# Factors regulating signal composition and task allocation in colonies of the ant *Myrmicaria eumenoides*

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Florian Lengyel M.Sc.
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Prüfungsausschuss:
Erstgutachter: Prof. Dr. D. von Holst
Zweitgutachter: Prof. Dr. K-H. Hoffmann
Prof. Dr. E. Komor (Vorsitzender)
Prof. Dr. R. Schobert
P.D. Dr. V. Stefanski

meinen Eltern

# Table of Contents

# 1. Introduction

Social insects, such as ants, are amongst the most dominant organisms in the world, despite their individual insignificance resulting from their small size. In fact, it has been estimated that ants constitute between “10 to 15 percent of the total animal biomass in most terrestrial environments, turn more soil than earthworms and play leading roles in the ecology as predators and scavengers” (Hölldobler and Wilson 1990). The approximately 8,800 ant species worldwide are classified into a single family – the Formicidae. They are highly eusocial, a complex form of social organisation characterised by cooperative brood-care, reproductive division of labour and overlapping generations (Wilson 1971). In addition, normally only one or more individuals reproduce, while all other workers have given up their own reproductive potential in an altruistic fashion to assist with the rearing of their sisters. How this can be evolutionary stable is explained by Hamilton’s (1964) rule; the altruistic gene will spread if the cost of giving up the own reproductive potential is less than the benefit of helping the related individual to reproduce. Importantly, a prerequisite for kin selection is the ability to discriminate between nestmates and non-nestmates, as well as reproductive individuals; this of course requires communication.

Olfaction is the oldest form of communication in the animal kingdom (Bradbury and Vehrencamp 1998), and the most widespread signalling mode amongst insects. Pheromones (compounds that elicit specific responses in conspecifics) are regarded as the most highly evolved form of chemical communication and play the central role in the organisation of ant societies (Hölldobler and Wilson 1990). Besides several other functions they are fundamental to the species and nestmate recognition systems.

Nestmate recognition is generally accepted to be responsible for colony integrity (e.g. Wilson 1971). The denial of access to all non-nestmates (both conspecific and alien) has been described as being “the first line of defence for a colony” (Vander Meer and Morel 1998). In order to facilitate recognition, each colony member must possess a label (i.e. a “bar code”) and be able to read and interpret the labels of other individuals (i.e. have a “bar code reader”). Additionally, a (perhaps plastic) label template must be stored in order to allow comparisons with the incoming signal to be made (reviewed in e.g. Hölldobler and Wilson 1990, Vander Meer and Morel 1998). Thus if the incoming signal (label) matches the template,

individuals will recognise each other as nestmates, and of course *vice versa* if the two do not match.

It is generally accepted that the nestmate recognition process involves a simple sweep of the antennae across the cuticle (Wilson 1971), although it has been suggested that when two ants encounter they may recognise each other from a very short distance (i.e. 1-2 cm) (Vander Meer and Morel 1998, Lenoir *et al.* 1999). Contact can be made with any part of the body indicating that the cues are present all over the cuticle. Therefore, the nestmate recognition cues have been suggested to come from chemicals present on the cuticle (reviewed by Bradshaw and Howse 1984).

The cuticle of insects is a heterogenous, membraneous outer skin, covered by a thin lipid layer consisting of fatty acids, alcohols, aldehydes, ketones and hydrocarbons (HCs) (Lockey 1988). In most social insects the HCs have been shown to constitute the largest fraction. A range of studies have indicated that these HCs are synthesised by oenocytes (reviewed by e.g. Romer 1991), and transported by haemolymph lipophorins to their epicuticular destinations (e.g. Schal *et al.* 1998b). Social insects are known to use long-chain cuticular hydrocarbons (CHCs) in chemical communication (reviewed by e.g. Howard 1993). Studies correlating aggressiveness with CHC profile differences indicate that they, not the polar components, are indeed responsible for nestmate and species recognition (Jutsum *et al.* 1979, Nowbahari *et al.* 1990, Dahbi and Lenoir 1998, Lahav *et al*. 1999, Thomas *et al*. 1999, Kaib *et al.* 2004). There has also been some direct experimental evidence (using purified CHC extracts) demonstrating the involvement of the CHCs in ant nestmate recognition [e.g. in *Cataglyphis niger* (Lahav *et al.* 1999), *Iridomyrmex purpureus* (Thomas *et al.* 1999), *Pogonomyrmex barbatus* (Wagner *et al.* 2000)]. Interestingly, a series of studies have manifested that the CHC profiles of individuals within a social insect colony are by no means uniform, and may vary temporally (Provost *et al.* 1993, Haverty *et al.* 1996, Nielsen *et al.* 1999), with sex, age and ovarian activity (Cuvillier-Hot *et al.* 2000), with reproductive status (Peeters *et al.* 1999) and diet (Liang and Silverman 2000), as well as with task (e.g. Kaib *et al.* 2000).

Individuals in social insect colonies perform several tasks. In many species lacking morphologically distinct castes, the tasks of individuals change throughout their lifetime (reviewed by e.g. Wilson 1971); this is termed the age-related division of

labour (or temporal polyethism). Generally in these species, tasks inside the nest (e.g. brood-tending) are executed early in life, while out-of-nest tasks, such as foraging, are performed later in life (Wilson 1971). This frequently observed correlation has led to the rather simplistic view that age is the sole factor responsible for the allocation of tasks. The division of labour system is vital in social insect colonies, as survival and reproductive output of a colony will depend on how well workers perform the different tasks that a colonial life cycle requires (Hartfelder and Engels 1998). The basic pattern of this has been described to be strikingly similar from species to species (Hölldobler and Wilson 1990), and has been most thoroughly investigated in the honey bee *Apis mellifera* (Hymenoptera) (e.g. Giray and Robinson 1994).

Studies that correlated the tasks performed with the CHCs of workers of *Camponotus vagus* (Hymenoptera) (Bonavita-Courgourdan *et al.* 1993) and of *Coptotermes formosanus* (Isoptera) (Haverty *et al.* 1996) gave first indications that workers of different task categories displayed different CHC profiles. Wagner *et al.* (1998) subsequently showed that CHC profiles within a *Pogonomyrmex barbatus* (Hymenoptera) colony may vary with the task performance (patrolling and foraging, nest maintenance) at the individual worker level. Kaib *et al.* (2000) showed the existence of temporal polyethism and task-specific CHC profiles in colonies of the ant *Myrmicaria eumenoides* (Hymenoptera). Furthermore, the intra-colonial CHC differences between nestmates of different task categories were shown to be far greater than the inter-colonial CHC differences between workers of the same task categories.

The factors regulating temporal polyethism in social insect colonies are as yet unidentified. However mounting evidence speaks for the involvement of endocrine factors such as ecdysteroids and juvenile hormones (JHs). For example, ecdysteroid titres are elevated in young *A. mellifera* and *Mellipona quadrifasciata* (Hymenoptera) workers, while older workers have much lower titres (Hartfelder *et al.* 2002). Thus high ecdysteroid titres are perhaps correlated with brood-tending, and low ecdysteroid titres with foraging.

Most of the evidence in favour of JHs playing a key role in the division of labour comes from research on *A. mellifera*. Numerous studies have demonstrated a clear correlation between haemolymph JH titres and the task performed; brood-tenders have low JH titres and foragers have high JH titres (e.g. Fluri *et al.* 1982,

Huang *et al.* 1994, Huang and Robinson 1995). Additionally, a series of topical application experiments showed that young bees treated with JH or JH analogue foraged precociously (e.g. Robinson 1985). However more recent studies have indicated that high JH titres may neither be a prerequisite for foraging, nor altering behavioural development (e.g. Sullivan *et al.* 1999, Schulz *et al.* 2002). Thus although the involvement of JH in the age-related division of labour is generally accepted, its exact role is still unclear.

Although the CHC biosynthesis pathways in insects have to a great deal been elucidated (reviewed by Blomquist *et al.* 1998), and the characteristics that are important for chemical communication have been identified, the endocrine factors regulating signalling have not yet been investigated in any Hymenopteran species. Evidence that CHC profiles may also be under the control of JHs and ecdysteroids comes from the endocrine regulation of insect pheromone biosynthesis pathways of several other orders. It appears that in Dipterans ecdysteroids regulate both pheromone and CHC biosynthesis. The pheromones of female *Musca domestica* (Diptera) are structurally similar to the epicuticular lipids of all insects (Blomquist *et al.* 1998), and their synthesis takes place through modifications of the pathways that produce cuticular lipids (Nelson and Blomquist 1995). Ovarian-produced ecdysteroids (possibly 20-hydroxyecdysone) have been shown to influence this pheromone biosynthesis by affecting the activities of one or several fatty acyl-CoA elongase(s) (Blomquist *et al.* 1994). In addition, results from studies on *Calliphora vomitoria* (Diptera) led Trabalon *et al.* (1994) to speculate that HC synthesis is under the direct control of ecdysteroids.

Conversely, in the Blattodean and Coleopteran species investigated to date, the current evidence implicates JH in the endocrine regulation of pheromone production. In many insect species HCs, or substances that are derived through modifications of the same general biosynthetic pathway (fatty acid biosynthesis), act as pheromones (e.g. the hydrocarbon-derived ketone contact sex pheromone of female *Blattella germanica* cockroaches: Chase *et al.* 1992). More then 40 years ago it was shown for the first time that female cockroaches require a functional corpora allata in order to produce sex pheromones (Barth 1961). Since then it has been demonstrated that in Blattodea (e.g. *B. germanica* – Schal *et al.* 1991), Coleoptera (e.g. *Ips pini* – Tillman *et al.* 1998) and Lepidoptera (e.g. *Pseudaletia unipuncta* – Cusson *et al.* 1994) one or more steps of the pheromone biosynthesis pathway are

induced by JH III (reviewed by Tillman *et al.* 1998). In sum, this makes JH a likely candidate for signalling regulation in the Hymenoptera as well.

The aim of this project is to contribute to our understanding of the factors, and to some extent also to the mechanisms, that govern signalling (CHC profile composition) and task allocation (i.e. the division of labour), as well as their relationship, in *Myrmicaria eumenoides* colonies. This involves:

1) The parallel recording of the CHC profile composition changes with behavioural development, and over time, during the entire lifetime of individual workers.
2) An in-depth analysis of the relationship between task performance and signal composition, and the function(s) served by this.
3) An investigation of the endocrinological factors (JHs, ecdysteroids) that may play a role in signalling. This involves a parallel assessment of the hormone titres with the CHC profiles displayed.
4) An investigation of the endocrinological factors (JHs, ecdysteroids) that may play a role in task allocation. This involves a parallel assessment of the hormone titres with behavioural development, as well as the CHC profiles displayed.

In order to shed some light on the endocrinological backgrounds of both task allocation and signalling, a simultaneous portrait of chemical, endocrinological and ethological results will be assembled; thus this amalgamation should allow a functional analysis of the possible underlying mechanisms involved in the causality of these important processes in *M. eumenoides*, and possibly other social insect colonies as well. To the best of my knowledge, this is the first time a study focusing on, and combining, ethology, chemistry and endocrinology has been carried out on a eusocial Hymenopteran species.

# 2. Materials and Methods

## 2.1. General

### 2.1.1. *Myrmicaria eumenoides*

*M. eumenoides* is a common African soil dwelling ant species, which is widely distributed in forests and plains south of the Sahara. This ecologically dominant ant is monogynous and preferentially builds subterranean nests amongst the roots of trees or shrubs. The population of these nests can rise to as many as 30,000 individuals within three years (Levieux 1983). They are highly aggressive towards anything alien, including non-nestmate conspecifics found in their foraging territories. The size of *M. eumenoides* workers ranges from 4 to 9 mm, with a dry weight of between 1.5 to 5.5 mg (Levieux 1983). The workers hunt alone or in small groups and their diet consists to large extent of arthropods (mainly termites and other ants), as well as seeds, fruits and honeydew collected from e.g. *Aleyrodidea spp.* (Homoptera) living in grasses or trees (Levieux 1983). *M. eumenoides* workers have been noted to use an exceptionally efficient recruitment communication system based on poison gland secretion to utilise and monopolise protein sources (Kaib and Dittebrand 1990).

### 2.1.2. *M. eumenoides* Focus Colony

The *M. eumenoides* focus colony was collected at Tiwi, in the Kwale District of Kenya, in June 1995, and includes one brood-producing, unwinged, female alate (queen). In the laboratory this colony was kept under constant climatic conditions (25 °C, 60 % relative humidity, 12:12 light/dark phase) in two transparent plastic boxes (20 cm x 10 cm x 6 cm) and a foraging arena (50 cm x 60 cm). The bottoms of the nestboxes are covered by an approximately 1 cm thick layer of plaster of Paris which is kept moist at all times. The nestboxes are covered with glass plates and are connected to each other, and the foraging arena by transparent silicon tubes (1 cm i.d.). The sides of the foraging arena are covered with Teflon (FLUON GP-1, ICI, Germany) to prevent the escape of the workers. The ants are provided with a constant diet of water and honey water *ad libitum* in the arena, as well as dead crickets (*Gryllus bimaculatus*) twice a week.

### 2.1.3. Marking of Focus Workers

In order to "track" individual workers during their entire lifetime (i.e. to create an ethogram for each focus worker), they needed to be individually distinguishable, as well as easily spotted, particularly in the densely populated nest area. Thus dots of various bright colours of acetone-based nail varnish in quantities around 0.3–0.6 µl were carefully applied to the thorax of callows within 48 hours of eclosion. This method was already successfully used by Kaib *et al.* (2000) to mark *M. eumenoides* workers on thorax and gaster. The longevity of the markings was improved by applying a layer of clear varnish on top of the primary marking. The callows were then kept in petri dishes for 30 to 45 min after application in order to vent the volatiles, and to allow the nail varnish to dry. The behaviour of the individuals was not affected in an obvious fashion by the markings.

### 2.1.4. Ethological Data Recording and Definitions

Focal sampling was carried out in order to create an individual ethogram of each focus *M. eumenoides* worker. The individuals were observed in the undisturbed colony for a duration of 5 min every 24 to 48 h after receiving their nail varnish markings. These observational bouts were carried out regularly during the entire lifetime of the workers. All observations took place during the light phase between 9.00 h and 19.00 h, with location, behaviour and thus also occupation (task) being noted. To enable the classification of CHC profile samples of individuals, three task categories (brood-tenders, "reserves" and foragers; these are superordinate terms as each comprises a range of characteristic behaviours) and a developmental category (callows) were clearly defined:

a) Callows are newly eclosed adult workers with a lightly pigmented and soft cuticle. They are less than 72 h old and mostly immobile on top of brood in the nestbox, and are not known to perform any tasks.

b) Brood-tenders are permanently located in nestbox close to brood and may be immobile amongst brood or be actively brood-tending (feeding/cleaning larvae of all ages, as well as callows, grooming/feeding the queen, help with eclosion, brood transport and inspection), and engaging in the grooming/feeding of other workers, or *vice versa.*

c) "Reserves" are located in peripheral areas of the nestbox, always away from brood and queen. They are mostly immobile (sitting motionless in one spot with antennae

either lowered or raised), often sitting in clusters in corners of the nest, but are occasionally mobile (moving around brood-free areas of nest antennating nestmates or surrounding areas, stopping occasionally for more detailed inspections); workers in this phase have no clearly assigned tasks, but may be recruited to virtually all tasks if the need (e.g. an environmental or predatory stress) arises.

d) Foragers perform scouting and foraging duties and are permanently located in the foraging arena (irrespective of whether a protein source is available in the arena), only occasionally entering the nest for very short periods of time. They are mostly mobile (move around arena with raised antennae), but may also display periods of immobility (sitting motionless, mostly within a cluster of nestmates, in a corner of the foraging arena).

### 2.1.5. Validation of Behavioural Development Recording Technique

To test if the recording technique to be used (see section 2.1.4.) was sufficient to create an ethogram that accurately reflected individual behavioural development, and thus the task performance of workers, or if regular "task–switching" occurred, a preliminary experiment was carried out. Eleven previously marked workers of various ages were chosen and observed once every half hour (for 5 min) during 10 h on three consecutive days. Thus 60 observations were recorded per individual, and the observed behaviour was categorised according to the defining properties of a particular task category (see section 2.1.4.a-d). These results allowed a "loyalty–to-task" percentage to be calculated.

### 2.1.6. Sampling Location

In order to find the optimum CHC sampling location, the head, thorax and gaster were investigated on the basis of the following criteria: 1) cuticular accessibility and size of surface area available for sampling, 2) ease of worker immobilisation in order to sample body part, and 3) presence of sufficient CHC concentration for representative samples. On the basis of 1) and 2) the gaster seemed to be the optimum sampling location, thus criterion 3) was tested on it using 15 individuals belonging to the categories callow, brood-tender and forager (five individuals in each category).

## 2.2. Cuticular Hydrocarbon (CHC) Investigations

### 2.2.1 Solid-phase Microextraction

Solid-phase microextraction (SPME) (Arthur and Pawliszyn 1990) was used to extract CHCs from live *M. eumenoides* workers. This method is based on the trapping of molecules on adsorbent–coated fibres, followed by thermal desorption of those molecules on insertion of the fibre directly into a GC injector (Millar 2002). Although SPME was developed primarily as a headspace sampling method, Malosse *et al.* (1995) modified it to study pheromone production in insects, and Monnin *et al.* (1998) used it for the first time to extract cuticular hydrocarbons (CHCs) from an ant.

In this study, the sampling process involved wiping the fibre over the cuticle of the worker. This allowed the precise extraction of the CHCs of one body part. This type of CHC extraction has been termed “contact SPME” (Millar 2002). The sampling sensitivity is increased since no solvent is used (Monnin *et al.* 1998). Most importantly, this sampling technique is non–invasive, thus not harming the focus workers and allowing repeated sampling of these live individuals in order to study the temporal dynamics of their CHC profiles. Additionally, the risk of contamination from other sources, e.g. exocrine glands, is lowered.

A fibre with a 7 µm non–polar polydimethylpolysiloxane (PDMS) coating (SUPELCO, Sigma-Aldrich Chemie GmbH, Munich, Germany) designed to extract compounds of high molecular weight was used. This has already been successfully used in other CHC profile studies of ants (e.g. Monnin *et al.* 1998, Cuvillier–Hot *et al.* 2000, Liebig *et al.* 2000, Dietemann *et al.* 2003). The ants were carefully immobilised using tweezers, and CHC profiles were obtained by rubbing the fibre back and forth exclusively on the fourth tergite of the gaster. The extraction of the CHCs was carried out in a standardised manner for a duration of 5 min. Care was taken that the fibre never made contact with the last tergites (VIIth and VIIIth) of the gaster to minimise the risk of contamination from Dufour’s, poison and pygidial gland secretions. This SPME-sampling procedure was not observed to have an obvious detrimental effect on the individual workers.

2.2.1.1. SPME Method Validation in *M. eumenoides*

To validate the SPME method the CHC profiles of individual workers obtained using this method were compared to the CHC profiles acquired using the "classical" solvent extraction in the same individuals. In the latter only the legs of the individuals were used to avoid contamination from other glands (the constituents from the tarsal glands do not influence the analysis; Kaib *et al.* 2000). Ten workers were at first SPME-sampled on the fourth gaster tergite, and then killed (by placing them in a freezer at –20 ºC for 5 min) before their legs were carefully removed using a scalpel. All six legs were immediately washed in 100 µl *n*-hexane (HPLC-grade; Merck, Darmstadt, Germany) for five minutes. The legs were subsequently removed and the extract was dried under a constant stream of nitrogen. The residue was then redissolved in 20 µl *n*-hexane and 1 µl of this solution was directly injected into the gas chromatograph.

2.2.1.2. Estimation of Optimum Sampling Time

The ideal SPME-sampling time was estimated by sampling nine workers three times (for 2, 5 and 10 min; in that order), each within a 5 h period. Samples were taken solely from the fourth tergite of the gaster. The optimal sampling time was estimated by monitoring the quantities (area count data resulting from the integration of the FID signal) obtained for ten randomly picked components (peaks) of various chain lengths present in the CHC profile of all individuals and samples.

2.2.1.3. Method Reproducibility

To estimate the reproducibility (or repeatability) of CHC profiles obtained by SPME-sampling, five workers were selected, and three SPME samples were taken from each one within a 4 h period. Sampling was carried out for 5 min and samples were all taken from the fourth tergite of the gaster. Ten components (peaks) of varying chain lengths and signal intensities present in all CHC profiles were randomly chosen and the area counts noted. The coefficients of variation (standard deviation divided by the mean) were used as measurements of the reproducibility. Both intra-individual and inter-individual coefficients of variation were calculated for each of the ten peaks on the basis of the area counts resulting from the integration of the FID signal (not the percentages of the total HC fraction).

2.2.1.4. Effect of Marking and SPME-sampling on Reintegration

The effect of marking and SPME-sampling on the reintegration of workers into the colony was investigated. Twenty workers that were marked 2-3 weeks previously and ten unmarked workers were removed from the foraging arena of the focus colony, and ten individuals (also previously marked) were taken from a different *M. eumenoides* colony. Next, half of the previously marked individuals from the focus colony were SPME-sampled, and the unmarked workers were individually marked. The other half of the previously marked individuals from the focus colony served as controls. Thus overall four groups consisting of ten workers each were used.

An hour after treatment workers of all four groups were then singly reintroduced into the foraging arena and observed for 10 min, then after 60 min, as well as 24 h later. An interaction was rated as a contact when a workers' antennae made contact with any part of the focus worker, grooming or trophallaxis took place or the focus worker was seized with the mandibles.

During the 10 min post-introduction, the short contacts (less than 2 s, i.e. consisting of inspection and investigation) and long contacts (longer then 2 s) by non-focus workers were noted. Additionally, the types of long contacts were recorded by instanteneous sampling every 12 s, as well as the time and mode (actively or passively) of leaving the foraging arena. These were categorised as follows:

a) Socioneutral: An inspection consisting of antennation of any body part(s) of the focus worker.

b) Sociopositive: Trophallaxis (in this case the unilateral exchange of alimentary liquid or grooming activities from nestmate to focus worker) and allogrooming (directed towards focus worker) interactions.

c) Socionegative: Aggressive acts such as immobilisation (seizing), biting and dismembering of the focus worker.

**2.2.2. GC-FID Analysis of CHC Profiles**

The samples were analysed using a Hewlett Packard 5890 Series II gas chromatograph (GC) equipped with a flame ionisation detector (FID). The split valve was closed for the first minute post injection and then reopened. The components were separated by a 30 m by 0.32 mm i.d. non-polar DB-1 (0.11 µm film thickness, J&W Scientific, Agilent Technologies GmbH, Waldbronn, Germany) fused silica capillary column. Helium was used as the carrier gas at a constant flow rate of 1.5 ml

$min^{-1}$. The injector and FID temperatures were set at 300 °C, and 320 ºC, respectively. The oven temperature started at 130 °C; this was held for 1 min before rising to 180 °C at 8 °C per min, and finally to 300 °C at 2 °C per min, resulting in a total run time of 66.25 min. This temperature program was chosen as a compromise between optimum peak separation and the processing of a reasonable number of samples per day. The septum was changed approx. every 15 injections to prevent any gas leaks possibly resulting from the use of the thick SPME injection needle. The SPME fibre was left in injection port for the entire duration of the run in order to recondition it prior to taking the next CHC sample. It is acknowledged that due to the limitation of the FID detector (lack of sensitivity for compounds with increasing chain lengths from approximately 34 carbons onwards) not all CHCs present may have been recorded.

To ensure consistency of elution times during the long time period of the study, a standardised *n*-alkane mixture from eicosane to hexatriacontane (ThetaKit®, AccuStandard, New Haven, USA) was sampled (of course also using SPME) at regular intervals between the CHC samples.

### 2.2.3. Data Aquisition

The peaks in chromatograms acquired by the FID signal were quantified using the HP ChemStation® software package. To compare the CHC profiles on an inter- and intra-individual basis, 51 HC components (chromatographic peaks) that were easily integrated and quantified in all individuals, and present in the vast majority of samples, were chosen. Peaks that could not be integrated and quantified but were present in minute quantities were allocated visually estimated values that were less than the set integration threshold levels, but higher than zero. Co-eluting components were treated as one component only for the purposes of these studies. Chromatograms of samples that had clearly been contaminated with poison gland secretion (characterised by a series of peaks eluting relatively early during a run) were not integrated and thus also not included in further analyses.

### 2.2.4. Component Identification

The structural identification of the CHCs of *M. eumenoides* was previously carried out by Kaib *et al.* (2000) using coupled gas chromatography/mass spectrometry (GC/MS). Thus the identification of the individual components

(chromatographic peaks) was in this case based on the retention times of standard substances (linear *n*-alkanes), as well as a comparison of the linear retention indices (LRI) of the CHCs with those previously identified. For the purposes of this study it was generally deemed sufficient to state the degree of saturation of the component rather than the name; there are however some exceptions.

2.2.4.1. Linear Retention Index

To enable the classification of the 51 components in many different CHC chromatograms, as well as to provide information about the equivalent chain length (ECL) of these components, the linear retention index (LRI) was calculated. This is the retention index of choice in temperature-programmed gas chromatography (McNought and Wilkinson 1997). On the basis of the standard mixture of *n*-alkanes the LRI for each of the 51 peaks was calculated. This index estimates the relative position of a component peak in a chromatogram by interpolating between the retention times of the neighbouring *n*-alkanes: $LRI = [(t_A - t_X)/(t_Y - t_X) + x] \times 100$, where x and y are the no. of C-atoms in the *n*-alkane series before and after component peak A, and $t_A$ is the corresponding retention time.

