Caste differentiation in lower termites

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To my family

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General Introduction

Termites, like all social insects, are characterized by the key elements of eusociality: overlap of two or more generations, care of brood by older generations and by a reproductive division of labor (Wilson 1971). Especially the latter is regarded as the hallmark of insect societies and predicates that within the colony only a few individuals reproduce while the vast majority foregoes own reproduction (Crozier and Pamilo 1996). This reproductive skew riddles researchers ever since. The occurrence of sterile castes seems to contradict Darwin's theory of natural selection where each individual should be selected to behave in such a way that maximizes its number of offspring (Darwin 1859). Nowadays, reproductive altruism is explained by Hamilton's inclusive fitness theory (Hamilton 1964) also termed kin selection theory (Maynard Smith 1964). Accordingly, altruistic behavior can be favored by evolution if the relatedness between donor (altruist) and beneficiary (recipient of the help) ranks higher than the ratio of costs to benefits. This means helping close relatives to maximize their reproductive success (gain of indirect fitness = benefits due to social action) countervails the loss of own reproduction (loss of direct fitness = costs due to loss of own offspring).

With the appearance of reproductive division of labor, a pronounced variety of morphological diverse castes has evolved in eusocial insects (Oster and Wilson 1978). Following kin selection theory this morphological variety is an example of phenotypic plasticity which is based on differential gene expression among individuals, whereby an identical genome produces alternate forms of morphology, physiology and behavior in response to environmental conditions (West-Eberhard 1998; Evans and Wheeler 1999; West-Eberhard 2005). These evolved adaptations in which a genome produces discrete phenotypes is called polyphenism (Evans and Wheeler 2001a; Nijhout 2003; Suzuki and Nijhout 2008). Social insects present one of the most striking examples of phenotypic plasticity in the form of their castes (Wilson 1971; West-Eberhard 1998; Evans and Wheeler 2001a). For example, most higher eusocial insects exhibit morphologically distinct reproducing and non-reproducing individuals, with reproductives differing from infertile workers for instance by size and ovarian development (Wilson 1971; Oster and Wilson 1978; Thorne and Traniello 2003). By contrast, in more simple societies (some primitive wasps and bees) individuals specialize as either workers or reproductives too, but the queen-worker dimorphism is less pronounced than in advanced eusocial species. Hence

castes differ mainly in social tasks and therefore are supposed to be the ancestral state of morphological castes (Wilson 1971; Oster and Wilson 1978). Termites reveal both types of this queen-worker dimorphism. While physogastric queens of higher termites (Termitidae e.g. Macrotermes and Odontotermes) demonstrate one of the most prominent examples of this dimorphism, neotenic queens (female replacement reproductives) of lower termites reveal only slight morphological differences to workers (Figure 1). Precisely the fact that neotenics differ from workers almost exclusively by traits linked to reproduction makes them an ideal subject to study differential gene expression in regard to reproductive division of labor.

Figure 1

Queen-worker dimorphism in termites. Left picture: Physiogastric queen of *Macrotermes herus* (© M. Leponce) with enlarged abdomen. Right picture: Neotenic queen (replacement reproductive; darker individual) of *Cryptotermes secundus*. Queens are shown in the center.

Lower termites

Amongst termites, drywood termites (Kalotermitidae) are especially suited to uncover the influence of molecular mechanisms controlling reproductive division of labor. Belonging to the lower termites, the Kalotermitidae take up a central position as they are the second diverging lineage within the termites (Legendre et al. 2008). Termites are a sister taxon to the subsocial woodroach genus *Cryptocercus* [Scudder] (Inward et al. 2007a; Legendre et al. 2008). The similarities between woodroaches and lower termites, such as feeding and nesting in decaying wood, transferring similar intestinal symbionts by proctodeal trophalaxis, living in overlapping generations and relying on biparental care (in termites only during colony foundation) provides evidence that eusociality in termites evolved in

dead trees (Thorne BL 1997; Korb 2007a; Klass et al. 2008). The group of lower termites, which do not forage because their wooden nest serves both as food source and shelter, is called “one-piece life type termites” (Abe 1987) or more recently “wood-dwellers” (Korb 2007a; Korb and Hartfelder 2008) and is regarded as the ancestral life type in termites (Thorne BL 1997; Korb 2007a; Korb and Hartfelder 2008; Legendre et al. 2008).

Beside its phylogenetic position, the high developmental flexibility of their worker caste is another characteristic of wood-dwellers. Here, so called false workers (formerly known as pseudergates, workers or helpers (Korb and Hartfelder 2008)) are large immatures that are temporarily arrested in their development (late larvae and nymphs) and develop from totipotent eggs (Noirot and Pasteels 1987; Roisin 2000; Korb 2007a). By molting either progressively, stationary or regressively they have the possibility to remain false workers, to develop into sterile soldiers or to follow one of the two possible pathways to become a reproductive. Taking the first one allows them to reproduce by developing into dispersing alates (winged sexuals) which found new colonies as primary reproductives. The second option circumvents the risky dispersal flight (Nutting 1969) and offers the possibility to potentially inherit the natal nest as a neotenic replacement reproductive if the same-sex reproductive of the colony dies or becomes unhealthy (Roisin 2000; Korb and Katrantzis 2004). As mentioned above neotenic reproductives almost look alike false workers. Unlike alates which express several traits for their dispersal flight, such as compound eyes, a dense sclerotization and wings, neotenics almost maintain their larval appearance (Weesner 1969). Requiring only a single molt, neotenics just exhibit a few imaginal characteristics including for instance functional gonads and a slight sclerotization of their cuticula which allows distinguishing them from other nestmates (Roisin 2000) (see Figure1).

