Ontogenetic and Individual Patterns of Volatiles in Honeybee Queens *Apis mellifera* and its Significance for the Acceptance of Queens in Honeybee Colonies

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Dedicated

to my father who passed away
before witnessing my achievement

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List of Abbreviation

QMP	Queen Mandibular Pheromone
9-ODA	9-keto-2-decenoic acid
9-HDA	9 (S) - hydroxy-2-(E)-decenoic acid
HOB	methyl p-hydroxybenzoate
HVA	4-hydroxy-3-methoxyphenylethanol
mm	Millimeter
µl	Microliter
CI	Confidence Interval
SD	Standard Deviation
CS+	Positive Conditioning Stimulus
CS-	Negative Conditioning Stimulus
ng	Nanogram
µg	Microgram
Kpa	Kilopascal
GC	Gas Chromatography
GC-MS	Gas Chromatography Mass Spectrometer
Min	Minute
Sec	Second
Hoh	Hohenheim
Aul	Aulendorf

1. General Introduction

Honeybees *Apis mellifera* L. are living as an integrated entity where individuals live and work according to the needs of the whole society (Winston, 1992). There are three types of individuals in a honeybee colony: the queen (the only egg-laying individual), 10-40 thousands non-reproductive workers and a few thousands drones. Each of the two female castes and the drones have their own distinctive role to perform in the honey bee society (Moritz & Southwick, 1992). Workers perform different tasks inside and outside the colony and the division of their labor is normally achieved via age polytheism but it can be adapted due to the external factors and the current need of the colony (Gary, 1992).

There is no central nervous system to coordinate the activities of the superorganism (Southwick, 1992). Instead, there is a fascinating regulatory system based on an effective communication network, where the queen is not only an egg-laying machine, but via its pheromones she plays a central role in this network (Winston, 1992). Life of a honey bee colony is much related to the queen; the presence of the queen maintains colony cohesion and stability. A close and regular contact and exchange of information exists between the members of the colony, especially between workers and between workers and queen (Simpson, 1979). These contacts include trophallaxis (food exchange), pheromone exchange by licking (Moritz & Southwick, 1992) and even acoustic communication through the “dancing language” (Seeley & Visscher, 2004). The workers exchange information concerning food sources, food availability in the hive (Naumann & Winston, 1992), kin recognition (Page, 1986), enemies, diseases, and presence of the queen (Oldroyed & Hunt, 1990). The queen produces pheromones which influence most of the activities of the colony. Through the primer effect of pheromones, the queen suppresses the reproduction of workers (Naumann, 1991), regulates juvenile hormone biosynthesis in workers (Kaatz *et al*. 1992) which, in turn, regulates workers’ division of labor (Lin *et al.* 2004). The pheromones releaser effects elicit retinue behavior (Wossler & Crewe, 1999b). The main queen pheromone is the mandibular gland pheromone (QMP) which is eliciting behavioral and physiological responses of bees and is important for the social organization (Engels *et al*. 1997). QMP is a five-component blend consisting of (E)-9-keto-2-decenoic acid (9ODA); the main component, 9 (R) - and 9 (S) - hydroxy-2-(E)-decenoic acid (9HDA), methyl p-hydroxybenzoate (HOB),

and 4-hydroxy-3-methoxyphenylethanol (HVA) (Callow *et al*. 1964, Slessor & Winston 1995). This blend may vary in the proportions of individual compounds but all five components are necessary to elicit the full range of worker responses to QMP (Moritz & Crewe, 1991). In addition to the QMP, the tergal gland secretions are considered as main semiochemicals which are released by the queen and spread on the cuticular body surface to evoke retinue behavior (Wossler & Crewe, 1999b), and as the secondary source of queen pheromones other than the mandibular glands secretions (Moritz & Crewe, 1988a). In addition, another semicochemical attraction system is located in the queen's head but not in the mandibular gland (Slessor *et al*., 1995). The natural acquisition of pheromonal compounds from the cuticular body surface of the queen is performed by antennating and licking of retinue workers which are functioning as messengers distributing the queen's pheromones within the colony (Engels *et al.*, 1993).

The pheromonal compounds of queens rapidly change in their quality and quantity after mating (Engels *et al*. 1997, Vaitkeviciene et al. 2006). Physiological changes are linked to the act of mating. Mated queens have bigger ovaries and are heavier in weight than virgin queens (Shehata & Townsend 1981). These changes are related to the role of the queens which is for virgin queens to attract drones for mating, while the task of mated egg-laying queens is to stabilize the colony (Apogaite & Skirkevieius, 1995).

Individual recognition mechanisms exist between queens and usually lead to fight until only one is surviving (Pflugfelder & Koeniger, 2003). Furthermore, virgin queens can recognize queen cells and selectively destroy the older queens' cells (Harano & Obara, 2004). There is also individual queen recognition by workers. To understand the mechanism of queen recognition and acceptance by workers, queen-workers interaction relations were investigated with focusing on queen's pheromonal compounds. For instance, the substances involved in the recognition of queen cells by workers were identified (Le Conte *et al.* 1995), the volatile compounds in virgin and mated egg-laying queens could be compared (Apesgaite & Skirkevius, 1995), and the volatile compounds present in both queens and workers were detected (Gilley *et al.* 2006). However, it is still not clear if the substance/substances responsible for queen recognition by workers are 9-ODA, tergite gland's secretion or the cuticular hydrocarbons. The fact is that each of them plays a role in the complex interactions between the queen and workers.

Another aspect, which should be taken in consideration when investigating queen recognition, is the genetic relationship among individuals. Page & Erickson (1980) found that recognition of queens appears to be associated with the genotype.

However, the fact that queens can be exchanged successfully demonstrates that workers are able to “learn” their queen. The cues for that “learning” are also unknown but it is obvious that a certain chemical pattern of the cuticle (odor or smack) is finally responsible for the recognition and acceptance as “own”. An interesting question is, whether the odorous patterns of the cuticle of different queens reflect the kin relation of these queens and whether worker bees are able to detect such kin-related differences. This would mean that worker bees accept a foreign queen which is closely related to their own queen better than a non-related one.

Herein, the objectives of this work are:

1. To have a better understanding of the bees’ behavior to “own” and “foreign” queens.
2. To evaluate the queen-workers interaction with the focus on the role of relatedness between “own” and “foreign” queens (sister, half sister and unrelated) and queen mating status (virgin – mated) in recognition and acceptance by honeybee workers.
3. To achieve these objectives, a specific bioassay is needed and should be established. This bioassay should reflect the complex social interactions between workers and queen and enable the observation of workers behavior to the queen.
4. To investigate the ability of bees to differentiate between queens through the application of the olfactory conditioning of the Proboscis Extension Reflex (PER) paradigm.
5. To investigate the relevance of queen kin and mating status to the volatile compounds through performing chemical analyses of queens’ body parts.

2. Honeybee Queen-Worker Interaction in a Bioassay

2.1. Introduction

The interaction between honeybee workers and their queen is ruled by pheromones of the queen which maintain the colony homeostasis (Engels, 1986) The main queen pheromone is the mandibular gland pheromone (QMP), which is eliciting behavioral and physiological responses of bees and is important for the social organization. In addition to the QMP, the tergal gland secretions are considered as the candidates for the semiochemicals, released by the queen and spread on the cuticular body surface, and as the secondary source of queen pheromones other than the mandibular glands secretions (Moritz & Crewe, 1988a). The natural acquisition of pheromonal compounds from the cuticular body surface of the queen is performed by antennating and licking of retinue workers which are functioning as messengers distributing the queen's pheromones within the colony (Engels *et al.*, 1993). As the mother of the colony and the only egg-layer, the queen suppresses reproduction of the workers through the primer effect of pheromones (Naumann, 1991; Kaatz, 1992; Beekman *et al*., 2004). The queen as the dominant parent manipulates its offspring to maximize its own fitness by transmitting her own genetic contribution to colony characteristics through her eggs (Alexander, 1974). Depending on the relatedness of the drone fathers, the common genes of the offspring range between 25-75% (Page *et al.* 1992). Due to this variation, it may not be easy to discriminate the kin recognition from the complicated nestmate recognition (Moritz & Crewe, 1988a). Clear individual recognition mechanisms exist between queens. This leads usually to fight until only one is surviving (Pflugfelder & Koeniger, 2003). Such contacts between different queens normally happen after swarming when 20-30 new queens are hatching within the colony. In addition, workers are clearly able to recognize their own queen. The mechanism for that recognition of "own" and "foreign" queen is not yet understood (Butz & Dietz, 1994). A consequence for the beekeeping practice is that beekeepers find difficulties to replace queens; it is difficult to verify that no other queen is existing in the hive (a queen may be overlooked). Besides, if the introduced queen is released from the cage too quickly after she is put into the hive, she may be killed (Leidlaw, 1992). In this case, the colony population can decline dramatically during queen replacement and for several weeks thereafter (Tarpy *et al*. 2000).

In this context, the main objectives of this chapter are:

1) To better understand the behavior of the bees to “own” and “foreign” queen.
2) To evaluate the queen-workers interaction with the focus on the role of relatedness between “own” and “foreign” queens (sister, half sister and unrelated) and queen mating status (virgin – mated) in recognition and acceptance by honeybee workers.
3) To achieve these objectives, a specific bioassay is needed and should be established. This bioassay should reflect the complex social interactions between workers and queen and enable the observation of workers behavior to the queen.

2.2. Materials and Methods

2.2.1. Location

All experiments were performed at the Apiculture State Institute (Landesanstalt für Bienenkunde) – University of Hohenheim in Stuttgart, Germany.

2.2.2. Queen Breeding

To obtain sister queens, inbred line brood combs were imported from Aulendorf (150 km to the south of Stuttgart) where the mother queen was artificially inseminated. The other source of brood combs was Heimsheim (near Stuttgart) where the mother queen was free mated. The third group of brood combs was from Hohenheim where the mother queen was free mated. One-day-old larvae from Aulendorf, Heimsheim and Hohenheim were grafted and put in plastic queen cups. The breeding frames were put in queenless colonies separately according to the source of larvae. 12 days later, 15 sister queens from Aulendorf, 12 half sisters (having the same mother) from Heimsheim and 20 half sisters (having the same mother) from Hohenheim were hatched. The queens from Hohenheim and Heimsheim served as unrelated to the sister queens. The queen breeding process was repeated to get the mated queens; 10 mated queens from Aulendorf, 10 mated queens from Heimsheim and 11 mated queens from Hohenheim were obtained.

2.2.3. Establishment of Nuclei Colonies in “Kirchhainer” Boxes

For the first set of tests, 15 mini colonies of the Kirchhainer type were prepared to receive the queens and honeybee workers.

Figure 2.1 Preparing the Kirchhainer boxes as nuclei colonies to receive honeybee workers and queens.

Sugar candy was used as a supporting source of feeding besides the collected nectar by foragers. After the queens had hatched, they were marked with small numbers and each one was put inside a Kirchhainer box with ~500 workers (half-full honey glass) from the breeding nest.

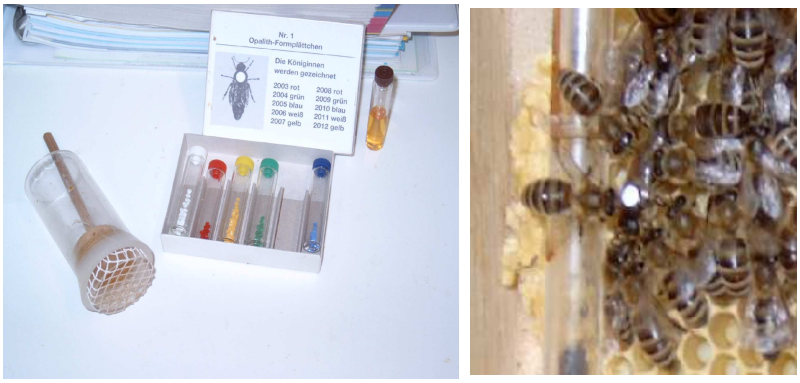

Figure 2.2 Marking of queens: Each queen was marked with a small number before putting her in the mini colony.

The established colonies were kept in darkness for 24 hours before setting them in their permanent location in the garden of the institute of apiculture. Two weeks later, the queens inside the mini colonies were laying eggs. At that point, the laboratory work started. The second set of tests required the preparation of 47 Kirchhainer boxes to receive the queens and bees where 16 of them were provided with a queen excluder covering the flight entrance to keep the queens unmated. The mated queens were used for the tests of the cage bioassay after starting laying eggs.

2.2.4. Development of the Bioassay: Petri Dish as a Primary Try of a Bioassay

A group of 30 workers with their own queen were put inside a Petri dish. A raw wax comb was used as a background. A small plate filled with sugar syrup was used for feeding. This simple bioassay was used to observe the interactions between workers-queen. A lot of disturbance happened during the replacement of queens. Therefore, it was not ideal for the aimed observation.

2.2.5. The Cage Bioassay

To obtain good observation conditions in the lab and avoid the disturbance occurred in the Petri dish, a small bee cage was used as the behavioral bioassay. The cage is used normally to test toxicity of pesticides on honeybees (Eppo, 1992). The box (13 ×12 × 6 cm) has a wooden frame and glass boards from both sides. To improve the conditions of observation and get an approximate situation to a normal honeybee colony, the design of the bioassay was modified by adding a wax comb, a feeding tube, and isolation boards to cover the cage from both wide sides and provide temperature isolation and darkness to the bees inside the bioassay when it is not in use.

Figure 2.3 The cage bioassay: A wooden box with the dimensions 13 ×12 × 6 cm provided with a piece of wax comb and a feeding tube. A glass board enables the observation of queen-workers interactions.

To perform a test in the lab, a queen and a group of about 30 of her own bees were taken from a Kirchhainer colony and put inside the cage bioassay. The feeding tube was filled with a mixture of sugar syrup and honey. The bees were left inside the cage over night to adapt to the new environment. The next day, the queens of two test-boxes were exchanged by introducing each one to the other cage bioassay through the upper hole of the cage. Workers' contacts behavior towards the foreign queen was observed and recorded. The contacts were divided into benign (antennating, touching with mandibles, forelegs, licking) and aggressive (Biting, stinging behavior). Each test lasted 125 minutes. This included 4 observations and 3 intervals in between. Each observation lasted five minutes. The intervals were for 15, 30 and 60 minutes respectively after the end of each observation. The queens were

returned back to their colonies after the test (24 hours period). 2 sets of introducing foreign queens were conducted:

2.2.5.1. Test of Foreign Mated Queens of two Different Kin Relations (Year 2005)

13 tests were conducted by using Hohenheim mated queens with kin relation (having the same mother) and 19 tests were conducted by using unrelated mated queens to the Hohenheim queens obtained from some beekeepers.

2.2.5.2. Tests of Foreign Queens of Defined Mating Status and three Different Kin Relations (Year 2006)

More details were taken into account in this set of tests; the queens were divided into two groups, namely, virgin and mated queens. Under each group there were 3 different kin relations between queens: sister queens, half sisters and unrelated queens. 20 tests were conducted for each kind of queens (virgin sister, virgin half sister, virgin unrelated, mated sister, mated half sister and mated unrelated). In total 120 tests were performed.

2.2.5.3. Tests of Queen's Extracts

Extra tests were performed by applying extracts of queen's head and abdomen over a living queen's abdomen and observing the contact behavior of workers to the own queen.

Extracting Protocol: A queen was partitioned into head, thorax and abdomen. Each part was put in a 1 mm flask with 0,4 mm pentane as solvent and kept in deep freezing (–25℃). 24 hours later, the extract was separated from the body part and kept again in deep freezing. Before applying the extract over a queen's abdomen in the cage bioassay, the flask was left open for a few hours in ambient temperature to allow the pentane to evaporate and reach the volume of 50 µl.

Application of Extracts: The process of preparing the test mentioned in paragraph 2.2.5 was repeated. The contact behavior of bees towards their queen has been observed for 10 minutes at the following situations:

- Without any application.
- Pentane application: 5 µl of pentane were dropped over the living queen's abdomen (Control).

- Extract application:

  1) Pentane extract of queen's head: 5 µl out of 50 µl extract amount were dropped over the living queen's abdomen.

  2) Pentane extract of queen's abdomen: 5 µl out of 50 µl extract amount were dropped over the living queen's abdomen.

The treated queen was immediately brought back to the cage bioassay. The observed and recorded contacts behavior of bees around the queen was divided into the following categories: Touching with antennae, with mandibles, with forelegs, licking, biting and stinging behavior. Two variations of queen equivalent in the extracts were used: The first variation was 0.1 queen equivalent. Number of observations was 44 including the control treatment with pentane and without any treatment. Observation time was 10 minutes. The second variation was 0.5 µl queen equivalent reached by evaporating the queen extract till 10 µl and dropping 5 µl of extract over the tested living queen. Number of observations was 32 including the control treatment with pentane only and without any treatment. Observation time was reduced to 5 minutes and repeated in different time intervals (15, 30 and 60 minutes respectively after the end of the previous observation). The extracts used in the tests were obtained from freshly hatched queens, two weeks old virgin queens, and mated egg-laying queens.

2.2.6. Statistical Analyses

Statistical tests were performed using SPSS software (Version 12). Non-parametric Mann-Whitney test and X^2- test were used.

2.3. Results

2.3.1. Introduction of Foreign Mated Queens of two Different Kin Relations (Year 2005)

When a foreign queen was introduced to the group of honey bees in the cage bioassay, different contact actions were performed by the workers. The contact behavior of workers were divided into “benign” (antennating, touching the queen’s body by forelegs and mandibles, and licking the queen’s body) and “aggressive” (biting and stinging the queen). Workers showed in general an aggressive reaction against the introduced foreign queens. However, there was significantly a stronger aggression against unrelated queens compared to the related queens (p=0.0004, Mann-Whitney test) and it was even fatal sometimes where the queen had not survived the aggression. The number of friendly contacts with the unrelated queens decreased significantly compared to the friendly behavior towards the related queens (p= 0.0004, Mann-Whitney test).