## 2.3. Endocrinological Investigations

### 2.3.1. Ecdysteroid Radioimmunoassay

A radioimmunoassay (RIA) was utilised to determine the total free ecdysteroid levels in whole body extracts of individual workers. The samples were processed as follows:

a) Extract Preparation: Individual workers were killed by placing them in the freezer at –20 ºC for 10 min, before being dried at 120 ºC for 24h. When the samples were lifted out of the oven they were immediately weighed to establish the dry weight. The samples were then crushed with a small amount of sea sand (Merck, Darmstadt, Germany) with a mortar and pestle, and transferred into a 2 ml Eppendorf Safe-Lock® (Eppendorf, Germany) tube. The extracts were then rinsed three times with 800 µl absolute MeOH (HPLC-grade; Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany), and twice with 800 µl of 70 % MeOH (each rinse involved vortexing the sample, then placing it in a sonic bath for 3 min, and finally centrifuging it at 13,000 x g for 3 min

before decanting off the supernatant). The supernatant was added to 5 ml of *n*-hexane (HPLC-grade; Merck, Darmstadt, Germany) for defatting, the resulting mixture was vortexed, put into a sonic bath for 10 min and then finally into a freezer (at –20 °C) for 20 min before centrifugation for 20 min (at 6000 x g and 4 °C). The hexane phase containing the lipids, as well as apolar impurities, was removed and the methanolic hypophase was decanted into a flask containing 200 µl $H_2O$ (HPLC-grade; Sigma-Aldrich, Taufkirchen, Germany). A rotary evaporator was used to remove the MeOH, before the samples were subsequently redissolved in 4 ml $H_2O$.

b) Solid-phase Purification: This and part c) were carried out as described by Lorenz *et al.* (1997): $C_{18}$ bonded silica disposable cartridges (SEP-PAK® Classic, Waters Assoc., Watford, UK) were rinsed with 10 ml MeOH (HPLC-grade; Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany), and then equilibrated with 10 ml $H_2O$ (HPLC-grade; Sigma-Aldrich, Taufkirchen, Germany) before the samples were loaded onto the cartridge. Polar impurities were removed with the water fraction, as well as by the elution with 4 ml of 25 % MeOH. The free ecdysteroids were eluted with 4 ml of 60 % MeOH, and only this fraction was collected and subsequently dried using a rotary evaporator.

c) Determination of Free Ecdysteroids Levels: The dried residues were redissolved in 600 µl RIA buffer solution (consisting of 0.1 M boric acid, 25 mM sodium tetraborate, 75 mM NaCl; pH adjusted to 8.4). Two duplicate aliquots of 200 µl were used for each extract subjected to the RIA, to these 100 µl of radiotracer solution (α-[23,24-$^3$H(N)] ecdysone (Dupont, Bad Homburg, Germany) dissolved in RIA buffer solution (with an activity of 180 Bq/100 µl buffer solution) was added along with 100 µl of antiserum (DBL-1; Trifolio-M GmbH, Lahnau, Germany). The resulting mixture was then vigorously vortexed and left for 2 h at room temperature, after which the reactions were abruptly stopped by the addition of 400 µl of cold saturated ammonium sulphate solution. The samples were again vortexed before being placed on an ice bath for 20 min, after which they were subjected to centrifugation (15,000 x g, 3 min) and the subsequent removal of the supernatant. The pellet (a thin white film) containing the antibody-bound ecdysteroids was redissolved in 1.7 ml scintillator solution [0.135:1 (v:v) water:scintillation fluid cocktail (Rotiszint 2211; Roth GmbH, Karlsruhe, Germany)]. The samples were then again vortexed and left at room temperature for 1 h before being measured in a liquid scintillation counter (TriCarb 2100 TR; Canberra Packard, Frankfurt, Germany).

In order to allow quantification, a standard curve was created using a series of known amounts of unlabelled ecdysone (Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany) instead of a worker extract. As the DBL-1 antiserum appears to be specific for the steroid nucleus (Reum and Koolman 1989), and thus cross-reacts with a wide range of free ecdysteroids, the results obtained are expressed as pg free ecdysteroid equivalents per mg dry weight.

### 2.3.2. Identification of the Primary Free Ecdysteroids

A review of published literature on several insect taxa had indicated that ecdysone and 20-hydroxyecdysone were also likely to be the primary free ecdysteroids in *M. eumenoides* workers (e.g. Rees 1989). Four pooled haemolymph samples (two taken from brood-tenders, and two from foragers) were used to confirm this (10 µl haemolymph per sample; between 15 and 30 individuals were used to obtain this amount). Workers of the required task categories were selected using the criteria outlined in section 2.1.4. The samples were extracted and processed as described in section 2.3.5., and then subjected to LC-MS analysis (section 2.3.6.); the latter however with some amendments.

In general both $[M+Na]^+$ and $[M+K]^+$ adducts are produced when the ecdysteroids are ionised in the haemolymph. Thus the following ions were monitored: *m/z* 503, 519 (20-hydroxyecdysone $[M+Na]^+$and $[M+K]^+$ adducts), *m/z* 487, 503 (ecdysone $[M+Na]^+$and $[M+K]^+$ adducts) and and *m/z* 333 (methoprene – the internal standard). The ratio of $[M+Na]^+$ to $[M+K]^+$ adducts was estimated to be approximately 1:0.5 for 20-hydroxyecdysone and ecdysone (S. Westerlund, personal communication).

The compounds were identified on the basis of the diagnostic ions mentioned above, the retention time comparisons with the standards, as well as a comparison with the retention time of the internal standard methoprene in each run. However, the quantification of the free ecdysteroids found in the samples was not carried out using this analytical method.

### 2.3.3. HPLC Fractionation of Free Ecdysteroids

In order to quantify the primary free ecdysteroids in workers of different task categories, the fractions containing ecdysone, 20-hydroxyecdysone and the sum of the other free ecdysteroids present in whole body extracts were collected using a

Micro-HPLC equipped with a UV detector (Eldex MicroPro, SunChrom, Friedrichsdorf, Germany). Extracts of individual workers were prepared as described in part a) of section 2.3.1., with the only difference being that the extract residue was redissolved in only 10 µl $H_2O$ (HPLC-grade; Sigma-Aldrich, Taufkirchen, Germany), of which 5 µl were then injected. The HPLC sample separation took place on a 250 x 2.1 mm $C_{18}$ reversed-phase column (ReproSil-Pur ODS-3, 5 µm; Dr. Maisch GmbH, Ammerbuch, Germany). A guard column ($C_{18}$; Phenomenex, Aschaffenburg, Germany) was installed on the column to prevent column blockage. The fractions were eluted using MeOH (HPLC-grade; Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany) in $H_2O$ (HPLC-grade; Sigma-Aldrich, Taufkirchen, Germany) at a gradient from 40 % to 80 % over a run time of 20 min (including a column equilibration time of 5 min at 40 % MeOH until the pressure had stabilised at the start of each run). The flow rate was kept unchanged at 200 µl/min, and the temperature was kept constant at 37 ºC in a column thermostat with forced air circulation (Spark, Emmen, The Netherlands) for the entire duration of a run. The wavelength of the UV detector was set at 242 nm which is the $\lambda_{max}$ for ecdysteroids due to the $\alpha$, $\beta$-unsaturated ketone present in all known zooecdysteroids (Morgan and Wilson 1989).

Using ecdysone and 20-hydroxyecdysone standards (Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany), the maximum elution time windows were determined to be 5.70 min to 6.10 min for 20-hydroxyecdysone and 8.15 min to 8.55 min for ecdysone. These fractions were then collected for each sample, along with a third fraction containing the remaining time windows, and thus all the other free ecdysteroids. Each of the three fractions collected for each sample was later dried in a rotary evaporator, and quantified using a RIA as described in section 2.3.1.

### 2.3.4. Selection of Individuals for Haemolymph JH Titre Determination

Since *M. eumenoides* workers contain only very little withdrawable haemolymph (on average 0.4 µl), pooled haemolymph samples were required for the identification, and quantification of JH (a minimum of 5 µl to 10 µl haemolymph is needed for LC-MS analysis). Each of the ten haemolymph samples of the task categories brood-tender, “reserve” and forager consisted of the pooled haemolymph of between 15 and 25 individual workers. Since the sheer number of workers needed did not allow the use of marked individuals, workers of the required task categories were selected using the criteria outlined in section 2.1.4.

Additionally, two samples were taken from female alates of another *M. eumenoides* colony available at the time. It was shown that female alates contained considerably more withdrawable haemolymph than workers (up to 4 µl), and thus the pooled haemolymph of five alates or less was enough for analysis.

### 2.3.5. Haemolymph Extraction and Sample Preparation

In order to determine the haemolymph JH titre the samples were extracted and processed as follows:

a) Sample Extraction: Individuals were immobilised using tweezers, and then using a scalpel a shallow, approximately 3 mm long, incision was made in the thorax. Gentle pressure was then applied with the tweezers allowing the haemolymph to ooze out of the incision. This was then collected with equilibrated glass capillaries (Lamag, VWR, Germany), the content to the nearest 0.1 µl was visually estimated and emptied into a conical vial containing 100 µl MeOH (HPLC-grade; Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany) - isooctane (HPLC-grade; Fluka, Taufkirchen, Germany) (1:1, v/v). The solvent-to-haemolyph ratio was at least 1:10 in all samples.

b) Sample Preparation: This was carried out according to the recently developed LC-MS method of Westerlund and Hoffman (2004). Methoprene (Isopropyl (*2E, 4E*)-11-methoxy-3,7,11-trimethyl-2,4-dodecadienoate; 95.6 % purity) (Fluka, Taufkirchen, Germany) serving as an internal standard was added to the vial containing the haemolymph sample. This mixture was then left at room temperature for 30 min before being vortexed for 20 s and subsequently centrifuged at 9,500 x g for 15 min in order to precipitate the proteins. The isooctane phase was then transferred into a new conical vial. The MeOH phase was again vortexed for 20 s and again centrifuged at 9,500 x g for 15 min before this too was drawn off and combined with the isooctane phase. These extracts were then concentrated to approx. 40-50 µl and subjected to analysis straight away, or stored at –20 °C until analysis.

The remaining residue (presumed to primarily consist of various proteins) from the JH extraction process was in three cases used to obtain chromatograms of internal (haemolymph) HCs. This involved dissolving the dried precipitate in 100 µl hexane (HPLC-grade; Merck, Darmstadt, Germany), 1 µl of which was then injected into the GC and further processed as described in sections 2.2.2. and 2.2.3.

### 2.3.6. LC-MS Analysis of Haemolymph JHs

LC-MS (liquid chromatography–mass spectrometry) analysis was performed on a Shimadzu mass spectrometer (LCMS-2010A) with an autosampler coupled to an Eldex MicroPro HPLC (Westerlund and Hoffmann 2004). The samples were seperated on a $C_{18}$ reversed-phase column (150 x 2.0 mm, ReproSil-Pur ODS-3, 5 µm; Dr. Maisch GmbH, Ammerbuch, Germany). Between 20 µl and 50 µl of each sample was injected by the Shimadzu Controller autoinjector. A guard column ($C_{18}$; Phenomenex, Aschaffenburg, Germany) was used to prevent column blockage, and elution took place using different gradients of $H_2O$ (HPLC grade; Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany) and MeOH (HPLC grade; Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany) at a flow rate of 200 µl/min and a total run duration of 15 min (0–5 min isocratic at 30 % MeOH (column equilibration time), 5–10 min from 30 % to 100 % MeOH, and 10–12 min isocratic at 100 % MeOH (run), 12–15 min from 100 % back to 30 % MeOH). However the chromatograms of the samples displayed in figures 16 and 17 were analysed prior to method optimisation, and thus a different HPLC gradient programme was used. In these cases the total run time was 30 min (0-5 min isocratic at 60 % MeOH, 5-8 min 60% to 80 % MeOH, 8-10 min isocratic at 80 % MeOH, 10-13 min 80 % to 90 % MeOH, 13-22 min 90 % to 100 % MeOH and 22-30 min from 100 % back to 60 % MeOH). The column temperature was kept constant at 37 °C in a column thermostat with forced air circulation (Spark, Emmen, The Netherlands) for the entire duration of the run.

The mass spectrometer was operated in electrospray ionisation (ESI) positive mode (mass range 2000), with a gas flow ($N_2$) of 3.76 L/min, a CDL temperature of 250 °C and a heat block temperature of 200 °C. The detector voltage was set to 1.5 kV, the probe high voltage to 4.5 kV, the CDL voltage to –5.0 V and the system was operated in Q-array scan mode. The following ions were monitored: *m/z* 289, 267 (JH III $[M+Na]^+$ and $[M+H]^+$ adducts), *m/z* 303, 281 (JH II $[M+Na]^+$ and $[M+H]^+$ adducts), *m/z* 317, 295 (JH I $[M+Na]^+$ and $[M+H]^+$ adducts) and *m/z* 333 (methoprene – the internal standard). The $[M+Na]^+$ adducts were primarily formed due to high abundance of $Na^+$ in insect haemolymph (Hoffman 1973), however at higher JH concentrations the $[M+K]^+$ adducts were also present.

The compounds were identified on the basis of the diagnostic ions mentioned above, the retention time comparisons with the standards, as well as a comparison with the retention time of the internal standard methoprene in each run.

### 2.3.7. Data Aquisition

The LC-MS peaks were integrated and peak area quantified using the "LC-MS Solution by Shimadzu" programme. The amounts of the compounds detected were quantified using the external standards method by monitoring the $[M+Na]^+$ adduct while using signal-to-noise ratios of 3 and 10 for the limit of detection (LOD) and the the limit of quantification (LOQ), respectively. At higher JH concentrations the areas of the $[M+Na]^+$ and $[M+K]^+$ adducts were totalled, however normally only $[M+Na]^+$ adducts were found. Due to the problem posed by the matrix effect the calibration curves had to be compiled by spiking *Gryllus bimaculatus* haemolymph samples with standards (standard addition method) (Westerlund and Hoffmann 2004). JH-free haemolymph could not be obtained as it is present at virtually all stages of insect development (Westerlund and Hoffmann 2004). Finally linear regressions were calculated for each compound (also using the "LC-MS Solution by Shimadzu" programme) in order to allow quantification.

### 2.3.8. JH III and Methoprene Application

In order to assess the effect of JH III, and its analogue methoprene, on task allocation (behavioural development) and signalling (CHC profiles) a series of exogenous application experiments were carried out. The methoprene and racemic JH III (methyl-(*2E,6E*)-10,11-epoxy-3,7,11-trimethyl-2,6-dodecadienoate; 94.2 % purity) (Fluka, Steinheim, Germany) were dissolved in acetone (HPLC grade; Sigma-Aldrich, Taufkirchen, Germany) and different dilutions were made up (methoprene 1.77 mmol; JH III 5 pmol, 50 pmol, 500 pmol and 5000 pmol per µl of solution). After the first CHC profile sample (serving as a reference profile) was taken, the JH III, methoprene or acetone solutions were regularly applied to focus workers twice a week. In order to do so, the workers were immobilised using tweezers and 1 µl of solution was applied to the head using a Hamilton syringe. Individual workers that had just 1 µl of acetone applied to their heads (at the same regular intervals) were used as controls. The focus workers were individually kept in petri dishes for 30 min after application of the solutions, to ensure all the acetone had evaporated, and the applied JH III and methoprene would not be removed and ingested by nestmates during allogrooming sessions.

In total 36 workers were used in this experiment; from each five or more CHC profile samples were taken. The control group consisted of six workers, the 5 pmol

JH III group of six workers, the 50 pmol JH III group of eight workers, the 500 pmol JH III category of five workers, the 5000 pmol JH III category of three workers and the 1.77 mmol methoprene category of eight workers. CHC profile samples were always taken at least three days after the last hormone, hormone analogue or acetone application.

## 2.4. Data Analyses

### 2.4.1. Statistics

All the statistics (e.g. coefficients of variation, significance tests, linear and non-linear correlations, as well as variance components analysis) were either carried out using the Statistica® 5 (1997 version) package or Microsoft Excel 97. Where the properties of the data sets allowed it (Lilliefors Normality test, and Levene's Homogeneity of Variances test passed), t-tests or ANOVAs were carried out. Where necessary, *post hoc* comparisons were then made using Tukey's HSD (honest significant difference) test. Finally, Rice's sequential Bonferroni corrections were then performed in order to eliminate possible effects of the multiple comparisons.

Where the assumptions of normal distribution and variance homogeneity were not met, the data were subjected to the non-parametric Kruskal-Wallis H-test, or a Kruskal-Wallis ANOVA. In this case the *post hoc* comparisons were carried out using the critical difference test for mean ranks: critical $\text{difference}_{0.05} = 2.394\sqrt{(N(N+1)/12*(1/n_1+1/n_2))}$, where N is the total number of subjects in the experiment, and $n_1$ and $n_2$ are the number of subjects in the groups compared. When the difference between a pair of rank means is greater than or equal to the right hand side of the above equation then they are significantly different at $p < 0.05$. However, one has to bear in mind that this is a highly conservative testing procedure, and thus the obtained results should be interpreted cautiously; existing significant differences might not be identified as such (K. Fiedler, personal communication).

The CNESS (chord normalised expected species shared) index was calculated using the COMPAH96 statistical package (Trueblood and Gallagher 1994). Multivariate analyses procedures and the calculation of the median index are described in detail in the subsections 2.4.1.1. to 2.4.1.2.

Importantly, for the analysis of CHC profile variation with task (behavioural development) (section 3.2.) all relevant figures (6-11) presented are based upon one data set consisting of 233 samples taken from 18 workers; however specific compositions are highlighted depending on the question posed. The same applies to the analysis of the free ecdysteroids (section 3.3.), where figures 13 and 15 are based on same data set, and to the application of JH III and its analogue (section 3.4.) where figures 20-22 are based on same data set.

2.4.1.1. Multivariate Analysis

The percentage contribution (relative concentration) of each component to the total CHC fraction (i.e. the total area count of 51 components) was calculated and entered into a data matrix. From this matrix a similarity index (Nei distance 072; see Nei 1972, and Nowbahari *et al.* 1990) for the pairwise comparison between each CHC profile was calculated using NT-SYS 2.02. Other similarity indices e.g. CNESS were also tested but generated almost identical distance matrices and hence were not used in further analyses. The Nei distances were then subjected to a principle components analysis (NT-SYS 2.02). Where it was deemed sufficient, only the first two principle components were chosen to display the results in a two-dimensional ordination. In addition group centroids were calculated from the first two principle components as mean Nei distance.

In general however the first three principle components were taken as an estimate of CHC variation. In this case the number of dimensions responsible for the estimate of CHC profile variation were reduced from three to one by calculating the Euclidean distances between the relevant profiles (Statistica 5 package (1997 version)). Preliminary analyses using City-block distances generated almost identical results (Statistica 5 package (1997 version)); thus only Euclidean distances were used for all analyses.

Depending on the question posed, the reference (or starting) point from which the Euclidean distances to other CHC profiles were calculated was either the first profile of an individual that was taken (within seven days of eclosion) or the centroid of the callow CHC profiles; in both cases equivalent to $x = 0$, $y = 0$, $z = 0$ in a three-dimensional ordination. Hence the resulting one-dimensional CHC variation measure, termed “chemical distance”, represents vectors from zero. Low chemical distances

stand for CHC profiles similar to the reference point, and *vice versa* for high chemical distances.

2.4.1.2. Median Index

To compare the relative quantities of the 51 components between different task categories an index of relative frequency using medians was chosen (Liebig *et al.* 2000). This is calculated as follows e.g. for component A in category X: median of component A in category X divided by median of component A in category X plus the median of component A in category Y. This was carried out for all 51 components, between each of the desired categories. This index ranges from 0 to 1, where 0.5 means that the relative concentrations are equal in the two categories being compared. If the index is less then 0.5 there is a lower concentration of the component in category X, and *vice versa* for values above 0.5.

# 3. Results

## 3.1. Results of Preliminary Investigations

### 3.1.1. Validation of Behaviour Recording Technique

Individual *M. eumenoides* workers belonging to the task categories brood-tender (workers actively brood-tending or resting amongst brood), "reserve" (workers with no clearly defined task located in peripheral areas of nestbox) and forager (workers permanently located in arena) were used to demonstrate over three consecutive days whether ants stayed "loyal" to their tasks, or if "task-switching" occurred. The percentage of consistency of task performance ranged from 83 % to 100 % amongst all individuals tested (n = 11), with a mean task consistency of over 97 %. Thus recording the behaviour once every 24 to 48 h was shown to be sufficient in order to create an accurate ethogram of an individual. Task-group related consistency differences were not observed.

### 3.1.2. The Effect of Marking and SPME-sampling on the Reintegration of Individuals

The reintegration of marked and SPME-sampled workers into the colony was examined in relation to untreated indigenous workers (controls) and non-nestmates. Twenty-nine of the SPME-sampled, control and marked workers (n = 10 per category) walked into the nestbox within 4 min of being placed into the foraging arena. In contrast, none of the ants in the non-nestmate category (n = 10) walked voluntarily into the nest, but seven were observed to be immobilised by the indigenous workers (seized with mandibles and held by one or more individuals) and carried into the nestbox. After 1 h all SPME-sampled, marked and control workers were mobile in arena or nestbox, while six of the non-nestmate workers were still being immobilised, three could not be located and one was mobile in the foraging arena. Twenty-four hours later all the SPME-sampled, marked and control workers were located in the nestbox or arena, either self-grooming or interacting with nestmates, but none of the non-nestmate workers were found. This shows clearly that all the workers indigenous to the colony survived regardless of whether they were marked or SPME-sampled.

However, in order to see if they were fully reintegrated the long contact (longer than 2 s) data from the nestbox was analysed (there was very little long contact data for the arena as all treated indigenous workers quickly left it). The results obtained are displayed in figure 1. No significant differences in the numbers of socioneutral events were found between the four categories (Kruskal–Wallis ANOVA; $n = 35$, $H = 1.113$, $p = 0.774$). This was expected since socioneutral events stand for inspection interactions, which are a prerequisite for both sociopositive (grooming/trophallaxis) and socionegative (aggression) events. However it is thus clear that socioneutral events cannot be used as a measure of reintegration (or lack of it), and will thus not be discussed in this context.

When the four categories were compared regarding the sociopositive events a significant difference was found (Kruskal–Wallis ANOVA; $n = 35$, $H = 17.11$, $p < 0.001$). No sociopositive event was recorded on a non-nestmate individual, however in the other three categories this was a common feature. Calculation of the *post hoc* critical difference values showed that this difference was significant (critical $\text{difference}_{0.05}$, $p < 0.05$) when control and non-nestmate workers were compared. No significant difference was found when the control workers were compared with the marked, and SPME-sampled workers (critical $\text{difference}_{0.05}$, $p > 0.05$, in both cases).

A global significant difference was found when the socionegative events occurring throughout the four categories were compared (Kruskal–Wallis ANOVA; $n = 35$, $H = 29.96$, $p < 0.001$). Socionegative events were only recorded on one marked ant, otherwise control, SPME-sampled and marked ants were not subjected to aggression (critical $\text{difference}_{0.05}$, $p > 0.05$ in all cases). The non-nestmate workers however were subjected to significantly higher levels of aggression (critical $\text{difference}_{0.05}$, $p < 0.05$).

In sum, the frequent occurrence of sociopositive events, and the lack of aggression directed towards the focus nestmate workers, demonstrates that marking and SPME-sampling does not effect the reintegration of the individuals into the colony.

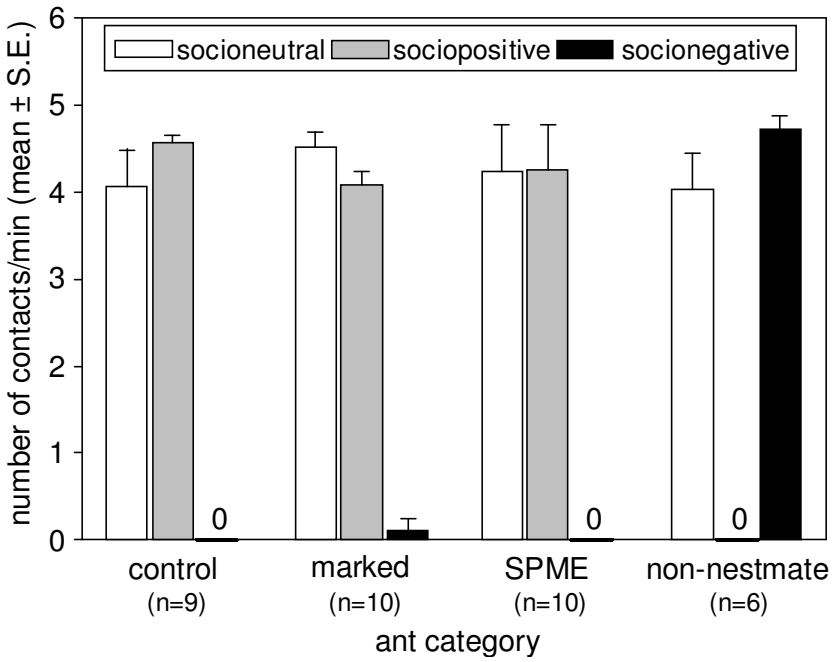


**Figure 1** A comparison of the mean sociorelevant (socioneutral, sociopositive and socionegative) events observed for four categories of focus ants during long contacts in the nestbox.