The number of neotenics varies between and within species of lower termites. In Mastotermitidae, Termopsidae and Rhinothermitdae this number can range from a pair to well over 150 neotenics per colony (Lenz 1994). However, only one pair of neotenics can be found in colonies of Kalotermitidae. This is due to a complex regulative process, which can proceed in differing modes. In the drywood termite *Cryptotermes secundus* [Hill] for example, a dead reproductive is generally replaced by a single false worker of the same sex that develops into a neotenic. By contrast, once colonies of the smallest known drywood termite *Cryptotermes cynocephalus* [Light] lose their reproductives, an excess of false workers molt into neotenics. The surplus, however, is eliminated during fights for the breeding position, leaving only one pair of reproductives (Lenz 1994).

Study subject

During recent years the drywood termite *Cryptotermes secundus* [Hill] was subject to intensive investigations of ultimate causes influencing caste development in lower termites. *C. secundus* belongs to the "wood-dwellers" (Korb 2007a; Korb and Hartfelder 2008) which spend their entire life in a single piece of wood. Termites of this life type do not forage outside the nest as they are living in their food. As a consequence, the colony dies when this bonanza type food source is depleted (Abe 1987; Korb 2007a). *C. secundus* is, like most members of the genus *Cryptotermes* [Banks], tropical in origin and occurs in Northern Australia in the region of Darwin (Northern Territory) where it inhabits indigenous mangrove trees like *Ceriops tagal* [Perr.] or *Avicennia marina* [Forsk.] (Miller and Paton 1983) (see Figure 2).

Figure 2

Darwin Mangrove. Natural habitat and collecting site of *Cryptotermes secundus*.

Colonies are established by pairs of alates (king and queen) which, after a nuptial flight, tandem running and dealation (breaking of their wings), seal themselves off in a suitable crack or crevice of a dead tree. The king stays with the colony over his entire life and periodically mates with the queen (Wilson 1971). Mature monogamous colonies of *C. secundus* consist of up to 400 individuals with false workers representing about 95% of all

colony members (Korb 2007b). It is assumed that false workers do not gain indirect fitness by taking care of the brood but rather the chance to inherit the natal nest as a neotenic reproductive is the decisive factor to stay (Korb 2007b). Also the number of young offspring present at the nest (Korb 2007b) does not seem to have an influence to abandon the clear safety advantages offered by the natal nest (Roisin 1999). Furthermore, the strategy to disperse and found a new colony is risky and rarely crowned with success (Nutting 1969; Nalepa and Jones 1991). The probability to found a new colony equals the chance to inherit the natal colony, with less than a 1% chance of success for any of the two possible pathways (Korb and Schneider 2007).

The wooden nest is the limiting resource and several investigations have shown that reduced food availability is one of the driving forces that induces false workers to choose the dispersal strategy (Korb and Lenz 2004; Korb and Schmidinger 2004). Thereby termites are able to assess wood size by using vibroacustic signals in order to ascertain the remaining food source (Evans et al. 2005). Additional factors influencing the decision to leave the natal nest are colony size (Lenz 1994; Korb and Lenz 2004; Korb and Schneider 2007) or high parasite pressure (Korb and Fuchs 2006). A strong genetic effect on caste determination, as recently described for the dampwood termite *Reticulitermes speratus* [Kolbe] (Hayashi et al. 2007), might be widespread throughout the termites but cannot be universal (Crozier and Schlüns 2008). Obviously it is not transferable to *C. secundus* or all other wood-dwellers and thus environmental control will be main mechanism regulating caste development. To coordinate the highly structured life in complex insect societies, nearly all activities are influenced by interactions with nestmates. This social regulation influences all areas of insects' life and is often mediated by chemical communication.

Fertility signaling

An unsolved enigma in lower termites and many other social insects is the mechanism that prevents totipotent colony members from reproducing when the queen is present. Unlike in social hymenoptera, where sex determination is based on relatedness asymmetries caused by haplodiploidy, workers gain indirect fitness by raising offspring (Wilson 1971), diploid false workers of *C. secundus* do not care for their brood at all (Korb 2007b). All the more astonishing seems the self-restrain of false workers as long as the queen is present and healthy. It was therefore suggested that the termite queen maintains her reproductive primacy by releasing chemicals which result in a reversible endocrine inhibition of the sexual development in false workers (Lüscher 1974; Brent et al. 2005), but so far it is not

proven whether these chemical cues are spread throughout the termite colony via proctodeal trophallaxis or olfactory (Bordereau 1985; Noirot 1990; Korb 2005).

In insect societies chemical communication plays an essential role for social organization. Cuticular hydrocarbons (CHCs) take a leading part in social interactions. They are involved in the recognition of nestmates (Lahav et al. 1999), task-specific differences (Kaib et al. 2000), and fertility (Peeters et al. 1999). Hereby the composition of CHC profiles varies within colony members in the quality, quantity and relative proportions of substances.

The ability of termites to identify nestmates according to their CHC profile is well known (Howard et al. 1982; Haverty et al. 1996; Bagneres et al. 1998; Dronnet et al. 2006). Furthermore caste-specific variations in CHCs have been described, but the chemical profile of fertile queens was not subject to investigation yet. However, the knowledge of characteristic CHCs which indicate the queen's fertility and health might help to explain the foregoing of worker reproduction as shown for other social insects (Heinze et al. 2002; Sledge et al. 2004; Sramkova et al. 2008).

In the honey bee it was shown that pheromones of the queen mediate gene expression in the worker brain (Grozinger et al 2003) and inhibit worker ovary development (Hoover et al 2003). In termites, however, the molecular basis underlying reproductive division of labor is unknown.