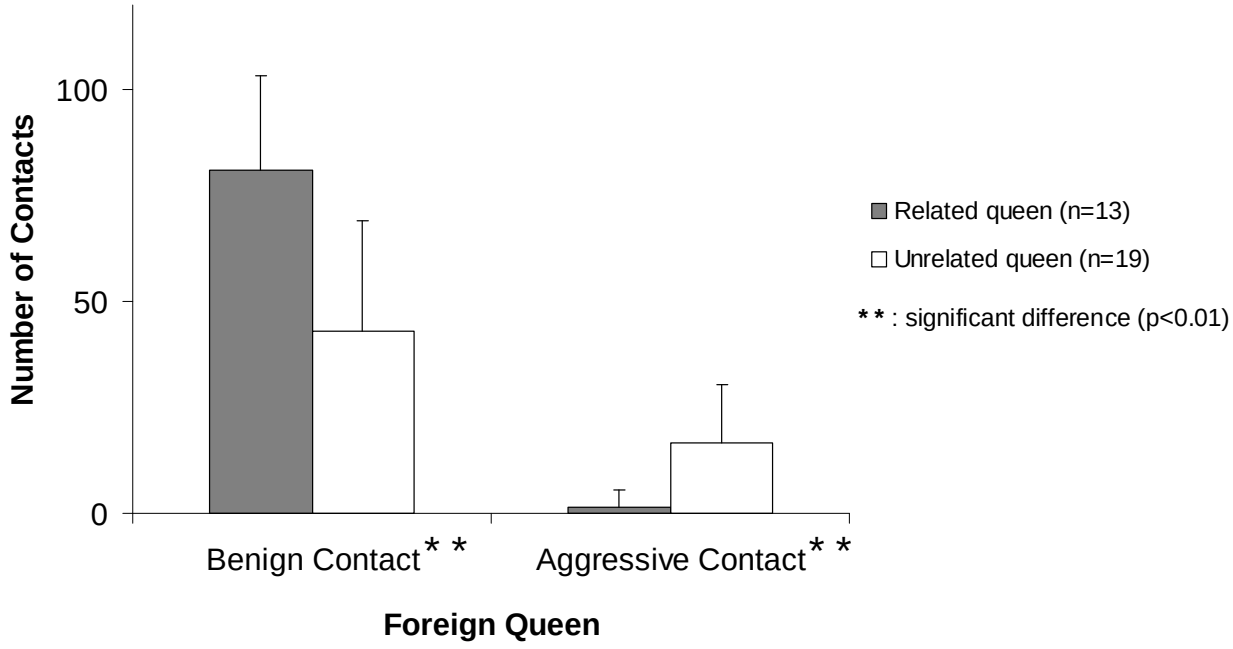

Figure 2.4 Average of total number of observed workers’ benign and aggressive contacts towards a foreign queen replaced their own queen inside a cage bioassay. 32 tests were performed by exchanging mated queens of two different kin relations to each other: related (having the same mother) and unrelated.

Related queens (13 half sisters) had all survived the aggressive behavior of workers. While 9 unrelated queens out of 19 had died. There was a significant difference in the survival rate of queens in respect to different kin relation ($p=0.003$, X^2 –test).

Each of the different contact behaviors of workers to the introduced foreign queen during the different time intervals was calculated and statistically analyzed. The number of friendly contacts of antennating and licking the unrelated queens significantly decreased compared to antennating and licking the related queens ($p< 0.05$, Mann-Whitney test) during the whole period of the test, while for the contacts with mandibles, the significant difference was only at the beginning of the test. Regarding the number of aggressive contacts, it increased significantly ($p< 0.05$, Mann-Whitney test) against unrelated queens compared to the number of aggressive contacts against related queens.

Table 2.1 Average number of workers' detailed reaction over time to a foreign queen replaced their own queen inside a cage bioassay. The numbers (0, 15, 30, 60) beside each contact behavior refer to the time interval prior to each observation. Exchanged queens had different kin relation to each other: 13 related queens having the same mother and 19 unrelated queens.

Contact behavior (in different time interval)	Related (n=13) Mean ±CI[1]		Unrelated (n=19) Mean ±CI[1]		P Mann-Whitney
Benign Contacts					
Antennae 0	7,1	1,6	2,6	1,5	0.001
Antennae 15	7,7	2	3,0	1,6	0.001
Antennae 30	6,7	1,7	3,4	1,9	0.008
Antennae 60	7,8	2,2	4,0	1,5	0.002
Mandibles 0	6,1	1,8	3,5	1,3	0.02
Mandibles 15	5,3	2	3,3	1,6	0.07
Mandibles 30	5,7	2,3	3,3	1,7	0.87
mandibles 60	5,0	1,6	3,6	1,7	0.13
Forelegs 0	4,5	1,2	3,2	1,2	0.15
Forelegs 15	3,7	1,4	1,9	1	0.03
Forelegs 30	3,3	1,4	1,8	1	0.07
Forelegs 60	3,0	1	3,0	0,9	0.88
Licking 0	5,6	2	2,2	1,2	0.005
Licking 15	4,5	1.4	1,3	1,0	0.000
Licking 30	2,4	1,0	1,1	1,0	0.02
Licking 60	2,5	0,9	1,9	1,3	0.08
Aggressive Contacts					
Biting 0	0,3	0,4	1,7	0,6	0.002
Biting 15	0	-	0,5	0,5	0.06
Biting 30	0,1	0,2	0,3	0,3	0.34
Biting 60	0,1	0,3	0,9	0,6	0.04
Stinging Behavior 0	0,2	0,4	3,4	1,6	0.002
Stinging Behavior 15	0,2	0,5	4,0	1,9	0.003
Stinging Behavior 30	0,1	0,3	3,5	1,8	0.003
Stinging Behavior 60	0,2	0.5	2,4	1,3	0.008

1: Confidence interval

2.3.2. Introduction of Foreign Queens of Defined Mating Status and three Different Kin Relations (Year 2006)

2.3.2.1. Benign Contacts

Concerning virgin queens, the closer the kin relation between the exchanged queens, the higher the number of benign contacts. Sister queens attracted workers more than half sisters and unrelated queens did. There was a significant difference between sisters and unrelated queens in the total number of benign contacts (p=0,005, Mann-Whitney test). In mated queens, there was a tendency of increasing benign contacts with sister queens compared to half sister queens and unrelated queens.

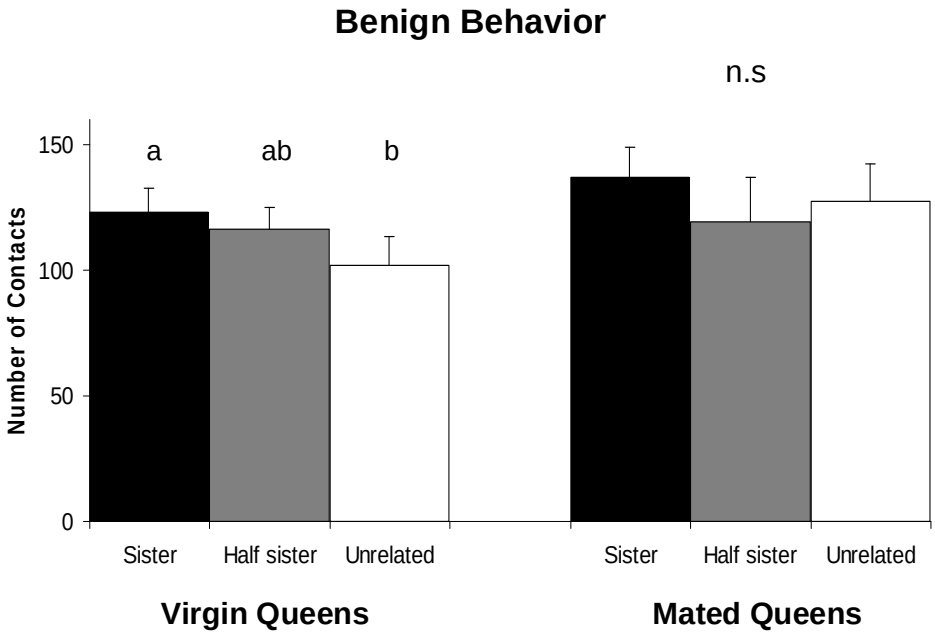

Figure 2.5 Average of total number of observed workers' benign contacts towards a foreign queen replaced their own queen inside a cage bioassay. 120 tests were performed by exchanging queens having different kin relations to each other: virgin/sisters, virgin/half sisters, virgin/unrelated, mated/sisters, mated/half sisters, mated/unrelated. Letters "a" and "b" describe the significant difference between queens according to Mann-Whitney test. "n.s" means not significant.

2.3.2.2. Aggressive Contacts

Against virgin queens, aggression increased by decreasing the level of kin relation. Workers were significantly more aggressive to unrelated queens compared to sister queens (p=0.03, Mann-Whitney). Against mated queens, there was a trend of increasing aggression against unrelated queens compared to sister and half sister queens. But there was no significant difference. Mated queens, in general, enhanced more aggressive contacts compared to the virgin queens as seen in figure (2.6).

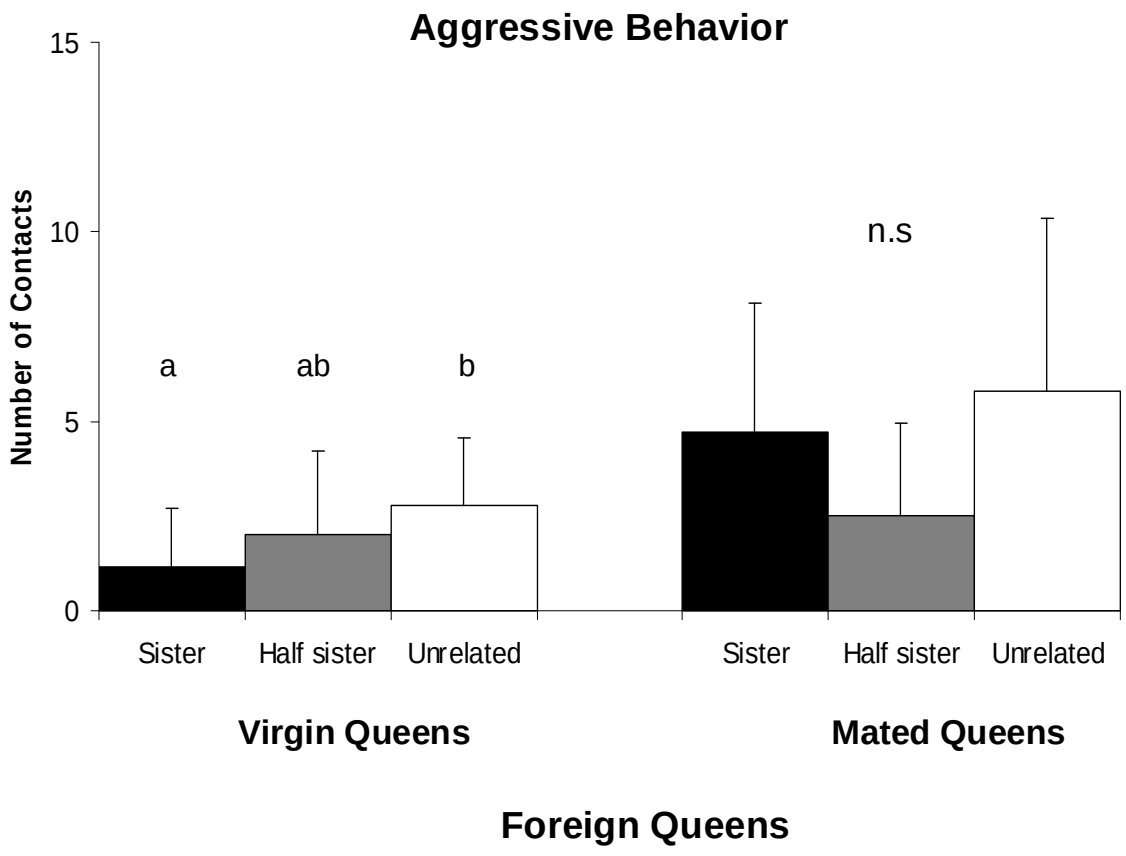

Figure 2.6 Average of total number of observed workers' aggressive contacts towards a foreign queen replaced their own queen inside a cage bioassay. 120 tests were performed by exchanging queens having different kin relations to each other: virgin/sisters, virgin/half sisters, virgin/unrelated, mated/sisters, mated/half sisters, mated/unrelated. Letters "a" and "b" describe the significant difference between queens according to Mann-Whitney test. "n.s" means not significant.

2.3.2.3. Duration of Aggressive Action

Each test, including 4 observations and 3 time intervals, lasted 125 minutes from the beginning of the first observation (at 0 minute) till the end of the last observation (at 125 minutes). During the tests, the duration of the aggressive action of workers against virgin queens was generally shorter than against mated queens. Against mated queens, aggression decreased generally with the time, significantly between the aggressive action of workers against virgin queens at the beginning of the test and at the end of it in half sisters and unrelated queens (p=0.009, 0.0004 respectively).

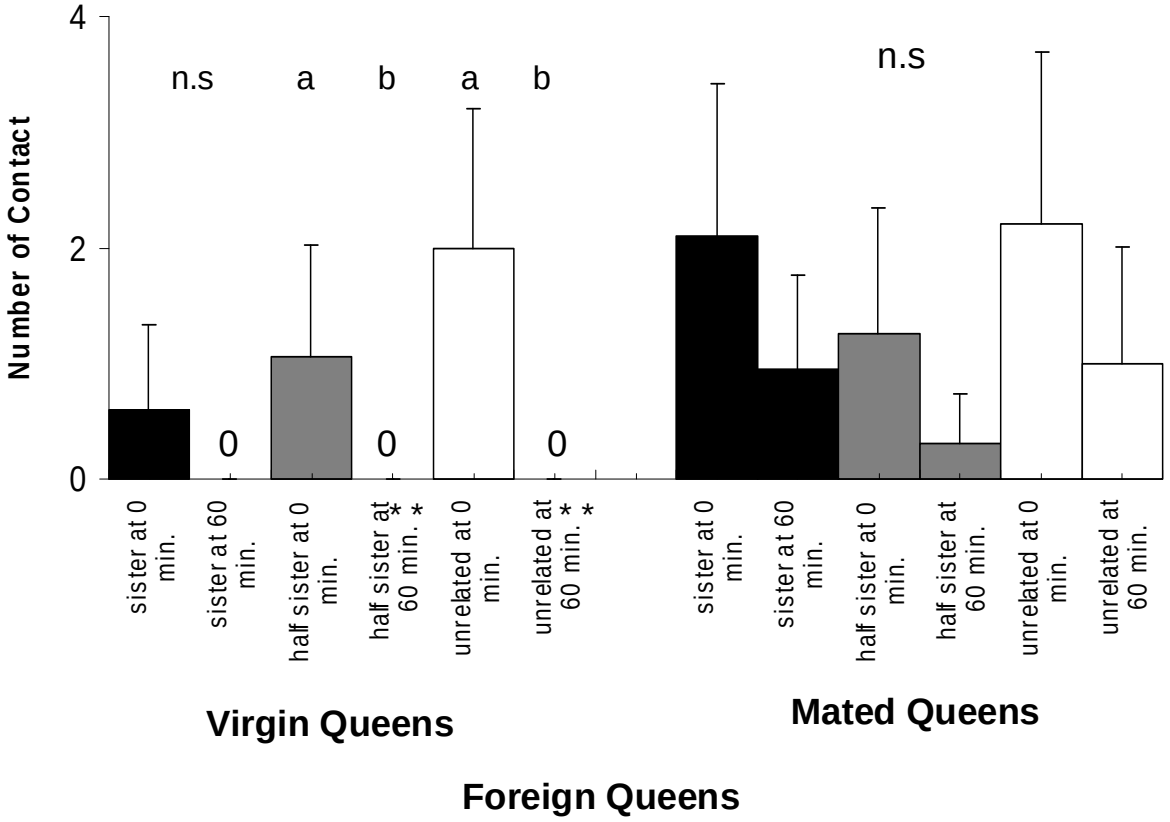

Figure 2.7 Average number of observed workers' aggressive contacts towards a foreign queen replaced their own queen inside a cage bioassay at the beginning of the test (0 min) and the end of the test (125 min). The exchanged queens have different kin relations to each other: virgin/sisters, virgin/ half sisters, virgin/ unrelated, mated/ sisters, mated/ half sisters, mated/ unrelated. (* *) refers to significant difference (p<0.01), "n.s" means not significant.

2.3.3. Application of Extracts

The number of workers contacts with the queen by licking her body surface was doubled when pentane or extracts were applied to the queens compared to the controls experiments with no application. No difference was found between pentane and extracts regarding the workers contacts of antennating, touching with mandibles and forelegs, licking and feeding the queen. There was a slight difference in the aggressive behavior of workers towards the treated queen, namely, biting and stinging behavior compared to the application of pentane. This result suggests that workers were excited due to the solvent odor. It was not clear whether the odor of a foreign queen's head or abdomen had been recognized by the workers when the extracts were applied over the queen's abdomen due to the low number of elicited aggressive contacts (Table 2.2). This indicated the need to increase the concentration of queen equivalent in the extract from 0.1 queen equivalent to 0.5 queen equivalent (Table 2.3).

Table 2.2 Average number of workers' benign and aggressive contacts towards their queen treated by (0.1 queen equivalent) pentane extracts of different body parts of a foreign queen inside a cage bioassay during 10 minutes.