### 3.1.3. SPME-sampling; Location and Validation

All the CHC samples were taken from the gaster. Preliminary examinations had shown that the gaster presents a large and rigid surface area that is easily sampled without harming the focus workers. The hair density on the gaster was visually determined to be lower than e.g. on the thorax, allowing the SPME fibre better contact with the cuticle, and thus with the CHCs in the lipid layer. Analyses of the chromatograms obtained by sampling the gaster of 15 individuals (of various task categories) showed that the gaster had sufficiently high concentrations of CHCs for the planned studies.

The SPME method was also validated in preliminary trials. The previously established hexane extraction of CHCs of legs (Kaib *et al.* 2000) was compared with the SPME extraction of gaster CHCs in individual workers. The results demonstrated that the same CHC profiles were obtained using the two methods, thus paving the way for all further investigations.

### 3.1.4. SPME-sampling Time

In order to determine the optimal SPME-sampling time, chromatograms obtained during 2, 5 and 10 min of sampling were examined. The results showed that during 5 min of SPME-sampling significantly more CHCs were adsorbed onto the fibre compared to 2 min of sampling (t-test: $p < 0.001$). However when 10 min samples were compared to 5 min samples no difference was found in the quantities of adsorbed CHCs (t-test: $p = 0.812$). This indicates that the adsorption of CHCs onto the fibre is nearly at the maximum after sampling for 5 min, thus this sampling time was used in all investigations.

### 3.1.5. Reproducibility of SPME-obtained CHC profiles

In order to assess the reproducibility of SPME-sampling, the coefficients of variation for ten components (peaks) were compared on an intra- and inter-individual basis. The results (figure 2) showed that the intra-individual variation ranged between 0.2 and 0.45. These values were always lower than the inter-individual variation which was shown to range from 0.6 to 0.8. Both the intra- and interindividual variation of the components was shown to increase only slightly with increasing chain length. This indicates that SPME is a suitable method to obtain representative CHC profiles in order to allow differentiation on both an intra- and interindividual level.

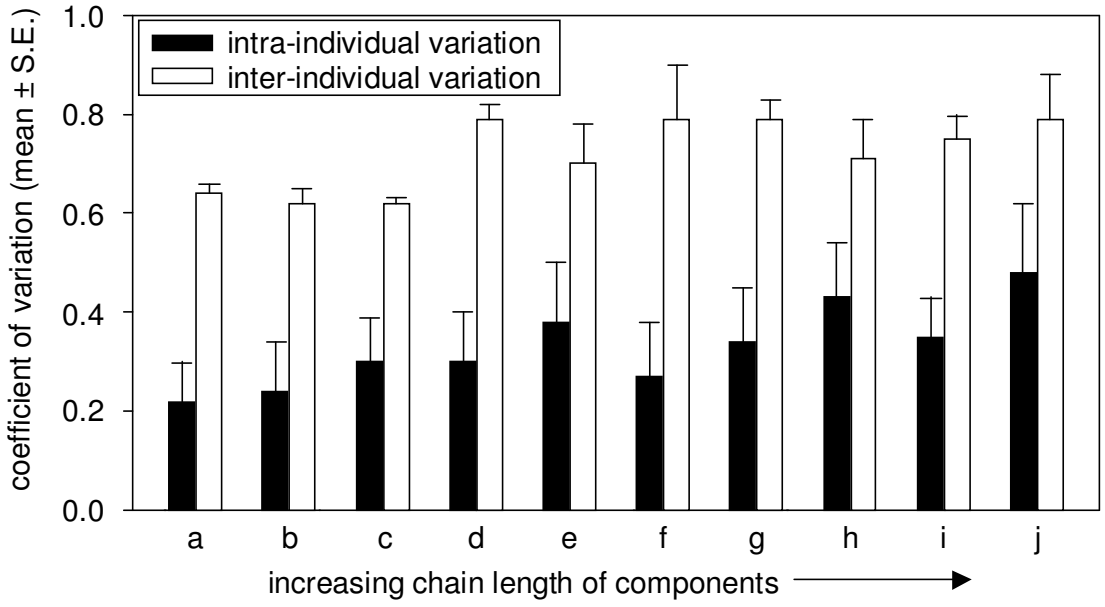


**Figure 2** A diagram of the reproducibility of SPME-obtained CHC profiles. The intra-individual (n = 5) and inter-individual (n = 5) variation is compared for ten components (labelled a to j) of varying chain lengths.

## 3.2. Assessment of CHC Variation with Task in Individual *M. eumenoides* Workers

### 3.2.1. Ethogram of the Focus Workers over Time

A general behavioural development pattern was observed among all focus workers (n = 18):

**a)** After eclosion the callows were observed to sit motionless amongst nestmates and brood for two to three days, during which they are frequently fed and groomed.

**b)** The workers then started to actively brood-tend no later then day three, and continued doing so up to week six to eight post-eclosion.

**c)** At this age, most workers were then observed to distance themselves from brood and queen, and move towards the peripheral areas of the nestbox. However, this was not a sudden behavioural change, as for a period of one to three weeks previously the workers were occasionally observed to brood-tend as well, thus making it look as if they had changed to "part-time" brood-tending. Overall however, this change was accompanied by a distinct shift from activity (feeding/cleaning/grooming) to inactivity. This is termed the "reserve" phase (Hölldobler and Wilson 1990). Depending on the individual, this phase was shown to last up to 13 weeks.

**d)** From about halfway through until the end of the "reserve" phase, the workers (n = 14) were shown to be recruited into the foraging arena when a protein source was placed there. However these foraging bouts only lasted for 6 to 8 h after which the individuals returned to the nest and resumed inactivity. Additionally when the nest was disturbed, these "reserves" were seen to help with brood transport to "safe areas", regardless of whether they had only recently turned into a "reserve", or had already started to occasionally participate in foraging (n = 16).

**e)** Finally, all workers were observed to eventually change to foraging duties which they then carried out until their point of death. A so-called "task-reversal" where a forager returned to brood-tending or "reserve" duties, was not observed. Foraging workers occasionally entered the peripheral parts of the nestbox and engaged in allogrooming and trophallaxis. However these bouts lasted only for 5 to 10 min, and foraging ants were never observed in the vicinity of the queen or brood.

### 3.2.2. Signalling (CHC) Change Assessment

Ninety-seven callows were marked with nail varnish (section 2.1.3.) within 48 h of eclosion and then returned to the colony. However only workers that retained the nail varnish marking for a period of time exceeding 15 weeks were included in the analyses (n = 18). All of these focus workers reached an age of more then 17 weeks, and in one case a worker reached an age of over 43 weeks and was sampled 21 times. The average number of samples taken from a focus worker was 13.

The CHC profiles of the focus workers were SPME-sampled during the first week post-eclosion, and from then on once every 10 to 14 days for the entire duration of their lifetime. All focus workers already had a darkly pigmented cuticle and were actively brood-tending when the first sample was taken. Parallel to the regular SPME-sampling, each individual worker was observed using focal sampling every second day in the undisturbed colony for 5 min (see section 2.1.4.).

The 18 focus workers were not SPME-sampled in the callow state, however the CHC profiles of callow workers (n = 11) were taken (within 48 h of eclosion) in a separate experiment. The callow CHC profiles obtained were compared with the first brood-tender and forager category CHC profiles of ten focus workers. Multivariate statistical analyses was carried out as described in section 2.4.1.1. Values presented are the group centroids (x, y coordinates) of a two dimensional ordination (not shown) consisting of the first two principle components that together contribute more then 90 % to the variance. Additionally, all measurements are given in mean values ± standard deviation.

The CHC profiles of the different workers in the callow state ($x = 0.264 \pm 0.007$, $y = 0.057 \pm 0.017$) were shown to be almost uniform compared to those of brood-tenders ($x = 0.206 \pm 0.040$, $y = 0.074 \pm 0.054$) and foragers ($x = 0.019 \pm 0.147$, $y = 0.130 \pm 0.061$). Due to this low variation the callows group centroid was taken as the uniform starting point in the further analysis of the quantitative CHC profile shift with task, and thus also in relation to age.

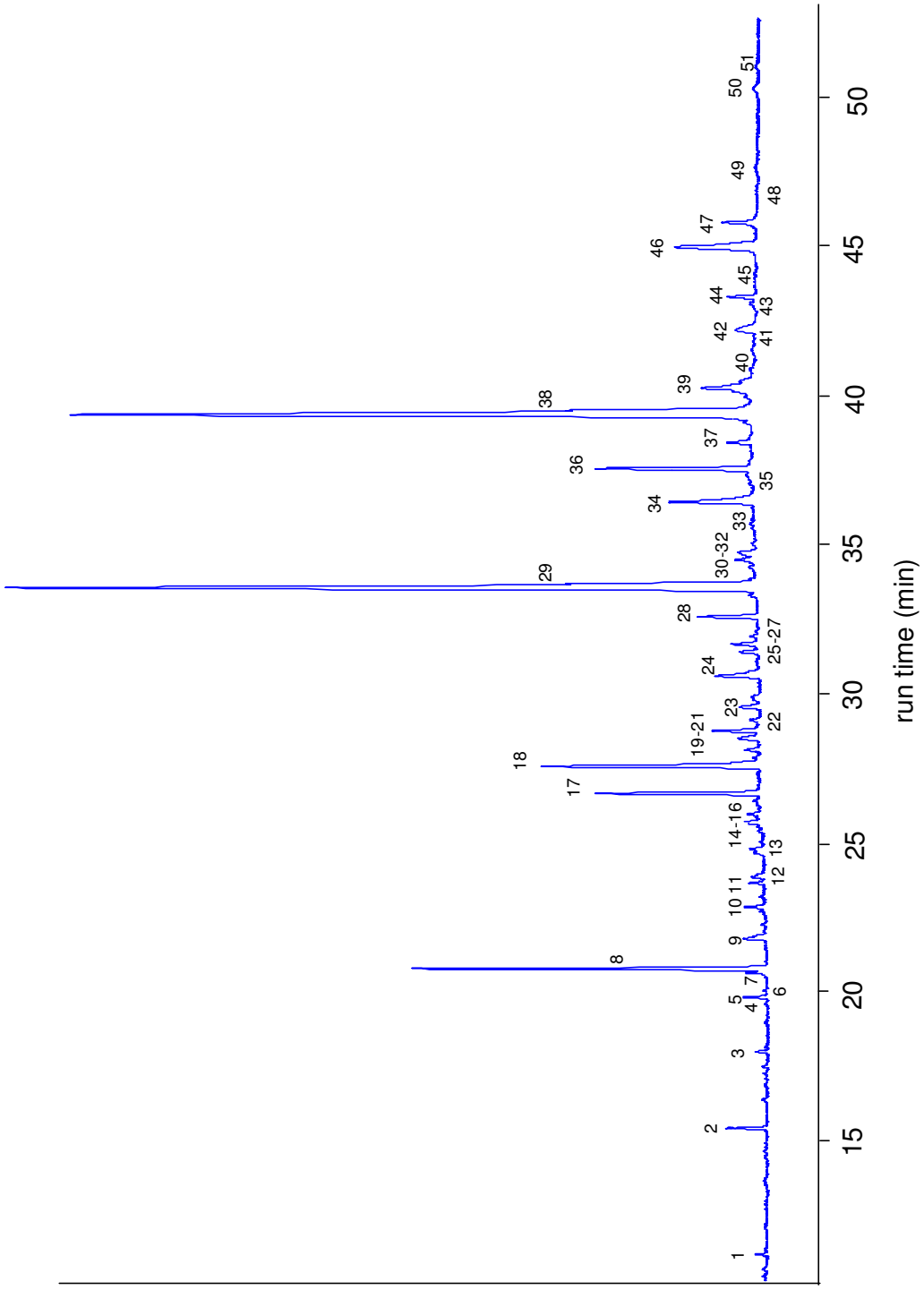


**Figure 3** Typical SPME-GC chromatogram of the CHCs of a brood-tending *M. eumenoides* worker. Numbers refer to the allocated peak numbers that are listed in table 1. The x-axis represents the retention time of the components, and the y-axis (no scale depicted) portrays the FID signal intensity.

| | | | relative concentrations (% of total CHCs) | | |
|---|---|---|---|---|---|
| **peak no.** | **LRI** | **sat. / unsat.** | **callow** | **brood-tender** | **forager** |
| | | | **(n = 11)** | **(n = 18)** | **(n = 18)** |
| 1 | 2104 | not allocated | 0.21 | 0.12 | 0.04 |
| 2 | 2300 | saturated | 1.13 | 0.83 | 0.10 |
| 3 | 2401 | saturated | 0.27 | 0.33 | 0.11 |
| 4 | 2458 | unsaturated | 0.18 | 0.10 | 0.00 |
| 5 | 2468 | unsaturated | 1.35 | 0.83 | 0.05 |
| 6 | 2476 | unsaturated | 0.16 | 0.11 | 0.00 |
| 7 | 2494 | not allocated | 0.06 | 0.08 | 0.03 |
| 8 | 2500 | saturated | 6.98 | 11.34 | 7.99 |
| 9 | 2535 | unsaturated | 0.77 | 0.57 | 0.08 |
| 10 | 2573 | unsaturated | 0.58 | 0.23 | 0.02 |
| 11 | 2600 | saturated | 0.19 | 0.33 | 0.68 |
| 12 | 2607 | not allocated | 0.38 | 0.18 | 0.00 |
| 13 | 2635 | unsaturated | 0.68 | 0.41 | 0.07 |
| 14 | 2664 | unsaturated | 0.12 | 0.09 | 0.04 |
| 15 | 2668 | unsaturated | 0.43 | 0.16 | 0.04 |
| 16 | 2677 | unsaturated | 0.41 | 0.23 | 0.05 |
| 17 | 2700 | saturated | 1.81 | 3.24 | 12.63 |
| 18 | 2734 | unsaturated | 6.18 | 4.51 | 1.58 |
| 19 | 2752 | unsaturated | 0.35 | 0.22 | 0.11 |
| 20 | 2764 | unsaturated | 0.64 | 0.47 | 0.26 |
| 21 | 2773 | unsaturated | 1.18 | 0.60 | 0.21 |
| 22 | 2784 | unsaturated | 0.35 | 0.17 | 0.06 |
| 23 | 2800 | saturated | 0.32 | 0.20 | 0.25 |
| 24 | 2833 | unsaturated | 1.48 | 1.14 | 0.60 |
| 25 | 2861 | unsaturated | 0.81 | 0.37 | 0.04 |
| 26 | 2869 | unsaturated | 1.15 | 0.69 | 0.13 |
| 27 | 2878 | unsaturated | 0.19 | 0.16 | 0.04 |
| 28 | 2900 | saturated | 0.86 | 1.82 | 3.39 |
| 29 | 2933 | unsaturated | 24.80 | 21.85 | 15.84 |
| 30 | 2956 | unsaturated | 0.18 | 0.11 | 0.08 |
| 31 | 2965 | unsaturated | 0.36 | 0.31 | 0.21 |
| 32 | 2974 | unsaturated | 0.58 | 0.55 | 0.60 |
| 33 | 3000 | saturated | 0.05 | 0.14 | 0.27 |
| 34 | 3032 | unsaturated | 2.91 | 2.59 | 2.38 |
| 35 | 3052 | unsaturated | 0.19 | 0.15 | 0.03 |
| 36 | 3070 | unsaturated | 5.84 | 4.97 | 2.62 |
| 37 | 3100 | saturated | 0.34 | 1.22 | 2.47 |
| 38 | 3132 | unsaturated | 24.59 | 23.47 | 26.88 |
| 39 | 3164 | unsaturated | 0.92 | 0.99 | 1.13 |
| 40 | 3200 | saturated | 0.02 | 0.09 | 0.16 |
| 41 | 3225 | not allocated | 0.09 | 0.29 | 0.22 |
| 42 | 3232 | unsaturated | 1.09 | 1.08 | 1.53 |
| 43 | 3243 | not allocated | 0.05 | 0.16 | 0.11 |
| 44 | 3271 | unsaturated | 0.99 | 1.14 | 2.33 |
| 45 | 3300 | saturated | 0.08 | 0.42 | 0.55 |
| 46 | 3331 | unsaturated | 5.62 | 5.68 | 9.37 |
| 47 | 3362 | unsaturated | 1.07 | 1.24 | 1.44 |
| 48 | 3443 | not allocated | 0.03 | 0.17 | 0.20 |
| 49 | 3473 | unsaturated | 0.03 | 0.04 | 0.16 |
| 50 | 3532 | unsaturated | 0.51 | 0.61 | 1.02 |
| 51 | 3563 | unsaturated | 0.15 | 0.30 | 0.41 |

**Table 1** The CHC composition of *M. eumenoides* callows, and of focus workers in different task phases (brood-tending, foraging). The median was used as the representative measure of relative concentration. Medians of brood-tender and forager phases were calculated by 1) calculating the medians for all samples taken during brood-tending, and foraging, for each individual and 2) then estimating the median across all individuals for each task category. The number of workers used in the analysis is noted below each category.

### 3.2.3. General Chemistry of the CHCs

The chain length of the CHCs found on the cuticle of workers ranged from 21 to 36 carbon (C) atoms. Although in general compounds with C-atom backbones of these lengths are considered long-chain, for the purposes of this study only CHCs with more then 29 C-atoms will be referred to as long-chain. All task groups were shown to have the same 51 CHCs, however in different relative quantities. A typical chromatogram of the CHCs of a brood-tending worker is displayed in figure 3.

The CHC profile of *M. eumenoides* workers was previously established to consist of a complex mixture representing a repeating homologous series of *n*-alkanes, alkadienes and alkenes, with methyl-branched components present only in minute amounts (Kaib *et al.* 2000).

For the purposes of this study it proved in most cases sufficient to divide up the CHCs into saturated and unsaturated components. Six quantitatively minor components (perhaps including some methyl-branched CHCs) could not be allocated, and were not included in any analysis based on those two groups. The *n*-alkanes (saturated) were shown to consist of a homologous series of even and odd numbered components with backbone lengths between 23 and 33 C-atoms (*n*-tricosane to *n*-tritriacontane). However, in terms of relative concentration, the odd carbon number CHCs were much more abundant than the even carbon number CHCs. The same general pattern was observed with respect to all the alkene and alkadiene (unsaturated) components. In terms of relative quantities the CHC profiles, independent of age and task, were shown to be dominated by unsaturated CHC components.

However the total relative quantities of saturated and unsaturated components (calculated from table 1) were shown to vary between callows, brood-tenders and foragers. Only 12 % of the CHC profile components in callows were saturated, while 86.8 % were unsaturated. The CHC profiles of brood-tenders were shown to consist of 20 % saturated and 76.1 % unsaturated components. The forager CHC profile however was made up of the largest saturated CHC fraction (28.6 %) and the smallest unsaturated CHC fraction (69.4 %).

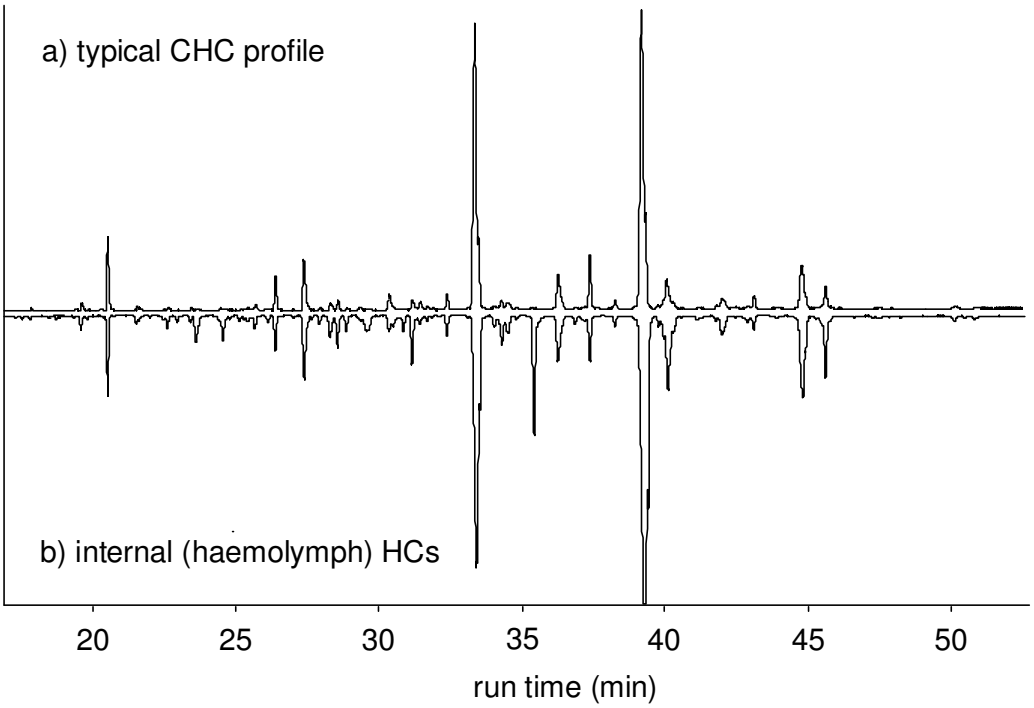


**Figure 4** Mirrored GC chromatograms of (a) typical CHCs of a brood-tender and (b) the haemolymph HCs extracted from 21 brood-tenders. The x-axis represents the retention time of the components, and the y-axis stands for the FID signal intensity. The chromatograms are not of a similar concentration and are depicted only to allow a qualitative, visual comparison between haemolymph and cuticular HCs.

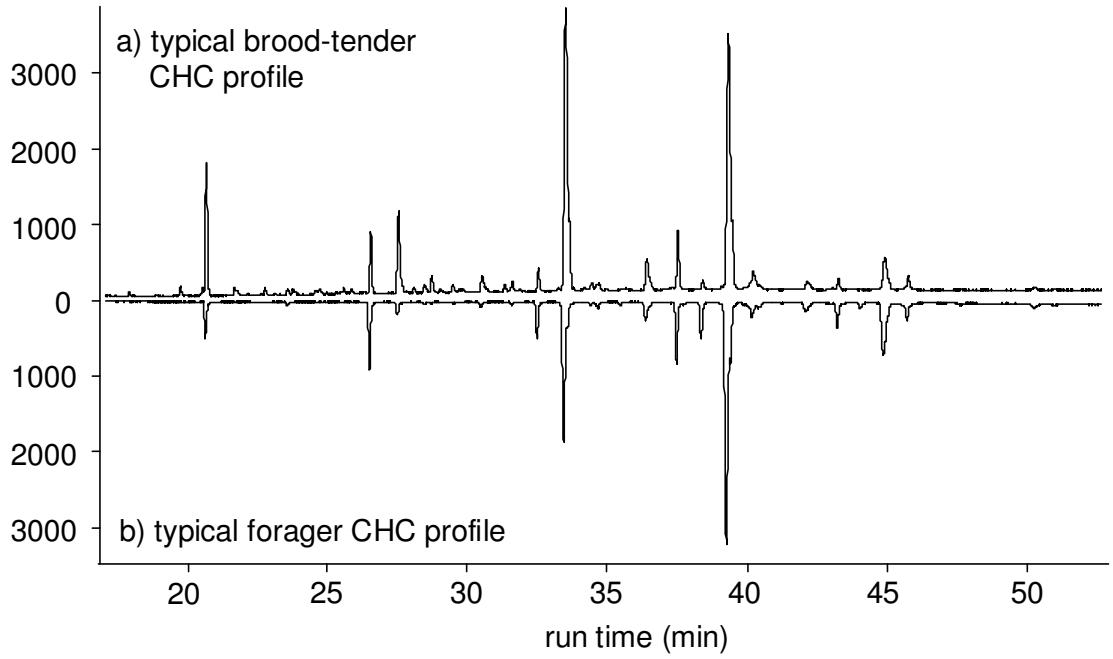


**Figure 5** Mirrored SPME-GC chromatograms portraying (a) a typical brood-tender profile and (b) a typical forager profile. The x-axis represents the retention time of the components, and the y-axis stands for the intensity of the signals obtained by the FID. Both CHC profiles portrayed are of a similar concentration.

### 3.2.4. Cuticular and Haemolymph HCs

A comparison of the external and internal HCs of brood-tenders was made (figure 4). The internal HCs were aquired from haemolymph constituents (presumed to primarily consist of lipids and including the HC transport proteins) deposited during the juvenile hormone extraction process (see section 2.3.5.). Although the analysis carried out was by no means in-depth, it showed that haemolymph and cuticular HCs (in this case both of brood-tenders) are qualitatively very similar. Quantitative comparisons could not be carried out.

### 3.2.5. CHC Profiles of Brood-tenders and Foragers

Mirrored chromatograms of CHC profiles typical of a brood-tender, and a forager are portrayed in figure 5. In total 51 individual peaks present in the vast majority of samples of all task categories were chosen as the basis for all further CHC change assessment. These components are listed in table 1.