Genomics

Genomic analysis enables the identification of entire sets of genes associated with a specific biological process. Insect genomics experienced a boost on the basis of more and more sequenced genomes during recent years. In social insects the now available whole genome of the honeybee *Apis mellifera* [Linnaeus] (The Honeybee Genome Sequencing Consortium 2006) plus the increasing number of cDNA libraries (Wang et al. 2007; Hoffman and Goodisman 2007) and the use of high-throughput sequencing (Toth et al. 2007) in other social insects, allows the identification of genes involved in the evolution of insect societies. In this regard, most insights were obtained from research on social Hymenoptera. Especially, the honeybee has emerged as one model organism for using genomics to study insect sociality (Evans and Wheeler 1999; Robinson 1999; Robinson et al. 2005; Wheeler et al. 2006; Amdam et al. 2006; Hunt et al. 2007; Toth and Robinson 2007; Page, Jr. and Amdam 2007). Nevertheless, as observed in other sequencing projects

previously, preliminary results disclose limitations of translating the genomic data into biological function.

Besides the possibility of genetic manipulation (e.g. gene silencing), interspecific comparisons might aid a functional evaluation of candidate genes. It is expected that molecular functions of many genes which regulate development are highly conserved across different species (Robinson et al. 2005; Toth and Robinson 2007). Due to their distinct heritage and their deviating biology, termites are an ideal model system for genomic analyses and offer a unique opportunity to take a deeper look inside evolutionary relationships by identifying both common and species-specific pathways across distantly related taxa. Termites have a rich history as a study subject (Krishna and Weesner 1969; Lüscher 1974; Noirot and Pasteels 1987; Myles TG 1988; Thorne BL 1997; Shellman-Reeve 1997; Roisin 2000; Abe et al. 2000; Thorne and Traniello 2003; Roisin 2006; Korb 2007a; Korb 2008) and are now receiving increased attention as a model to study the molecular building blocks of caste development. Termite false workers are totipotent immature stages and have the genetic information for all castes present in their genome. During caste differentiation genes are expressed in a caste specific manner. Termite research mainly concentrated on the development of soldiers (Miura et al. 1999; Miura and Matsumoto 2000; Scharf et al. 2003; Hojo et al. 2005; Koshikawa et al. 2005; Scharf et al. 2005a; Scharf et al. 2005b; Cornette et al. 2006; Zhou et al. 2006a; Zhou et al. 2006c). Termite soldiers, however, are an exclusive caste with no equivalent in other social insects (Noirot and Pasteels 1987; Noirot 1990), hence they are not suited for comparative studies. Reproductives, on the other hand, are studied throughout eusocial taxa due to their important rank within the colony, which makes them most suitable for this kind of research.

Aim of this thesis

Whereas social Hymenoptera were investigated intensively concerning the evolution of eusociality in molecular terms, our knowledge of termites is in the fledgling stages in this regard. Especially the proximate underpinnings of known ultimate factors are largely missing. I was therefore interested to investigate the proximate mechanisms underlying a key transition in insects' evolution of social life – the reproductive division of labor. The first two publications deal with the identification of genes involved in this important characteristic of insects' sociality. The conceptual design of the third publication was targeted to shed light on the long-lasting question about the chemical mechanisms involved in reproductive inhibition of lower termites. Therefore I analyzed cuticular hydrocarbon profiles in correlation to the reproductive status of individuals.

Publication 1

Molecular basis for the reproductive division of labour in a lower termite

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Neotenic queen and false worker of *C. secundus*

Publication 1

Tobias Weil, Michael Rehli and Judith Korb

2007

Research Article

Molecular basis for the reproductive division of labour in a lower termite

BMC Genomics 2007, 8:198-206

Background: Polyphenism, the expression of different phenotypes with the same genetic background, is well known for social insects. The substantial physiological and morphological differences among the castes generally are the result of differential gene expression. In lower termites, workers are developmentally flexible to become neotenic replacement reproductives via a single moult after the death of the founding reproductives. Thus, both castes (neotenics and workers) are expected to differ mainly in the expression of genes linked to reproductive division of labour, which constitutes the fundamental basis of insect societies.

Results: Representational difference analysis of cDNAs was used to study differential gene expression between neotenics and workers in the drywood termite *Cryptotermes secundus* (Kalotermitidae). We identified and at least partially cloned five novel genes that were highly expressed in female neotenics. Quantitative real-time PCR analysis of all five genes in different castes (neotenics, founding reproductives, winged sexuals and workers of both sexes) confirmed the differential expression patterns. In addition, the relative expression of these genes was determined in three body parts of female neotenics (head, thorax, and abdomen) using quantitative real-time PCR.

Conclusion: The identified genes could be involved in the control and regulation of reproductive division of labour. Interestingly, this study revealed an expression pattern partly similar to social Hymenoptera indicating both common and species-specific regulatory mechanisms in hemimetabolous and holometabolous social insects.

Background

Social insects (termites and social Hymenoptera, such as ants, some bees, and wasps) are the exemplars of social life. They are characterized by a reproductive division of labour in which only a few individuals within a colony reproduce (queen/s, and king/s in termites), while the large majority helps in raising offspring (workers, in termites additionally soldiers). This caste system is a result of phenotypic plasticity; i.e. different castes generally arise from environmentally induced differential gene expression (Hamilton 1964; Crozier and Pamilo 1996; Evans and Wheeler 1999).