Application	Antennae[1] Mean ±SD		Mandibles[1] Mean ±SD		Forelegs[1] Mean ±SD		Licking[1] Mean ±SD		Feeding[1] Mean ±SD		Biting[2] Mean ±SD		Sting. Bhvr.[2] Mean ±SD	
No application (n=8)	30.4	11.6	15.6	7.5	6.6	3.9	6.0	2.2	1.4	1.1	0	-	0	-
Pentane only (n=7)	26.0	5.0	18.3	6.5	9.1	3.9	17.4	7.9	1.3	1.0	0	-	0	-
Head mated (n=10)	25.7	7.0	13.9	6.7	7.6	2.8	15.6	4.6	1.2	0.8	0.3	0.9	0	-
Head virgin (n=8)	26.1	13.1	15.2	6.1	8.1	3.1	13.6	5.4	1.2	1.6	0.2	1.1	0	-
Abdomen mated (n=8)	27,6	10.4	19.4	7.0	11.0	3.6	18.4	5.8	1.4	1.2	0.5	0.8	0,13	0.4
Abdomen virgin (n=3)	29,3	4.2	15.3	5.7	13.7	2.1	21.7	4.6	1.7	0.6	0.7	1.6	0	-

1: Benign contacts, 2: Aggressive contacts

By increasing the concentration to a half queen equivalent, there was still no difference between the application of abdomen extract and pentane as control. By applying head extracts, there was a tiny difference in the number of licking the queen's body and in biting just after introducing the treated queen. But the harmful

effect of pentane (solvent) over the living queen's body had made the queen nervous and panicky. The queen's reaction could somehow influence the obtained results of the experiment and thus made them not suitable for performing further analyses.

Table 2.3 Average number of workers' benign and aggressive contacts towards their queen treated by (0.5 queen equivalent) pentane extracts of different body parts of a foreign queen inside a cage bioassay during 5 minutes.

Application	Antennae[1]		Mandibles[1]		Forelegs[1]		Licking[1]		Feeding[1]		Biting[2]		Sting. Bhvr.[2]	
	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD
No application (n=2)	12,8	3,5	5,0	3,3	3,1	2,4	2,9	1,1	0,3	0,5	0	-	0	-
Pentane only (n=2)	8,6	3,3	4,3	2,4	3,8	1,8	4,1	3,3	0,1	0,4	0	-	0	-
Head mated (n=2)	12,4	2,7	6,9	2,9	5,6	2,4	7,8	4,4	0,4	0,5	0,4	0,7	0	-
Abdomen mated (n=2)	9,1	3,8	7,6	3,7	2,8	1,3	3,5	2,9	0,5	0,8	0	-	0	-

1: Benign contacts, 2: Aggressive contacts

3. Proboscis Extension Reflex

3.1. Introduction

The results of the cage bioassay tests demonstrated different reactions of honeybee workers towards the introduced foreign queens. This was due to the different kin relations between queens on the one hand, and due to the individual behavior of workers on the other hand. The objectives of this chapter are to emphasize the role of queen kin and mating status in queen recognition by workers and to investigate the individual ability of bees to differentiate among queens. To achieve these objectives, and since the recognition cues are olfactory (Vaitkeviciene et al. 2006), there was a need to apply the olfactory conditioning of the Proboscis Extension Reflex (PER); a well established paradigm to study the bees' learning abilities in the laboratory (Brandes & Menzel, 1990). Inside a honeybee hive, the social structure of the colony cannot survive without communication which requires fundamental abilities of learning a certain message and remembering this message when communicating it to nest members at a later time (Moritz & Southwick, 1992, Geber *et al*., 1998). Out of the hive, honeybees have the ability of associative learning (Giurfa & Malun, 2004). The combination of odor, color, and form of flowers facilitate searching for nectar. The PER technique, based on the learning and remembering abilities of honeybees, has been employed by some companies in different applications such as the detection of smuggled goods, explosives and drugs at airports and borders check points (www.inscentinel.com), where the bees serve as sniffer dogs. In apiculture researches, the PER had many applications. For instance, it was used to investigate the ability of honeybees to discriminate between old and new wax (Froeholich *et al*. 2000) and to differentiate among different cuticular hydrocarbons (Chaline, *et al*. 2005). Nevertheless, no study has been done on discrimination of queens' odors by workers using the PER. Therefore, applying this paradigm to investigate the ability of bees to differentiate between queens of different kin relations (sister – unrelated) and different mating status (virgin– mated) was the main purpose in this chapter.

3.2. Materials and Methods

3.2.1. Queens

Two sources of queens have been used for the PER experiments:

Sister Queens: An open brood comb was obtained from Aulendorf. Their mother queen had been artificially inseminated, with the mother‘s number: 19-1-3604-2004, and father’s number: 02-503-3363-1999 from Bavaria. One-day old larvae from the Aulendorf comb were grafted into the plastic cups of 2 breeding frames (60 larvae). Each frame was inserted in an orphan colony. 12 days later, 24 of the hatched queens were moved into nuclei colonies. 12 nuclei colonies were provided with queen excluders to keep the queens unmated.

Unrelated Queens: This group of queens were obtained from Hohenheim from a free mated mother queen and served as unrelated to the Aulendorf queens. One-day old larvae from an open brood comb were grafted into 2 breeding frames. 24 hatched queens were put in nuclei colonies where 12 of them were provided with queen excluders to keep the queens unmated.

3.2.2. Test Bees

About 30 honeybee workers were collected randomly from a brood comb without the use of smoke and kept to starve for about 3 hours in a glass covered with a holed lid. The hungry bees were put in a plastic bag and were chilled in a freezer till motionless. Each bee was inserted in a 1.5 ml open tipped top Eppendorf tube where the head and forelegs were free. A tape was wrapped behind the bee’s head to prevent the bee from fleeing, but without hindering a free right-left movement of the head at the same time. Sugar syrup (2 molar) was prepared to be used for conditioning. The restrained bees were placed on a table with 4 cm distance from each other. Bee positions were numbered.

3.2.3. PER Conditioning

The queen used for conditioning was inserted in a 2 ml Eppendorf tube with an opened tip. Under the tube opening, two pins were inserted into the tube to form a barrier aimed to prevent the queen from coming closer to the opening and blocking it with her head, to avoid any possible contact between the queen and the tested honeybee, and to allow only the queen’s odor to pass. Each hungry honeybee restrained in the Eppendorf tube was conditioned with the queen’s odor by bringing

the queen tube close to the bee antennae without touching them for 5 seconds. 2 seconds later, the antennae were touched with the sugar syrup. The hungry bee would respond in an unconditioned reflex to the sugar syrup by extending the proboscis towards the sugar syrup (unconditioned stimulus US) and licking it (3 seconds were given). The same process was repeated with the other bees. 5 learning acts were performed for each bee within 10 minutes interval between each conditioning/learning. By the first learning, the bee, which reacted spontaneously to the queen odor by extending the proboscis, was discarded from the test. By the second learning, when a bee reacted to the odor (conditioned stimulus CS), it had been immediately rewarded by offering a droplet of sugar syrup to enhance the conditioned reflex to the odor and thus the learning process (CS+). The same has been done by the third till the last learning.

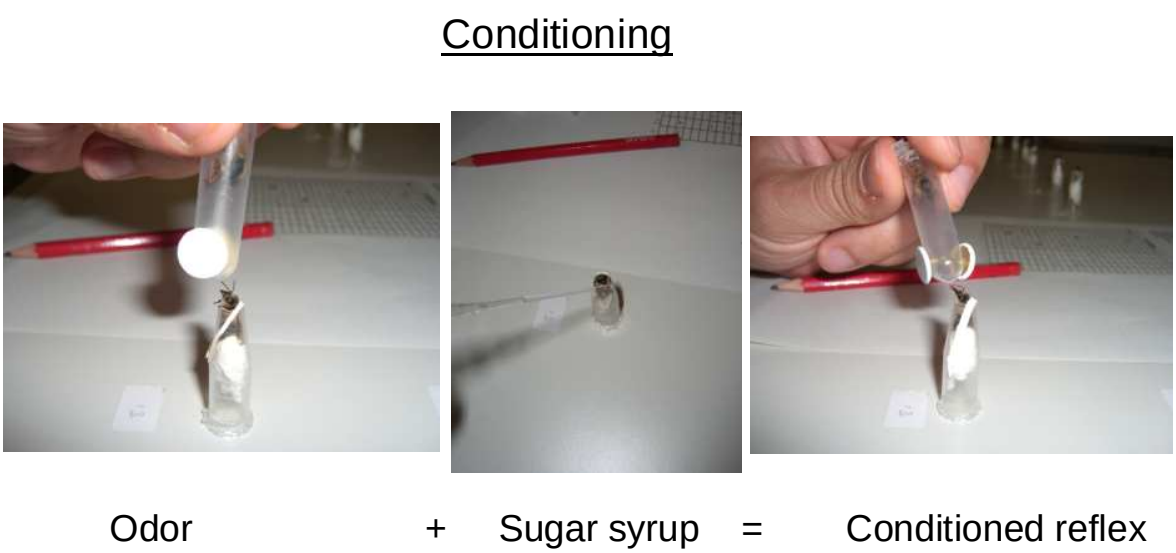

Figure 3.1 Conditioning (learning):When a worker is offered a queen's odor while tasting sugar syrup, she learns and remembers this odor and subsequently, she exposes the proboscis just to the presentation of the odor without the sugar syrup.

3.2.4. Testing

Another queen had been used to test whether the bees could discriminate between two different queens. 4 testing acts were performed by offering another's queen odor to the test bees without rewarding with sugar syrup (CS-). The reaction of each bee in learning and testing was immediately recorded on the data sheet. Different combinations of conditioning and testing have been applied (Table 3.1).

Table 3.1 Queens used in the PER tests to differentiate between queens having different kin relation to each other (sister or unrelated) and by different mating status (virgin or mated).

Conditioning [1]CS+	Testing [2]CS-
Virgin queen	Virgin – sister queen
Virgin queen	Virgin – unrelated queen
Virgin queen	Mated – sister queen
Virgin queen	Mated – unrelated queen
Mated queen	Mated – sister queen
Mated queen	Mated – unrelated queen
Mated queen	Virgin – sister queen
Mated queen	Virgin – unrelated queen

1: Conditioned stimulus + sugar syrup reward, 2: conditioned stimulus without reward

3.2.5. Statistical Analyses

X^2- test (SPSS software version 15) was conducted to compare between the response of workers at the last conditioning (learning) act and at the first testing act.

3.3. Results

3.3.1. Learning-Testing Curves

The learning – testing curves were drawn for each test. In learning curves, where queen's odor was associated with sugar syrup (CS+), the percentage of PER was recorded and drawn for all learning acts. At the first learning, it was always 0% PER to the queen's odor. The percentage was increasing at each repetition till it reached the saturation stage at the 4^{th} or 5^{th} learning. Not all bees have the same learning ability. The different learning curves contain differences in the PER percentages, but they all have the same tendency. In testing curves, the bees reacted differently when the queen's odor changed. As no sugar reward was associated with the queen's odor (CS-), the testing curve was, in most cases, declining towards the right direction as the percentage of PER was decreasing (Figure 3.2, 3.3).

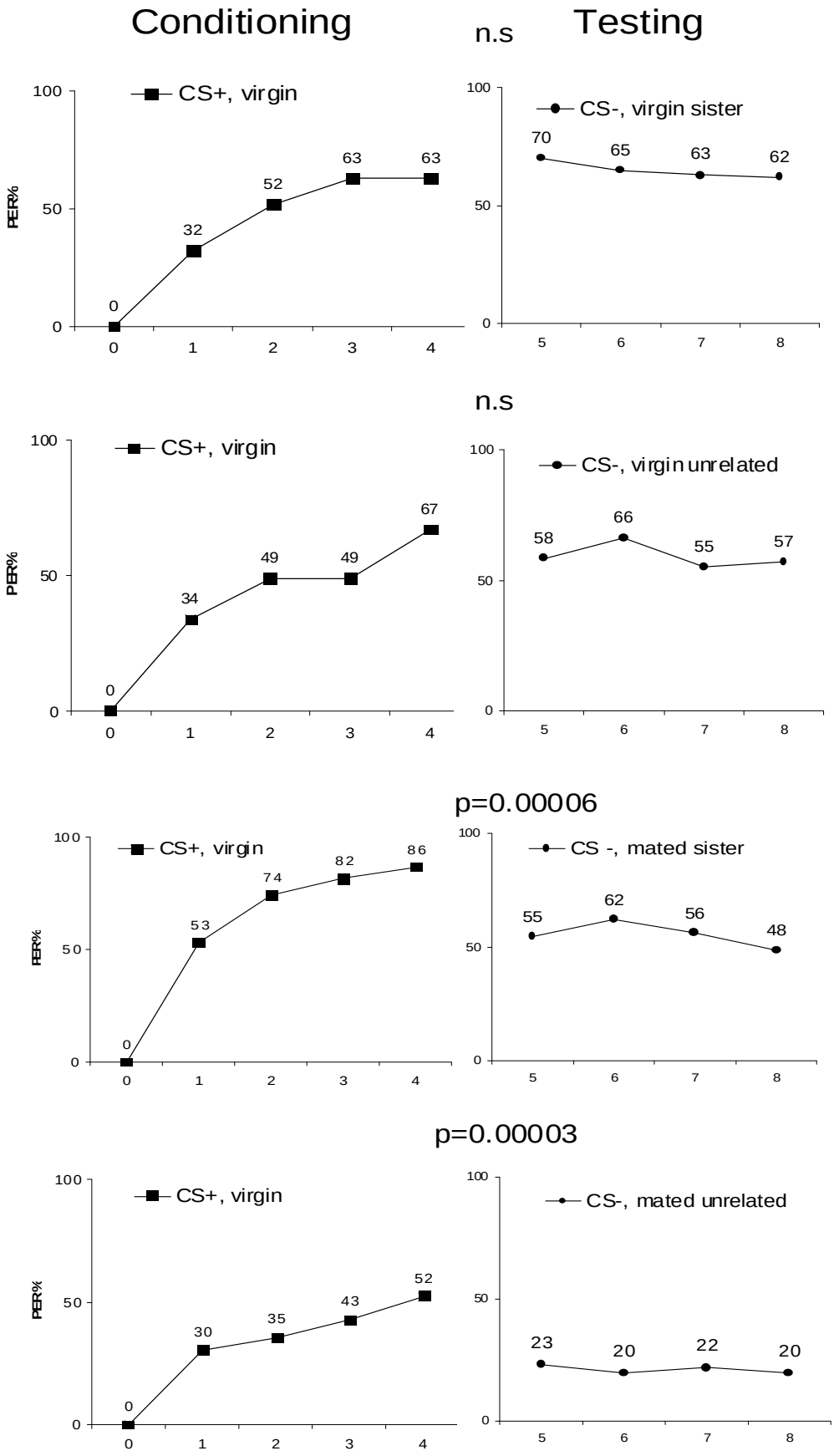

Figure 3.2 The learning – testing curves represent the percentage of PER at each repetition of conditioning (learning) a virgin queen's odor (CS+) and at each repetition of testing another queen's odor (CS-).

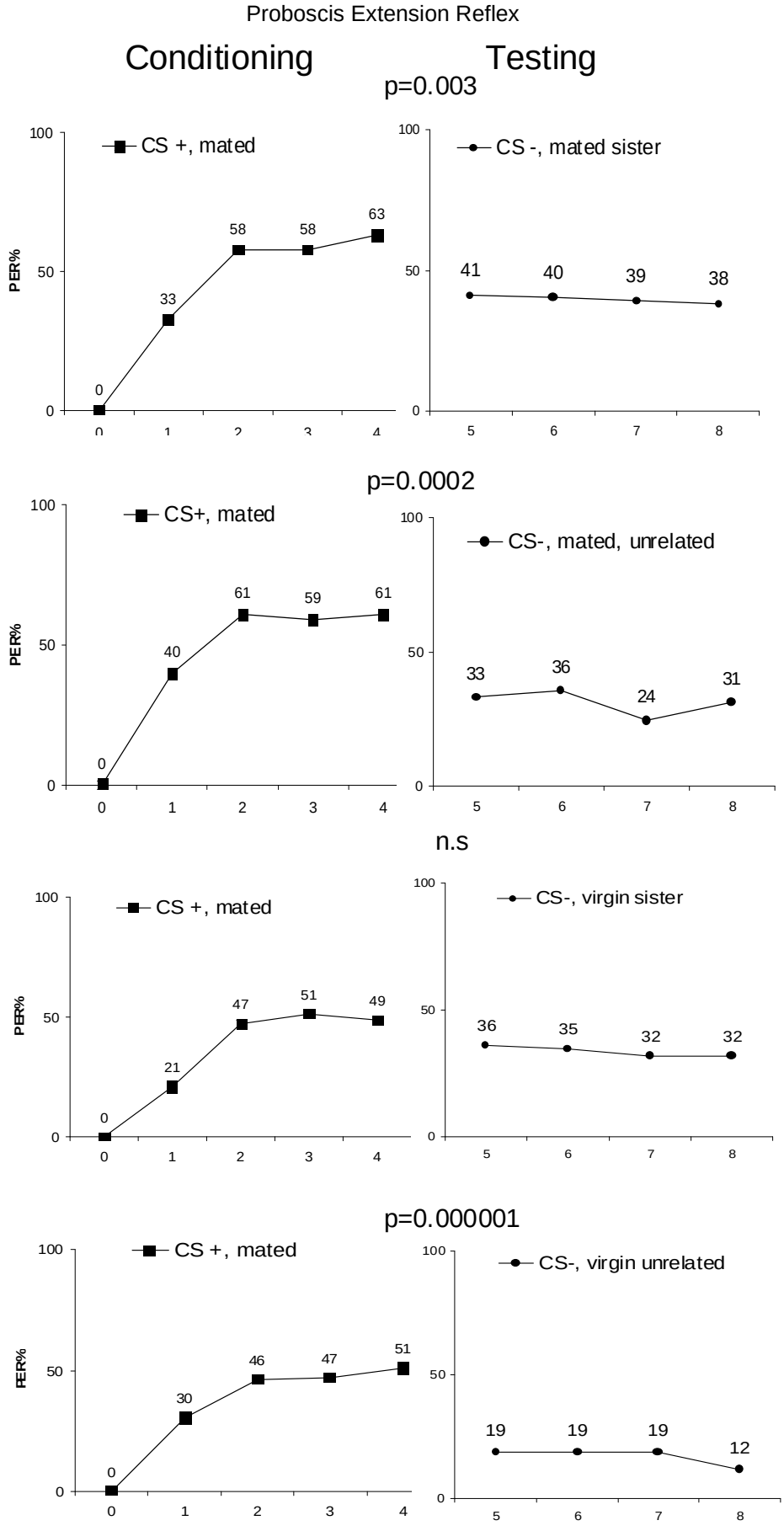

Figure 3.3 The learning – testing curves represent the percentage of PER at each repetition of conditioning (learning) a mated queen's odor (CS+) and at each repetition of testing another queen's odor (CS-).