In order to display and examine task-specific signalling, the ethograms of ten individual workers were correlated with their CHC profiles. The obtained task-specific chromatograms were analysed as described in section 2.4.1.1.. The results (figure 6) showed that the brood-tender and forager task categories are separated on the basis both of principle component (PC) axis 1 (Kruskal-Wallis H-Test; $n = 20$, $H = 13.16$; $p < 0.001$) and PC axis 2 (Kruskal-Wallis H-Test; $n = 20$, $H = 14.29$; $p < 0.001$). The variation explained by the first two PCs exceeded 95 %. This showed that brood-tender CHC profiles are clearly separated from forager CHC profiles on the basis of quantitative differences alone. Additionally, when the first two PCs were plotted against each other, the correlation of the two different task categories taken from the same individuals showed that the direction of CHC change is highly similar.

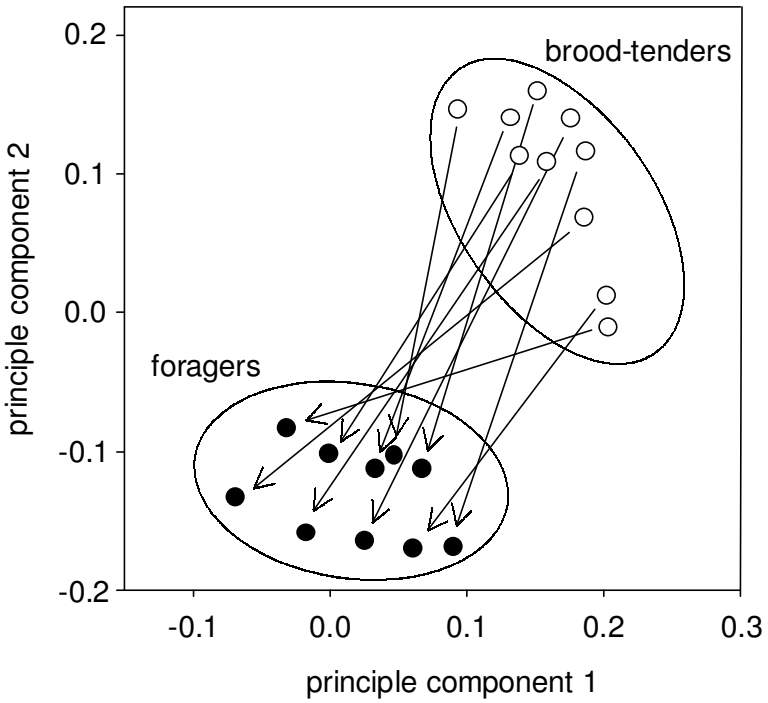


**Figure 6** The task-related CHC profiles, and profile changes in 10 randomly chosen focus workers. The points shown represent the centroids of PC1 and PC2 scores of all samples taken during active brood-tending, and foraging, in each of the 10 individuals. Arrows indicate the direction of the CHC profile changes of individual workers. The ellipses serve only as visual aids and have no statistical significance.

### 3.2.6. CHC Change with Task and Age in Individual Workers

In order to document the CHC profile changes with task and age, the chemical distances (i.e. an estimate of the magnitude of the CHC profile changes) from a callow-type CHC profile to each of the CHC profiles taken from the focus workers were calculated as described in section 2.4.1.1., and are depicted in figure 7. The data points shown thus represent individual samples taken from 18 focus workers at regular time intervals after eclosion for a time period lasting up to 35 weeks. The mean chemical distance across all CHC samples displayed in the ordination was calculated for each task category. All measurements are given in mean values ± standard deviation.

An explicit trend regarding the behaviour and CHC profile changes over time in individual ants was demonstrated to exist. Workers observed to carry out brood-tending duties, such as grooming, cleaning and trophallaxis, in general possessed similar CHC profiles. The samples taken in this phase (n = 46) were characterised by low chemical distances, and varied only slightly (0.151 ± 0.075).

After six to eight weeks most individuals became more and more inactive and moved towards the peripheral areas of the nestbox. This marked the start of the "reserve" phase. This is, both in terms of CHC profile and task (i.e. behavioural development), an intermediate stage between the two principle task categories. The CHC profile samples taken during this phase (n = 110) showed a marked increase, as well as a higher variation of chemical distances (0.328 ± 0.140). The duration of this phase varied greatly amongst individuals (roughly between 40 and 120 days) under constant environmental and dietary conditions.

All focus workers started to actively forage between day 66 and 165 post eclosion (120.5 ± 24.7 days). Thus the onset of active foraging is by no means uniform but varies from worker to worker. CHC profiles taken from workers in this task phase were characterised by high chemical distances, and their relatively high variation (0.390 ± 0.170). These high chemical distances portray the magnitude of the CHC profile changes from a callow-type to a forager-type CHC profile.

The parallel change of CHC profile with task in five focus workers is depicted in figure 8. This demonstrates that the CHC profiles of individuals change in a highly similar fashion with task, and consequently to a certain extent also with age. On the basis of these five randomly chosen individuals it was demonstrated that the transition of CHC profiles from a brood–tender-type profile to a forager-type profile is a continuous process that is synchronously accompanied by conspicuous behavioural changes.

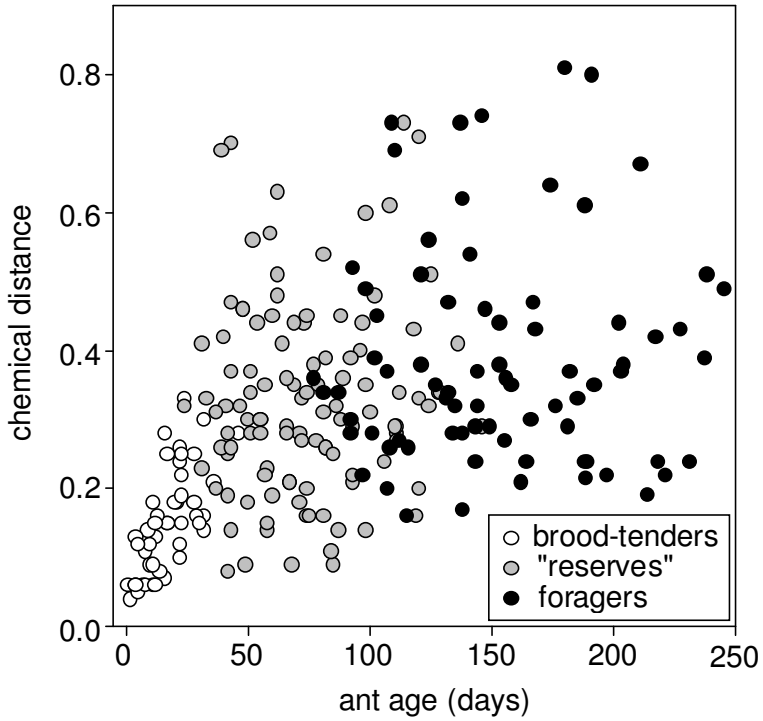


**Figure 7.** The variation of CHCs in *M. eumenoides* as a function of age and task. This ordination portrays all CHC samples taken (n = 233) from all focus ants (n = 18) over a time period of up to 35 weeks per individual. The chemical distance is the euclidean distance to the callows group centroid; this was calculated to show the intra-individual variation present.

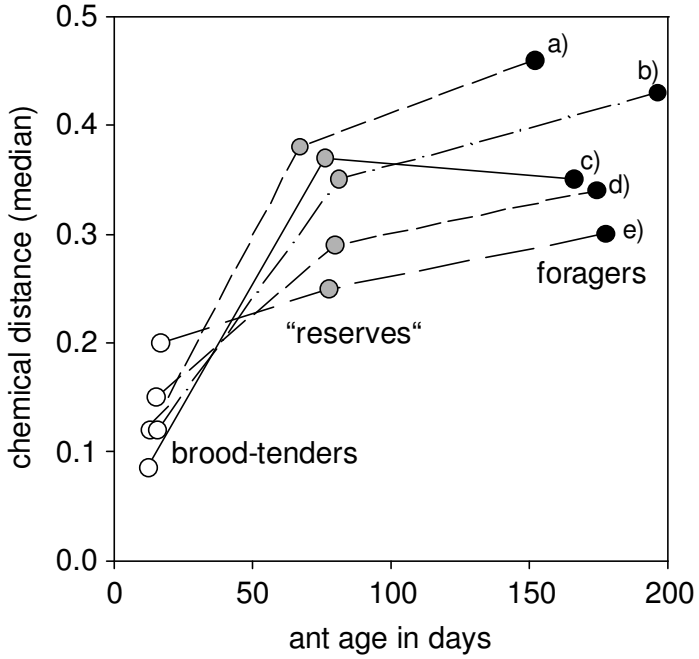


**Figure 8** The CHC profile change in five workers (labelled a to e) over time, from brood-tender to forager via the "reserve" phase. The data points represent the mean number of days post eclosion plotted against the median chemical distances (the euclidean distances calculated from the callows group centroid) of all CHC profile samples taken from each individual in that task category. The median values, quartiles and number of CHC profile samples in each category for each individual are shown in table a1 in the appendix.

### 3.2.7. Analysis of CHC Chain Length and Behavioural Development

3.2.7.1. Callows *versus* Brood-tenders

The median index (see section 2.4.1.2) was calculated to analyse differences based on chain length between callow-type and brood-tender-type CHC profiles. The equivalent chain length (obtained from calculation of the linear retention index) plotted against the median index showed that the two groups differed in the relative quantities of components in a chain length-dependent manner. A negative correlation ($n = 51$, Pearson's $r = -0.53$, $p < 0.05$; $y = 1.198 - 0.00024 * x$) was found between median index and equivalent chain length of the components. This means the relative concentration of the compounds decreases with increasing chain length, and that callows have more short-chain (and less long-chain), and brood-tenders more long-chain (and less short-chain) CHCs.

This general trend was also seen when the saturated and unsaturated components were analysed separately (figure 9). A negative correlation was observed for the saturated compounds (n = 11; y = 1.336 - 0.00035 * x) (figure 9a). With the exception of only two components (*n*-tricosane and *n*-octacosane), the relative quantities of the *n*-alkanes were always lower in callows than in brood-tenders i.e. they possessed a median index value of less then 0.5. The unsaturated components also showed a similar, strong negative correlation of median index with equivalent chain length (n = 34; y = 1.198 - 0.00021 * x) (figure 9b). In this case the median index values ranged between 0.4 and 0.7, thus indicating the presence of only minor quantitative differences.

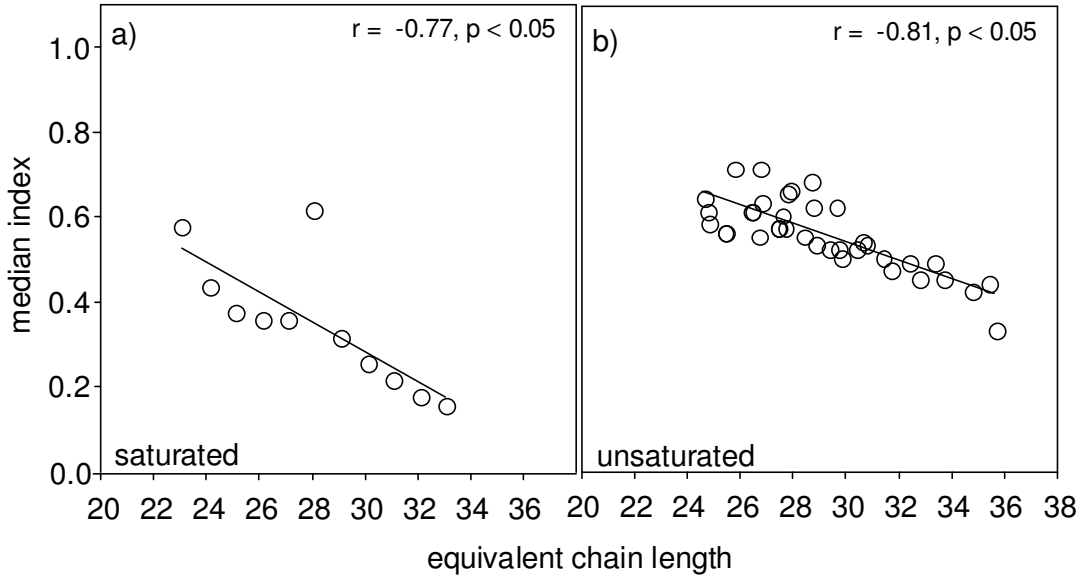


**Figure 9** A comparison of the total saturated (a) and total unsaturated (b) components of callow (n = 11) and brood-tender (n = 18; per focus worker one sample taken during brood-tending phase was randomly chosen) CHC profiles. The median index (calculated for callows *versus* brood-tenders) is plotted against the equivalent chain length. The median index represents the relative quantities of the compounds in the two categories.

3.2.7.2. Brood-tenders *versus* Foragers

To analyse the chain length differences between brood-tender-type and forager-type CHC profiles the median index was calculated. The equivalent chain length of each of the compounds was plotted against the median index (calculated

for brood-tenders *versus* foragers). The values obtained ranged from 0.2 to 1.0, thus indicating the existence of substantial quantitative differences between brood-tenders and foragers. The two task groups were subsequently shown to differ in the relative quantities of components in a chain length dependent manner. The plot of all components demonstrated the presence of a negative correlation; the relative concentrations of the compounds decrease with increasing chain length ($n = 51$, Pearson's $r = -0.66$, $p < 0.05$; $y = 1.859 - 0.00042 * x$). This means that in general brood-tenders have higher relative quantities of short-chain, and foragers higher relative quantities of long-chain CHCs.

This pattern is present at a finer scale if the saturated and unsaturated components are analysed separately (figure 10). The concentrations of the saturated components (*n*-alkanes) (figure 10a) with a backbone chain length of 24 to 28 C-atoms decrease rapidly with increasing chain length, however this stabilises at a median index value of around 0.4 for CHC components with more then 29 C-atoms. Overall there is also a negative correlation ($n = 11$; $y = 1.589 - 0.00046 * x$), and the median index values lie between 0.2 and 0.9.

The unsaturated components (alkenes, alkadienes) also show a negative correlation with increasing chain length ($n = 34$; $y = 2.405 - 0.00059 * x$) (figure 10b). The median index here ranges from 0.2 to 1.0 thus indicating the high degree of variation present at the individual component level between the two task categories.

To summarise, short-chain saturated and unsaturated components are present at higher relative quantities in brood-tenders, and at lower relative quantities in foragers. Thus in turn, foragers have higher relative quantities of long-chain saturated and unsaturated CHCs than brood-tenders.

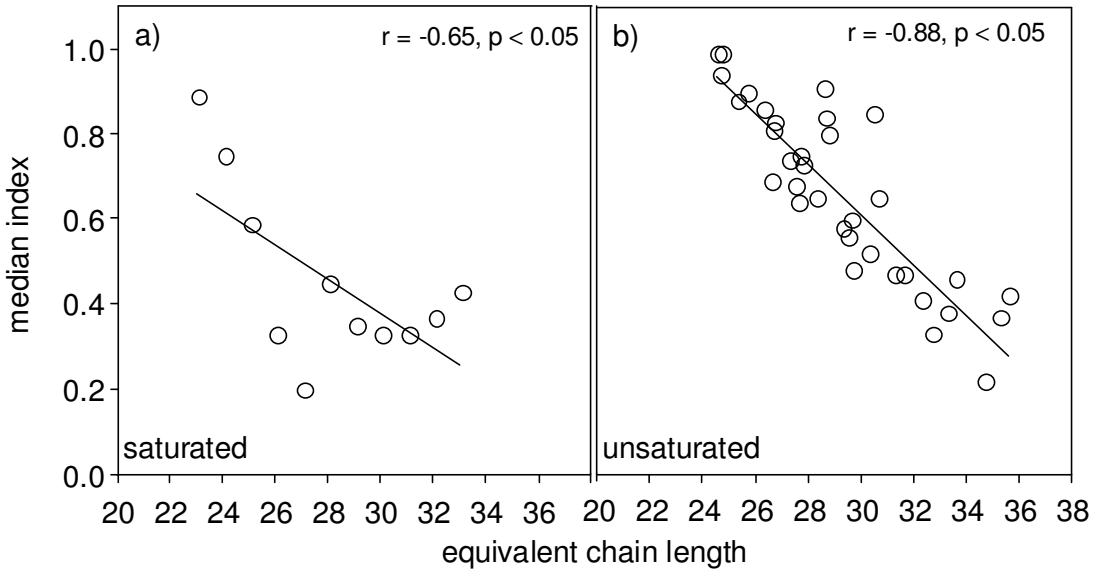


**Figure 10** A comparison of the total saturated (a) and total unsaturated (b) components present in brood-tender and forager (n = 18 in both cases; per focus worker one sample taken during each task phase was randomly chosen) CHC profiles. The median index (calculated for brood-tenders *versus* foragers) is plotted against the equivalent chain length (obtained from linear retention index). The median index represents the relative quantities of the compounds in the two categories.

### 3.2.8. Analysis of Task-related CHC Component Differences

A "variance components analysis" was carried out in order to investigate the contribution of individual components to the variation between brood-tender-type and forager-type CHC profiles. To allow comparisons with the results obtained in section 3.4.4.1. to be made, the first ten components (in order of magnitude of contribution to the variance) of the 51 CHC components were first of all assessed. Together these components contributed more then 98 % to the variance between brood-tender-type and forager-type CHC profiles (four *n*-alkanes; 64.9 %, four alkadienes; 29.1 %, two alkenes; 4.6 %) (figure 11).

However, the top five ranked components were shown to already cumulatively contribute more than 87 % to the variation between brood-tender-type and forager-type CHC profiles. Thus an analysis was carried out to investigate if it was possible to differentiate between brood-tender and forager CHC profiles solely on the basis of the relative quantities of these five components. A new, independent sample set consisting of five foragers and five brood-tenders was taken from the focus colony

and the CHC profiles were analysed using both five and 51 components to enable a comparison to be made.

In both cases, PC analysis resulted in the separation of the CHC profiles of the two task groups primarily along axis 1. This summarised around 90 % of the variation present between brood-tender and forager CHC profiles (figure 12a,b). The significance of this separation was statistically tested. In the analysis involving 51 components (figure 12a), a significant difference was shown to exist (Kruskal-Wallis H-test; n = 10, H = 6.81, p = 0.009) between the two groups. The analysis of only the five components (figure 12b) yielded an identical result; the significant difference between the two groups was again demonstrated (Kruskal-Wallis H-test; n = 10, H = 6.81, p = 0.009). These results show clearly that even on the basis of quantitative differences of only five CHC profile components, the brood-tender and forager groups can be differentiated between just as well as with a 51 component analysis.

Overall, these results suggest that primarily the major components (i.e. the components present in higher relative concentrations), and some components present in medium relative concentrations, but not the minor components are responsible for the differentiation between brood-tender and forager CHC profiles. For example, *n*-heptacosane (17), triacontadiene (29) together contribute over 72 % to the variation between brood-tender and forager CHC profiles (figure 11). However they also constitute 25.1 %, and 28.5 % of the total extracted CHCs of brood-tenders and foragers, respectively. Overall, the ten most important components (in terms of % contribution to the total variation between brood-tenders and forager CHC profiles) also make up 79.2 % of the total sampled CHCs of brood-tenders, and 85.1 % of the total sampled CHCs of foragers. These results additionally suggest that the components involved do not need to belong to the same chemical family.

Thus in sum only a few components of a whole CHC bouquet are required for differentiating between brood-tender-type and forager-type CHC profiles. These are to large extent components present in high relative concentrations which probably do not need to belong to the same chemical family.

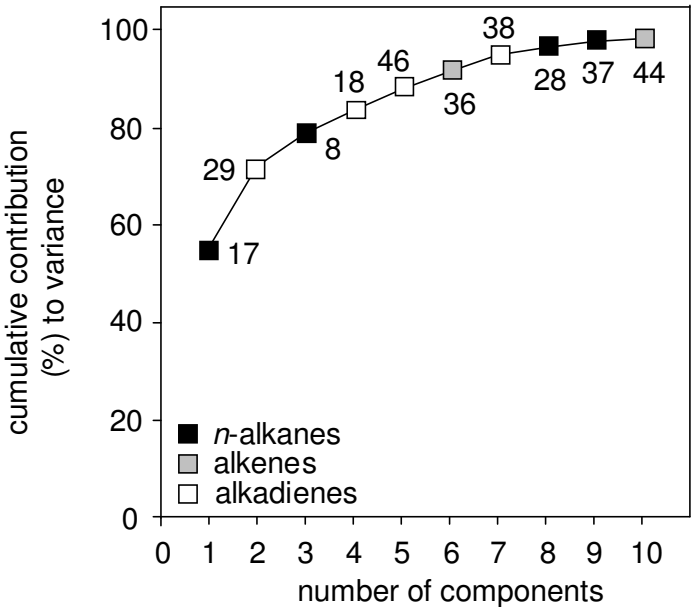


**Figure 11** Saturation curve portraying the cumulative contribution to the variance of the ten most important components that differentiate between brood-tender-type and forager-type CHC profiles (n = 18 in both cases; per focus worker one sample taken during each task phase was randomly chosen). The number allocated to each component in table 1 are displayed above the data points. These components are (in the order of the magnitude of contribution to variance): 17) *n*-heptacosane, 29) triacontadiene, 8) *n*-pentacosane, 18) octacosadiene, 46) tetratriacontadiene, 36) hentriacontene, 38) dotriacontadiene, 28) *n*-nonacosane, 37) *n*-untriacontane and 44) tritriacontene.

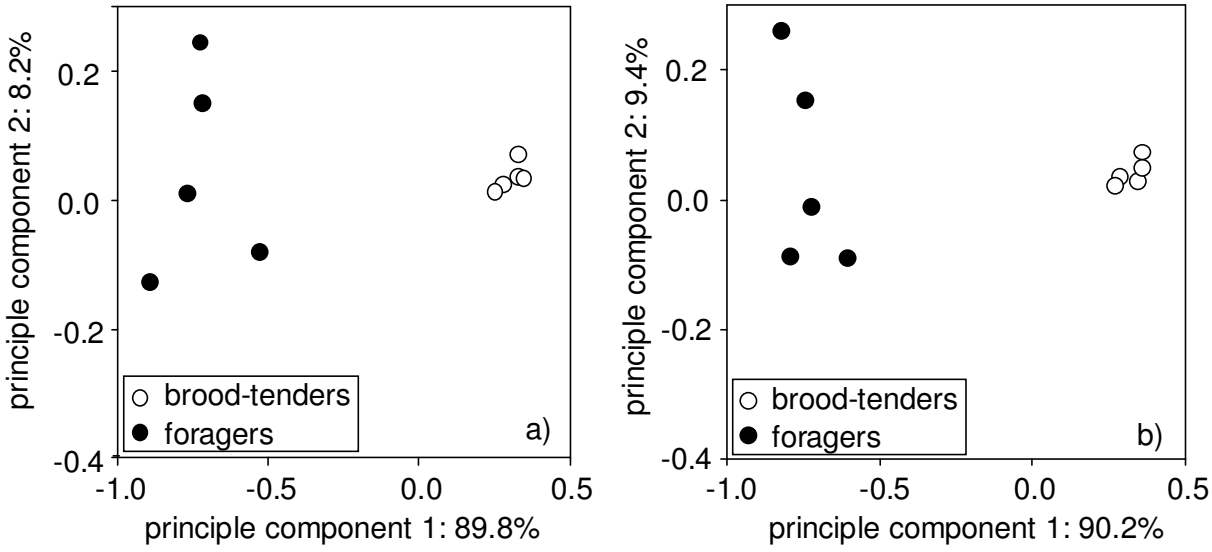


**Figure 12** The separation of 10 CHC profiles belonging to the two main task groups on the basis of (a) 51 components and (b) 5 components, both as percentages of the total CHC fraction.

### 3.3. Ecdysteroids: A Role in Task Allocation and Signalling in *M. eumenoides*?

#### 3.3.1. The Total Free Ecdysteroid Content of Individual Workers

Only the free ecdysteroids were investigated as these are generally accepted to be the biologically active molecules. Using a radioimmunoassay, the total free ecdysteroid content of individual workers was measured in pg ecdysteroid equivalents per mg dry weight. All measurements are given in mean values ± standard deviation. The total free ecdysteroid content was found to be highest in callows (862.2 ± 145.0) (figure 13). Statistical analysis excluding the "callows" category (as this is not relevant in the context of this investigation), revealed the presence of significant global differences in the total free ecdysteroid content between the different task, and age categories tested (Kruskal-Wallis ANOVA; $n = 40$, $H = 14.75$, $p = 0.022$). *Post hoc* tests showed that the amount of the total free ecdysteroids was significantly higher in one week old brood-tenders (420.6 ± 137.6) than in brood-tenders aged two to four (149.1 ± 34.4), six to eight (148.8 ± 45.1) and over 18 weeks (176.8 ± 49.3), as well as the "reserves" (179.4 ± 92.4) (critical $difference_{0.05}$; $p < 0.05$ in all cases). Although a similar trend seemed to be evident, no significant difference was found between one-week old brood-tenders and 10 to 12 week old brood-tenders (209.5 ± 70.1), as well as foragers (206.5 ± 42.8) (critical $difference_{0.05}$; $p > 0.05$ in all cases). When the total free ecdysteroids of brood-tenders older then one week were compared to each other, as well as the "reserve" and foragers category, no significant difference was found (Kruskal-Wallis ANOVA; $n = 35$, $H = 5.06$, $p = 0.408$).