In termites, caste polymorphism is the result of a highly flexible postembryonic development which is especially pronounced in wood-nesting species. Here, workers (sometimes also called pseudergates, false workers or helpers due to their flexible development Roisin 2000) develop from totipotent eggs and have the possibility (i) to become winged sexuals (alates) that disperse from the nest and found their own colony as primary reproductives; (ii) to reproduce in the natal nest as neotenic replacement reproductives when the same-sex reproductive of the colony dies, or (iii) to develop into sterile soldiers that defend the colony (Figure 1). The development into each of these castes requires different numbers of moults; several for alates, one for neotenics and two for soldiers. As an alternative, individuals can remain as workers in the nest by moulting stationarily (moulting without change of the external morphology) or regressively (returning to morphological characters of an earlier instar). Research in termites so far concentrated on the development of soldiers (Miura et al. 1999; Scharf et al. 2005a; Cornette et al. 2006; Zhou et al. 2006a; Zhou et al. 2006c). Termite soldiers are a unique caste with no equivalent in other social insects (Noirot and Pasteels 1987; Noirot 1990). A comparison of differential gene expression between reproductives and workers may, however, allow the identification of common principles and differences in the regulation of reproductive division of labour between social insect taxa.

We specifically addressed the question of what characterizes a queen by comparing gene expression profiles between workers and female reproductives in the drywood termite *Cryptotermes secundus*. In termites, neotenic replacement reproductives are especially suited for this purpose because they differ from workers only by traits linked to reproduction, while confounding traits that are developed by winged sexuals for the dispersal process (e.g. compound eyes, wings) are not expressed. Our analysis revealed a number of interesting genes that are primarily expressed in neotenic replacement

reproductives and may be involved in processes controlling or maintaining the reproductive division of labour.

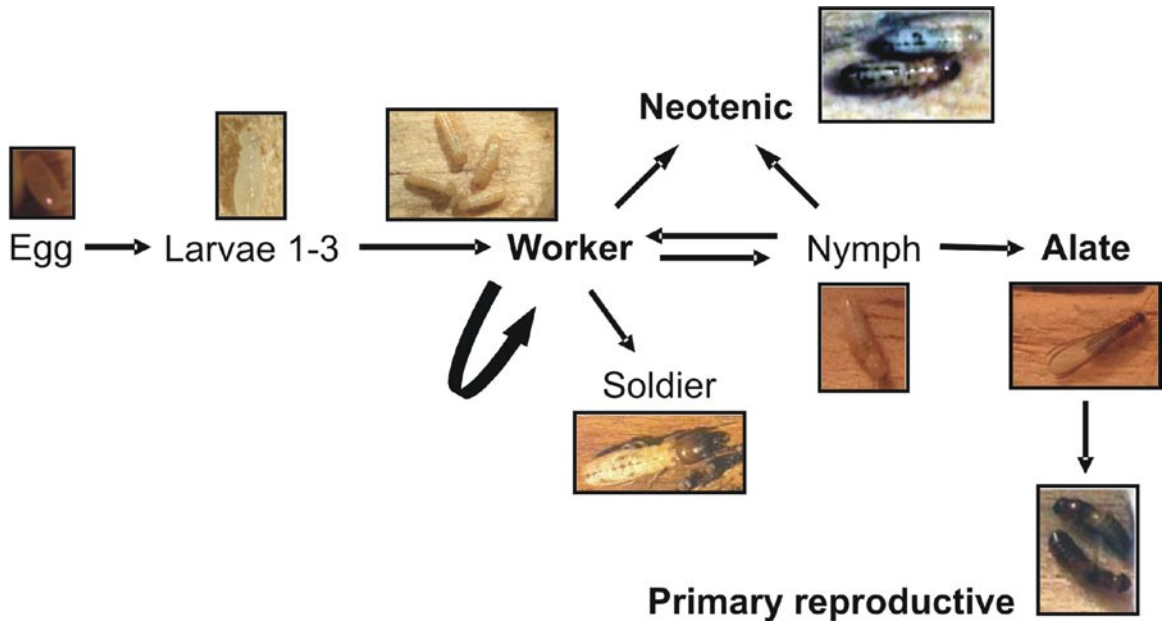

Figure 1

Developmental Pathways of *Cryptotermes secundus*. Caste differentiation in lower termites reflects larval polyphenism as reproductives are the only adults. Workers which develop from totipotent eggs have the potential (i) to remain workers by moulting stationarily (moulting without change of the external morphology) or regressively (returning to morphological characters of an earlier instar), (ii) to develop into sterile soldiers that defend the colony, (iii) to become alates that disperse from the nest and found an own colony as primary reproductives, or (iv) to reproduce in the natal as neotenic replacement reproductives when the same-sex reproductive of the colony dies. The development from a worker into a neotenic requires only a single moult. Bold letters indicate castes used for analysis.

Results

Identification of caste-specific transcripts in female neotenics

The limited publicly available information on genome or cDNA sequences of the drywood termite *Cryptotermes secundus* (Kalotermitidae) restricts the number of possible screening techniques for differential gene expression analysis. We chose to compare termite castes using the representational difference analyses of cDNA (cDNA-RDA) approach because it

is independent of sequence knowledge and requires relatively small amounts of mRNA. To identify genes that are specifically expressed in female neotenics, we initially performed a cDNA-RDA using female neotenics as tester cDNA and workers of both sexes as driver cDNA. The difference product of the third round was shotgun cloned and 187 randomly picked clones were validated using reverse dot blot hybridization with labelled tester and driver cDNAs. A representative dot blot hybridisation of representational difference products is shown in Figure 2.

