Variation in the Components of Head Extracts of Workers and Queens of *Apis mellifera intermissa* Buttel-Reepen

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Quantitative analysis of nine major components of the head extracts of workers and queens of *Apis mellifera intermissa* was carried out. Queen and worker component patterns were distinct. The patterns of both castes changed with age, and the worker patterns were also affected by being reared in the absence of a queen.

The genetic variance of the component composition of the extracts was found to be higher in the queens than the workers. This may have functional significance in the recognition by workers of their own queen.

Introduction

Social control *via* chemical signals in insects is exemplified in the honey-bees by the mandibular gland signals of the queens [1]. These have been shown to have a variety of effects both on workers and on the production of additional female reproductives [2]. Chemical analyses of the secretions of this gland have indicated that it is dominated by the presence of the fatty acid (*E*)-9-ketodec-2-enoic acid (the "queen substance", ODA) [3, 4] which was initially thought to be specific to the queen caste. Subsequent analyses of mandibular gland extracts from queens and workers have demonstrated that the composition of the secretions is affected by age, caste and race [5]. Since the composition of the secretions is affected by all these factors, its expression in the female castes of honey-bees needs to be defined more rigorously.

Removal or loss of a queen from a honey-bee colony can result either in the rearing of a new queen if conditions within the colony permit such replacement or in the production of laying workers from the group of young workers present in the colony [6]. The laying workers may act as "false queens" in that they can elicit retinue behaviour in their nest-mates and may produce mandibular gland secretions that mimic the patterns of components found in queens [5].

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Apis mellifera intermissa Buttel-Reepen is the honey-bee race native to the western Mediterranean African coast [7]. It is a bee which produces laying workers very rapidly on the removal of the queen [8]. In this characteristic it resembles the Cape honey-bee *(A. m. capensis)* in which laying workers also develop rapidly. Many *capensis* workers in queenless colonies produce a mandibular gland secretion in which ODA predominates [9, 10]. Since *intermissa* workers have behavioural similarities to *capensis*, we decided to investigate the secretions of the mandibular glands of the female castes of *intermissa* in order to determine whether the speed with which laying workers develop is related to changes in the composition of this secretion.

Materials and Methods

Queens of *A. m. intermissa* were obtained from feral colonies in Sejenane, Tunisia. At the start of the experiment, these queens were three months old and had been naturally mated in Tunisia. They were established in colonies of *A. m. carnica* at the Institut für Bienenkunde in Oberursel. The brood produced by these queens was removed from the colonies when the cells were sealed and the workers allowed to emerge in an incubator maintained at 33 °C and 65% RH.

Workers that were 24 h old were sampled from groups of nest-mates emerging in the incubator. In all other cases, individual young (≤ 24 h old) *intermissa* workers were placed in a cage with 80 car-

niolan bees *(A. m. carnica)*. The cages were supplied with food, placed in an incubator and the *intermissa* workers harvested at 2, 4, 8, 12, 16 and 21 days after the establishment of the worker groups. Individual *intermissa* workers that had previously been marked with paint were removed from queenright colonies when they were 5, 13, 16 and > 21 days old. The frequency of sampling in this case was reduced from that of the workers in the *carnica* groups, since the rate of changes in the composition of the extracts was not expected to be as great. Sampling of the workers consisted of removing them from their cage or colony, placing them in a refrigerator at 4 °C until they were immobile and then removing the head and placing it in 500 µl of dichloromethane.

Queens of *intermissa* were reared by grafting the eggs produced by the queens from Tunisia into queenless carniolan colonies. The sealed queen cells were placed in an incubator and the queens allowed to emerge in the incubator. They were then placed in three frame colonies of carniolan bees where they were allowed to age until harvested in the same manner as the workers. Since the genetic relationships of the queens were known, the genetic variance of the secretions could be estimated.