To summarise, only callows and one week old brood-tenders have elevated levels of free ecdysteroids, and thus all individuals older than one week have similar levels of free ecdysteroid irrespective of age or task performed. Thus there is no correlation between the division of labour and the total amount of free ecdysteroids in *M. eumenoides* workers.

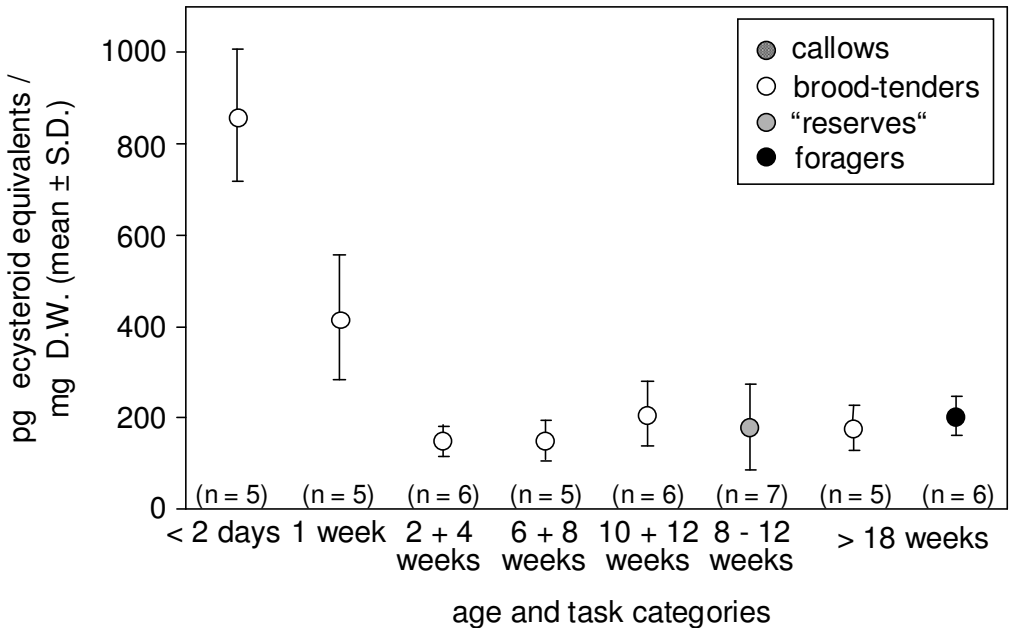


**Figure 13** A comparison of the total free ecdysteroid content (mean ± S.D.) of callows, brood-tenders (of various age categories), "reserves" and foragers. See section 3.3.1. for statistical analysis.

### 3.3.2. Identification of the Primary Ecdysteroids

Using coupled LC-MS, ecdysone and 20-hydroxyecdysone were identified to be the primary ecdysteroids present in pooled haemolymph samples of both brood-tending and foraging workers. Both are of course free ecdysteroids. There is also some evidence (on the basis of e.g. fragment mass, retention times and characteristic steroid peak shape) that other ecdysteroids (free or conjugated), or ecdysteroid-type substances, were also present in the samples, however in much smaller quantities. Although it was not possible to identify them, there were indications that two of these substances were 3-epiecdysone and 3-epi-20-hydroxyecdysone; epimerisation (to the 3α-form) is generally regarded as a procedure to inactivate the highly active 3β-forms (Kaplanis *et al.* 1979).

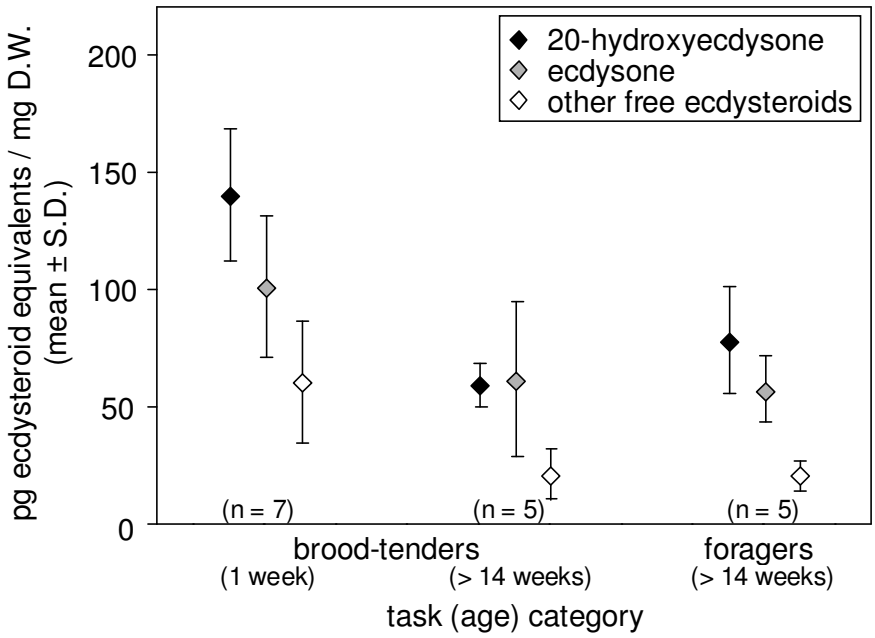


**Figure 14** Comparison of ecdysone, 20-hydroxyecdysone and the sum of the other free ecdysteroids in whole body extracts of brood-tenders aged one week, and older than 14 weeks, as well as of foragers also older than 14 weeks. See section 3.3.3. for statistical analyses.

### 3.3.3. Breakdown of Primary Free Ecdysteroids

No task-related differences were found in the content of the total free ecdysteroids of workers. Thus the next step involved breaking down the sum of these free ecdysteroids into its primary components in order to investigate whether any task-related differences were present at this level. The levels of ecdysone and 20-hydroxyecdysone, as well as the total amount of other free ecdysteroids present, were determined in whole body extracts of young (1 week old) and "old" (older than 14 weeks) brood-tenders, as well as foragers (also older than 14 weeks). Again all measurements were made in pg ecdysteroid equivalents per mg dry weight, and are given in mean values ± standard deviation. The results are depicted in figure 14.

Analysis of the whole body content of 20-hydroxyecdysone in workers in the three categories revealed the presence of a significant difference (Kruskal-Wallis ANOVA; n = 17, H = 11.99, p = 0.002). A *post hoc* test subsequently showed that one week old brood-tenders contained significantly more 20-hydroxyecdysone (140.1 ± 28.2) than both "old" brood-tenders (59.1 ± 9.0) and foragers (78.1 ± 22.7) (critical

difference$_{0.05}$ $p < 0.05$ in both cases), while no difference was found between "old" brood-tenders and foragers (critical difference$_{0.05,}$ $p > 0.05$).

A similar pattern was found regarding the ecdysone fraction in the whole body extracts of workers. Again a global significant difference was determined (Kruskal-Wallis ANOVA; n = 17, H = 7.14, p = 0.028), and subsequent *post hoc* tests showed that one week old brood-tenders (100.9 ± 29.9) contained significantly more ecdysone than "old" brood-tenders (61.5 ± 32.9) (critical difference$_{0.05}$ $p < 0.05$) but not foragers (57.5 ± 14.36) (critical difference$_{0.05}$ $p > 0.05$). However the value obtained for foragers just exceeded the significance limit (values obtained: one week old brood-tenders = 14, "old" brood-tenders = 4.6, foragers = 6.4; critical difference$_{0.05}$ = 7.0). As the *post hoc* test used is highly conservative (see section 2.4.1.), the result is nevertheless regarded as presenting a clear trend. No difference was found between the ecdysone levels of "old" brood-tenders and foragers (critical difference$_{0.05}$, $p > 0.05$).

Statistical analysis of the fraction containing the sum of the other free ecdysteroids present resulted in a global significance (Kruskal-Wallis ANOVA; n = 17, H = 11.67, p = 0.003). *Post hoc* tests demonstrated clearly that one-week old brood-tenders (60.6 ± 25.9) contained significantly more of this fraction than both "old" brood-tenders (21.0 ± 11.1) and foragers (21.0 ± 5.9) (critical difference$_{0.05}$ $p < 0.05$, in both cases). No difference was found between "old" brood-tenders and foragers (critical difference$_{0.05}$, $p > 0.05$).

To summarise, there are no significant differences in the levels of any of the three fractions between "old" brood-tenders and foragers. Hence there appears to be no correlation between the levels of any of the three fractions and the task performed by the workers.

### 3.3.4. Total Free Ecdysteroid Content and Signalling (CHCs)

The existence of a correlation between the total free ecdysteroid content and the CHC profiles of *M. eumenoides* workers was also investigated. The total free ecdysteroid content of individuals (determined using a RIA; data is the same as presented in section 3.3.1., and the CHC profiles were analysed as described in section 2.4.1.1.) in various behavioural development categories was plotted against their CHC profiles (figure 15).

Callows and one week old brood-tenders have elevated total free ecdysteroid levels and similar, low chemical distances. All workers older than one week have similar, low total free ecdysteroid levels, but CHC profiles of highly variable chemical distances; thus no correlation was found. This relationship between total free ecdysteroid levels and CHC profiles is best described as the exponential regression function: $y = 173.99 + \exp(6.53 + (-19.37) * x)$.

In sum, this demonstrates that, aside from callows and one week old workers, there is no correlation between the total free ecdysteroid content and the CHC profile of individual workers regardless of behavioural development phase. This indicates that free ecdysteroids may not play a role in signalling in *M. eumenoides* colonies.

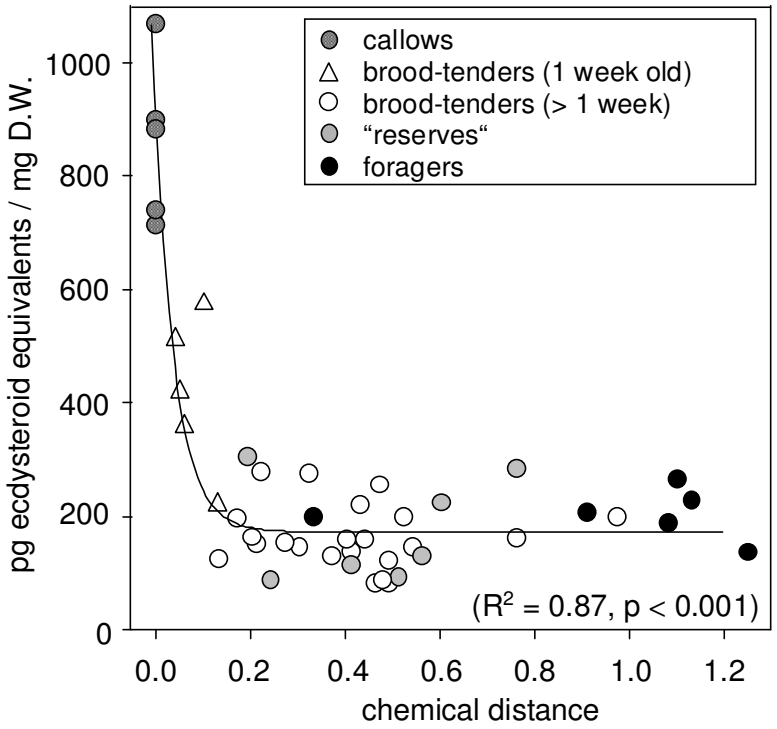


**Figure 15** The total free ecdysteroid levels of 40 workers, and five callows, plotted against the chemical distance of their CHC profiles (in each case the euclidean distance to the callows group centroid). The data points portray individual scores, and the black line represents the regression line.

## 3.4. Juvenile Hormones: A Role in Task Allocation and Signalling in *M. eumenoides*?

### 3.4.1. Juvenile Hormone (JH) Identification and Haemolymph Titres

LC-MS was used to measure haemolymph JH titres (see sections 2.3.6 and 2.3.7. for details). In the pooled samples taken from female alates both JH I and JH III was identified (figure 16). Although quantification could not be carried out (due to technical problems), it was clearly shown that based on raw area counts alone more JH I than JH III was present in female alate haemolymph samples.

Only JH III was identified in the pooled haemolymph samples of all workers (figure 17). The JH III titres were measured in pg/µl haemolymph, and all values are given as mean ± standard deviation. The JH III titres of each of the different task categories are depicted in figure 18. An analysis of variance revealed the existence of a statistically significant difference amongst the three task categories (ANOVA; $n = 30$, $F = 6.45$, $p = 0.005$). *Post hoc* tests showed that foragers ($n = 10$, $32.6 \pm 10.9$) had significantly higher JH III haemolymph titres (Tukey's HSD; $p = 0.003$) than brood-tenders ($n = 10$, $15.4 \pm 7.5$). The JH III titres of the "reserves" ($n = 10$, $23.2 \pm 13.0$) were shown to be of an intermediate value between the titres of brood-tenders and foragers. This difference was not statistically significant when brood-tenders and "reserves", or foragers and "reserves" were compared (Tukey's HSD; $p = 0.24$ and $p = 0.143$, respectively).

In sum, this demonstrates the existence of a task-related difference in JH III haemolymph titres. This consists of a correlation between low JH III titres and brood-tending, and a similar correlation between high JH III titres and foraging activities in *M. eumenoides* workers.

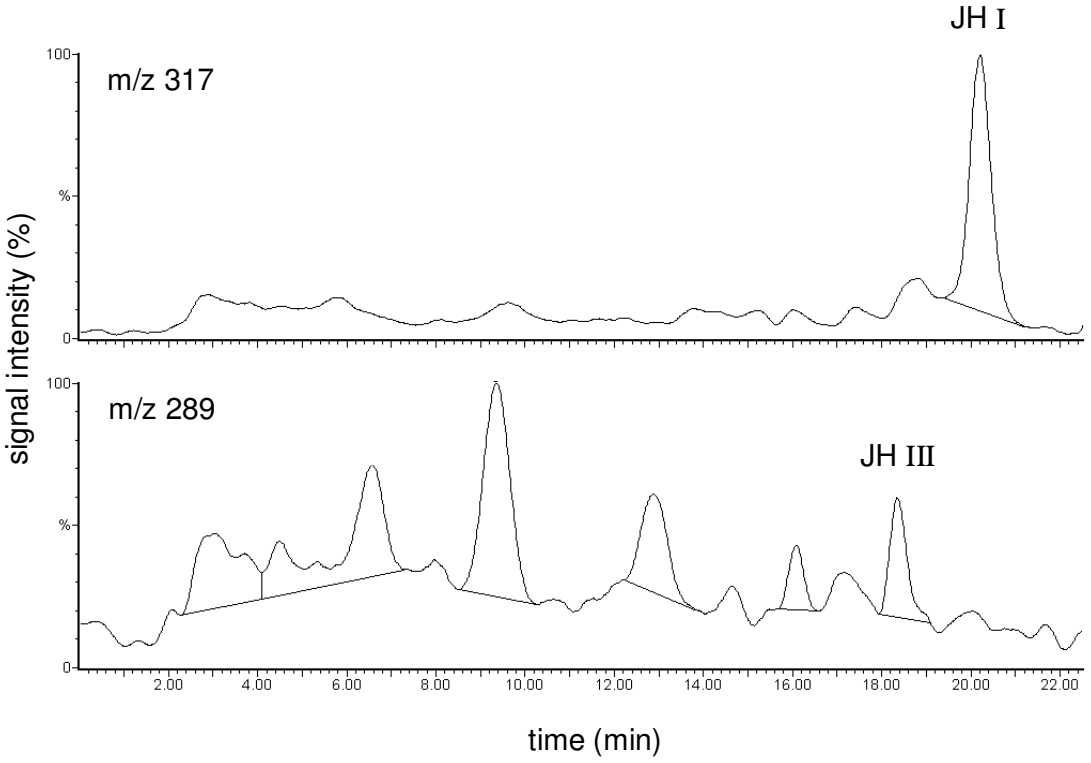


**Figure 16** LC-MS chromatograms of the ions representing the JH I and JH III $[M+Na]^+$ adducts in a pooled haemolymph sample of female *M. eumenoides* alates.

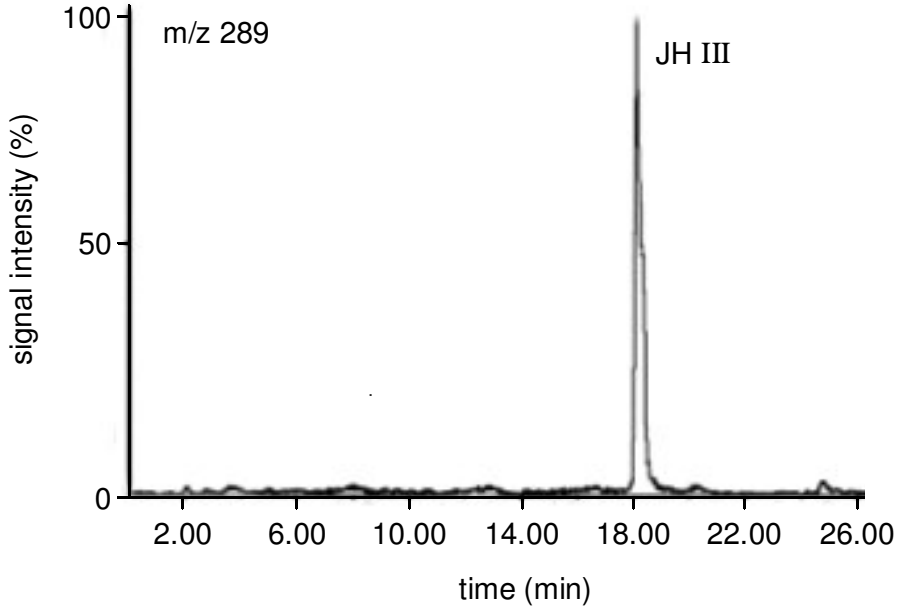


**Figure 17** LC-MS chromatogram of the ion representing the JH III $[M+Na]^+$ adduct in a pooled haemolymph sample of *M. eumenoides* workers carrying out foraging duties.

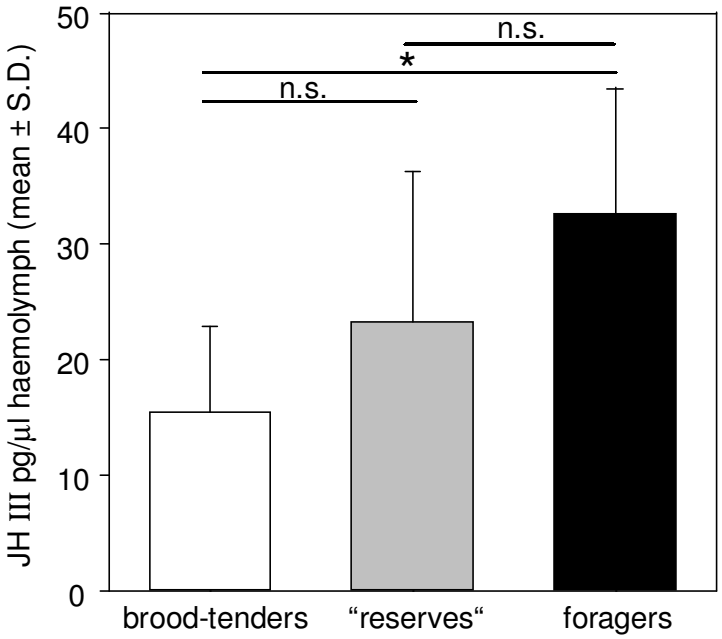


**Figure 18** The JH III titres (mean ± S.D.) in the haemolymph of brood-tenders, “reserves” and foragers. Each category is represented by ten pooled samples (see section 2.3.4.). The asterisks above the bars denote statistical significance at $p < 0.05$, while n.s. stands for non-significant differences.

### 3.4.2. The Impact of Application Experiments on Worker Life Expectancy

During the course of the investigations it emerged that the application experiments had detrimental effects on the longevity of the participating focus workers. This was of course assessed; all measurements made are given as mean values ± standard deviation. The life expectancy of laboratory-kept *M. eumenoides* workers lies roughly between 180 to 230 days (own unpublished observations). Here even acetone (used as a control) seriously reduced the life expectancy of workers. the control ants (n = 6) lived for an average of 63 ± 15.9 days post first application (the first application always took place within seven days of eclosion).

The longevity of the focus workers was shown to decrease further with increasing JH III concentration. At a concentration of 5 pmol JH III (n = 6) individuals have an average life expectancy of 49.5 ± 15.9 days after the first application, at 50 pmol JH III (n = 8) 41.5 ± 13.8 days, at 500 pmol JH III (n = 5) this decreases to 34.0 ± 18.5 days and at 5000 pmol JH III (n = 3) it is down to only 22.0 ± 7.0. The application of 1.77 mmol methoprene (n = 8) lowers the life expectancy of individual worker to a mean of 39.0 ± 13.5 days after the first application.

Due to this reduced life expectancy, none of the focus workers survived long enough to carry out foraging duties. Thus the effects of the application of the native hormone and its analogue were only investigated with reference to the task categories brood-tender and “reserve”.

### 3.4.3. The Impact of Exogenous JH III and Methoprene on Task Allocation

The effects of the administration of exogenous JH III and methoprene on task allocation were examined. The mean number of days after the first application a focus worker started to show “reserve” behaviour was taken as a representative measure of the effect of the applied substance on the division of labour. The results are portrayed in figure 19. The application of JH III (at any of the applied concentrations) as well as its analogue methoprene, did not result in any observed changes in task; no difference was found between the control, hormone- and hormone analogue-applied workers (ANOVA; n = 32, F = 0.613, p = 0.656).

It was not possible to determine whether there were differences in the number of days spent carrying out “reserve” duties (i.e. long term effects) due to the

differential life expectancies of individuals in different application brackets; this would not have allowed a conclusive analysis (see section 3.4.2.).

None of the focus individuals (in any category of application) were observed to forage at any point, despite (especially in the case of the application of 1.77 mmol methoprene) displaying CHC profiles characteristic of foragers. However on three occasions it was noted that after antennation, nestmates picked up actively brood-tending individuals (two individuals treated with 50 pmol JH III, one individual treated with 1.77 mmol methoprene) in the nest and carried them out into the foraging arena. They were then immediately released. All individuals involved in this had received topical applications for four to six weeks previously, and displayed forager-type CHC profiles.

In sum, this indicates that neither repeated applications of JH III, nor its analogue methoprene had any effect upon pacing the development from brood-tender to "reserve". This lack of a correlation indicates that JH III may not induce the behavioural shift from brood-tender to "reserve"

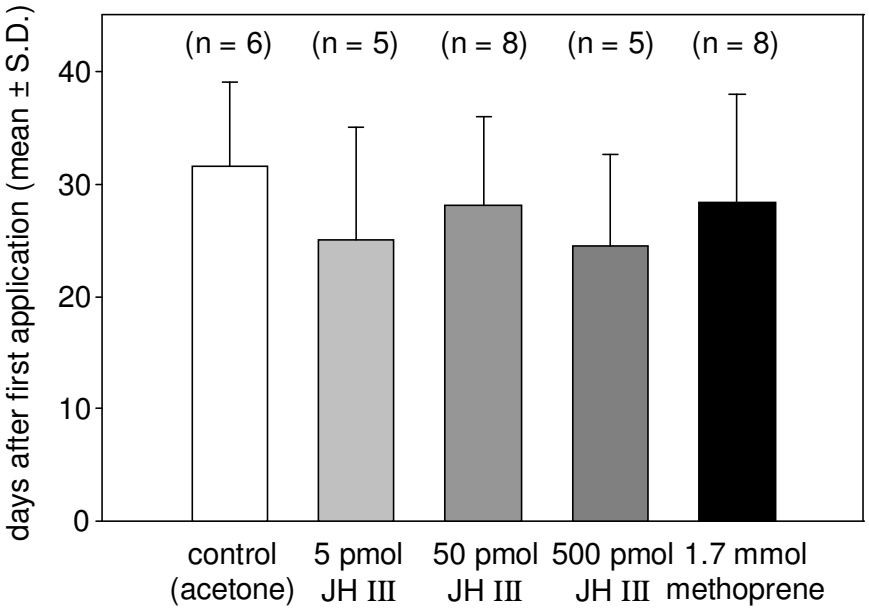


**Figure 19** A comparison of the number of days post first application individual workers started to show "reserve" behaviour in five application categories, as well as the controls. See section 3.4.3. for statistical analysis; no differences were found between the control group, and any of the application groups.

### 3.4.4. The Impact of Exogenous JH III and Methoprene on Signalling

The effects of the application of JH III and methoprene on the CHC profiles were examined, and documented in terms of days post first application of the native hormone and its analogue. The results are portrayed in figure 20. Analyses of the five different time brackets were carried out, and the results showed the presence of significant differences from day 11 onwards in comparison to the acetone controls: Kruskal-Wallis $ANOVA_{1-10}$; n = 24, H = 2.77, p = 0.431, Kruskal-Wallis $ANOVA_{11-20}$; n = 32, H = 15.26, p = 0.004, Kruskal-Wallis $ANOVA_{21-30}$; n = 32, H = 13.97, p = 0.007, Kruskal-Wallis $ANOVA_{31-40}$; n = 23, H = 8.96, p = 0.029, Kruskal-Wallis $ANOVA_{41-60}$; n = 19, H = 9.34, p = 0.025). *Post hoc* tests were carried out in order to localise the significant differences between treatment and control groups.