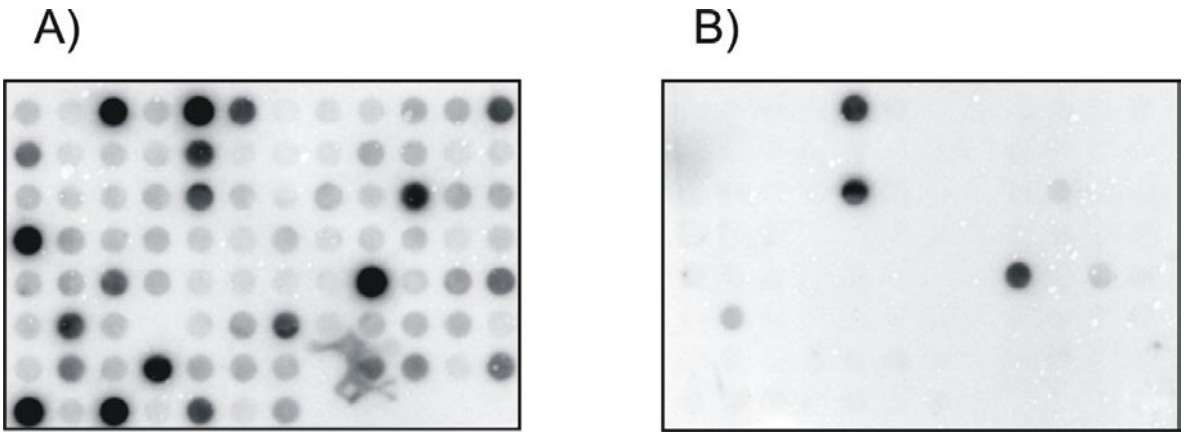

Figure 2

Reverse Dot Blot of cloned RDA fragments. A representative reverse dot blot of the RDA difference products is shown. Cloned inserts of randomly picked clones were PCR-amplified, denatured and dot-blotted onto two duplicate nylon transfer membranes and hybridized to radioactively labelled female neotenic (A) and worker (B) representations.

Thirty five out of 38 sequenced fragments with highly specific signals in reverse dot blot hybridization were derived from termites and most likely belonged to three independent genes that were named *Neofem1 – Neofem3*. To identify additional fragments we performed a second RDA where we suppressed the seven initially identified, highly overrepresented fragments by adding them in excess to the driver population. Additional 192 randomly selected clones were picked and analysed as above. Sequencing of 52 clones revealed 8 novel fragments including five fragments that most likely belonged to three different genes (*Neofem4*, *Neofem5* and a putative transferrin homolog). Seven sequences were most likely of non-termite origin. Mapping of individual fragments was done by a series of inter-fragment PCRs and by 3'- and 5'-RACE-PCRs, confirming the initial

assignment of the identified fragments to six genes. Complete transcripts were obtained for two genes (*Neofem1 and Neofem2*), partial 3'- or 5'-sequences were obtained for all other genes. Overrepresentation of the putative transferrin cDNA ([GeneBank: EF029058]) could not be validated by quantitative real-time PCR (qRT-PCR, data not shown). Physical maps of the remaining five genes indicating the location and number of cloned RDA fragments are shown in Figure 3.

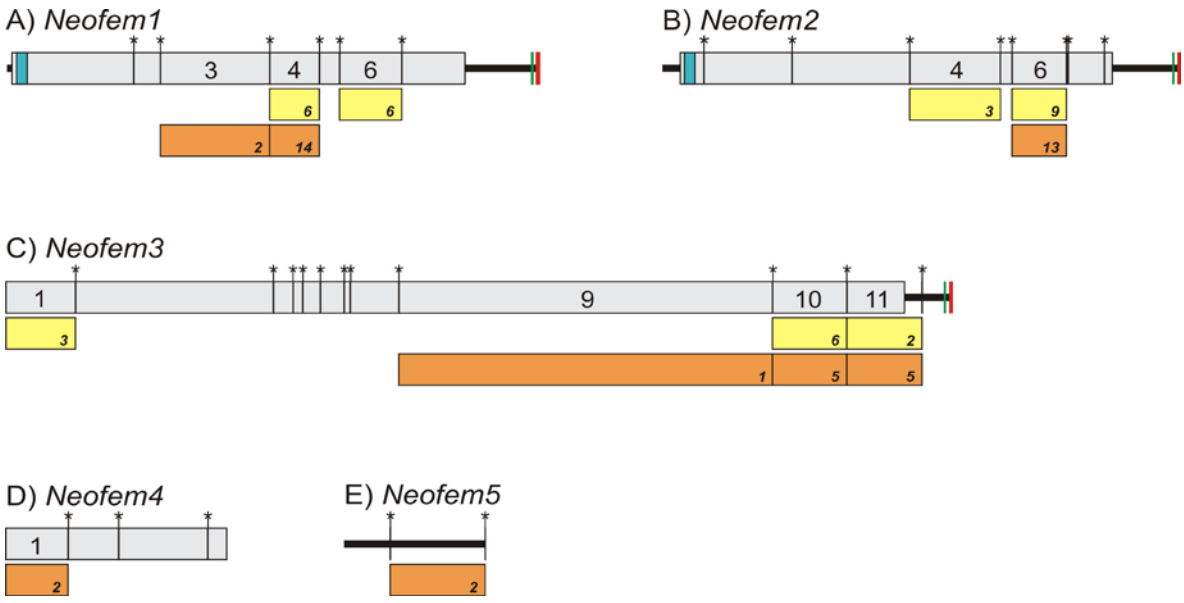

Figure 3

Physical map of *Neofem* genes. Physical maps depicting Neofem 1-5 genes (A-E) and the isolated RDA fragments; bold line: cDNA strand; grey bar: open reading frame; numbers; *Dpn* II fragment; yellow bar: *Dpn* II fragment obtained from the first RDA; orange bar: *Dpn* II fragment obtained from the second RDA; turquoise bar: signal peptide; green bar: poly-adenylation-signal; red bar: poly-A-tail; asterisk: *Dpn* II restriction site; italic numbers: number of individual sequenced fragments.