The head extracts were analyzed by removing the head from the solvent, and then evaporating the solvent just to dryness with a stream of N_2. The residue in the vial was then redissolved in 20 µl of internal standard solution (containing octanoic acid and tetradecane as the two internal standards) to which was added 20 µl of bis-(trimethylsilyl)trifluoroacetamide (BSTFA). The mixture was allowed to react for a minimum of 10 min at room temperature before being analyzed gas chromatographically.

The internal standard solution was made up by accurately weighing approximately 1 mg amounts of standard *n*-octanoic acid and tetradecane on a Mettler BE 22 microbalance and placing these in a vial with 4 ml dichloromethane. This produced a solution of known composition with a concentration of approximately 200 ng/µl.

The standard solution that was used for the measurement of the relative mass ratios [11] was produced by accurately weighing amounts (± 1 mg) of the following: tetradecane, octanoic acid, decanoic acid, methyl *p*-hydroxybenzoic acid, *p*-hydroxybenzoic acid, 2-(3-methoxy-4-hydroxyphenyl)ethanol, (*E*)-9-ketodec-2-enoic acid and 10-hydroxydecanoic acid, and placing these in a vial with 4 ml dichloromethane. The relative mass ratios (RMR) of each of these compounds was measured relative to tetradecane. Mean RMR's for each compound were determined from 18 successive runs of the standard solution. This standard solution was run each day, before analyses of bee extracts took place, to ensure that RMR's were within the limits of variability (mean CV of the standard compounds was 11%) found in the series of standard runs. These RMR's were used to calculate the amount of each of the compounds in the bee extracts. Since standards for the hydroxy acids (*E*)-9-hydroxydec-2-enoic acid, (*E*)-10-hydroxydec-2-enoic acid and 8-hydroxyoctanoic acid were not available, the RMR of the hydroxy acid, 10-hydroxydecanoic acid, was used to estimate the amounts of each of these compounds present in the extracts.

Gas chromatographic analyses were performed on a Hewlett-Packard 5890 gas chromatograph fitted with a split-splitless inlet and a 25 m × 0.32 mm methyl silicone coated fused silica capillary column. The split-splitless injection technique was used with hexane as the solvent plug. The carrier gas was hydrogen at a flow rate of 1 ml/min and the oven temperature was programmed as follows: 60 °C for 1 min then programmed at 50 °C/min to 110 °C, then 3 °C/min from 110 °C to 220 °C and held at 220 °C for 10 min. Chromatograms were recorded and peak areas quantified with an HP 3392A recording integrator. Peaks were identified by their retention times relative to the two internal standards and their identity was confirmed by GC-MS using a HP-5890 gas chromatograph interfaced to an HP-5970 mass selective detector.

Statistical analyses were performed using the programs in SAS for Personal Computers, Version 6.

Results

Workers

The major components of the mandibular gland secretions of this race of honey-bee are given in Table I. These secretions are characterized by the presence of a variety of components that are common to the mandibular gland secretions of honeybees. The extracts from the queenless workers are dominated by the production of (*E*)-9-hydroxydec-2-enoic acid (HDA9) until they are 16 days old. Large quantities of 10-hydroxydecanoic acid (HDAA) and (*E*)-10-hydroxydec-2-enoic acid (HDA) are also present. The (*E*)-9-ketodec-2-enoic acid (ODA)

which is the predominant component of queen extracts is present in these workers by the age of 4 days and is present in relatively large quantities at 8 days old. Examination of the standard deviations indicates that the composition of individual extracts is highly variable.

The extracts from workers obtained from queenright colonies exhibit some differences in composition from those of the queenless workers. These consist of: 1) in most cases HDA is the major component of the extracts and 2) HDA9, although prominent, is never quantitatively the most prominent component. Fig. 1 gives a graphic representation of changes in the chemical composition of the head extracts of the queenless worker bees as they age. These workers produce secretions that are variable in composition and rich in the number of components present. Bees of 16 days and older had mixtures dominated by HDA. Queenright worker bees produce secretions (Fig. 2) of a more uniform composition in which the three acids HDA9, HDAA and HDA predominate. In both cases there are overall quantitative increases with age.