3.3.2. Differentiation of Queens / PER Statistics

Workers could neither discriminate between the volatile odors of sister virgin queens, nor the unrelated ones (p= 0,294, 0,189 respectively). The results indicate, as seen in figure (3.2), that mating status provokes clear discrimination cues where the volatile odors of mated queens were significantly differentiated regardless the kin relatedness among the queens (p=0.003, 0.0002 for sister queens and unrelated queens respectively). In addition, workers could significantly discriminate between the learned odor of virgin queens and the tested odor of mated queens in both sister and unrelated (p< 0,000) and between the learned odor of mated queens and the tested odor of unrelated virgin queen (p< 0,000). Whereas they could not discriminate between the learned odor of mated queens and the tested odor of sister virgin queens (p=0,129).

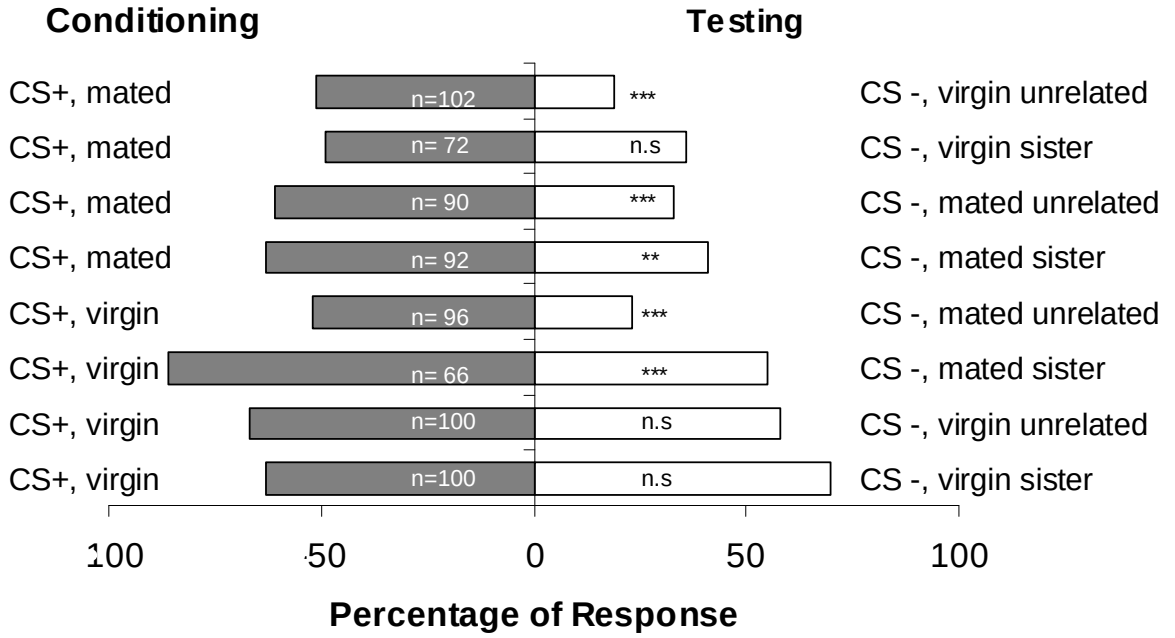

Figure 3.4 The percentage of workers' response (PER) to the queen's odor by the last learning act (left) and the first testing act (right). The difference in response was statistically analyzed using X^2-test. The significant difference means workers could differentiate between the odors of different queen. (**) refers to the significance level (p<0.01), (***) refers to the significance level (p<0.001).

4. Chemical Analyses

4.1. Introduction

In many social insects, it has been proved that nest-mate recognition has a chemical basis such as in the European hornet *Vespa crabro* (Ruther *et al*. 2002), in the paper wasp *Polistes dominulus* (Dani *et al.* 2004), and in honeybees *Apis mellifera* (Breed *et al.* 2004, Dani *et al.* 2005). Honeybee queen recognition by workers and queen-workers interaction relations were investigated with the focus on analyzing the components of the queen's glands secretions such as the secretions of the mandibular gland (Slessor *et al*. 1988, Winston and Slessor, 1998, Engles *et al*. 1993), secretions of tergite glands (Espelli *et al.* 1990, Wossler and Crewe, 1999a) and secretion of Dufour's gland (Gozansky *et al.* 2001). By the help of chemical analysis, steps forward were done on the way of understanding the mechanism of queen recognition and acceptance by workers. For instance, the substances involved in the recognition of queen cells by workers were identified (Le Conte *et al.* 1995), the volatile compounds in virgin and mated egg-laying queens could be compared (Apesgaite & Skirkevius, 1995), and the volatile compounds present in both queens and workers were detected (Gilley *et al*. 2006). However, it is still not clear if the substance/substances responsible for queen recognition by workers are 9-ODA, tergite gland's secretion or the cuticular hydrocarbons. The fact is that each of them plays a role in the complex interactions between the queen and workers. Another important factor, which should be taken in consideration when investigating queen recognition, is the genetic relationship among individuals. Page & Erickson (1980) found that recognition of queens appears to be associated with the genotype. In the cage bioassay, the contact behavior of workers to foreign queens was observed. In the PER tests, the ability of workers to learn and differentiate volatile odors of foreign queens was investigated. The objectives of this chapter are to help getting a better interpretation of the obtained results of the cage bioassay and PER and to check if the kin relation of queens is a determinant for the supposed recognition cues volatiles. To achieve these objectives, chemical analyses of queens' body parts with different kin relation and mating status were performed.

4.2. Materials and Methods

4.2.1. Queen Extracts

4.2.1.1. Extracting Protocol

A queen was partitioned into head, thorax and abdomen. Each part was put in a 1 mm flask with 0,4 mm dichloromethane as solvent and kept in deep freezing (–25℃). 24 hours later, the extract was separated from the body part and kept again in deep freezing. Before using the extract for analysis, the flask was left open for a few hours in ambient temperature to allow the dichloromethane to evaporate and reach the required extract volume of 100 µl.

4.2.1.2. Fractionating of Abdomen Extracts

In order to separate the extracts' polar from non-polar components, abdomen extracts were fractionated in silica column by adding 200 µl of the extract to the silica column ended by glass wool which allows the extract only to pass from the bottom opening. 500 µl of dichloromethane were poured over the extract + the silica column. By using a syringe, the air was pumped in the column to accelerate the outgoing of extract and solvent. The fractionated extract was held in a new flask which was left open in ambient temperature to allow the extract reaching the volume of 100 µl before injecting 1.5 µl of it in GC.

4.2.1.3. Adding an Internal Standard

Nonadeconoic acid methyl ester (Methyl nonadeconoate) (1g) was purchased from Fulka company to use it as an internal standard in abdomen extracts. A stock solution of methyl nonadeconoate was prepared by adding 10 mg of the standard into 10 ml dichloromethane (1 mg/ ml). 1 ml of the stock solution was taken into another vessel and diluted with 9 ml of dichloromethane to reach the concentration of 1:10 (0.1 mg/ ml = 100µg/ ml = 100 ng/ µl). 10 µl of the methyl nonadeconoate standard were added to the fractionated queen abdomen extract. (1000 ng standard in ca. 100µl extract). 1.5 µl of extract + standard were injected in the GC.

4.2.2. Analysis of Extracts

Dichloromethane extracts of queens' body parts (head, thorax and abdomen) were analyzed by a combined gas chromatography-mass spectroscopy using a Shimadzu GC-17A gas chromatography equipped with a 30 m fused silica capillary column of 0.25 mm i.d. coated with DB-5 and coupled with a GCMS-QP 5050A mass spectrometer. Gas flow was 1.5 ml/min. Linear velocity: 44.7 cm/sec. Split ratio: 16. Total flow: 28.2 ml/min. Column inlet pressure: 92.3 kpa. Progress time: 74 min. Scan interval: 0.5 sec from a mass number 35-450. Sampling time: 0.25 min. Injection temperature: 250°C Interface temperature: 280°C. Control mode: Spiltless.

Temperature program:

	Rate	Temp.	Hold time
1	-	60	2
2	4	300	12

1.5 µL of each extract was injected in the GC. The retention time of peaks was compared with the retention time of standard hydrocarbons.

Analyzed extracts of:

- 1-day-old virgin queens' head and abdomen
- 8-days-old virgin queens' head and abdomen
- 150-days-old mated queens' head and abdomen

4.3. Results

4.3.1. Abdomen Extracts (Polar Fractions)

4.3.1.1. Identification of Substances

The identified substances in the polar fraction of abdomen extracts of sister queens at different ages and physiological states (1 day old virgin, 8 days old virgin, and 150 days old mated queens) analyzed by the GC are listed in the table (4.1) and numbered as illustrated in the chromatograms (Figures 4.1, 4.2, 4.3).

Table 4.1 List of the identified substances in polar fractions of abdomen extracts of 1-day and 8-day old virgin sister queens and 150-days old mated sister queens and their quantities in ng.

No.	Component	abdomen 1 d virgin n= 7		abdomen 8 d virgin n= 7		abdomen 150d mated n= 5	
		Mean	SD	Mean	SD	Mean	SD
1	n-Nondecene	140	63	24	63	0	-
2	n-Nonadecane	204	67	0	-	0	-
3	Decyl octanoate	1081	516	44	117	0	-
4	n-Heneicosane	750	192	330	92	47	105
5	Decyl decanoate	2265	456	285	460	0	-
6	n-Tricosene	466	177	719	307	143	197
7	n- Tricosane	1571	297	2814	665	1332	1001
8	Dodecyl decanoate	1402	528	192	232	58	129
9	n.i	0	-	107	101	58	131
10	Tetradecyl octanoate	698	418	0	-	0	-
11	n-Pentacosene	265	85	1034	362	348	313
12	n-Pentacosane	813	359	2460	543	1169	1072
13	Tetradecyl decanoate	1252	638	199	190	103	143
14	n.i	0	-	275	79	0	-
15	Tetradecyl octanoate	813	511	0	-	0	-
16	n-Heptacosane	1296	537	1500	294	1848	1324
17	Methyl heptacosane	274	106	0	-	0	-
18	n.i	650	508	137	155	0	-
19	Hexadecyl decanoate	1616	895	367	250	0	-
20	Octadecyl decanoate	219	198	0	-	0	-
21	n-Nonacosane	1042	389	274	149	1193	783
22	Octadecenyl decanoate	1386	713	538	105	445	425
23	Octadecenyl decanoate	373	232	112	116	430	315
24	Octadecyl decanoate	915	711	207	334	0	-
25	Eicosenyl octanoate	638	470	753	203	203	454
26	n- Hentriacontene	661	304	1038	1426	1232	974
27	n- Hentriacontene	490	213	1326	1356	247	228
28	n-Hentriacontane	457	158	1110	790	1724	906
29	Eicosenyl decanoate	962	555	875	663	283	632
30	Tetradecyl tetradecanoate	566	663	2654	1893	2318	1909
31	Eicosyl decanoate	771	591	0	-	821	843
32	n-C33-alkene	756	367	628	618	548	604

n.i: Not identified substance

4.3.1.2. Chromatograms of Abdomen Extracts

A) Chromatograms of Queens' Abdomens of Different Kin

The gas chromatograms of abdomen extracts for virgin sister queens illustrate the high similarity between the closely related queens when comparing the numbered peaks of identified substances in both chromatograms (Figure 4.1 - A & B). In unrelated queens, the hydrocarbons pattern changed in queens originating from Hohenheim (Figure 4.1 - C) compared to queens originating from Aulendorf (Figure 4.1 - A & B).

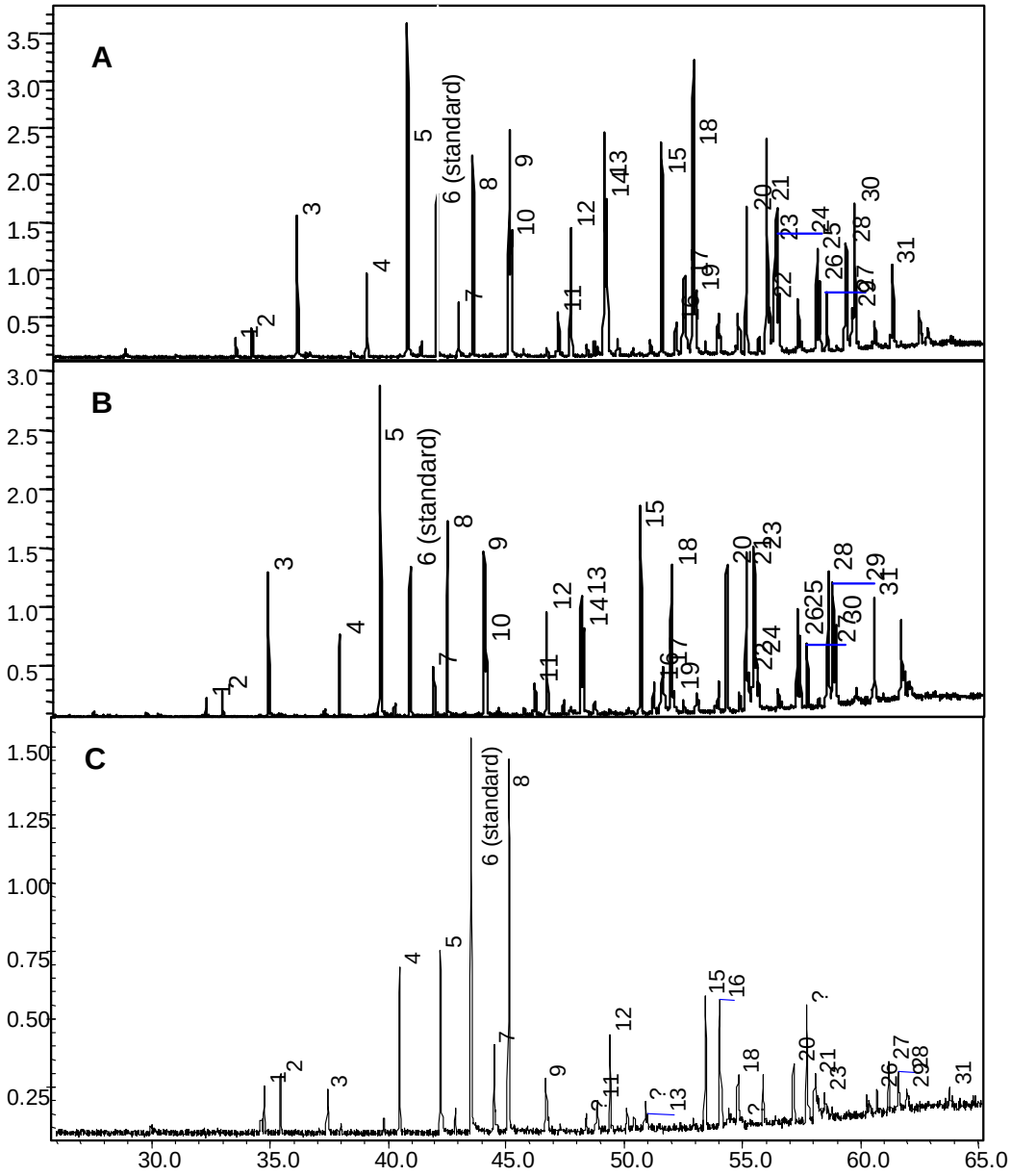

Figure 4.1 Chromatograms of abdomen extracts for one-day-old virgin sisters (A & B) and unrelated (A & C, B& C) queens (polar fraction) analyzed by GC-MS. Identified substances and the internal standard are numbered.

B) Chromatograms of Virgin/ Mated Queens

The gas chromatograms of abdomen extracts for sister queens of different ages show a different quantitative pattern of the identified substances when comparing between one-day-old virgin queen (Figure 4.2 - A), eight-day-old virgin queen (Figure 4.2 - B) and five-month-old mated queen (Figure 4.2- C).