The *Neofem1* gene encodes a putative polypeptide of 558 amino acids. Its N-terminus comprises a signal peptide suggesting that the *Neofem1* gene product is secreted. A comparative sequence analysis using the BLAST-X algorithm suggests similarity to genes of the esterase-lipase family, in particular to genes of *Tribolium castaneum* and *Apis mellifera* that are similar to an uncharacterized *Drosophila* gene ortholog. The putative 532 amino acid gene product of the *Neofem2* gene also contained a signal peptide. Similarity

Table 1

Gene	Size (bp)	No. of clones	Identity match by BLASTX [species]	Accession no.	Local identity (%)	Score (bits)	*e*-value
Neofem1	1970	28	PREDICTED: similar to CG4382-PA [Tribolium castaneum]	XP_974072	47	520	1E-145
			PREDICTED: similar to CG4382-PA [Apis mellifera]	XP_393293	46	493	1E-137
			juvenile hormone esterase [Aedes aegypti]	EAT39446	47	485	3E-135
Neofem2	1918	25	beta-glucosidase [Neotermes koshunensis]	BAB91145	50	510	8E-143
			male-specific beta-glycosidase [Leucophaea maderae]	AAL40863	48	504	7E-141
			PREDICTED: similar to CG9701-PA [Tribolium castaneum]	XP_972437	48	475	3E-132
Neofem3	3502	22	Vitellogenin 1 precursor (Vg-1) [Periplaneta americana]	Q9U8MO	32	592	3E-167
			Vitellogenin [Athalia rosae]	BAA22791	29	512	4E-143
			Vitellogenin 2 precursor (Vg-2) [Periplaneta americana]	Q9BPS0	28	449	5E-124
Neofem4	817	2	family 4 Cytochrome P450 [Coptotermes acinaciformis]	AAC03111	46	270	7E-71
			Cytochrome P450 4C1 (CYPIVC1) [Blaberus discoidalis]	P29981	38	217	4E-55
			Cytochrome P450 [Aedes aegypti]	EAT35570	40	550	9E-55
Neofem5	525	2	PREDICTED: similar to guanylate cyclase OlGC-R2 [Danio rerio]	XP_688499	52	32,2	8,70

Table 1 (previous page)

Description: BLASTX results that show best hits against the non-redundant NCBI database, including species in [], GenBank accession number, local identity (%), score (bits) and E-score value. Further the sequence length (*Neofem1* and *Neofem2* = full length cDNAs; *Neofem3 – Neofem5* = partial cDNAs) and the number of cDNA clones from RDA are shown.

searches identified homologies of the *Neofem2* gene product to members of the glycosyl hydrolase family 1, in particular for a beta-glucosidase gene of the termite *Neotermes koshuensis* (Tokuda et al. 2002) and a male-specific β-glycosidase of the Madeira cockroach *Leucophaea maderae* (Cornette et al. 2003). The partial sequence of *Neofem3* showed the highest sequence similarity to the Vitellogenin 1 precursor (Vg-1) sequence of the American cockroach *Periplaneta americana* (Tufail et al. 2000) which serves as a precursor of egg-yolk proteins. The putative *Neofem4* gene product is closely related to family 4 cytochrome P450 enzymes (CYP4) from arthropods, with highest similarities to CYP4U1 from the Australian termite *Coptotermes acinaciformis* [GenBank:AAC03111] and to CYP4C1 of *Blaberus discoidalis* (Lu et al. 1999). No homologies were found for the *Neofem5* gene fragment. Table 1 summarizes the sequence analysis of all these genes. Complete nucleotide sequences were submitted to GenBank [GenBank:EF029054 - EF029059].

Quantitative expression analysis of the Neofem1-5 genes

To validate and further analyse the expression of *Neofem*1-5 genes, we performed qRT-PCR using RNA-samples derived from different termite castes (neotenics, primary reproductives, winged sexuals and workers of both sexes). To be able to normalize the expression data, we initially cloned gene fragments of putative house keeping genes (*18S rRNA*, *β-actin* and *hexamerin*) and designed primers for qRT-PCR. The suitability of putative reference genes was evaluated by using the *BestKeeper* software (Pfaffl et al. 2004). The comparison revealed that *18S rRNA* was the most stable reference gene ($N = 21$, $r^2 = 0.60$, $P = 0.001$). Figure 4 shows relative expression levels of the five *Neofem* genes that met the selection criteria of the initial RDA – their expression was generally much higher in female neotenics as compared with workers. In line with the order of appearance and the fragment abundance in the initial RDAs, expression levels of the *Neofem1 – Neofem3* genes in female neotenics were up to four orders of magnitude higher

in female neotenics than in workers. As expected, the difference in gene expression was less pronounced in the two genes identified exclusively in the second RDA (*Neofem4* – *Neofem5*). While *Neofem1* (homologous to esterase-lipase) and *Neofem2* (homologous to glycosidase) genes were almost exclusively expressed in female reproductives (neotenics and primaries), the gene homologous to vitellogenin (*Neofem3*) was expressed in all reproductives. Expression of the P450 homolog (*Neofem4*) was highest in female neotenics. The unknown transcript (Neofem5) was detected in all castes and showed highest expression in females.