In order to determine the degree to which variability in the composition of the extracts was genetically determined, the secretions of individual bees of 16 and 21 days old were analyzed. They were chosen, since their secretions were not undergoing the ontogenetic changes seen in bees of younger ages. There was a total of sixteen bees in these two age classes. They were the progeny of four different queens, thus they could be divided into four groups of four sisters. The composition of the extracts of these sixteen individuals were subjected to a principal components

Table I. Composition of the components present in the head extracts of *A. m. intermissa* workers of various ages in queenless groups of bees and from queenright colonies. DEC = decanoic acid, MEBENZ = methyl *p*-hydroxybenzoate, OHBENZ = *p*-hydroxybenzoic acid, ODA = (*E*)-9-ketodec-2-enoic acid, MHPE = 2-(3-methoxy-4-hydroxyphenyl)-ethanol, HDA9 = (*E*)-9-hydroxydec-2-enoic acid, HDAA = 10-hydroxydecanoic acid, HDA = (*E*)-10-hydroxydec-2-enoic acid, HOA = 8-hydroxyoctanoic acid.

Age [days]	n		ng Component present								
			DEC	MEBENZ	OHBENZ	ODA	MHPE	HDA9	HDAA	HDA	HOA
Queenless workers											
1	8	$\bar{x}$	119	8	23	0	9	453	161	141	244
		s	76	22	25	0	25	244	207	173	121
2	8	$\bar{x}$	141	23	0	0	12	531	330	68	295
		s	63	33	0	0	33	394	443	125	346
4	8	$\bar{x}$	106	20	0	164	0	1903	3014	0	960
		s	91	40	0	432	0	2825	6985	0	1306
8	8	$\bar{x}$	540	64	8	662	0	1972	829	524	1345
		s	1115	95	22	1640	0	2618	710	754	2667
12	8	$\bar{x}$	169	61	22	58	24	3134	2051	1870	1999
		s	23	102	41	66	68	2488	1942	3386	1944
16	7	$\bar{x}$	2170	45	8	126	209	1249	2212	3219	273
		s	2407	87	23	108	339	780	1105	4770	205
21	10	$\bar{x}$	389	10	19	113	21	2057	1703	3007	1229
		s	692	31	45	99	43	1080	1473	4615	731
Queenright workers											
5	3	$\bar{x}$	135	0	0	30	0	889	1668	2457	398
		s	118	0	0	52	0	576	1110	1283	690
13	4	$\bar{x}$	137	14	0	226	22	714	1346	949	197
		s	14	27	0	129	44	350	1061	425	113
16	7	$\bar{x}$	150	15	0	179	42	2843	1939	3418	1781
		s	26	27	0	111	75	3133	995	2682	2316
21	5	$\bar{x}$	178	7	5	351	99	3642	3210	6520	1831
		s	53	18	10	134	72	2436	1346	8177	1591