No significant changes were noted resulting from the application of JH III at a concentration of 5 pmol in all five time brackets (critical $difference_{0.05}$, $p > 0.05$ in all cases). Although the application of 50 pmol JH III resulted in increased chemical distances in all age brackets, these differences were significant only from days 21 to 30 (critical $difference_{0.05}$, $p < 0.05$), and from days 41 to 60 (critical $difference_{0.05}$, $p < 0.05$). For days 31 to 40, the value obtained was below the critical difference value (critical $difference_{0.05}$, $p > 0.05$), and thus non-significant (values obtained: control = 6.1, 5 pmol JH III = 9.8, 50 pmol JH III = 13.5, 1.7 mmol methoprene = 16.6; critical difference value = 9.0). Although statistical significance (at the $p < 0.05$ level) was thus not reached, probably due to the highly conservative nature of the *post hoc* test used (see section 2.4.1.), this result is nevertheless viewed as presenting a clear trend. No differences in CHC profiles were found as a result of the application of 500 pmol JH III (critical $difference_{0.05}$, $p > 0.05$) in the two age brackets tested.

Methoprene at a concentration of 1.77 mmol was shown to result in significantly higher chemical distances from day 11 post first application onwards (critical $difference_{0.05}$, $p < 0.05$, in all cases). Additionally, this precocious shift towards chemical distances similar to forager-type CHC profiles is also evident independent of age in terms of task (figure 21). In comparison to the acetone treated controls the application of 1.7mmol methoprene resulted in significantly different CHC profiles in both brood-tender (Kruskal-Wallis H-test; n = 46, H = 9.85, p = 0.002) and “reserve” (Kruskal-Wallis H-test; n = 43, H = 22.21, $p < 0.001$) categories.

These results in sum suggest that the CHC composition, and thus signalling, in *M. eumenoides* workers is under the endocrinological control of juvenile hormone. The application of JH III, as well as its analogue methoprene, resulted in a precocious shift towards forager-type profiles in a time-, and also concentration-dependent manner without effecting the behavioural development.

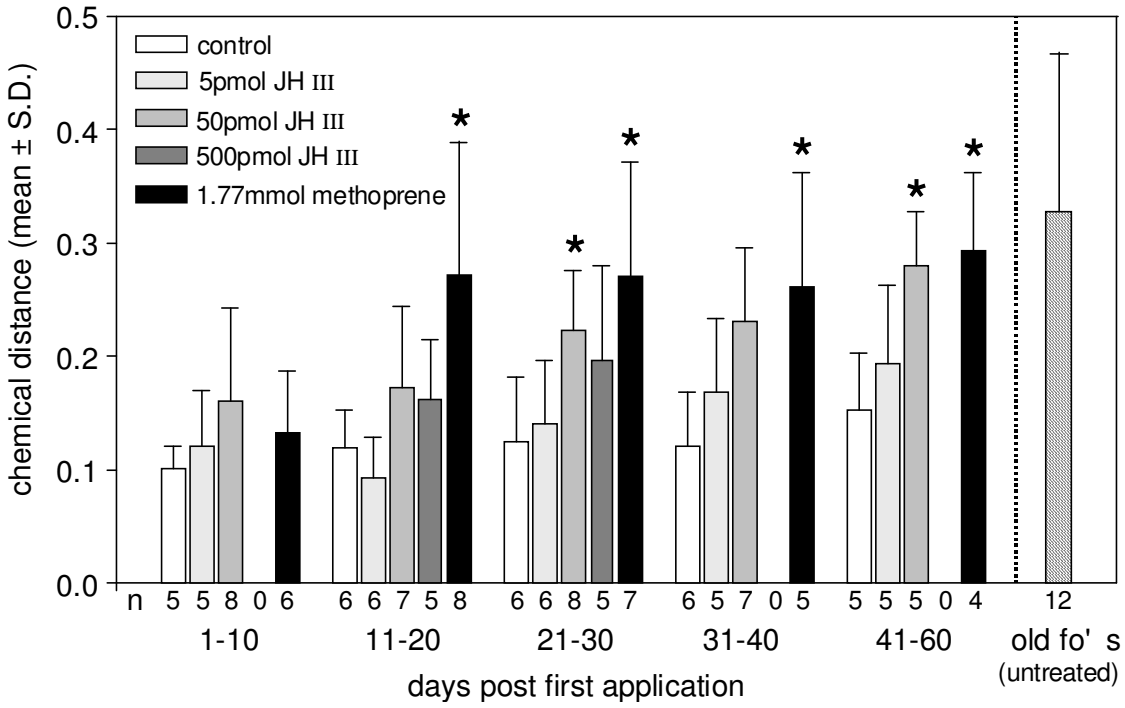


**Figure 20** The effects of the application of 5pmol, 50 pmol and 500 pmol JH III, as well as 1.7 mmol methoprene, on the CHC profiles of individuals as a function of time. The chemical distance is the euclidean distance to the first CHC profile taken from a focus worker. Thus the mean chemical distance of CHC samples in each time bracket was firstly calculated per individual, then for each application group. The number of individuals used in the analysis is denoted in each time bracket for each application category. The asterisks indicate differences at the $p < 0.05$ level between that specific application group and the acetone control group in the same time bracket. The “old foragers” (“old fo’s”) group (at the very right) was included for visual comparisons only.

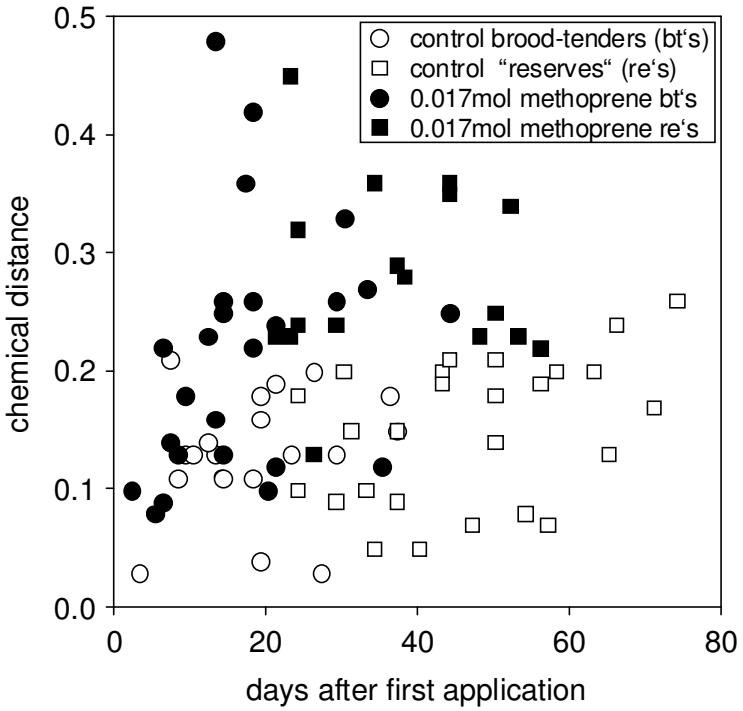


**Figure 21** The effects of the application of 1.7 mmol methoprene on the CHC profiles of individuals in comparison to the controls (acetone) group. Each individual in each application group (controls = 6, 1.7 mmol methoprene = 8) is represented by several CHC profiles in the diagram. The chemical distance is the euclidean distance to the first CHC (reference) profile taken from each individual before the applications commenced. A diagram comprising all four application categories is presented in the appendix (figure a2).

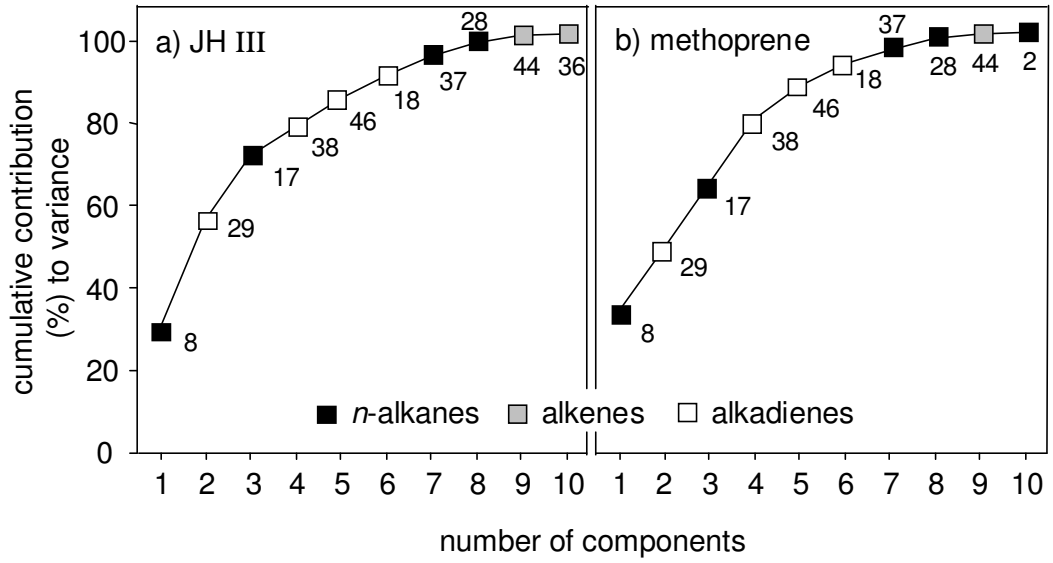


**Figure 22** Cumulative saturation curves depicting the ten components that contribute most to the variance between (a) CHC profiles of the controls (brood-tending phase; n = 12) and CHC profiles of JH III (50 pmol) treated workers ("reserve" phase; n = 12), and (b) CHC profiles of the controls (brood-tending phase; n = 12) and CHC profiles of methoprene (1.7 mmol) treated workers ("reserve" phase; n = 12). Above each component the number allocated to it in table 1 is indicated.

3.4.4.1. Analysis of Application-induced Signalling Changes

Variance components analyses was used to investigate the contribution of individual components to the variance between CHC profiles of brood-tenders (controls), and the precocious forager-type CHC profiles of JH III (50 pmol used as the representative contribution) and methoprene treated workers in the "reserve" phase.

The results showed that the CHC profiles of the controls can be differentiated from the CHC profiles of JH III and methoprene treated workers on the basis of only ten components (see figure 22). These were shown to contribute more than 98 % (JH III; 98.3 %, methoprene; 98.9%) to the variance between the tested groups, and were almost identical for both JH III- and methoprene-altered CHC profiles. Although one component was different, in both cases this contributed least (of the ten components) to the variance between the tested groups. In fact, only five (both

saturated and unsaturated) components were shown to cumulatively contribute 82.5 % (JH III) and 86.0% (methoprene) to the variance between the control CHC profiles and the application-induced forager-type CHC profiles. These results are highly similar to those obtained during the analysis of task-related signalling differences (see section 3.2.8.) of non-treated workers.

Thus the control (brood-tender) CHC profiles can be differentiated from ("reserve") CHC profiles altered through JH III and methoprene application on the basis of only a few components. Moreover, these components do not belong to the same chemical family and are the same for JH III and its analogue methoprene.

# 4. Discussion

## 4.1. Project Niche

Using solvent extraction, Kaib *et al.* (2000) demonstrated the existence of task-specific CHC profile differences in *M. eumenoides* workers. Furthermore, these differences appeared to be age-related only in workers that left the nest and turned to foraging duties. The investigations were however hampered by the fact that solvent extraction was used to obtain the CHC profiles; this is an invasive, and hence destructive sampling technique. Therefore, only one CHC profile sample per worker, representing one task category (behavioural development phase), was available, and the global picture had to be constructed using single samples from numerous individuals across all behavioural development stages. Although the results obtained by Kaib *et al.* (2000) were conclusive, the CHC profile changes with task at the individual worker level could of course not be documented.

Therefore the first goal of this project was to overcome this problem, and explore the temporal dynamics of individual worker CHC profiles in relation to the tasks performed. To the best of my knowledge, this type of parallel “tracking” has not been carried out before to investigate signalling (CHCs) and task allocation in the Formicidae. For this purpose the contact SPME method was used to extract CHC profiles. This method has one huge advantage over other commonly used CHC sampling techniques; since it is non-destructive it allows repetitive sampling of focus individuals over time. In the last five years or so, the SPME method has been used successfully to monitor CHC profile changes in several ant species over long periods of time (e.g. Liebig *et al.* 2000, Dietemann *et al.* 2003).

Although some studies on the endocrinological control of the age-related division of labour have been carried out (primarily on the honey bee *Apis mellifera* e.g. Robinson 1985, Robinson *et al.* 1989, Hartfelder *et al.* 2002), signalling was never investigated in relation to this. Conversely, task-specific CHC profiles have been evidenced in several ant species (e.g. Wagner *et al.* 1998, Kaib *et al.* 2000, Greene and Gordon 2003) but without any parallel assessment of the possible endocrinological factors governing them. Thus this project is unique in the way that chemistry (signalling) and behavioural development (i.e. task allocation) are investigated not only in relation to each other, but also with respect to the likely

causal endocrinological factors (ecdysteroids, juvenile hormones) in a eusocial Hymenopteran species.

## 4.2. Peripheral Results

### 4.2.1. CHC Investigations

This study utilised the SPME method for the first time to extract CHCs from workers of the species *M. eumenoides*, and thus it had to be validated to ensure that representative CHC profiles could be obtained in this way. Since most of the exocrine glands located in the gaster open at the VIIth and VIIIth tergites (Hölldobler and Wilson 1990), the risks of contamination were minimised by taking all SPME samples exclusively from the dorsal side of the fourth tergite. Preliminary results showed that the CHC profiles obtained by both hexane extraction and SPME-sampling were qualitatively the same; this makes it highly unlikely that the SPME samples were contaminated.

The optimum SPME-sampling time was estimated to be 5 min. The results indicated that the non-polar PDMS coating of the fibre is almost fully saturated with CHCs after 5 min. To compromise between damage and distress to the ants, and method efficacy, it was decided to use this sampling time in all investigations. This is identical to the one used by Liebig *et al.* (2000) in their study of CHCs of the ponerine ant *Harpegnathos saltator* (Hymenoptera).

The reproducibility of the profiles obtained using SPME was also validated. The intra-individual variation was always shown to be much lower than the inter-individual variation; both increased only very slightly with increasing chain length of the components. In addition the SPME-sampled individuals were demonstrated to be treated as nestmates on return to the colony, thus SPME-sampling was shown not to effect the colonial reintegration of individuals. Together this shows that the SPME method is adequate to monitor individual CHC profiles changes over time, and to allow the grouping of samples according to profile similarity.

Since the division of labour in social insect colonies is not characterised by individuals switching back and forth between various tasks (when the colony is not subjected to external stress), observing the focus individuals for 5 min every 24 to 48 h was proven sufficient to reflect the behavioural development categories of the

individual workers over time. Thus the ethograms created could be correlated with the chemical (CHC profile) data to assess changes over time and with task. Even during periods of task-transition such as from brood-tender to “reserve”, and from “reserve” to forager, where individuals may exhibit behaviour characteristic of two task categories for a period of time, the number of observational samples taken are sufficient to reflect this.

### 4.2.2. JHs of Female Alates

It is generally accepted that JH III is the principle form of JH found in Hymenoptera, and that only the Lepidoptera possess JH I and JH II as well as JH III (Gäde *et al.* 1997). Although quantification was not possible, the fact that JH I, along with JH III, was found in the haemolymph of female *M. eumenoides* alates is fascinating as it is the first time that JH I has been identified in Hymenoptera, and only the third time it has been found outside the Lepidoptera; the presence of JH I was previously only documented in the cockroach *Nauphoeta cinerea* (Blattodea) (Lanzrein *et al.* 1975), and in *Gryllus bimaculatus* (Ensifera) (Westerlund and Hoffmann 2004).

JH is generally regarded as a key component in flight and migratory behaviour that acts by either directly or indirectly mobilising nutrient reserves (Kumaran 1990). After the prenuptial flight in the eusocial Hymenoptera, the wings are shed and the ability to fly is lost. It has been put forward that JH I in particular plays a key role in regulating the flight mechanisms (e.g. Rankin 1991). This hypothesis would explain the absence of JH I in the haemolymph of *M. eumenoides* workers, and its presence in female alates.

### 4.2.3. The Impact of Application Experiments on Worker Life Expectancy

The concentrations of JH III applied (ranging from 5 pmol (1.33 fg/µl solution applied) to 5000 pmol (1.33 pg/µl solution applied) were roughly an order of magnitude lower than the actual JH III haemolymph titres determined using LC-MS (ranging from 16 pg to 33 pg/µl of haemolymph). The reason why the JH III was applied at these lower concentrations was the reduced life expectancy relative to the acetone treated controls. Although the solutions at higher concentrations were still lower than the actual titres determined, these did not allow the individual workers to

survive long enough to fulfil the aims of the experiment. Hence the JH III was applied at lower concentrations that proved to be less detrimental.

Acetone was used as solvent as well as the control, as it has been demonstrated that acetone treatments have no effects on the behavioural development of *A. mellifera* workers (Robinson 1985). However the acetone application alone was shown to reduce the life expectancy of individual workers. The toxic effects of organic solvents at the cellular level are well known, but only poorly documented. Nevertheless, it is generally agreed that the cell membrane is the primary target, and that changes in permeability lead to increased mortality (e.g. Stevens and Hofmeyr 1993).

The increased mortality resulting from the application of high doses of JH III has been documented before (in the Aphidae: e.g. Kuhr *et al.* (1973); in the cat flea *Ctenocephalides felis* (Siphonoptera): Meola *et al.* (2001); and in *Manduca sexta* (Lepidoptera): Orth *et al.* (2003)). It has been suggested that this toxic effect of JH is closely correlated with the concentration of the unbound hormone (Orth *et al.* 2003). A possible explanatory model for this is that after passing through the cuticle, JH enters the haemolymph resulting in a localised flash saturation of juvenile hormone binding proteins (JHBP). A certain proportion of the unbound hormone is then deactivated by esterases, while the rest, due to its lipophilic and hydrophobic properties, is thought to preferentially associate with membrane lipids (but not with the putative JH membrane receptors where the JHBP-JH complex is thought to dock), and in doing so alter their physical properties such as permeability (Barber *et al.* 1981). This of course could result in a concentration-dependent increase in mortality. Thus, the statement that it may be a “pharmacological side-effect rather than a physiological consequence of normal hormone action” (Herman and Tatar 2001) makes sense, since under normal circumstances the concentration of unbound hormone in the haemolymph has been shown to be extremely low. In *Blattella germanica* (Blattodea) for example, the amount of JH present in the haemolymph is four times less than the maximum binding capacity; this suggests that virtually all JH must be bound to a JHBP (probably lipophorin) even during titre peaks (Sevala *et al.* 1997).

The JH analogue methoprene was applied at a very high concentration (1.77 mmol or 0.55 ug/µl). It has also been previously noted to result in increased mortality at high concentrations e.g. in *Apis cerana* (Hymenoptera) (Huang *et al.* 2001). Again,

this higher mortality is probably based on effects on membranes. Research on the salivary glands in *Galleria mellonella* (Lepidoptera) revealed that JH analogues change ionic conductivities possibly by interacting with the membrane phospholipids (Baumann 1969). It is conceivable that this also led to the relatively short life spans of workers in this study.

### 4.3. Analyses of the Behavioural Development of Individual Workers

All focus workers carried out brood-tending duties early on in life, and then went through the so-called "reserve phase" before eventually changing to foraging duties. This confirmed the presence of a functional age-related division of labour system in *M. eumenoides* colonies. The transition from brood-tender to forager was shown to be continuous and to change in an analogous manner. This correlation between age and task has been shown to occur in a large number of ant species (reviewed by Hölldobler and Wilson 1990). Although the results obtained in this study may suggest that the division of labour is age-dependent rather than age-related, this is clearly not the case. Kaib *et al.* (2000), in a study involving 326 workers, showed that in colonies of *M. eumenoides* many individuals carry out brood-tending duties during their entire lifetime (and that these individuals keep their brood-tender-type CHC profile). The phenomenon of workers persevering with brood-tending for their entire life has been previously noted e.g. in the ant *Formica sanguinea* (Hymenoptera) by Dobrzanska (1959).

Brood-tending duties were on average carried out six to eight weeks post eclosion, and the workers associated closely with the brood and queen even during periods of inactivity. The "reserve" phase (Hölldobler and Wilson 1990) workers generally stayed in the peripheral areas of the nest away from brood and queen, and were mostly immobile and inactive. However several weeks prior to turning into foragers they were observed to become more mobile, and to be recruited into the foraging arena for periods lasting up to six hours when a protein source was placed there. This general type of behaviour was also noted in the harvester ant *Pogonomyrmex barbatus* (Wagner *et al*. 2001) where the transition from interior work to exterior work was shown to involve short bursts of exterior work. Perhaps these trips condition the workers for the future exterior work.

Despite their apparent high level of inactivity, *M. eumenoides* workers in the "reserve" transitional phase probably have multiple chores. In addition to their involvement in protein sourcing, the "reserves" were observed to participate in both brood transport and brood-tending during colony disturbances, and probably additionally perform guarding duties (Otto 1962). They occupy the peripheral areas of the nest, which ensures that any intruders must actively make their way past them on the way to the brood and queen; any nestmates re-entering the nestbox were always inspected closely by the nearest "reserves". The "reserves" thus provide colonies with a pool of workers that flexibly allocate to where they are most needed.

The time required for the transition from interior to exterior work was shown to vary from worker to worker. This parallels findings by Otto (1962) who described that in *Formica polyctena* (Hymenoptera) colonies the transition does not occur suddenly, but via a transitional phase of variable duration. In light of the constant colony conditions (environmental, dietary etc.) that this study was conducted under, it is difficult to find a viable explanation for this phenomenon.

It was shown that once foraging duties were resumed these were then carried out until death in all focus workers. No workers were observed to revert to interior work, which under certain circumstances has been shown to occur in *Pogonomyrmex barbatus* (Hymenoptera) (Gordon 1989) and in *A. mellifera* workers (Robinson *et al.* 1992). The foraging workers were observed to enter the peripheral areas of the nest (occupied by "reserves") sporadically for short periods of time. On several occasions they were seen to offer food to nestmates (they were antennating nestmates and had a droplet of food between the opened mandibles), and in all observed cases they were inspected and then groomed.

## 4.4. The CHCs of *M. eumenoides* Workers

### 4.4.1. CHC Chemistry, Changes in Workers and Possible Functions

The focus workers were all shown to have the same qualitative CHC composition, made up of non-volatile saturated (*n*-alkane) and unsaturated (alkene and alkadiene) components with a backbone ranging from 21 to 36 C-atoms. However minute quantities of methyl-branched CHCs were probably also present (see Kaib *et al.* 2000), but could not be allocated in this study. Irrespective of task,

the CHC profiles were shown to be dominated by unsaturated components. This is an unusual CHC composition in as far as in most other investigated ant species the CHC profiles consisted primarily of methyl-branched hydrocarbons (e.g. Cuvillier-Hot *et al.* 2001, Wagner *et al.* 2001, Dietemann *et al.* 2003).

The CHC profiles of the focus *M. eumenoides* workers were shown to adjust quantitatively throughout their life in the same continuous fashion with the tasks carried out. Since all of these individuals eventually changed from brood-tending to foraging duties, the CHC profile changes are, in part, also correlated with age.

Callows were shown to possess highly similar CHC profiles. As they are put into an environment that is distinctly hostile to anything alien, they have to prove otherwise in order to survive (Jaisson 1985). Since the callow CHC profiles were shown to be only quantitatively different to those of adult workers, it is tempting to suggest that this qualitative blend of CHCs acts as the “colony membership card”.

The change from a callow-type to a brood-tender-type, and on to a forager-type CHC profile was shown to be characterised by an increase in saturated, and a decrease in unsaturated components as well as decrease in short-chain, and an increase in long-chain CHCs. In fact, this CHC profile transition could be described as a continuum. This parallels results by Cuvillier-Hot *et al.* (2001) who demonstrated that, in the ant *Diacamma ceylonense,* all callow workers initially have the same profile, however with time this changes to a forager-type profile. The fact that all the transitions are continuous makes it likely that a common mechanism is responsible for the CHC differences between the categories.

The brood-tenders were shown to have relatively similar CHC profiles and group together, while the CHC profiles of the “reserves” and the foragers varied greatly. In the case of the “reserves”, the observed variation may be explained by the fact that these represent a transitional phase in a continuous developmental pathway, and thus range from one extreme (brood-tender-type CHC profile) to the other (forager-type CHC profile). The CHC profiles taken from individuals whilst foraging also varied greatly, but in light of the constant environmental and dietary conditions it is very difficult to find an explanation for this observed variability.

The fundamental function of the lipid-rich surface of the cuticle is to act as an anti-desiccatory barrier (Blomquist *et al.* 1998), and it is generally accepted that CHCs play a key role in this by reducing the cuticular permeability (e.g. Hadley *et al.* 1987, Hadley 1994). It is thought that *n*-alkanes melt at slightly higher temperatures

than alkenes and alkadienes with similar C-atom backbones (Gibbs and Pomonis 1995, Gibbs 1998), and that waterproofing increases with chain length; numerous studies have correlated chain length with water loss rates (e.g. Toolson 1984, Hadley and Schultz 1987). In fact, the properties of the callow-type CHC profile alone suggest that it is a highly effective passive barrier to water loss. This indicates that the observed CHC changes with progressive behavioural development are most likely not necessary to adjust the water loss rates.