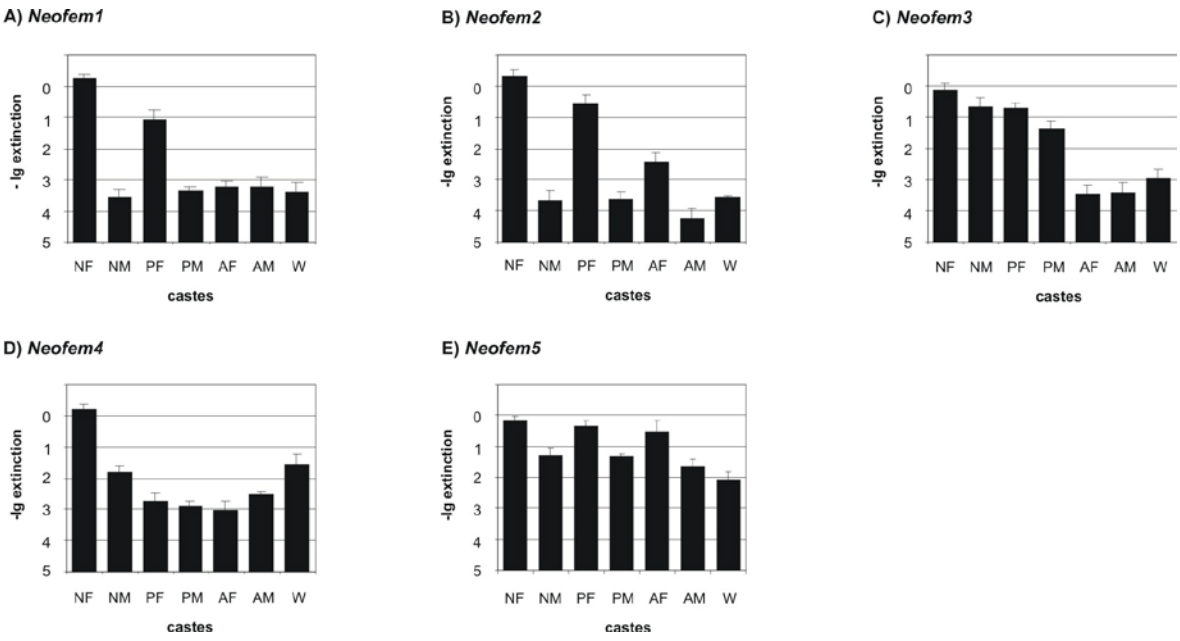

Figure 4

Quantification of *Neofem* genes in different castes. Relative expression levels of *Neofem* 1-5 genes (A – E) in different castes measured by qRT-PCR. The Y-axis is on negative $\log_{10}$ scale indicating the gene expression levels and the calculated errors (SD), for female neotenics (NF), male neotenics (NM), female primary reproductives (PF), male primary reproductives (PM), female alates (AF), male alates (AM) and workers (W) of both sexes.

To determine, where the five neotenic-specific genes are expressed in female neotenics, total RNA was prepared from different body parts (head / caput, thorax, and abdomen) and was analysed by qRT-PCR. As shown in Figure 5, four of the five genes were expressed primarily in the termite head. The gene *Neofem3* was detected almost equally in all three body parts.

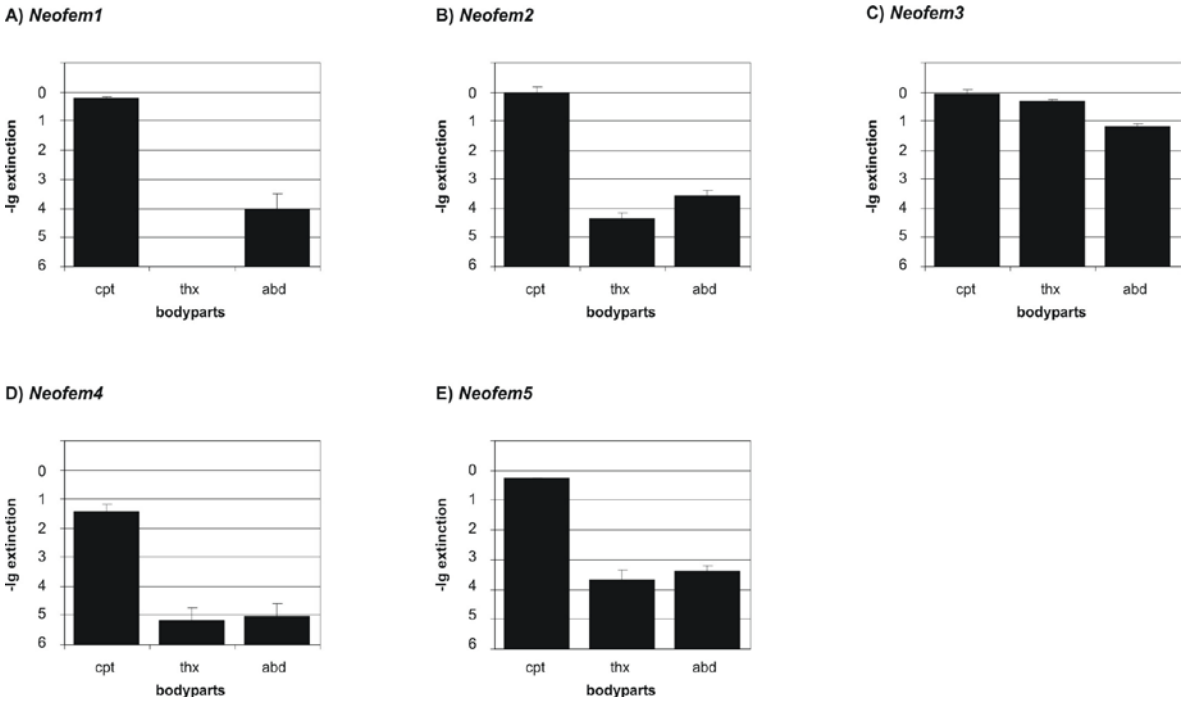

Figure 5

Quantification of *Neofem* genes in different body parts. Relative expression levels of *Neofem* 1-5 genes (A –E) for different body parts measured by qRT-PCR. The Y-axis is on negative $\log_{10}$ scale indicating the gene expression levels and the calculated errors (SD). cpt: caput, thx: thorax and abd: abdomen.