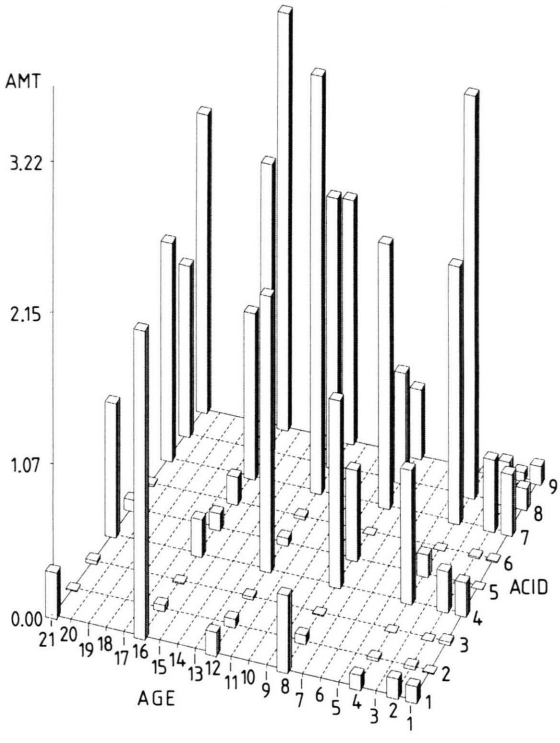

Fig. 1. Patterns of component composition in bees of various ages from queenless groups. The X-axis indicates the age from emergence of the group of bees, the Y-axis the amount of each acid in μg, and the Z-axis identifies the particular component by number. These compounds are numbered as follows: 1) decanoic acid, 2) methyl *p*-hydroxybenzoate, 3) *p*-hydroxybenzoic acid, 4) 8-hydroxyoctanoic acid, 5) (*E*)-9-ketodec-2-enoic acid, 6) 2-(3-methoxy-4-hydroxyphenyl)ethanol, 7) (*E*)-9-hydroxydec-2-enoic acid, 8) 10-hydroxydecanoic acid, 9) (*E*)-10-hydroxydec-2-enoic acid.

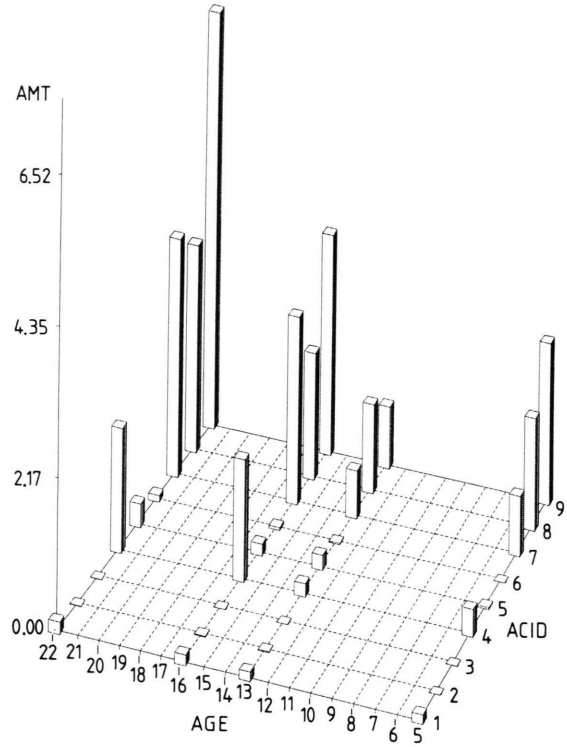

Fig. 2. Patterns of component composition in bees of various ages from queenright groups. The X-axis indicates the age from emergence of the group of bees, the Y-axis the amount of each acid in μg, and the Z-axis identifies the particular component by number. These acids are numbered as follows: 1) decanoic acid, 2) methyl *p*-hydroxybenzoate, 3) *p*-hydroxybenzoic acid, 4) 8-hydroxyoctanoic acid, 5) (*E*)-9-ketodec-2-enoic acid, 6) 2-(3-methoxy-4-hydroxyphenyl)ethanol, 7) (*E*)-9-hydroxydec-2-enoic acid, 8) 10-hydroxydecanoic acid, 9) (*E*)-10-hydroxydec-2-enoic acid.

analysis (PCA) in order to determine whether the secretions produced by sisters formed clusters. None were formed.

The first factor that was extracted by the PCA accounted for 28% of the variability in component composition. This factor was then used to perform an analysis of variance (ANOVA) on the four groups of sisters. The means of the groups were not significantly different from each other (Duncan's Multiple Range Test). The results of the ANOVA were used to estimate the genetic variance of the chemical secretions. For this limited set of data, 33 ± 33% of the total variance was due to genetic effects [12, 13].