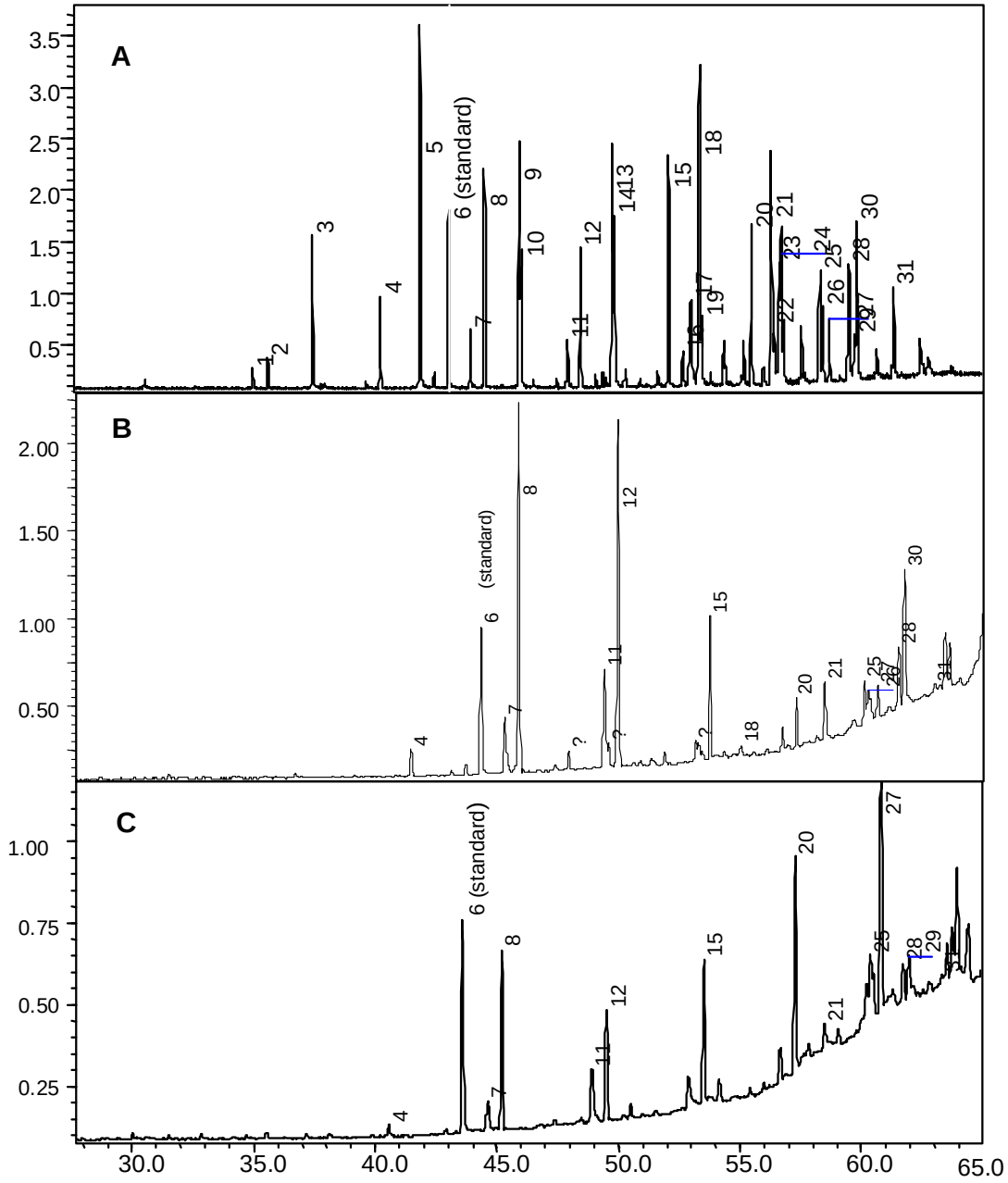

Figure 4.2 Chromatograms of abdomen extracts for sister queens of different age and mating status: one-day-old virgin queen (A), eight-day-old virgin queen (B) and mated queen (C) (polar fraction) analyzed by GC-MS. Identified substances and the internal standard are numbered.

4.3.1.3. Nei Distances

To quantify the differences of chemical compositions among abdomen extracts of different queens, a matrix of Nei- distance was applied (Nei, 1972). The value of Nei distance ranges between 0 and 1. The chemical distance increases when the calculated value is getting lower (towards 0) and decreases when the calculated value is getting higher (towards 1). By considering the 0 values of substances analyzed by the GC and calculated by the Nei distance method, the chemical distance of sister queens was close to 1 (0.92±0.01), increased to 0.77±0.07 in half sisters and to 0.66±0.06 in unrelated queens.

Table 4.2 Chemical distance between abdomen extracts of sister queens, half sister queens and unrelated queens at age of one day. (According to 32 identified components in abdomen extracts).

sister queens		half sisters		unrelated (Aulendorf: Hohenheim)		unrelated (Hohenheim: Aulendorf)	
Mean	SD	Mean	SD	Mean	SD	Mean	SD
0,93	0,05	0,84	0,11	0,60	0,19	0,74	0,06
0,92	0,04	0,85	0,13	0,76	0,14	0,74	0,07
0,93	0,03	0,69	0,18	0,64	0,19	0,88	0,05
0,93	0,05	0,73	0,25	0,60	0,19	0,50	0,08
0,92	0,04	0,73	0,26	0,73	0,18	0,50	0,09
0,89	0,03			0,69	0,13		
0,93	0,04			0,62	0,21		
0,92	**0,01**	**0,77**	**0,07**	**0,66**	**0,06**	**0,67**	**0,17**

The clustering of Nei distances of the components of abdomen extracts of queens analyzed by GC-MS (Figure 4.3) demonstrates clearly both the differentiation between unrelated queens and the similarity of sister queens.

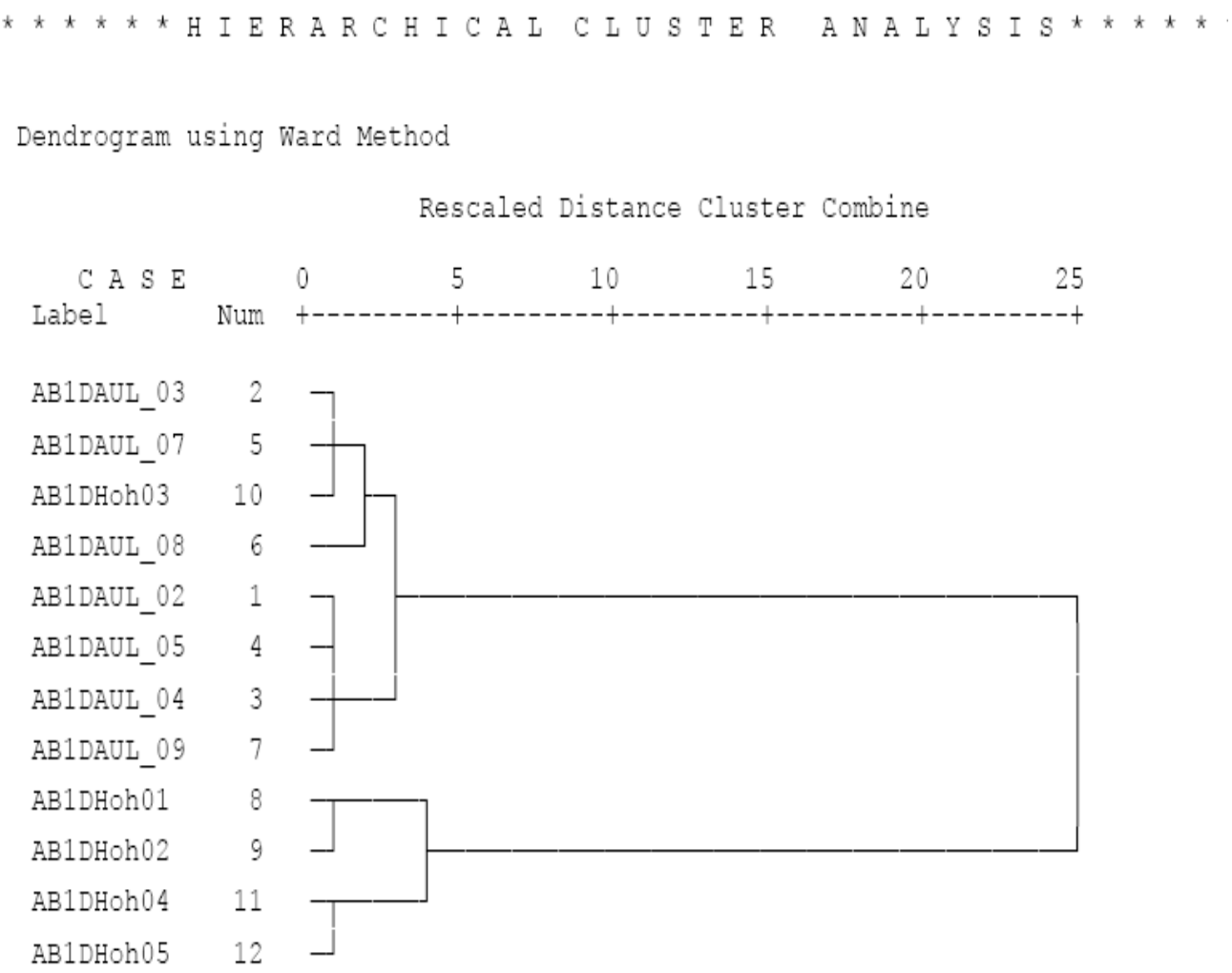

Figure 4.3 Hierarchical cluster analysis based on Nei distance for extracts of queens' abdomens analyzed by GC-MS where AB1DAUL_n: one-day old virgin sister queens from Aulendorf, AB1DHoh_n: one-day old virgin half sister queens from Hohenheim. Aulendorf queens are unrelated to the Hohenheim queens.

The multidimensional scaling (Figure 4.4) gives a clear picture for the distribution of queens in groups according to their kin to each other. The sister queens are close to each other and obviously far from the unrelated queens (with one exception, namely, AB1Dhoh03). The half sister queens have variations in their distance from each other.

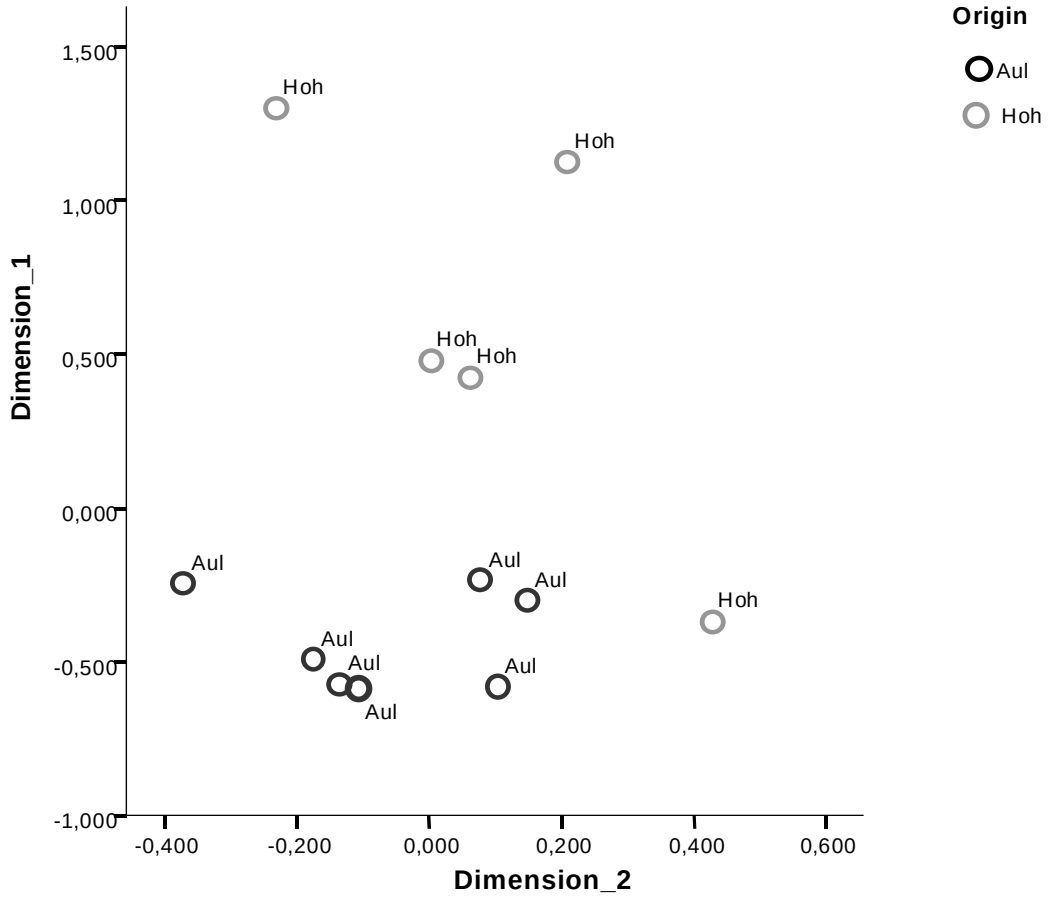

Figure 4.4 Multidimensional scaling of extracts of queens' abdomens analyzed by GC-MS. The queens are from different regions: (Aul) sister queens from Aulendorf and (Hoh) half sister queens from Hohenheim. Aulendorf queens are unrelated to Hohenheim queens.

The Nei distance was measured for sister queens at different ages. The one-day-old virgin sister queens had the lowest chemical distance (0.92±0.01) compared to the eight-days-old virgin sisters (0.71±0.15) and 150 days old mated egg-laying queens (0.72±0.10). The increase of the chemical distance was clear between virgin queens at different ages (0.50±0.06) and between virgin queens and mated queens (0.46±0.07) (Table 4.3).

Table 4.3 Chemical distance between abdomen extracts of sister queens in different ages.

v1d[1]		**v1d x v8d**		**v1d x m150d**		**v8d[2]**		**v8d x m150d**		**m150d[3]**	
Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD
0,91	0,05	0,49	0,09	0,47	0,06	0,72	0,22	0,67	0,17	0,58	0,10
0,92	0,04	0,51	0,11	0,43	0,09	0,75	0,17	0,68	0,19	0,81	0,10
0,94	0,04	0,57	0,10	0,54	0,05	0,78	0,17	0,61	0,07	0,74	0,08
0,91	0,04	0,52	0,09	0,48	0,05	0,79	0,16	0,66	0,19	0,74	0,19
0,90	0,03	0,45	0,11	0,37	0,08	0,81	0,12	0,62	0,18		
0,91	0,05	0,56	0,11	0,55	0,08	0,41	0,12	0,67	0,11		
		0,41	0,09	0,37	0,05			0,32	0,12		
0,92	**0,01**	**0,50**	**0,06**	**0,46**	**0,07**	**0,71**	**0,15**	**0,60**	**0,13**	**0,72**	**0,10**

1:one-day-old/virgin; 2: eight-days-old/virgin; 3: 150 days old/mated (m150d)

The clustering of Nei distances of the components of abdomen extracts of sister queens analyzed by GC-MS (Figure 4.5) demonstrates clearly the differentiation between queens of different ages and mating status.

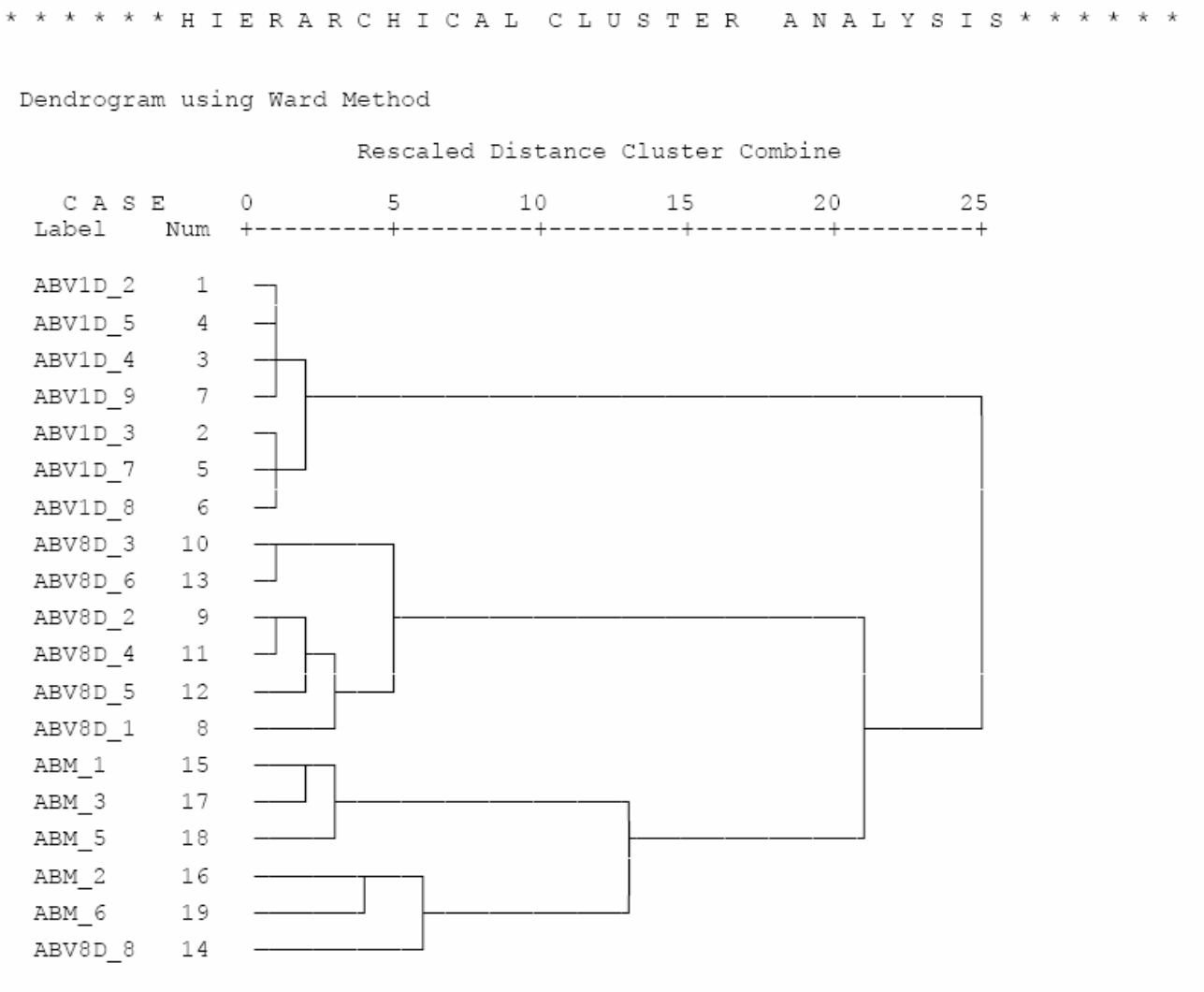

Figure 4.5 Hierarchical cluster analysis based on Nei distance for extracts of queens' abdomens analyzed by GC-MS. The queens are sisters but of different ages and mating status where ABV1D_n: one-day old virgin queen, ABV8D_n: eight-day old virgin queen, ABM_n: mated egg-laying queen

The multidimensional scaling (Figure 4.6) shows the distribution of sister queens in groups according to their age and mating status. Except for one individual case, there are 3 distinct groups; one for the one-day old queens, the other for the eight-day old queens, and the last for the five-month old queens. Concerning the mating status, there are two levels, the first for the virgin queens of one- and eight-day old, and the second for the mated queens.