Thus, if the cardinal function of these CHC profile adjustments does not lie in providing dessication prevention, they most likely serve other purposes. In the last decades, much research has focused upon the role of CHCs in chemical communication. They have been demonstrated to act as sex, recruitment and alarm pheromones, and to function as territorial marking and defence secretions (reviewed by Howard 1982), as well as recognition cues. Nestmate recognition is of particular importance in social insects, and it has been demonstrated several times in various ant species (e.g. Lahav *et al.* 1999, Thomas *et al.* 1999, Wagner *et al.* 2000) that CHCs play a key role in this. However all the evidence points towards other levels of information, such as what task is being performed, also being encoded in the CHC compositions. The variety of CHC structures such as chain length, and number and positions of double bonds allow multiple information processing in chemical communication (Lucas *et al.* 2004); the CHCs of the ant *Diacamma ceylonense* have already been demonstrated to contain information that may be used at four different identification levels (Cuvillier-Hot *et al.* 2001).

### 4.4.2. The CHCs of Workers Inform Task Decisions

The results obtained in this study show that there are quantitative CHC profile differences that vary with task in individual *M. eumenoides* workers. Thus, the CHC profile of a worker carrying out brood-tending duties is much more similar to the CHC profiles of other brood-tending workers than its own CHC profiles during the “reserve” or forager phases later in its life. Of course this also applies to CHC profiles of individuals carrying out foraging duties, or in the “reserve” phase. This clearly demonstrates the existence of task-group specific CHC compositions in *M. eumenoides* colonies.

Since the behavioural development of individual workers was shown to change synchronously with the CHC profile, it implies that the latter accurately

reflects an individuals' task at a given moment in time; in other words "task signalling" takes place. A similar correlation between ethological and chemical (CHC) data with respect to the division of labour has also been observed in colonies of the ant species *Camponotus vagus* (Hymenoptera) (Bonavita-Courgourdan *et al.* 1993) and *Pogonomyrmex barbatus* (Hymenoptera) (Wagner *et al.* 1998).

Importantly, there are indications that this is not just correlative evidence, but that these task-specific signals are perceived by nestmates. Thus the CHC differences are likely to allow a worker to determine the tasks performed by nestmates during brief antennal encounters. Using behavioural tests, Greene and Gordon (2003) demonstrated that workers perceive, and respond to, task-related CHC differences in the red harvester ant *Pogonomyrmex barbatus*. In colonies of the ponerine ant *Pachycondyla inversa* (Hymenoptera), the CHCs of fertile queens and dominant workers are characterised by the branched 3,11-diMe$C_{27}$ component. Using coupled GC-EAG, D'Ettorre *et al.* (2004) showed that the antennae of ordinary workers perceived this key component. This provided the first evidence that certain components in a CHC profile, and not just the total CHC bouquet, are detected, and recognised by workers. Thus it is highly interesting that there was also some direct behavioural evidence that the CHCs not only inform task decisions in *M. eumenoides* colonies, but that workers can interpret task-specific CHC profiles. On several occasions it was noted that JH III and methoprene treated workers displaying forager-type CHC profiles but carrying out brood-tending duties in the nestbox were picked up and carried into the foraging arena by nestmates. This indicates that on the basis of their CHC profiles they were recognised as foragers which "did not belong into the nest but into the foraging arena", and that brood-tenders reacted accordingly to rectify this situation. Similar results, however on the opposite process, were obtained by Bonavita-Cougourdan *et al.* (1993) who described a "retrieving behaviour" in colonies of the ant *Camponotus vagus*; brood-tenders removed from the nestbox and left in the arena were carried back to the nest by foragers. This led the authors to speculate that this behaviour is due to the "two subcastes being able to recognise each other".

Analysis of task-specific CHC profile differences in *M. eumenoides* workers revealed that five components (two of which are saturated and three unsaturated) contributed nearly 90 % to the variance between brood-tender and forager CHC profiles. Additionally it was demonstrated that brood-tender and forager CHC profiles

can be separated equally as well on the quantitative differences of these five components alone as on the basis of all 51 CHCs. In fact, only two components, *n*-heptacosane and triacontadiene, contributed more than 72 % to the total variance between brood-tenders and foragers CHC profiles. This indicates that "task signalling" perhaps only requires a few components of both saturated, and unsaturated nature.

These findings parallel results obtained during research on fertility signalling in the Formicidae, where there are also indications that only a few components are involved, and that these do not need to belong to the same chemical family. In the primitive ant *Myrmecia gulosa* (Hymenoptera) two components of the CHC profiles (3-methyl-pentacosane and 9-pentacosene) were shown to differentiate reproductives from non-reproductives (Dietemann *et al*. 2003). In the queenless ant *Diacamma ceylonense* several substances (*n*-$C_{29}$ and some methyl $C_{25}$ and $C_{27}$) were shown to discriminate between callows, foragers and egg-layers (Cuvillier-Hot *et al*. 2001). Similarly, in *Vespa crabro* (Hymenoptera) queens were shown to differ from workers in only four substances (two *n*-alkanes, one alkene and one methyl-branched alkane) (Butts *et al.* 1995). In the ponerine *Harpegnathos saltator* (Hymenoptera) it was shown that a methyl-branched alkane (13,23-diMe-$C_{37}$) was present in high quantities in egg-layers but not in infertile workers and queens; egg-layers are additionally characterised by higher relative amounts of long-chain CHCs (Liebig *et al.* 2000).

Compellingly, the results obtained in this study indicate that primarily the major components, along with a few components present in medium relative concentrations, but definitely not the minor components, play the key role in "task signalling" in *M. eumenoides*. Similar results were obtained in the four fertility signalling studies mentioned in the above paragraph. In each study the CHCs involved could be classed as major components in at least in one or more of the categories investigated. This is intriguing in light of the results obtained by Kaib *et al.* (2004) from work on the termite *Macrotermes subhyalinus* (Isoptera), who suggested that nestmate recognition is based on components present only in lower concentrations, and mostly on unsaturated HCs. Hence this raises the possibility that the minor compounds signal colony membership (i.e. function as nestmate recognition cues), and the major components reflect the current task (in some ant species fertility) category of the individual worker.

## 4.5. The Endocrinological Background of Task Allocation and Signalling

Most of the results discussed in this section are based upon three levels of information: chemical (CHC profile), ethological (behavioural development) and endocrinological (hormonal) data. This resulted in the build up an almost three-dimensional picture of the factors surrounding both task allocation and signalling. The registration of the hormonal parameters firstly involved a measurement of the relevant haemolymph titres or whole body levels. If the results indicated the presence of a hormonal correlation with either task and/or signalling, the substance in question was subsequently exogenously administered and the effects monitored in order to provide some direct, rather than just correlative evidence.

### 4.5.1. Do Ecdysteroids Play A Role in Task Allocation and Signalling?

Ecdysone and 20-hydroxyecdysone were identified to be the primary free ecdysteroids in pooled haemolymph samples of workers, with possibly some other ecdysteroids (free or conjugated) also present in minute quantities. It was anticipated that ecdysone and 20-hydroxyecdysone would be present, since *M. eumenoides* is an omnivorous ant species and thus has a large dietary intake of cholesterol, the "building block" from which ecdysone is synthesised (Rees 1985).

Whole body extracts (commonly used to determine individual ecdysteroid titres in small insects - Smith 1985) were used to determine total free ecdysteroid levels of individual workers. It is acknowledged that the total free ecdysteroid levels obtained this way may not necessarily reflect the physiologically relevant haemolymph titres of these individuals. This is because ecdysteroids taken up into various tissues, or excreted into the gut, may contribute to the measurements (Kaplanis *et al.* 1980). However these putative storage and inactivation conjugates in general are either less or more polar molecules than the free ecdysteroids (Hoffmann and Lorenz 1997); and thus should have been removed during sample purification (section 2.3.1.). Additionally, in all insect species investigated to date, unmetabolised free ecdysteroids that are not required are generally rapidly excreted (e.g. Thiry and Hoffmann 1992, Blackford *et al.* 1997; also reviewed by Dinan 1997). In sum, this suggests that the recorded whole body total free ecdysteroid levels should at the very least be indicative of physiologically relevant titre changes.

The total levels of free ecdysteroids in whole-body extracts were shown to be elevated only in callows and one week old workers. Irrespective of task, no differences in total free ecdysteroid levels were found between workers aged two weeks and above. When the primary free ecdysteroids were analysed separately, again increased levels of ecdysone and 20-hydroxyecdysone (as well as the sum of the other free ecdysteroids present) were only found in one week old workers, and no differences were located in older workers regardless of task, thus providing evidence that there is no correlation between whole-body ecdysteroid levels and task.

Similarly, no correlation between free ecdysteroid content and CHC profile was found in any individual worker aged two weeks and older. As the CHC profiles of individuals display marked changes over the time period where the free ecdysteroid levels remain static at a low level, this would suggest strongly that ecdysteroids do not play a role in signalling in the ant *M. eumenoides*.

Hartfelder *et al.* (2002) measured the haemolymph ecdysteroid titres in *A. mellifera* and the stingless bee *Mellipona quadrifasciata* (Hymenoptera). It was noted that in both species only workers less then four days old had significantly higher haemolymph ecdysteroid titres; in workers older than four days the titres dropped and were thereafter shown to remain constant at a low concentration, thus paralleling the results obtained on *M. eumenoides* workers. Additionally, the Hartfelder *et al.* (2002) study provides some indirect evidence that ecdysteroids do not play a role in task allocation. Although no reference to task was made, the haemolymph ecdysteroid titres of five and twenty day old *A. mellifera* workers were measured. Workers of this species generally start to forage between fifteen and twenty days post eclosion (Hartfelder *et al.* 2002), thus both brood-tending and foraging task groups are likely to be represented here. Although this is not mentioned by the authors, these results thus suggest that in *A. mellifera* there is also no correlation between ecdysteroid levels and task. Similar results were obtained by Robinson *et al.* (1991) who found that haemolymph ecdysteroid titres of both nurses and foragers from a queenright *A. mellifera* colony were below the detection limit of the RIA utilised (65 fmol). This led the authors to suggest that ecdysteroids are not involved in the age-related division of labour.

In addition, there is some evidence that age-related CHC profiles exist in *A. mellifera* workers, and that nurses and foragers probably have different CHC profiles (Blomquist *et al.* 1980, Francis *et al.* 1989). Together with the results obtained by

Robinson *et al.* (1991) and Hartfelder *et al.* (2002) (both detailed in the above paragraph), this provides indirect evidence that in *A. mellifera,* as in *M. eumenoides*, there is also no correlation between ecdysteroid levels and signalling. This indicates that ecdysteroids do not play the key role in the signalling system of eusocial Hymenopterans, unlike in some Dipteran species where there is some speculative evidence that ecdysteroids are involved in the regulation of pheromone biosynthesis pathways e.g. *Musca domestica* (Blomquist *et al.* 1998) and *Calliphora vomitoria* (Trabalon *et al.* 1994).

The most likely explanation for the high levels of free ecdysteroids in callows and one week old workers is that they are due to the internal remnants from the very broad peak that occupies the period of pupal-adult development in holometabolous insects (Steel and Vafopoulou 1989), and larval-adult development in hemimetabolous insects (Gäde *et al.* 1997). For example, in the cockroach *Periplaneta americana* (Blattodea), high free ecdysteroid haemolymph titres were observed directly after adult emergence in both males and females, and these were then shown to decrease over the following five days (Weaver *et al.* 1984).

To summarise, the lack of a correlation between total free ecdysteroid levels and the behavioural development, as well as CHC profiles of individual workers would therefore indicate that ecdysteroids do not play a role in the division of labour nor signalling in social insect colonies.

### 4.5.2 Does JH III Play a Role in Task Allocation?

Only JH III was identified in the haemolymph of *M. eumenoides* workers, and foragers were shown to have significantly higher haemolymph JH III titres than brood-tenders. These findings parallel results from a range of previously published studies of eusocial Hymenopterans. In the haemolymph of the bumble bee *Bombus terrestris*, JH III was the only homologue identified (Bloch *et al.* 2000). Only JH III was identified in the haemolymph, and the concentration of it was shown to increase with age in workers of a malaysian *Diacamma spp*. (Hymenoptera) ant (Sommer *et al.* 1993); however there was no reference to task in that particular study. In the haemolymph of workers of *A. mellifera* (e.g. Robinson 1987, Huang *et al.* 1991) and the closely related bee *Apis cerana* (Hymenoptera) (Huang *et al.* 2001) only JH III has been identified, and the titres were demonstrated to be higher in foragers than in brood-tenders (and thus also to a certain extent to be age-related).

Additionally, the *M. eumenoides* “reserves” (the middle aged workers) have a JH III titre which is intermediate between that of brood-tenders and foragers. This further parallels results obtained on *A. mellifera*. The haemolymph JH III titres in that species have been shown to be primarily governed by the rates of biosynthesis (Huang *et al.* 1991). Consequently, the “bee equivalents” of the “reserves” (the middle aged bees consisting of wax producers, food storers, guards and undertakers) were shown to have rates of JH biosynthesis that were intermediate between those of nurses and foragers (Huang *et al.* 1994).

These results in sum strongly suggest that in *M. eumenoides,* and other eusocial Hymenopteran species, common mechanisms regulate the age-related division of labour, and that JH III is likely to play a substantial role in this.

### 4.5.3. The Impact of Exogenous JH III and Methoprene on Task Allocation

The application of the JH III (at all applied concentrations), as well as the JH analogue methoprene, did not result in any observable changes in behavioural development, thus indicating that JH III may not induce the behavioural shift from brood-tender to “reserve”. Thus it is questionable whether JH III plays a role in task allocation in *M. eumenoides* colonies. This result was unexpected since a range of comparable studies on *A. mellifera* (e.g. Robinson 1985, Robinson *et al.* 1989, Sasagawa *et al.* 1989) had shown that the application of JH III and its analogue resulted in precocious behavioural development. However, there is a range of possible scenarios that offer explanations for the results obtained in this study of *M. eumenoides*.

Firstly, the most obvious explanation for the lack of observed behavioural changes may be that JH III does not play the key role in the age-related division of labour in *M. eumenoides* colonies. Most evidence from research in this field however indicates that a link between JH III and task allocation is present. Compellingly, several recent studies on *A. mellifera* have linked both high JH III titres and elevated brain levels of octopamine to foraging (e.g. Schulz *et al.* 2002). Both are assumed to interact to influence the onset of foraging, however octopamine is thought to act more proximally than JH III which appears to exert its influence over a comparatively long period of time (Schulz and Robinson 2001). It has even been suggested that JH III

may only act as a behavioural pacemaker, and is not required for foraging behaviour (Robinson and Vargo 1997, Sullivan *et al.* 2000).

The long-term effects of the applications could not be recorded due to the application-related premature death of the focus workers (section 3.4.2.). At concentrations of 50 pmol JH III, and 1.7 mmol methoprene, workers survived for an average of just over 45 days (roughly 40 days post first application). Previous investigations (not involving the application of hormones) had shown that in the mean individual workers started foraging around day 120 post eclosion (see section 3.2.6.). Thus it cannot be ruled out that the applications could have resulted in precocious behavioural development changes, which may have become observable several weeks later had some of the focus workers survived for longer periods of time.

Secondly, it is conceivable that the concentrations of the applied JH III were too low to have any effect on task allocation, as they were much lower than the actual determined haemolymph JH III titres. One has to also bear in mind that it is generally accepted that JH does not act in a concentration-dependant manner, but during discrete critical periods (Gäde *et al.* 1997), inferring that the actual JH titres are immaterial, and it is only important whether they are above or below a certain threshold level during these critical periods. In terms of task allocation this may mean that if JH III is present at a sufficiently high concentration during a critical period, the behavioural switch occurs, and if the concentration is below the threshold level then no switch occurs. Thus in light of the low concentrations applied, and the presumably rapid metabolism and breakdown of applied JH (e.g. JH I applied to *Bombyx mori* (Lepidoptera) has been shown to have a half-life of between six to ten hours (Gilbert and Schneiderman 1958)), it is plausible that the required threshold level was not reached during the critical periods, and thus no precocious behavioural development switch took place.

The same may apply to individuals topically applied with methoprene. The regular application of methoprene did not result in any observable behavioural development changes. This contradicts results obtained during studies of *A. mellifera (*Schultz *et al.* 2002) and the tropical wasp *Polybia occidentalis* (Hymenoptera) (O'Donnell and Jeanne 1993) where methoprene application was shown to induce precocious foraging. In the case of *A. mellifera* it is however well documented that the dose of methoprene required to consistently cause precocious foraging is 200 µg (e.g. Robinson 1985, Robinson *et al.* 1989, Withers *et al.* 1994); in this study only

0.55 µg methoprene was applied twice a week. It is thus possible that the concentration of methoprene applied was too low to effect the behavioural development of the *M. eumenoides* workers.

Thirdly, it has to be taken into account that it is not known how well the topical application of both JH III and methoprene mimicked the normal increase age- and task-related increase in hormone titre. Staal (1975) lists eight primary factors, each of which may play a decisive role in determining the resulting activity of a topically applied compound (e.g. receptor fit, resistance to enzyme degradation, feedback loops etc.). Hence the outcome of any topical JH and JH analogue application experiment is governed by numerous factors that are partially still unidentified, and may differ on an individual as well as species level. Additionally, the physiological changes that accompany the behavioural changes are to a great extent unknown; it is for example possible that the shift requires new neurons to be formed in the brain complex. Thus, despite the failure to induce behavioural development changes by JH III and JH analogue topical application in this study, one cannot rule out that JH III plays a perhaps even major role in task allocation.

### 4.5.4 Does JH III Play a Role in Signalling?

To my knowledge, this was the first time that JH III was investigated with regard to signal composition in a eusocial Hymenopteran. A strong correlation was shown to exist between the CHC profiles and JH III titres of *M. eumenoides* workers. Although it was not possible to determine the relationship in individual workers (too little haemolymph for individual JH III titre determination – see section 2.3.4.), this was demonstrated by the existence of brood-tender-type and forager-type CHC profiles, and the associated significantly different haemolymph JH III titres (of pooled samples) of the two task groups.

Numerous studies on *A. mellifera* workers have documented that the haemolymph JH III titre and the task performed are closely correlated (e.g. Robinson *et al.* 1989, Huang and Robinson 1995). Interestingly, research on *A. mellifera* has also indicated that age- and thus also task-related CHC profiles may also exist. Results obtained by Francis *et al.* (1989) indicate that adult workers possess the same qualitative blend of CHCs, but that nurses and foragers have quantitatively different CHC profiles. In another study, the percentage of saturated CHCs, as well

as short-chain CHCs, has been shown to increase dramatically in an apparently age-related fashion (Blomquist *et al.* 1980). This would in turn indicate the existence of a correlation between JH III titre and task-specific CHC profiles in *A. mellifera* similar to the one manifested for *M. eumenoides*. Thus these results suggest that in eusocial Hymenoptera JH III plays a role in signalling. Compellingly, JH has already been implicated in the endocrine regulation of pheromone production in some Blattodean (e.g. Schal *et al.* 1991) and Coleopteran (e.g. Tillman *et al.* 1998) species.

### 4.5.5. Exogenous JH III and Methoprene alters Signalling

The applications of JH III and its analogue methoprene were shown to result in a general, precocious shift of the CHC composition towards a forager-type profile. This is the first time that conclusive evidence has been presented showing that the CHC compositions, and thus the chemical "barcodes" in a eusocial Hymenopteran species, are governed by JH III. Furthermore, this CHC shift was shown to take place independent of the behavioural development of the individual workers.

In terms of time, the CHC profile alterations were noticable within the first week post application, however the first significant differences were only found between two to three weeks after the first application. This demonstrates that the changes take place in a continuous fashion, the effects intensify with increasing number of applications, and that even the first one or two applications already alter the CHC profile within a short period of time. This type of JH III-governed, rapidly induced effect has been noted before e.g. by Dickens *et al.* (2002) who showed that the topical application of JH III to male Colorado potatoe beetles (*Leptinotarsa decemlineata*; Coleoptera) increased the pheromone production eightfold within 24 hours of application.

In addition, these results demonstrate that in *M. eumenoides* methoprene acts in an identical fashion to the native hormone itself. This was anticipated since a number of studies have previously shown that methoprene mimics the actions of true JH III in *A. mellifera* (reviewed by Bloch *et al.* 2002). In addition, research on *Drosophila melanogaster* (Diptera) has even suggested that JH and methoprene may even induce the same molecular mechanisms needed to activate gene expression (e.g. Ashok *et al.* 1998, Restifo and Wilson 1998).

In-depth analysis of the CHC profile changes showed that only ten of the 51 CHC components constituted over 98 % of the variance between the reference CHC profiles (of acetone-applied workers in the brood-tending phase) and the precocious forager-type profiles (for both JH III and methoprene applied workers). Furthermore, these were shown to be essentially the same ten components that were demonstrated to summarise the differences between brood-tender-type and forager-type CHC profiles observed during the course of the normal (non-manipulated) behavioural development (see section 3.2.9.). This manifests that the exogenous hormone application induced changes mimic the normal CHC composition change with behavioural development that was demonstrated to take place over a much longer period of time.

Intriguingly, the behavioural observations showed that JH III and methoprene treated individuals displaying forager-type CHC profiles but carrying out brood-tending duties were removed from the nest by (brood-tending) nestmates. This is highly interesting as it shows that only the CHC profile, not the behaviour of individuals changed as a result of the applications, and this imbalance was noticeable to not just the human observer, but to the nestmates of the focus workers as well.

These results fit very well into the current state-of-the-art model where foragers of e.g. *A. mellifera* are characterised by both high haemolymph JH III titres and brain octopamine levels; it has been demonstrated that high JH III titres are not necessary for foraging behaviour (Sullivan *et al.* 2000), while octopamine has been shown to determine the induction of foraging in *A. mellifera* workers independent of JH III (Schulz *et al.* 2002). Furthermore it was demonstrated that octopamine does not have an effect on HC biosynthesis and distribution in the ant *Camponotus fellah* (Boulay *et al.* 2000). Thus JH III might serve a dual function by perhaps only pacing behavioural development (Sullivan *et al.* 2000), but playing the key role in the CHC composition (signalling) of workers.

No studies focusing upon the endocrine regulation of signalling (CHC composition) in eusocial Hymenoptera have as yet been carried out, however Tillman *et al.* (1999) stated that “rather than developing and dedicating an entirely unique set of enzymes for pheromone biosynthesis, insects appear to have evolved to add one or a few tissue-specific auxiliary or modified enzymes that transform the products of “normal” metabolism to pheromone compounds of high stereochemical and

quantitative specificity". The available evidence from research on biosynthetic pathways and endocrine regulation of pheromone production in model species of Blattodea and Coleoptera (reviewed by Tillman *et al.* 1999) allows the supposition that JH III directly influences the CHC profile composition in *M. eumenoides* workers. This hypothesis is supported by several observations: a) a strong correlation exists between signalling and JH III titres in *M. eumenoides*; the CHC composition of individual workers changes in a continuous fashion, with the so-called "reserves" having both CHC profiles and JH III titres that are intermediate between those of brood-tenders and foragers, b) the CHC profiles of the workers change rapidly after the application of JH III and methoprene, and c) the magnitude of the effects on the CHC profiles is dose-dependent. Further but indirect evidence is provided by Huang *et al.* (1994) who showed that the rates of JH III biosynthesis by the corpora allata (which are known to be highly correlated with the haemolymph JH III titre) increase continually with age in *A. mellifera* workers.

There are some indications that JH III probably alters the CHC profile via one of two mechanisms. Firstly, it is conceivable that JH III governs the HC synthesis processes, perhaps involving a receptor-mediated gene induction for key biosynthetic enzymes (Tillman *et al.* 1999). In female *Blattella germanica* (Blattodea) the *in vivo* synthesis of the hydrocarbon-derived ketone sex pheromone 3,11-dimethylnonacosan-2-one and its subsequent accumulation on the cuticle is highly correlated with the *in vitro* synthesis of JH III by the corpora allata (Schal *et al.* 1991). It has thus been suggested that the pheromone production is governed by the JH III-induced enzymatic conversion of the HC precursor to the oxygenated sex pheromone (e.g Schal *et al.* 1994, Schal *et al.* 2003)

In the pine engraver *Ips pini* (Coleoptera) it was shown that the *in vivo* incorporation of radiolabelled acetate into the aggregation pheromone ipsdienol increased with topical JH III application (Tillman *et al.* 1998). Subsequent studies revealed that the JH III most likely exerts its effect by increasing the mRNA transcription and/or transcription stability for the key reductase enzyme (HMG-CoA reductase). This enzyme catalyses the first step in the *de novo* mevalonate biosynthesis pathway. In male *Dendroctonus jeffreyi* (Coleoptera) topically applied JH III was shown to stimulate HMG-CoA reductase expression 30-fold in both a dose and time dependent manner (Tittiger *et al.* 2003). However, the exact mechanisms of

this have to date not been elucidated. Additionally JH III may to a lesser extent also effect HMG-CoA synthase, a possible regulatory enzyme which acts just upstream of HMG-CoA reductase in the mevalonate pathway (Tillman *et al.* 1999). Interestingly, the sesquiterpenoid JH III is itself a product of the mevalonate pathway (Seybold and Tittiger 2003).