Queens

The results of the analysis of queen head extracts are summarized in Table II and Fig. 2. The same series of components is present in the queens as in the workers. The queens are distinguished by an order of magnitude difference in the total amount of components produced and by the predominance of ODA in the mixture once they are more than four days old. As can be seen from Fig. 3, the composition of the secretion undergoes rapid changes in the relative proportions of the constituent compounds as well as a rapid increase in the total amount of components present over the first four days after

Table II. Composition of the components present in the head extracts of *A. m. intermissa* queens of various ages. DEC = decanoic acid, MEBENZ = methyl *p*-hydroxybenzoate, OHBENZ = *p*-hydroxybenzoic acid, ODA = (*E*)-9-ketodec-2-enoic acid, MHPE = 2-(3-methoxy-4-hydroxyphenyl)ethanol, HDA9 = (*E*)-9-hydroxydec-2-enoic acid, HDAA = 10-hydroxydecanoic acid, HDA = (*E*)-10-hydroxydec-2-enoic acid, HOA = 8-hydroxyoctanoic acid.

Age [days]	n		ng Component present								
			DEC	MEBENZ	OHBENZ	ODA	MHPE	HDA9	HDAA	HDA	HOA
0	5	$\bar{x}$	133	18	137	4,650	100	4,057	416	1429	735
		s	183	39	212	8,749	152	3,824	597	2299	1427
1	5	$\bar{x}$	768	257	731	10,793	466	27,895	597	7202	3848
		s	672	221	879	6,642	540	18,495	707	9207	3608
2	4	$\bar{x}$	301	184	236	40,761	0	18,855	3873	4801	207
		s	231	233	208	9,250	0	14,725	6172	9602	246
4	5	$\bar{x}$	231	29	64	31,017	0	10,070	631	2008	1188
		s	201	71	107	24,701	0	6,304	999	3586	1056
8	5	$\bar{x}$	221	36	20	26,454	0	8,707	174	0	1894
		s	118	50	45	24,420	0	6,824	247	0	1775
12	4	$\bar{x}$	42	18	0	34,753	0	5,597	1356	4554	1053
		s	85	37	0	13,923	0	5,217	1958	2907	1602
16	5	$\bar{x}$	113	34	23	14,574	103	3,265	154	1166	1683
		s	64	38	32	9,478	120	764	122	2284	1229
>21	13	$\bar{x}$	152	2060	5	34,334	758	8,281	1541	4737	1519
		s	31	2225	19	17,880	1028	6,903	1724	5698	1347

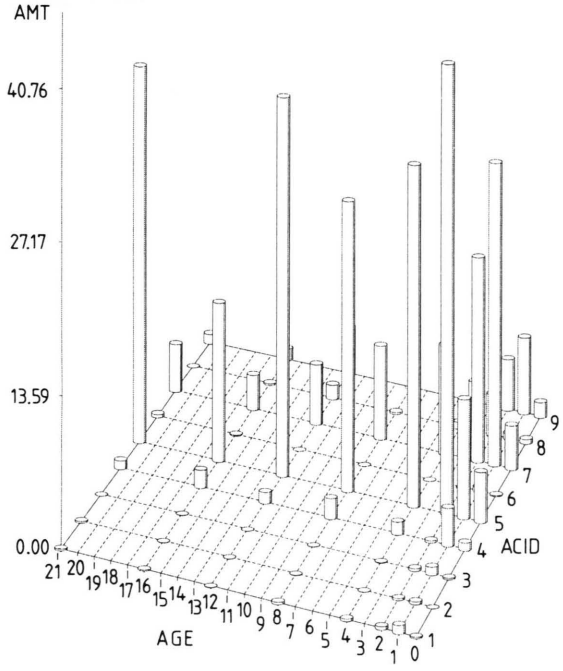

Fig. 3. Patterns of component composition in queens of various ages. The X-axis indicates the age of the group of bees, the Y-axis the amount of each acid in μg, and the Z-axis identifies the particular component by number. These acids are numbered as follows: 1) decanoic acid, 2) methyl *p*-hydroxybenzoate, 3) *p*-hydroxybenzoic acid, 4) 8-hydroxyoctanoic acid, 5) (*E*)-9-ketodec-2-enoic acid, 6) 2-(3-methoxy-4-hydroxyphenyl)ethanol, 7) (*E*)-9-hydroxydec-2-enoic acid, 8) 10-hydroxydecanoic acid, 9) (*E*)-10-hydroxydec-2-enoic acid.

emergence. After 4 days of age, the extracts are dominated by the presence of ODA with a significant contribution of HDA9. At all ages, the queen extracts can be readily distinguished from those of workers from queenright colonies and those kept in queenless groups.