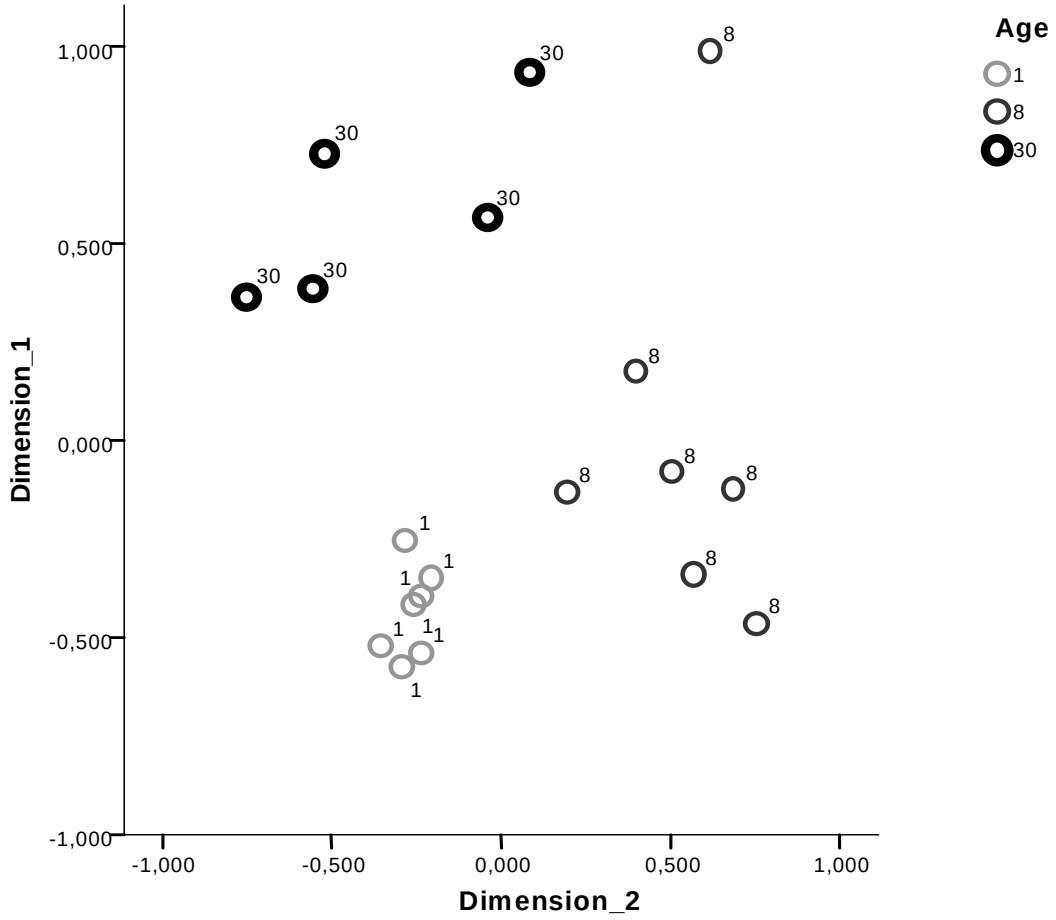

Figure 4.6 Multidimensional scaling of extracts of queens' abdomens analyzed by GC-MS. The queens are sisters but with different age groups and mating status; 1: one-day old/virgin, 8: eight-day old/virgin, 30: five-month old/mated.

4.3.2. Head Extracts

Gas chromatograms of extracts of queens' heads analyzed by GC show clear differences in the amount of 9-ODA between virgin (Figure 4.7 - A & B) and mated queens (Figure 4.7 - C). There is also an increase in the amount of 9-ODA in virgin queens when comparing between one-day old virgin queen and eight-day old virgin queen.

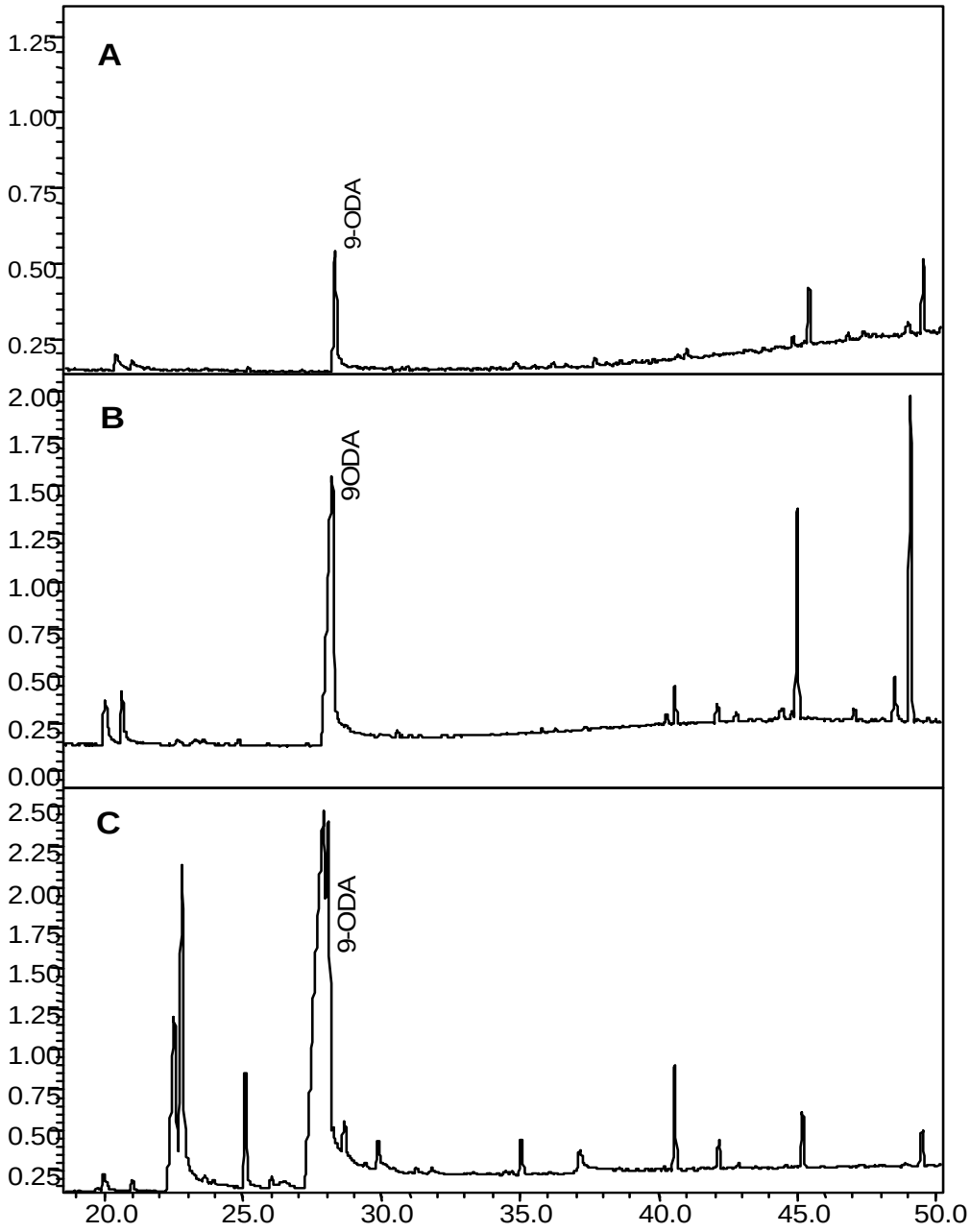

Figure 4.7 Chromatograms of heads extracts for sister queens of different ages and mating status analyzed by GC-MS where (A): for one-day-old virgin queen, (B): for eight-day-old virgin queen, and (C): for 5-month mated queen.

5. Discussion

Honey bees (*Apis mellifera* L.) are eusocial insects living together in a colony which functions as a super-organism with a central role for the queen in the society (Southwick, 1992). The queen produces pheromones which influence most of the activities of the colony. The queen-workers direct interactions are known as “Retinue Behavior” where workers gather in a ring around the queen and form a court around her. This court is present at all times and more obvious when the queen is not moving (Butler, 1973). The queen’s pheromones are acquired mainly through antennating and licking contacts of workers in the retinue (Slessor *et al*., 1995) and then distributed within the hive by messenger workers (Moritz & Crewe, 1988b).

While a lot of work has been performed on the primer and releaser effect of certain queen pheromones (Butler, 1973; Vierling & Renner, 1977; Moritz, 1988; Espellie, 1990; Krieg, 1994; Pankiw *et al,* 1994; Slessor *et al*.,1995; Wosslor & Slessor,1999b) it is still unknown how the workers distinguish their own queen from foreign ones. The fact that queens can be exchanged successfully by protecting the foreign queen for some days demonstrates that workers are able to “learn” their queen. It is likely that a certain chemical pattern of the cuticle (odor or taste) is finally responsible for the recognition and acceptance as “own”.

In this context, this work has three different objectives:

- To better understand the bees’ behavior to “own” and “foreign” queen and to quantify certain behavioral traits of the queen-workers interaction.
- To study the learning ability for own and foreign queens by the use of the Proboscis Extension Reflex (PER) in order to have a tool for future tests of odorous compounds.
- To compare the cuticular pattern of queens of different origin.

5.1. Contacts Behavior Inside a Cage Bioassay

For studying and quantifying the behavior of workers toward a foreign queen, there was a need for a bioassay which enables the record of workers behavior to the queen without an inhibition of the complex social interactions between queens and workers. Several laboratory bioassays were used in apiculture researches to test the pheromone effects (Krieg, 1994), to quantify the responsiveness of workers to queen’s mandibular pheromones “QMP” (Pankiw *et al,* 1994), to determine the nature of the stimuli involved in queen-queen recognition (Pflugfelder, Königer, 2003), to

observe the recognition of queen cells by honeybee workers (Le Conte, 1995) and to compare between kin relations of honeybee workers and their odors (Getz *et al*., 1986). The previous mentioned bioassays were mostly performed under artificial conditions with lures or dummies and a very limited number of bees. None of them could reflect the social behavior of the bees and the queen workers interactions. Therefore, I established a new laboratory bioassay and developed it to fulfill the requirement of measuring queen-workers interaction under more natural conditions. The final design of the cage bioassay had succeeded, to a big extent, in achieving its goals. Consisting of a small wooden box with a glass front, a wax comb, feeding tube, 30-40 worker bees and a queen, it provided a suitable environment for the bees during the period of the test. A clear indication for this is, that the bees inside the cage bioassay showed a typical retinue behavior around the queen and reactions towards foreign queens. With the newly developed cage bioassay I could have the smallest unit which can show most of the behaviors occur within the colony. Besides the retinue behavior, bees had social contacts with each other like antennating and exchanging of feed (trophallaxis). Mated queens, being left in the cage bioassay over night, had mostly laid eggs. Workers stored honey in comb cells and rebuilt damaged cells in the comb. With all these behaviors, the work with the bioassay could be extended for days.

In all tests of introducing a foreign queen in the cage bioassay, workers recognized the introduced foreign queen and behaved differently from the normal case, mostly with aggression. The aggressive reaction of workers to the introduced foreign queen was sometimes fatal. In the first set of tests, 9 queens out of 32 had not survived. The dead queens were all unrelated to the resident queens. There was a significant difference in the survival rate between unrelated queens compared to related queens.

Therefore, the question arise whether the recognition of a foreign queen by workers depends on the kin relation of own and foreign queen. Moretto *et al.* (2004) analyzed the maternal influence on acceptance of virgin queens and concluded that acceptance rate of virgin queens varied due to the genetic origin of the queen. This conclusion is in agreement with Wossler and Crewe (1999b) who recognized variations in the virgin queen acceptance rate caused by the genetic origin of queens. In the second set of tests, I focused in detail on two issues: the influence of queens' kin relatedness and mating status on their acceptance by workers. According

to kin relation, three types of queens were bred and established in small nuclei: sisters received from an inbred line at Aulendorf, half sisters and unrelated ones. It should be mentioned that only the kin relation between queens but not between worker bees and queens was tested in this thesis. The results of these tests showed a higher acceptance rate of sister queens compared to the unrelated queens, which was significant in virgin queens but not in the mated ones. The benign contacts towards half sisters were intermediate between the sister and the unrelated queens but the differences were not significant, neither between sisters and half sisters, nor between half sisters and unrelated queens. However, their intermediate position between the sisters and unrelated queens on the one hand, and the significant difference between the sisters and unrelated queens on the other hand supports the assumption of the role of queen kin to each other in recognition and acceptance by workers, at least in the virgin ones. But also in the mated queens, there was a trend of increasing benign contacts and decreasing aggressive behaviors when sister queens were compared to unrelated queens. This tendency is confirmed if the results from the tests with mated queens from the first set of experiments in 2005 are considered: In these trials the differences in behavior toward related and non-related queens, respectively, were even significant. However, the relatedness of the queens in this set of tests were not defined as exactly as in the later on experiments of 2006. The better acceptance of related queens could be due to the learning ability of workers to their own queen and using her as the major referent (Page *et al.* 1986). What the related queens have in common could minimize the differences detected by workers between their own queen learnt as a referent and her sister. In mated queens, the amount of volatile gland products including queen mandibular gland pheromones (QMP with 9-ODA as main component) are significantly higher than in virgin ones (Pankiw *et al.* 1996). This probably superposes the effect of kin related patterns which can be detected by the workers in virgin queens. The GC analyses performed by Apogaite (1995) detected lower 9-ODA in the extracts of virgin queens compared to the extracts of mated egg-laying queens. This may also be the reason, that the aggressive reactions were, in general, stronger against mated queens compared to virgin queens. However, Moretto *et al.* (2004) mentioned that mated queens are usually better accepted than are virgin queens and the use of virgin queens is more applicable because of the difficulties in producing big quantities of mated queens.

Unlike the previously mentioned bioassays, in which dummies with queen's extracts were applied, in the newly developed cage bioassay, a living queen was directly used without caging. After removing the resident queen, a foreign queen was introduced and the contact behavior of workers towards the introduced foreign queen was directly observed and successfully recorded. For the first time, workers reaction to an own queen in comparison to a foreign queen of different kin could be quantified. Nevertheless, some limitations for using the cage bioassay have to be mentioned:

- The test is very time consuming. The process took about 24 hours starting from preparation of the tests until the end of it. Not included is the time needed to establish queens in "Kirchhainer" nuclei boxes and maintain these "mini colonies" till the next test.
- Bees did not always react the same way. There was an individual behavior where specific bees attacked the queen whereas other workers didn't pay attention to the foreign queen. To overcome this issue, there is a need to use defined bees but this would be again more time and work needed.
- Another difficulty is that a slight manipulation of the own queen, in order to measure separately the effect of different components, does not elicit a distinct and measurable change of worker behavior.

This may have been contributed to the problems in measuring the effect of different volatile extracts of foreign queens. In former studies on queen-workers interactions, extracts of queens' pheromones were applied on glass lures to inspect their effects on the retinue behavior of bees (Pankiw *et al*., 1996; Wossler & Crewe, 1998; Slessor *et al*. 1998). In this study, there was a try to test extracts of foreign queens which were applied over the abdomen of the test queen. The idea of utilizing a living queen instead of lures was to observe the queen-workers interaction after adding the foreign odor over the own queen. In most cases, a slight increase in the aggressive behavior of workers towards the treated queen was observed. However, there was no difference between the application of pentane alone and extracts regarding workers contacts with the queen. The result has not changed by increasing the concentration from 0.1 to 0.5 queen equivalents in the extract. This suggests that workers were excited due to the solvent odor. Another problem was the harmful effect of pentane (solvent) trickling over the living queen's body which made the queen nervous and panicky. As these effects could not be avoided, these sets of experiments have not been extended.

5.2. PER – Differentiation of Queens' Odors

Honeybees form a typical model for the study of learning and memory at the behavioral, cellular and molecular level (Menzel, 1999). Olfactory conditioning of constrained bees had been used to characterize the learning process in bees (Brandes & Menzel, 1990). Therefore, the "Proboscis Extension Reflex" (PER) of honeybees was used as a biosensor to test their ability to differentiate between queens of different kin and mating status. The following questions should be answered: Can honeybees recognize their own queen from a foreign queen? And if yes, what are the cues involved in this recognition?

