickly in the absence of the queen ensuring colony survival by rearing a new queen.

QMP retinue response does not appear to be associated with QMP influences on JH titer and foraging ontogeny, likely because division of labor is influenced by a hierarchy of factors in a variety of social and extra-colonial contexts. The activator-inhibitor model suggests that workers also can influence division of labor (Huang and Robinson, 1995), and worker mandibular glands produce a number of components that could act as primer pheromones (Plettner, 1995). QMP may be used to modulate division of labor in much the same way that forage-age workers inhibit young workers from becoming foragers, such that the primer influence is used to regulate ergonomic structure.

QMP is efficiently used for at least two primer functions in colonies, the inhibition of queen rearing and regulation of worker temporal division of labor. Additional functions of this queen substance will undoubtedly be elucidated by future studies.

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