In light of the fact that in *M. eumenoides* workers the transition from brood-tender-type to forager-type CHC profile composition involves only quantitative changes, it is thus possible that the HC precursor conversion rates, i.e. the activities of specific enzymes, are regulated by haemolymph JH III titres. Hence a model where the activities of enzymes responsible for the conversion of the HC precursor to long-chain HCs are promoted, and/or the activities of enzymes converting the HC precursor to short-chain HCs are inhibited by increasing JH III titers, is conceivable.

The second postulated regulatory mechanism is at the HC transport level. High density lipophorin (HDLp) has a specific, high-affinity JH III binding site in Hymenoptera and several other orders (Sevala *et al.* 1997). Thus one of its major functions is thought to be the facilitation of the transport of the hydrophobic JH III from the site of synthesis to target tissue(s), as well as protecting it from enzymatic degradation (Whitmore and Gilbert 1972). In addition, HDLp has been hypothesised to play a key role in the transportation of HCs from the site(s) of synthesis to the cuticular surface (reviewed in Schal *et al.* 1998b). In the termite *Zootermopsis nevadensis* (Isoptera) (Sevala *et al.* 1999, Sevala *et al.* 2000), nearly all HCs in the haemolymph were shown to be bound to the HDLp, while other proteins contained only trace amounts of HCs. Additionally, in *Reticulitermes flavipes* and *Reticulitermes lucifugus* (Isoptera) all haemolmph HCs were recently shown to associate exclusively with HDLp (Fan *et al.* 2004). In *Musca domestica* (Diptera) more then 90 % of the haemolymph HCs were shown to be bound to HDLp (Schal *et al.* 2001). In *Blattella germanica,* HDLp has been shown to be involved in HC and pheromone transportation to the cuticle (Gu *et al.* 1995), and it has additionally been suggested that in this species virtually all the circulating JH III must be bound to HDLp (Sevala *et al.* 1997). HDLp is generally considered a reusable shuttle that does not enter cells (e.g. Chino and Kitazawa 1981, Van Heusden *et al.* 1991). However, the uploading of HCs into HDLp is only poorly understood, and their downloading to e.g. the cuticle has not been studied in any insect (reviewed by Schal *et al.* 2003).

Importantly, HDLp has been shown to play a major role in the tissue specific deposition (unloading) of HCs in the tiger moth *Holomelina aurantiaca* (Lepidoptera) (Schal *et al.* 1998a). In this species short-chain volatile sex pheromone components and long-chain epicuticular HC are simultaneously synthesised by adult females, and both are loaded onto haemolymph HDLp. The sex pheromone components are then deposited in sex pheromone glands near the ovipositor, while the long-chain HC are shuttled to the cuticle and thus appear on it. A similar role for HDLp has been suggested for the house fly *Musca domestica* (Diptera) (Schal *et al.* 2001). Compellingly, a superficial analysis showed that the *M. eumenoides* haemolymph HCs were qualitatively highly similar to the CHCs. These results parallel the findings by Soroker *et al.* (1995, 1998), who noted an internal pool of HCs, similar to the CHCs, in workers of the ant species *Pachycondyla apicalis* (Hymenoptera). Moreover, it was recently demonstrated that in the ant *Pachycondyla villosa* (Hymenoptera) (Lucas *et al.* 2004) the HDLp-bound HCs are qualitatively and quantitatively similar to the CHCs.

Since in *M. eumenoides* individuals carrying out different tasks have altered relative proportions of the same CHC*s*, HDLp may play a major role in the regulation of HC externalisation. In light of the specific, high-affinity JH III binding site present on HDLp (e.g. Trowell 1992, Sevala *et al.* 1997), it is thus conceivable that JH III regulates the loading of HCs into HDLp, thus resulting in the task-specific HC profiles. Tentative evidence favouring this mechanism is provided by Lucas *et al.* (2004) who demonstrated that in *P. villosa* workers the HDLp-bound HCs are already qualitatively and quantitatively similar to the CHCs. Of course JH III might also govern the tissue-specific unloading (deposition) of HCs from HDLp, or even play a role in both processes. In addition, it cannot be ruled out that JH III-effected HDLp may rule post-depositional processes such as selective abrasion and selective reabsorption (internalisation) of HCs.

However these hypotheses are still only speculative, as to-date nothing is known about the possible mechanisms of how JH III might affect HDLp to bring about the changes. One possibility is that the JH III, upon binding, results in a conformational change of the HDLp. It is conceivable that the altered conformation in turn effects the affinity of the HDLp for specific HCs; thus at high JH III titres, perhaps long-chain, saturated components are taken up rather than short-chain, unsaturated components. Interestingly, at normal physiological titres of JH, only a fraction of the

high affinity JH binding sites in the haemolymph are occupied (e.g. Goodman and Gilbert 1978, Sevala *et al.* 1997). This suggests that there is “room for manoeuvre” and that rising JH III titres could be quickly translated into the appropriate CHC profile composition; this of course fits in well with results obtained in this study. In addition it is also plausible that JH III also affects the rate of HC transport. In sum however, there is merely speculative evidence that signalling in *M. eumenoides* workers is controlled by JH III-mediated HDLp transport of the CHCs, and only further research on this complex subject will determine whether this is really the case.

## 5. General Conclusion and Outlook

The primary aim of this study was to contribute to our knowledge of the regulation of age-related division of labour (task allocation) and signalling in *M. eumenoides* colonies. Since the results have shown that task and signalling are tightly linked, the results obtained in these investigations must not be viewed individually, but as pieces of a puzzle. In total, and including results obtained in numerous previous studies they allow a hypothesis of the control of the age-related division of labour in *M. eumenoides* colonies to be put forward. It is to some extent based upon the hypothesis advanced by Schulz *et al.* (2002) for the division of labour in *A. mellifera* colonies; workers are proposed to have different response thresholds to task-related stimuli.

Since ants and other social insects usually reside in dark, enclosed nest areas, the task-related stimuli are most likely of a tactile and olfactory nature (Schulz *et al.* 2002). Thus the semiochemicals involved need to have a high information content but be of a low volatility in order to minimise sensory habituation (Howard and Blomquist 1982); and the prime candidates for this are of course the CHCs. Recent direct behavioural evidence showed that when two nestmates encounter, their CHCs allow each other's task to be recognised and interpreted (Greene and Gordon 2003). In the current study it was shown that the tasks of individual *M. eumenoides* workers changed analogously to their CHC profiles, and these changes were strongly correlated with haemolymph JH III titres; this indicates the presence of a task-based recognition system perhaps mediated by JH III.

The primary use of such a recognition system is likely to be that it allows each worker to take a subsample (based on encounter rates) of how many workers are involved in the various tasks. Rates of brief antennal contact (involving the perception of CHCs) have been demonstrated to influence task decisions in the ant *Pogonomyrmex barbatus* (Gordon and Mehdiabadi 1999). In such a scenario, it is possible that that the "reserves" with their intermediate CHC profiles may communicate how many "spare" workers exist at that moment in time, or how many potential foragers are "in the pipeline". The outcome of this sampling procedure could then be translated into an endocrine signal, with JH III being the prime candidate for this. The brain-corpora allata complex has been hypothesised to convert internal and

environmental stimuli into endocrine signals (Wyatt and Davey 1996). It is likely that this step would be dependent upon, and thus regulated by, the threshold level of the individual worker to the relevant task-related stimulus (Beshers *et al.* 1999). Threshold levels are likely to be age-related, and/or dependent on e.g. patrilines (demonstrated in *A. mellifera*; e.g. Breed *et al.* 1990).

The next step, should the outcome of the sampling procedure have determined that e.g. the colony lacked foragers, would involve an increased rate of JH synthesis. Increasing JH titres are in turn likely to set off a cascade of events acting on HC synthesis and/or transport mechanisms (thus effecting signalling), and possibly brain octopamine levels (thus perhaps altering the behavioural development; Schulz *et al.* 2002). Differential threshold levels for these two target processes may exist. To complicate matters, a feedback mechanism may also be in place at this point; octopamine, thought to act as a neuromodulator, has been shown to alter response thresholds to key chemical stimuli in *A. mellifera* (Mercer and Menzel 1982) and in moths (Pophof 2000), and probably also adjusts the responsiveness to key olfactory stimuli that sets off the performance of certain tasks (Schulz and Robinson 2001).

In sum, this set-up would allow individual workers to assess the colony needs using CHCs as simple, localised cues, and to adjust their own behavioural development and signal composition accordingly. This would in sum result in a dynamic network that highly efficiently regulates the colony's behaviour (Greene and Gordon 2003) without any central or hierarchical control.

Although this type of scenario may constitute the backbone of the age-related division of labour in eusocial Hymenopterans, it is likely that other environmental and social cues also play a role in the "task decisions" made, along of course with the individual attributes of each worker. Thus further research is required to increase our knowledge of this highly fascinating and equally as complex subject. Although the possibilities of further work are sheer endless, particular emphasis should be placed upon three approaches which would help to clarify the overall picture.

Firstly, callows of *M. eumenoides* could be allatectomised by the surgical removal of the corpora allata, or the destruction of this organ using precocene (this has been shown to atrophy the corpora allata in *Solenopsis invicta* - Burns *et al.* 2002). The behavioural development and signalling patterns could then be monitored over the entire lifetime of the individuals. It would be expected that in the absence of

JH III workers will commence foraging later, and possibly produce less CHCs and/or keep the brood-tender-type CHC profile. Furthermore, JH III and/or methoprene could be applied to allatectomised callows, and again the behavioural development and signalling monitored over time; this should result in the removal of the effects of the allatectomy.

Secondly, foragers could be allatectomised and the impact of this monitored over time. If, as in *A. mellifera*, JH III only plays a role in pacing behavioural development but is not necessary for foraging (Sullivan *et al.* 2000), a so-called "task reversal" back to "reserve" or brood-tending behaviour should not take place. Additionally, it would be fascinating to monitor the effects of this allatectomy on signalling; would this result in alterations in the quantity and/or quality of CHCs?

Thirdly, octopamine could be applied to young brood-tending workers and the impacts of this monitored over time. It is anticipated that this would lead to a vast increased in the pace of behavioural development, and thus precocious "reserve" behaviour, as well as foraging (as shown for *A. mellifera* by Schulz and Robinson 2001). Of course the signalling should also be monitored during the course of this investigation.

## 6. Summary

The integrity of social insect colonies is facilitated by nestmate recognition. A defining characteristic of social insect colonies is their elaborate division of labour system. Cuticular hydrocarbons (CHCs) have long been implicated in nestmate recognition. In the African ant *Myrmicaria eumenoides* they also appear to vary with task. The factors regulating the task allocation (i.e. division of labour), as well as the CHC profiles (i.e. signalling) are still not well understood. Both external and internal (endocrine) factors have both been postulated in the past. Most of the evidence to-date suggests that juvenile hormones (JHs) and ecdysteroids are the prime endocrine control candidates.

The aim of this project is to investigate how signalling and task allocation are dynamically governed in *M. eumenoides* colonies. Firstly, the CHC profiles of individual workers in relation to their task (behavioural development phase) over their life span are assessed. Secondly, the influences of the two endocrinological factors (JHs, ecdysteroids) hypothesised to play a role in task allocation, as well as signalling, are investigated.

1) The behavioural transition from callow to brood-tender, and on to forager was shown to be continuous and to change in a synchronous manner in all individual *M. eumenoides* focus workers. In addition, all workers were shown to go through the "reserve" phase (characterised by a high degree of inactivity) before changing to foraging activities.

2) The CHC profiles of all individual workers were shown to change parallel to the behavioural development transition noted above. Overall, the CHC profile change is only quantitative, and characterised by a decrease in short-chain, and a simultaneous increase in long-chain components, as well as an increase in saturated, and a decrease in unsaturated CHCs.

3) Direct behavioural evidence, and the strong correlation of signalling with task suggests that in *M. eumenoides* the CHC profiles inform task decisions. Only five components were shown to contribute more then 90% to the variance between brood-tender-type and forager-type CHC profiles. This suggests that perhaps

"task signalling" requires only a few CHCs. These may be of a saturated and/or unsaturated nature, but must be of a high or medium high relative concentration.

4) The levels of ecdysone and 20-hydroxyecdysone (found to be the main free ecdysteroids in workers), as well as the sum of the total free ecdysteroids in workers, are only high after eclosion, and then fall to a low, steady level within two weeks. Thus no correlation was found between the above and signalling, or task.

5) Interestingly, JH I was found (only) in the haemolymph of female *M. eumenoides* alates: this is the first time this JH homologue has been identified in the Hymenoptera. It is hypothesised that JH I plays a role in either flight or migratory behaviour, and is thus absent from worker haemolymph.

6) Foragers were demonstrated to have significantly higher JH III titres than brood-tenders. Exogenous JH III and JH analogue application however did not result in an observable change in, or acceleration of, the behavioural development of workers. This may however be due to the much reduced longevity of the applied workers, and the hence decreased observational period.

7) The changes from a brood-tender-type to a forager-type CHC profile are accelerated in a concentration-dependent manner by the application of JH III, and JH analogue. This appears to take place independent of task changes, resulting in e.g. brood-tending workers that display clear forager-type CHC profiles. Thus these results demonstrate for the first time that signalling in Hymenoptera is strongly influenced by JH. Several possible pathways for the action of JH III are discussed.

8) Finally, it is suggested that workers assess the colony needs using CHCs as simple, localised cues. The CHCs would thus play a key role in efficiently regulating a colony's behaviour. Several promising approaches were also detailed with respect to further work on this topic.

## 7. Zusammenfassung

Die Erkennung von Nestgenossen ermöglicht sozialen Insekten, die Integrität einer Kolonie zu erhalten. Unter den Signalen für Kolonieerkennung spielen Profile kutikulärer Kohlenwasserstoffe (KKW) eine tragende Rolle. Bei der afrikanischen Ameisenart *Myrmicaria eumenoides* variieren diese KKW Profile zwischen Arbeiterinnen mit unterschiedlicher Tätigkeiten. Der physiologischen Hintergrund für diese chemische Änderung ist bisher ebensowenig bekannt wie die Verhaltensänderung der einzelnen Ameise. Es wurden hierfür externe als auch interne (endokrine) Faktoren postuliert. Auf der Basis bisheriger Untersuchungen ist es wahrscheinlich, daß bei den letzteren die Juvenoide und Ecdysteroide eine tragende Rolle spielen.

Ziel dieses Projektes ist es, zu untersuchen wie bei *M. eumenoides* Arbeitsteilung und Signalstoffe dynamisch geregelt werden. Dazu wird erstens die Modifizierung der KKW Profile mit der Tätigkeit bei individuellen Arbeiterinnen erfasst, und zweitens die endokrinen Faktoren (Juvenilhormone, Ecdysteroide) untersucht, die möglicherweise Verhaltensänderungen und Änderungen der KKW Profile bedingen.

1) Die Verhaltensveränderungen der frisch geschlüpften Ameisen erst zum Innendienst, und dann weiter zum Außendienst sind kontinuierlich und verändern sich gleichermaßen in allen Arbeiterinnen. Dieser Übergang involviert auch immer die sogenannte, durch Untätigkeit geprägte “reserve” Phase.

2) Die Arbeiterinnen besitzen nach dem Schlüpfen und während der anschließenden Tätigkeiten im Nest gleichartige und sich nicht verändernde KKW Profile. Diese verändern sich jedoch kontinuierlich Richtung Außendienstprofil parallel zur Aufnahme von Außendiensttätigkeiten. Zusammenfassend wurde gezeigt, daß nur quantitative Veränderungen der KKW stattfinden, und diese von einer Abnahme kurzkettiger, und einer simultanen Zunahme langkettiger KKWs, sowie eine Zunahme von gesättigten, sowie einer simultaner Abnahme von ungesättigten KKWs geprägt werden.

3) Die klare Korrelation von KKW Profilen mit der Tätigkeit als auch eine Reihe von Verhaltensbeobachtungen lassen darauf schließen, daß die KKW Profile der Arbeiterinnen möglicherweise Nestgenossen über deren Tätigkeit informieren ("task-signalling"). Die Varianz zwischen Innendienst-Typus und Außendienst-Typus des KKW Profil wird zu mehr als 90 % von nur fünf Komponenten getragen. Deshalb liegt die Vermutung nahe, daß dieses "task-signalling" nur auf einigen, wenigen Komponenten basiert. Diese können gesättigt und/oder ungesättigt sein, müssen aber in hoher, oder zumindest in mittlerer relativer Konzentration im KKW Profil vertreten sein.

4) Die Konzentrationen von Ecdyson und 20-Hydroxyecdyson sowie der Gesamtgehalt der freien Ecdysteroide sind nur nach dem Schlupf hoch. Bei allen Arbeiterinnen die älter als zwei Wochen sind, unterscheiden sich die Konzentrationen nicht. Es ergab sich somit keinerlei Zusammenhang zwischen den freien Ecdysteroiden und den KKW Profilen bzw. Tätigkeit.

5) In der Hämolymphe von weiblichen *M. eumenoides* Geschlechtstieren wurde JH I identifiziert. Dies ist der Erstnachweis dieser Substanz bei den Hymenopteren. Es wird postuliert daß JH I nur eine Rolle beim Fliegen oder in der Migration (bei Lepidopteren) spielt, und deshalb nicht in der Hämolymphe von Ameisen-arbeiterinnen vertreten sein sollte.

6) Außendiensttiere weisen einen höheren natürlichen JH III Hämolymphtiter als Innendiensttiere auf. Es konnten aber keine JH III oder Methopren applikationsbedingten Verhaltens- oder Tätigkeitsänderungen (bzw. Beschleunigung des Aufgabenwechsels) festgestellt werden. Allerdings besteht durchaus die Möglichkeit, daß durch die applikationsbedingte reduzierte Lebensdauer der Beobachtungszeitraum zu sehr eingrenzt wurde, und dadurch mögliche später auftretende Auswirkungen nicht mehr beobachtet werden konnten.

7) Die regelmäßige Applikation von JH III und Methopren, einem JH-Analog, resultierte in einer konzentrationsabhängigen, beschleunigten Veränderung des KKW Profils in Richtung Außendienstprofil. Da dies offensichtlich unabhängig von

Tätigkeitsänderungen stattfand, konnten z.B. Innendienstarbeiterinnen mit Außendienst-typischen KKW Profilen beobachtet werden. Diese Ergebnisse liefern die ersten Hinweise, daß JH bei den Hymenopteren kutikuläre Signalstoffe stark beinflusst. Verschiedene mögliche Pfade dieser wahrscheinlichen JH Steuerung werden postuliert.

8) Letztlich wird postuliert, daß die KKWs als tätigkeitsbedingte Stimuli agieren, und dadurch die Anzahl und der Bedarf an Arbeiterinnen innerhalb einer Kolonie besser eingeschätzt werden kann. Das würde bedeuten, daß die KKWs eine Hauptrolle bei der dynamischen Arbeitsteilung zwischen Nestgenossen spielen. Zuletzt wird eine Reihe von weiterführenden Untersuchungen beschrieben.

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# 9. Appendix

| | | brood-tenders | "reserves" | foragers |
|---|---|---|---|---|
| a) | median chem. dist. | 0.12 | 0.38 | 0.46 |
| | lower quartile | n.a. | 0.32 | 0.35 |
| | upper quartile | n.a. | 0.46 | 0.72 |
| | n | 3 | 6 | 7 |
| | mean age /category | 13 | 67 | 151.8 |
| b) | median chem. dist. | 0.12 | 0.35 | 0.43 |
| | lower quartile | n.a. | 0.34 | 0.42 |
| | upper quartile | n.a. | 0.44 | 0.54 |
| | n | 2 | 9 | 10 |
| | mean age/category | 15.5 | 81 | 196.3 |
| c) | median chem. dist. | 0.08 | 0.37 | 0.35 |
| | lower quartile | n.a. | 0.3 | 0.28 |
| | upper quartile | n.a. | 0.41 | 0.61 |
| | n | 2 | 9 | 6 |
| | mean age/category | 12 | 76.1 | 166 |
| d) | median chem. dist. | 0.15 | 0.29 | 0.34 |
| | lower quartile | n.a. | 0.22 | 0.29 |
| | upper quartile | n.a. | 0.48 | 0.36 |
| | n | 3 | 7 | 8 |
| | mean age/category | 12.7 | 79.7 | 174.2 |
| e) | median chem. dist. | 0.21 | 0.25 | 0.29 |
| | lower quartile | n.a. | 0.19 | 0.24 |
| | upper quartile | n.a. | 0.32 | 0.35 |
| | n | 2 | 8 | 8 |
| | mean age/category | 16.5 | 77.7 | 177.7 |

**Table a1** The median chemical distance values, quartiles as well as number of samples in each task category for each individual worker depicted in figure 8 (showing the CHC profile change in five workers over time).

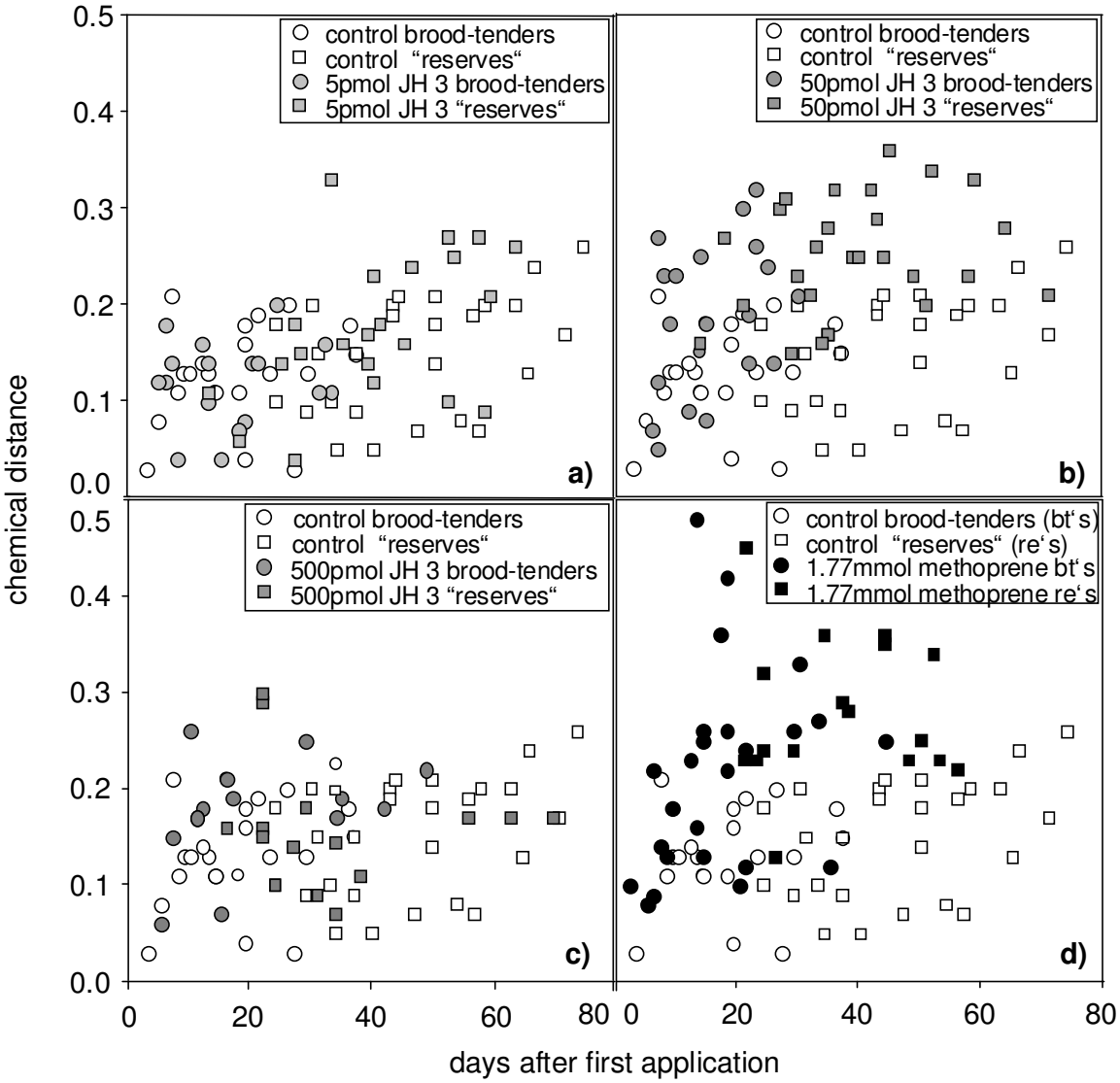


**Figure a2** A depiction of the complete data sets displaying the effects of the application of 5, 50 and 500 pmol JH III, as well as 1.7 mmol methoprene on the CHC profiles of individuals in comparison to the controls (acetone) group. Each individual in each application group (controls = 6, 5 pmol JH III = 5, 50 pmol JH III = 8, 500 pmol JH III = 5, 1.7 mmol methoprene = 8) is represented by several CHC profiles in the diagram. The chemical distance is the euclidean distance to the first CHC (reference) profile taken from each individual before the applications commenced.

# Erklärung

Hiermit erkläre ich, daß ich diese Arbeit selbständig verfaßt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt habe.

Ferner erkläre ich, daß ich weder an der Universität Bayreuth, noch anderweitig versucht habe, eine Dissertation einzureichen oder mich der Doktorprüfung zu unterziehen.

Bayreuth, im Oktober 2004