Since workers respond most strongly when they are exposed to the odor of living queens as to the odor of queen's glands extracts (Moritz & Crewe, 1988), and since queen's differentiation is based on olfactory signals (Vaitkevicinene *et al.* 2006), a living queen was used as the source of odor in the PER tests instead of body extracts. The same types of queens were used in the PER tests as in the cage bioassay. By applying the classical olfactory conditioning (PER) of worker honeybees, the obtained results proved that workers could learn the queen's odor when they were trained on either mated or virgin queens. The gradual increase in the learning curve, for instance in virgin queen from 0, 30, 50, 60 till 65%, is a good indication of learning the odor.

The results revealed clear differences in the cues used for the "learning" of individual mated and individual virgin queens, respectively: The workers could significantly discriminate between the learned odor of a mated queen and any other mated queen irrespective of the relatedness. In mated queens the bouquet has an individual specificity but obviously no kin specific pattern. Probably, the huge amount of many volatile gland products (including the main component 9-ODA) makes each mated queen "unique". In contrast, worker bees cannot discriminate at all virgin queens from each other. It seems that virgin queens' volatile compounds were not strong enough or the volatile "bouquet" is neither individual specific nor kin specific to be differentiated. As the worker could only use volatile substances for the associative learning one can conclude that the volatile "bouquet" of virgin queens does contain neither an individual nor a kin specific pattern. But in the cage bioassays the worker could recognize whether an introduced foreign queen was related to the own queen or not, at least in the virgin queens. Such recognition must, therefore, depend on volatile substances of the cuticle of virgin queens which are non or low volatile and

perceived by licking. This interpretation is in agreement with differentiation tests which depended on direct contact, where workers could differentiate between virgin queens of different races (Levchenko *et al.* 1995). Additionally, Vaitkevicinene *et al*. (2006) found that virgin queens use volatile odor labels as recognition cues.

If learned and tested queens were of different mating status, the worker bees could significantly discriminate between such individuals (except for learned odor of mated queens/ tested odor of related virgin queens). Again, this is not surprising as the differences in pheromone composition between mated and virgin queens are enormous (Apogaite & Skirkevieius, 1995; Apogaite, 2003).

In many social hymenopteran insects, it has been found that cuticular hydrocarbons contain recognition cues as in the termite *Macrotermes subhyalinus* (Kaib *et al.* 2004), the termite *Hodotermes mossambicus* (Kirchner & Minkley, 2003) and in the European hornet *Vespa crabor* L. (Ruther *et al*. 2002). In honeybees, it is believed that cuticular hydrocarbons play a role in worker-worker interaction and nestmate recognition (Dani *et al*. 2005). Workers can discriminate between most of the cuticular hydrocarbons (Chaline *et al*. 2005).

To check the role of queen's cuticular hydrocarbons in queen recognition by workers, the performed PER tests could be used for future tests of queen's extracts, fractions of extracts, mixture of certain substances (for instance hydrocarbons) which was not possible in the cage bioassay.

5.3. Chemical Analyses

The chemical analyses should prove whether typical patterns exist on the cuticle of the queens which are related to kin and, therefore, could be used for kin-related recognition cues. The general retinue behavior of workers around the queen is one visible example of the queen-workers interactions. Several works investigated the retinue behavior of honeybees around their queen in association with analyses of queen's head and abdomen glands secretion such as the secretions of the queen mandibular gland, (Slessor *et al*. 1988, Winston & Slessor, 1998, Naumann *et al.* 1991) and the secretion of the tergite glands (Espellie *et al.* 1990, Wossler & Crewe, 1999). Retinue behavior reflects the loyalty of workers to their own queen. If bees have loyalty to own queen, it means they can recognize their own queen and differentiate it from others (Page, 1986).

Focusing on the role of queen kin relation and mating status in foreign queen recognition and acceptance by workers, queens of different kin (sisters and unrelated), mating status (virgin, mated) and different ages (1 day to 5 months) were extracted and the obtained extracts were analyzed using GC-MS methods. Extracts of abdomens were chosen for the measure of the "chemical distance" (Cvacka *et al.* 2006) between queens of different types because the abdomen has the largest surface area compared to head and thorax and retinue workers normally have more contacts with the queen abdomen than with the head and with thorax (Alkattea & Rosenkranz, 2004). Furthermore, the texture and chemistry of the queen body surface is crucial for the release of pheromones (Moritz & Crewe, 1991).

32 substances were identified and used for the statistical comparison of the odor pattern of different queens. The results showed a significantly higher concordance in the chemical pattern within sister queens compared to non related ones. The "chemical distance" was increasing as the level of queen kin was decreasing. Cluster analyses of the Nei distance and multidimensional scaling clearly confirmed the differentiation between unrelated queens and the similarity of sister queens. Using the same method, a clear differentiation between queens of different ages and mating status could be also demonstrated.

To analyze specific compounds which could be responsible for kin and mating status, one first have to take into account that the composition of queens' cuticular hydrocarbons differed with age (Apogaite, 2003). The results of the GC analyses showed that quantities of substances decreased in eight-days-old virgin queens' extracts compared to one-day-old virgin queens' extracts in 19 out of 32 identified substances, from which 6 substances disappeared or were in very tiny quantities that the GC could not detect them. The quantities of 13 substances increased in extracts of eight-day-old queens compared to one-day-old queens' extracts. The obtained results of the GC showed that the quantities of 20 substances in mated queens' extracts decreased compared to eight-day-old virgin queens' extracts, from which 9 substances disappeared (very tiny amounts and not detected by the GC) while the quantities of 6 substances increased. Most of the decreased substances were alkenes. The increased substances from one day old to mated through 8 days old were heptacosane and hentriacontane. The differences in the composition of hydrocarbons between virgin and mated queens come in agreement with the analyses done by Apogaite (2003) and Gilley *et al.* (2006). It was proved that

honeybees are able to discriminate between individual cuticular hydrocarbons (Chaline *et al*. 2005) but no data exist on the ability of bees to discriminate mixed hydrocarbons.

Specific compounds were found to be either in virgin or mated queens such as the ester compounds (decyl decanoate, decyl dodecanoante and decyl tetradecanoate) which are specific for virgin queens (Wossler & Crewe 1999b).

The chromatograms of head extracts showed a small amount of the 9-ODA in virgin queens. The combination between the results of the GC analyses with the results of the cage bioassay and the results of the PER conditioning support the direction that virgin queens' recognition cues are acquired via the direct contact such as licking and touching with mandibles and forelegs. Since bees are strongly attracted to the abdominal gland secretions via direct contact (Vierling & Renner 1977), then also the recognition cues should be located in the abdomen. The chromatograms of head extracts clearly showed an increasing in the amount of 9-ODA and other fatty acids after mating.

The differentiation of mated queens in the PER tests could be due to the huge amount of polar compounds like fatty acids including the 9-ODA. This could be also the reason for the long duration of the aggressive behavior in the experiments of introducing mated foreign queens to the cage bioassay.

5. 4. Conclusion

The newly developed cage bioassay was a good tool to observe the workers-queen interaction. Its design allows to show most of the social behaviors occur within the colony. The contacts behavior of workers to the own and foreign queens could be observed and quantified. The results presented in this work confirmed with 3 different approaches that workers are able to learn their own queen with different learning cues depending on the mating status of the queen. In virgin queens it could be demonstrated for the first time that the kin relation between different queens can be recognized, presumably by low or non-volatile substances. The chemical analysis confirms that the cuticular pattern of queens could be used by the worker bees for the differentiation not only according to age but also according to kin.

The results presented here may also be used in the beekeeping practice when queens in managed colonies are exchanged. The results obtained on the recognition of kin specific pattern may explain why certain queens (especially imported ones from

other countries) are often badly accepted (Moretto *et al*., 2004; Rohdes *et al*., 2004). A better understanding of the mechanisms for the individual queen recognition in bees, in general, could help to avoid queens losses during the exchange of queens .

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7. Summary

Activities of honeybees *Apis mellifera* L. colony are coordinated by an effective communication network in which the queen plays a central role by controlling behavior and reproduction of workers through pheromones. Most pheromones are produced in the mandibular (QMP) and tergal gland and distributed over the queen's cuticle. The acquisition of these pheromones from the cuticular body surface of the queen is performed by antennating and licking of the retinue workers. Workers of a colony are able to recognize their own queen. Foreign queens which are introduced without protection are normally killed by the workers.

While a lot of work has been performed on the primer and releaser effect of certain queen pheromones, it is still unknown how the workers distinguish their own queen from foreign ones. The fact that queens can be exchanged successfully by protecting the foreign queen for some days demonstrates that workers are able to "learn" their queen. It is likely that a certain chemical pattern of the cuticle (odor or taste) is finally responsible for the recognition and acceptance as "own". In this context, this work has three different objectives:

- To better understand the bees' behavior to "own" and "foreign" queens and to quantify certain behavioral traits of the queen-workers interaction.
- To study the learning ability for own and foreign queens by the use of the Proboscis Extension Reflex (PER) in order to have a tool for future tests of odorous compounds.
- To compare the cuticular pattern of queens of different origin.

In all three approaches, virgin and mated queens and queens of different kin relation to each other were reared and established in Kirchhainer nuclei colonies. These queens were compared due to the following hypothesis: If the workers perceive their own queen by a distinct smell and if closely related queens have a more similar chemical pattern on the cuticle, then a related foreign queen should be easier "learned"/ accepted than a non related one.

For this purpose, first a specific bioassay had to be developed and established to enable the record of workers behavior to the queen without an inhibition of the complex social interactions between queens and workers. This "cage bioassay" consists of a small wooden box with a glass front, a wax comb, 30-40 worker bees and a queen. For the tests, the own queen of this mini-colony was removed and a

foreign queen was introduced. For a period of about 2 hours certain aggressive and benign actions, respectively, of the workers toward the queen were recorded.

In the first set of tests, queens of different kin relations were compared. The results showed, in general, an aggressive reaction against the introduced foreign queens. However, there were clear lower benign and stronger aggression behaviors against unrelated queens compared to the related ones. Some of the unrelated queens were even killed. However, these differences were only significant when virgin queens were exchanged but not when mated queens were used. Concerning the duration of the aggressive action of workers, aggression generally decreased between the beginning and the end of the test; again, this was significant only in the experiments with virgin queens. This indicates, that at least in virgin queens the individual recognition by the worker bees depends on a kin specific odorous pattern of the queen.

The same types of queens used in the cage bioassays were used for the learning experiments. A classical olfactory conditioning (PER) of worker honeybees was applied by using a living queen as the source of odor. Hereby the queen was offered in a way that the worker bees could not touch the body surface. The gradual increase in the learning curves was a good indication that the workers are able to learn the queen's odor and, therefore, can be used as a kind of "biosensor". After having learned a queen's odor, the conditioned workers were tested by offering virgin and mated queens, respectively, with defined kin relation to the queen used for the conditioning before. The results revealed clear differences in the cues used for the "learning" of individual mated and individual virgin queens, respectively. The workers could significantly discriminate between the learned odor of a mated queen and any other mated queen irrespective of the relatedness. In contrast, worker bees could not discriminate virgin queens from each other. As the worker could only use volatile substances for the associative learning, one can conclude the following: In virgin queens the volatile "bouquet" is neither individual specific nor kin specific. In mated queens the bouquet has only an individual specificity. Probably, the huge amount of many volatile gland products (including the main component 9-ODA) makes each mated queen "unique". But as in the cage bioassays the worker could recognize whether an introduced virgin queen was related to the own queen or not, these recognition must depend on non volatile substances of the virgin queens cuticle which are perceived by licking.

If learned and tested queens were of different mating status the worker bees could significantly discriminate between such individuals (except learned odor of mated queens/ tested odor of related virgin queens). This is not surprising because the GC-MS analysis confirmed the huge differences in the odorous pattern (and here mainly the volatile polar gland products) between virgin (= young) and mated (=elder) queens.

From the same types of queens used for the cage bioassays and PER, queens' heads and abdomens were extracted in a solvent and the obtained extracts were analyzed using GC-MS. From the extracts of queen abdomens 32 substances (hydrocarbons and polar compounds) were identified and chosen to calculate the "chemical distance" between queens of different kin relation (sister vs. unrelated) and between sister queens having different ages and mating status. For that purpose, a matrix of Nei-distances was applied as a measure for the similarity of different patterns. The results showed a significantly higher concordance in the chemical pattern within sister queens compared to non related ones. The "chemical distance" increased from sister queens over half sister to non-related queens. Cluster analyses of the Nei distance and multidimensional scaling clearly confirmed the differentiation between unrelated queens and the similarity of sister queens. Using the same statistical methods, also a clear differentiation between queens of different ages and mating status could be demonstrated.

The results presented in this work confirmed with 3 different approaches that workers are able to learn their own queen with different learning cues depending on the mating status of the queen. In virgin queens it could be demonstrated for the first time that the kin relation between different queens can be recognized, presumably by low or non-volatile substances. The chemical analyses confirmed that the cuticular pattern of queens could be used for the differentiation not only according to age but also according to kin.

8. Zusammenfassung

Das Sozialverhalten innerhalb eines Bienenvolkes (*Apis mellifera* L.) wird durch ein wirkungsvolles Kommunikationsnetzwerk koordiniert, in dem Königinnenpheromone eine zentrale Rolle bei der Kontrolle von Verhalten und Reproduktion der Arbeiterinnen spielen. Die meisten Pheromone werden in der Mandibel- und der Tergittaschendrüse gebildet. Die Perzeption dieser Pheromone durch die Arbeiterinnen erfolgt durch Betasten mit den Antennen und Lecken an der Kutikula der Königinnen. Die Arbeiterinnen eines Volkes sind in der Lage ihre eigene Königin zu erkennen. Ungeschützt ins Bienenvolk eingebrachte fremde Königinnen werden normalerweise von den Arbeiterinnen getötet. Es gibt zahlreiche Untersuchungen zu Primer- und Releaser-Effekten von bestimmten Bestandteilen des Königinnenpheromons. Nach wie vor ist aber nicht bekannt, wie die Arbeiterinnen zwischen ihrer eigenen und fremden Königinnen unterscheiden. Die Tatsache, dass Königinnen erfolgreich ausgetauscht werden können, nachdem man sie einige Tage lang durch einen Käfig geschützt hat, zeigt, dass die Arbeiterinnen in der Lage sind, den Geruch der Königin zu lernen. Vermutlich ist ein bestimmtes chemisches Muster der Kutikula (Duft oder Geschmack) für die Erkennung und Akzeptanz als „eigen“ verantwortlich.

Diese Arbeit hat daher drei verschiedene Zielsetzungen:

- das Verhalten von Bienen gegenüber eigenen und fremden Königinnen besser zu verstehen und bestimmte Verhaltensmerkmale der Interaktion zwischen Königin und Arbeiterinnen in einem Biotest zu quantifizieren
- mit Hilfe des Rüssel-Reflexes (PER) zu prüfen, ob fremde und eigene Königinnen anhand von Duftstoffen „erlernt“ werden können
- die Zusammensetzung der Kutikuladuftstoffe von Königinnen unterschiedlicher Verwandtschaft und Paarungsstatus zu untersuchen

Für alle drei Ansätze wurden unbegattete und begattete Königinnen mit unterschiedlichen Verwandtschaftsverhältnissen zueinander aufgezogen und in Kirchhainer Begattungskästchen gehalten.

Der Arbeit lag die folgende Hypothese zu Grunde: Wenn Arbeiterinnen ihre Königin anhand eines eindeutigen Dufts wahrnehmen und verwandte Königinnen ein ähnlicheres chemisches Muster besitzen, sollte der Duft einer verwandten

Königinnen leichter gelernt oder akzeptiert werden, als der einer nicht verwandten Königin.

Zunächst war es notwendig, einen speziellen Biotest zu entwickeln und zu etablieren, der eine quantitative Erfassung des Verhaltens der Arbeiterinnen gegenüber der Königin erlaubt, ohne die sozialen Interaktionen zwischen Königinnen und Arbeiterinnen zu stören. Dieser „Käfig-Biotest“ besteht aus einer kleinen Holzkiste mit einer Glasscheibe, einer Wabe mit 30-40 Arbeiterinnen und einer Königin. Für die Versuche wurde die eigene Königin dieses Mini-Volkes entfernt und stattdessen eine fremde Königin eingesetzt. Für einen Zeitraum von 2 Stunden wurden dann bestimmte aggressive und „freundliche“ Handlungen der Arbeiterinnen gegenüber der Königin aufgezeichnet.

In der ersten Testreihe wurden Königinnen mit unterschiedlichem Verwandtschaftsgrad zur eigenen Königin verglichen. Die Ergebnisse zeigten grundsätzlich eine aggressive Reaktion gegenüber der eingesetzten, fremden Königin. Jedoch gab es deutlich weniger freundliches und vermehrt aggressives Verhalten gegenüber Königinnen, die mit der ursprünglichen Königin nicht verwandt waren im Vergleich zu verwandten Königinnen. Diese verwandtschaftsspezifischen Unterschiede waren in allen Versuchen vorhanden, jedoch nur dann signifikant, wenn unbegattete Königinnen ausgetauscht wurden, nicht aber bei begatteten Königinnen. Während der Versuchsdauer nahm das aggressive Verhalten der Arbeiterinnen im Allgemeinen ab. Auch hier zeigte sich eine Signifikanz nur bei Versuchen mit unbegatteten Königinnen. Das weist darauf hin, dass zumindest bei unbegatteten Königinnen die individuelle Erkennung durch Arbeiterinnen auf einem verwandtschafts-spezifischen Duftmuster beruht.