The genetic variance of the particular mixture of components was estimated with a sib analysis in two groups of sister queens. The extracts obtained from these two sets of individuals were analyzed and then the component composition subjected to a PCA. The two sets of individuals fell into two clusters, one of which was distinct and the other dispersed, indicating that the composition of the secretions can be similar within a group. The first factor (which accounted for 62% of the variability in component mixtures) was then used in an ANOVA. The factor means of the two groups were significantly different from each other (Duncan's Multiple Range Test) and the results of this analysis were used to estimate the genetic

variance of the component mixtures which was 47 ± 90% of the total variance.

Discussion

The results presented here are an extensive investigation of the component composition of the head extracts of a particular race of honey-bee. They supply empirical evidence of the nature and degree of variability present in these exocrine products.

The *A. m. intermissa* worker mandibular gland secretions exhibit changes which are related both to age and to the presence or absence of a queen (Fig. 1 and 2). The effect of age is largely quantitative, with an increase in total amount of components produced. The workers were placed in two kinds of experimental situation, a single *intermissa* in a group of heteroracial workers (queenless condition) and *intermissa* workers from a normal *intermissa* colony (queenright condition). The effect of queenlessness is similar to that found for other races of honey-bees [10, 14], the composition of the secretions becomes more variable and ODA is found as a component of many of the extracts. Where these results differ from those obtained previously, is in the production of ODA by workers from queenright colonies. It appears that the production of this component is neither caste specific nor necessarily controlled by presence or absence of a queen in the group.

Under queenless conditions, workers can assume a reproductive role with a number of behavioural attributes associated with it. They can be laying workers which are characterized by the ability to produce eggs. In addition, some laying workers may exhibit some of the characteristics of a queen in that they elicit court formation and suppress ovarial development in other workers [14]; they are called false queens [15]. The fact that *capensis* workers invariably act as false queens when placed in groups of heteroracial workers [14], correlated with their ability to produce component mixtures in which ODA predominates [9, 10], suggested that there might be a connection between the composition of the component mixture produced by the mandibular glands and the behavioural characteristics of the false queens. The finding that both queenright and queenless *intermissa* workers produce ODA, indicates that the relationship between behavioural categories and the chemical composition of a secretion is not straight forward. However, although both *intermissa* and *capensis* develop laying workers rapidly [8], the component mixtures produced by their mandibular glands are distinctly different. *Capensis* is the only race of honey-bees in which thelytokous parthenogenesis is known to occur commonly [20] and to produce component mixtures in which ODA is the dominant component. *Intermissa* on the other hand has workers which produce mixtures similar to those found in workers of other races [10].

The queen secretions exhibit a pattern which is similar to that of the queens of other races in that it is dominated by the component ODA [14]. Furthermore, the composition changes with age, with an initial period of rapid change (Fig. 3) over the first 4 days after emergence followed by relative stability after that time. The secretions of *intermissa* queens have been shown to be more similar in composition to European races of honey-bees than to the two other African races that have been investigated [16].

The estimate of the genetic variance component of the variability in the *intermissa* head extract mixtures, although fairly large, was subject to a large standard error and has to be interpreted with great care. Secretions which show a large genetic variance have the potential to be used as labels for kin recognition, which has been repeatedly claimed for honey-bees [19]. Our analysis of the component mixtures of two groups of related queens shows similar patterns of variability to that found previously for related queens [17]. Such findings supply a possible mechanism for the behavioural observation by Breed [18] that groups of workers accepted sisters of their own queens more readily than queens that were unrelated to them. However, in spite of pattern similarity among related queens, the high error of the estimate for genetic variance makes it impossible, in the absence of data on the ability to worker bees to discriminate between mixtures of similar composition, to determine the significance of mandibular gland secretions for kin recognition.

Acknowledgements

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