Für die Lernversuche wurden ebenfalls Königinnen unterschiedlicher Verwandtschaft verwendet. Die lebenden Königinnen wurden hier als Duftquelle für eine klassische olfaktorische Konditionierung der Arbeiterinnen eingesetzt. Dabei wurde die Königin so angeboten, dass die Arbeiterinnen den Körper der Königin nicht berühren konnten. Die Lernkurven zeigen deutlich, dass die Arbeiterinnen fähig sind, den Geruch der Königin zu lernen und daher als eine Art „Biosensor“ eingesetzt werden können. Nachdem sie den Geruch der Königin gelernt hatten, wurden den konditionierten Arbeiterinnen zum Test begattete und unbegattete Königinnen angeboten, die einen unterschiedlichen Verwandtschaftsgrad mit der zuvor für die Konditionierung eingesetzten Königin hatten.

Die Ergebnisse zeigen, dass sich die Signale, die für das Lernen des individuellen Dufts von begatteten und unbegatteten Königinnen verantwortlich sind, deutlich unterscheiden. Die Arbeiterinnen konnten unabhängig von der Verwandtschaft signifikant zwischen dem gelernten Geruch einer begatteten Königin und dem anderer begatteter Königinnen unterscheiden. Im Gegensatz dazu konnten die Arbeiterinnen unbegattete Königinnen nicht voneinander unterscheiden. Da die Arbeiterinnen in diesem Versuch nur flüchtige Substanzen für das assoziative Lernen nutzen konnten, lässt sich Folgendes daraus schließen: bei begatteten Königinnen ist das volatile „Bouquet“ weder auf individueller Ebene noch in Bezug auf die Verwandtschaft spezifisch. Nur bei begatteten Königinnen hat dieses Bouquet eine individuelle Spezifität. Möglicherweise macht die große Menge an flüchtigen Drüsensekreten (einschließlich der Hauptkomponente 9-ODA) die begattete Königin „einzigartig“. Da die Arbeiterinnen aber in den Versuchen mit dem Käfig-Biotest erkennen konnten, ob eine zugesetzte unbegattete Königin verwandt mit der eigenen war oder nicht, muss diese Erkennung auf nicht-volatilen Substanzen der Kutikula beruhen, die durch direkten Kontakt aufgenommen werden müssen. Wenn gelernte und getestete Königinnen einen unterschiedlichen Begattungsstatus hatten, konnten die Arbeiterinnen mit einer Ausnahme signifikant zwischen diesen Individuen unterscheiden. Dies ist nicht überraschend, da die GC-MS-Analyse die großen Unterschiede im Duftmuster von unbegatteten (=jungen) und begatteten (=älteren) Königinnen bestätigt.

Von allen Versuchsgruppen wurden Extrakte von Kopf und Abdomen angefertigt und anschließend im GC-MS analysiert. Insgesamt wurden 32 Substanzen des Königinnen-Abdomens analysiert und die „chemische Distanz“ von Königinnen unterschiedlicher Verwandtschaft (Schwestern im Vergleich mit nicht verwandter Königin) und von Schwester-Königinnen unterschiedlichen Alters und Begattungsstatus berechnet. Hierzu wurde die „Nei-Distanz“ zur Messung der Ähnlichkeit der verschiedenen Muster herangezogen. Die Ergebnisse zeigten zwischen Schwesterköniginnen eine signifikant höhere Übereinstimmung im chemischen Muster als zwischen nicht-verwandten Königinnen.

Die „chemische Distanz“ nahm innerhalb der Gruppen von Schwester-Königinnen über Halb-Schwestern zu nicht-verwandten Königinnen zu. Cluster-Analysen der Nei-Distanz und eine multidimensionale Skalierung bestätigen deutlich die Unterschiede zwischen nicht verwandten Königinnen und die Ähnlichkeit von

Schwesterköniginnen. Mit denselben statistischen Methoden konnte außerdem ein deutlicher Unterschied zwischen Königinnen verschiedenen Alters und Begattungsstatus gezeigt werden.

Die in dieser Arbeit dargelegten Ergebnisse bestätigen mit drei unterschiedlichen Ansätzen, dass Arbeiterinnen in der Lage sind, den Duft ihrer eigenen Königin zu lernen, wobei die Erkennungssignalen in Abhängigkeit des Begattungsstatus variieren. Bei unbegatteten Königinnen konnte erstmalig nachgewiesen werden, dass die Verwandtschaft zwischen verschiedenen Königinnen vermutlich anhand schwer flüchtiger Substanzen erkannt wird. Die chemische Analyse beweist, dass das Kutikulamuster der Königinnen nicht nur für die Unterscheidung des Alters, sondern auch für die Unterscheidung der Verwandtschaft herangezogen werden kann.

9. Appendices

Appendix 9.1 Average number of workers' benign contacts towards foreign virgin queens of different kin relation (sister, half sister, unrelated) during each observation. The numbers (0, 15, 30, 60) beside each contact behavior refer to the time interval prior to each observation.

	Benign Contacts (Virgin queens)		
Observation	sister	half sister	unrelated
0	41,35[a] (±5,92)	40,40[a] (±6,14)	34,90[a] (±6,54)
15	29,65[a] (±3,33)	29,10[a] (±3,27)	25,45[a] (±4,33)
30	27,05[a] (±4,64)	23,85[a] (±3,09)	22,20[a] (±4,22)
60	24,75[a] (±2,91)	23,00[ab] (±2,96)	19,50[b] (±2,81)
Total	122,80[a] (±9,58)	116,35[ab] (±8,54)	102,05[b] (±11,56)

Appendix 9.2 Average number of workers' benign contacts towards foreign mated queens of different kin relation (sister, half sister, unrelated) during each observation. The numbers (0, 15, 30, 60) beside each contact behavior refer to the time interval prior to each observation.

	Benign Contacts (Mated queens)		
Observation	sister	half sister	unrelated
0	42,80[a] (±5,96)	36,60[a] (±5,33)	40,90[a] (±5,95)
15	34,85[a] (±4,10)	29,80[a] (±5,86)	32,05[a] (±5,03)
30	30,95[a] (±4,15)	27,05[a] (±4,80)	26,45[a] (±4,37)
60	28,55[a] (±3,57)	25,55[a] (±4,55)	27,90[a] (±3,07)
Total	137,15[a] (±11,67)	119,00[a] (±17,96)	127,30[a] (±15,08)

Appendix 9.3 Average number of workers' aggressive contacts towards foreign virgin queens of different kin relation (sister, half sister, unrelated) during each observation. The numbers (0, 15, 30, 60) beside each contact behavior refer to the time interval prior to each observation.

	Aggressive Contacts (Virgin queens)		
Observation	sister	half sister	unrelated
0	0,60[a] (±0,73)	1,05[ab] (±0,97)	2,00[b] (±1,20)
15	0,40[a] (±0,60)	0,50[a] (±0,62)	0,65[a] (±0,63)
30	0,15[a] (±0,31)	0,45[a] (±0,94)	0,15[a] (±0,31)
60	0[a] -	0[a] -	0[a] -
Total	1,15[a] (±1,56)	2,00[ab] (±2,23)	2,80[b] (±1,78)

Appendix 9.4 Average number of workers' aggressive contacts towards foreign mated queens of different kin relation (sister, half sister, unrelated) during each observation. The numbers (0, 15, 30, 60) beside each contact behavior refer to the time interval prior to each observation.

	Aggressive Contacts (Mated queens)		
Observation	sister	half sister	Unrelated
0	2,10[a] (±1,32)	1,25[a] (±1,09)	2,20[a] (±1,49)
15	1,05[a] (±0,94)	0,55[a] (±0,63)	1,50[a] (±1,32)
30	0,60[a] (±0,49)	0,40[a] (±0,49)	1,10[a] (±1,04)
60	0,95[a] (±0,82)	0,30[a] (±0,43)	1,00[a] (±1,01)
Total	4,70[a] (±3,43)	2,50[a] (±2,45)	5,80[a] (±4,57)

Appendix 9.5 Average number of workers' biting and stinging behavior against queens of different kin and mating status at the beginning of the test (0 min.) and at the end of the test (125 min.)

Queen	Aggressive Behavior	At 0 min.	At 125 min.
Virgin sister	Biting	0,25[a] (±0,58)	0[a] -
	Stinging	0,35[a] (±0,44)	0[a] -
Virgin half sister	Biting	0,6[a] (±0,58)	0[b] -
	Stinging	0,45[a] (±0,44)	0[b] -
Virgin unrelated	Biting	1,15[a] (±0,73)	0[b] -
	Stinging	0,85[a] (±0,59)	0[b] -
mated sister	Biting	0,8[a] (±0,52)	0,35[a] (±0,38)
	Stinging	1,3[a] (±0,83)	0,6[a] (±0,49)
Mated half sister	Biting	0,5[a] (±0,44)	0,1[a] (±0,21)
	Stinging	0,75[a] (±0,68)	0,2[a] (±0,33)
Mated unrelated	Biting	1,1[a] (±0,69)	0,45[a] (±0,44)
	Stinging	1,1[a] (±0,82)	0,55[a] (±0,58)

Appendix 9.6 Number of positive responses and errors made by workers during applying the olfactory PER by conditioning and testing with odors of different queens.

Test	Positive response	Error	Sign.	Differentiation
Virgin –Virgin sister	63/ 70	37/ 30	0.294	no
virgin – Virgin unrelated	67/ 58	33/ 42	0.189	no
Virgin – Mated sister	57/ 36	9/ 30	0,00006	yes
Virgin – Mated unrelated	50/ 22	46/ 74	0,00003	yes
Mated – Mated sister	58/ 38	34/ 54	0,003	yes
Mated – Mated unrelated	55/ 30	35/ 60	0,0002	yes
Mated – Virgin sister	35/ 26	37/ 46	0,129	no
Mated – Virgin unrelated	52/ 19	50/ 83	0,000001	yes

Appendix 9.7 Nei distance for 1 day old virgin sister queens. (32 identified substances in queen abdomen extracts).

			GC/MS-run /Filename	AB1DAUL_02	AB1DAUL_03	AB1DAUL_04	AB1DAUL_05	AB1DAUL_07	AB1DAUL_08	AB1DAUL_09
			body part	Abdomen	Abdomen	Abdomen	Abdomen	Abdomen	Abdomen	Abdomen
			mating status	Virgin	Virgin	Virgin	Virgin	Virgin	Virgin	Virgin
GC/MS-run/Filename	body part	mating status	Code	1	2	3	4	5	6	7
AB1DAUL_02	Abdomen	Virgin	1		0,88	0,95	0,98	0,88	0,85	0,96
AB1DAUL_03	Abdomen	Virgin	2			0,90	0,88	0,99	0,92	0,93
AB1DAUL_04	Abdomen	Virgin	3				0,97	0,90	0,91	0,92
AB1DAUL_05	Abdomen	Virgin	4					0,88	0,87	0,95
AB1DAUL_07	Abdomen	Virgin	5						0,91	0,94
AB1DAUL_08	Abdomen	Virgin	6							0,84
AB1DAUL_09	Abdomen	Virgin	7							
			GC/MS-run /Filename	AB1DAUL_02	AB1DAUL_03	AB1DAUL_04	AB1DAUL_05	AB1DAUL_07	AB1DAUL_08	AB1DAUL_09
			body part	Abdomen	Abdomen	Abdomen	Abdomen	Abdomen	Abdomen	Abdomen

Appendix 9.8 Nei distance for 1 day old virgin sister queens and 1 day old virgin unrelated queens. (32 identified substances in queen abdomen extracts).

			GC/MS-run /Filename	AB1DAUL_02	AB1DAUL_03	AB1DAUL_04	AB1DAUL_05	AB1DAUL_07	AB1DAUL_08	AB1DAUL_09	AB1DHoh01	AB1DHoh02	AB1DHoh03	AB1DHoh04	AB1DHoh05
			Body part	Abdomen	Abdomen	Abdomen	Abdomen	Abdomen	Abdomen	Abdomen	Abdomen	Abdomen	Abdomen	Abdomen	Abdomen
			Mating	Virgin	Virgin	Virgin	Virgin	Virgin	Virgin	Virgin	Virgin	Virgin	Virgin	Virgin	Virgin
GC/MS-run/Filename	Body part	Mating	Code	1	2	3	4	5	6	7	8	9	10	11	12
AB1DAUL_02	Abdomen	Virgin	1		0,90	0,96	0,98	0,89	0,86	0,96	0,68	0,66	0,85	0,41	0,40
AB1DAUL_03	Abdomen	Virgin	2			0,90	0,89	0,99	0,92	0,93	0,82	0,83	0,92	0,62	0,62
AB1DAUL_04	Abdomen	Virgin	3				0,98	0,90	0,91	0,92	0,72	0,71	0,88	0,45	0,45
AB1DAUL_05	Abdomen	Virgin	4					0,89	0,88	0,95	0,68	0,66	0,86	0,42	0,40
AB1DAUL_07	Abdomen	Virgin	5						0,91	0,95	0,79	0,79	0,96	0,55	0,54
AB1DAUL_08	Abdomen	Virgin	6							0,84	0,75	0,78	0,81	0,53	0,56
AB1DAUL_09	Abdomen	Virgin	7								0,68	0,67	0,91	0,41	0,41
AB1DHoh01	Abdomen	Virgin	8									0,98	0,78	0,87	0,88
AB1DHoh02	Abdomen	Virgin	9										0,76	0,90	0,92
AB1DHoh03	Abdomen	Virgin	10											0,51	0,49
AB1DHoh04	Abdomen	Virgin	11												0,96
AB1DHoh05	Abdomen	Virgin	12												

Appendix 9.9 Nei distance for 1 day old virgin, 8 days old virgin and 150 days old mated sister queens (32 identified substances in queen abdomen extracts).

		GC/MS-run /Filename	ABV1D_2	ABV1D_3	ABV1D_4	ABV1D_5	ABV1D_7	ABV1D_8	ABV1D_9	ABV8D_1	ABV8D_2	ABV8D_3	ABV8D_4	ABV8D_5	ABV8D_6	ABV8D_8	ABM_1	ABM_2	ABM_3	ABM_5	ABM_6
		body part	Abdomen	Abdomen	Abdomen	Abdomen	Abdomen	Abdomen	Abdomen	Abdomen	Abdomen	Abdomen	Abdomen	Abdomen	Abdomen	Abdomen	Abdomen	Abdomen	Abdomen	Abdomen	Abdomen
		mating status	Virgin	Virgin	Virgin	Virgin	Virgin	Virgin	Virgin	Virgin	Virgin	Virgin	Virgin	Virgin	Virgin	Virgin	mated	mated	mated	mated	mated
GC/MS-run/Filename	body part	mating status	Code	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
ABV1D_2	Abd	Virgin	1		0,88	0,95	0,98	0,88	0,85	0,96	0,41	0,50	0,53	0,47	0,66	0,46	0,40	0,45	0,50	0,40	0,55	0,43
ABV1D_3	Abd	Virgin	2			0,90	0,88	0,99	0,92	0,93	0,42	0,54	0,50	0,53	0,71	0,46	0,37	0,47	0,43	0,35	0,54	0,33
ABV1D_4	Abd	Virgin	3				0,97	0,90	0,91	0,92	0,51	0,58	0,59	0,55	0,76	0,54	0,45	0,54	0,55	0,52	0,60	0,47
ABV1D_5	Abd	Virgin	4					0,88	0,87	0,95	0,48	0,53	0,55	0,49	0,71	0,49	0,42	0,50	0,50	0,44	0,55	0,43
ABV1D_7	Abd	Virgin	5						0,91	0,94	0,36	0,46	0,46	0,45	0,66	0,39	0,34	0,40	0,37	0,30	0,47	0,29
ABV1D_8	Abd	Virgin	6							0,84	0,49	0,59	0,58	0,56	0,77	0,54	0,40	0,56	0,60	0,48	0,65	0,47
ABV1D_9	Abd	Virgin	7								0,32	0,41	0,45	0,39	0,60	0,36	0,34	0,35	0,39	0,32	0,46	0,33
ABV8D_1	Abd	Virgin	8									0,88	0,56	0,87	0,75	0,77	0,29	0,89	0,61	0,74	0,67	0,43
ABV8D_2	Abd	Virgin	9										0,72	0,98	0,91	0,84	0,53	0,98	0,61	0,64	0,73	0,45
ABV8D_3	Abd	Virgin	10											0,71	0,83	0,91	0,48	0,71	0,53	0,61	0,63	0,55
ABV8D_4	Abd	Virgin	11												0,88	0,87	0,45	0,95	0,56	0,63	0,71	0,45
ABV8D_5	Abd	Virgin	12													0,86	0,55	0,90	0,52	0,54	0,69	0,43
ABV8D_6	Abd	Virgin	13														0,28	0,83	0,57	0,68	0,71	0,56
ABV8D_8	Abd	Virgin	14															0,51	0,31	0,24	0,32	0,20
ABM_1	Abd	mated	15																0,58	0,63	0,68	0,42
ABM_2	Abd	mated	16																	0,85	0,88	0,84
ABM_3	Abd	mated	17																		0,71	0,73
ABM_5	Abd	mated	18																			0,86
ABM_6	Abd	mated	19																			

Lebenslauf

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Französisch	gut
Chinesisch	Grundkenntnisse

Tagungen

2008	Vortrag: Sniffer bees” can honeybees learn the odor of queens of different kin relation? 55. Arbeitsgemeinschaft der Bieneninstitute, Berlin – Deutschland
2007	Vortrag: Influence of the kin relation of a foreign queen on the acceptance of honeybee workers in a laboratory bioassay, 54. Arbeitsgemeinschaft der Bieneninstitute, Veitshöchheim- Deutschland
	Vortrag: Individual queen recognition in honeybees *Apis mellifera*: Role of mating status and queen kin relation, Treffen der Chemoökologen aus BW, Ulm – Deutschland.
2005	Poster: Individual queen recognition by honeybee workers *Apis mellifera* within a laboratory bioassay, 52. Arbeitsgemeinschaft der Bieneninstitute, Halle – Deutschland