Discussion

The ability of *Cryptotermes secundus* workers to develop into neotenic replacement reproductives after a single moult offers the unique possibility to study differential gene expression during caste differentiation. In this study, we compared the transcriptomes from female neotenics and workers of both gender using RDA to identify novel neotenics-specific transcripts.

We were able to identify five genes that were highly overrepresented in female neotenics of the drywood termite *C. secundus*. Four of these five genes were overexpressed in the head. Expression of the genes *Neofem1* and *Neofem2* of *C. secundus* was highly specific for female reproductives. Both genes are predicted to encode secretory proteins that are specifically expressed in the heads of female neotenics. The open reading frame of *Neofem1* encodes a putative esterase-lipase which shows the highest similarity to yet uncharacterized proteins of the red flour beetle *Tribolium castaneum* and the honey bee

Apis mellifera which are putative orthologs of the *Drosophila* protein CG4382-PA. In addition, two juvenile hormone esterases (JHE) of the mosquito *Aedes aegypti* are closely related to the *Neofem1* protein sequence. Most interestingly, the *Apis mellifera* homolog (GB16889, [GenBank: XP393293]) was found in the brain of adult female worker honey bees (Claudianos et al. 2006) and is closely related to a moth integumental carboxyl/cholinesterase which is implicated in pheromone processing (Ishida and Leal 2002; Claudianos et al. 2006).

Neofem2 showed highest similarity to a digestive β-glycosidase from the salivary glands of the termite *Neotermes koshuensis* (Tokuda et al. 2002). Insect glycosidases are known to include, amongst others, digestive and pheromone degrading enzymes (Tokuda et al. 2002; Cornette et al. 2003). However, the lack of expression in males suggests a sex specific function. Thus *Neofem2* is presumably not a digestive enzyme. Rather the close match to Lma-p72 protein of the Madeiran cockroach (Cornette et al. 2003), which is sex specifically expressed in the abdominal glands of male cockroaches to attract females, may indicate a pheromonal function.

The *C. secundus Neofem4* protein is closely related to family 4 cytochrome P450 enzymes (CYP4) from arthropods, with highest similarities to an uncharacterized termite CYP4 from *Coptotermes acinaciformes* and to CYPIVC1 from *Blaberus discoidalis* (Blattodea; Bradfield et al. 1991). Cytochrome P450 enzymes of insects are generally associated with the metabolism of endogenous substrates or hormones, and with detoxification (summarized by Feyereisen 1999). In termites and social Hymenoptera, some cytochrome P450 enzymes are expressed in a caste specific manner (Evans and Wheeler 1999; Evans and Wheeler 2001b; Liu and Zhang 2004; Cornette et al. 2006; Judice et al. 2006). Contrary to these studies on Hymenoptera and on *Coptotermes acinaciformes* that all revealed highest expression levels in non-reproducing castes, *Neofem4* of *C. secundus* was overexpressed specifically in female neotenics. In termites, cytochrome P450 enzymes are involved in metabolic pathways (*C. acinaciformis*) or insecticide resistance (*Mastotermes darwinensis*) (Falckh et al. 1997; Cornette et al. 2006). However, the specific expression of *Neofem4* in the head of female neotenics suggests that *Neofem4* is involved in the metabolism of endogenous substrates like ecdysteroids or JH rather than insecticide resistance.

The gene *Neofem3* is the only gene that is distributed almost equally in all body parts of female neotenics. It showed highest similarities to insect vitellogenins (Vgs), specifically to Vg1 of the American cockroach *Periplaneta americana* and a Vg of the

turnip sawfly *Athalia rosae*. In most insect species vitellogenins are synthesized extraovarially in female fat body cells as large precursor proteins of vitellin (the major yolk protein of insects). Vgs are secreted into the haemolymph and then incorporated into developing oocytes (Wheeler 1996; Amdam et al. 2003). High expression levels of Vg in female reproductives (primaries and neotenics) were expected because of their ovarian activity. The elevated Vg expression in male reproductives may be explained by the function of Vgs as storage proteins (Wheeler 1996). Recently it was shown that functionally sterile nursing honey bee workers utilize vitellogenin to produce royal jelly to feed larvae (Amdam et al. 2003). The above findings suggest that an ancestral reproductive protein, Vg, was repeatedly co-opted in different social species to serve different functions in different castes. Thus, Vg seems to function as an important developmental protein.

Conclusion

We isolated and characterized five genes that were up-regulated in female replacement reproductives compared to non-reproducing workers of the drywood termite *Cryptotermes secundus* (Kalotermitidae). Interestingly, potential homologues of some of these genes appear to be expressed in different insect species, hemimetabolous as well as holometabolous, in a caste- and species-specific manner. Especially, pheromone-processing genes and Vg emerge as major players that were repeatedly exploited in social evolution of insect societies.

Methods

Chemicals

All chemical reagents used were purchased from Sigma-Aldrich (Taufkirchen, Germany) unless otherwise noted. Oligonucleotides were synthesized either by Metabion international AG (Martinsried, Germany) or by Carl Roth GmbH (Karlsruhe, Germany). Sequences of all Oligonucleotides are given in Additional Table 1.

Termites

Complete termite colonies (*Cryptotermes secundus*) were collected in mangroves around Darwin (NT, Australia) and held in climate chambers at 27°C and a relative humidity of 70% (for details see Korb and Schmidinger 2004). Primary reproductive and alates were taken from these colonies.

To obtain neotenic reproductives, big colonies were split and groups of at least 15 workers were placed together in new *Pinus radiata* wood blocks (16x4x4cm³). After about two weeks neotenic reproductives developed which were removed together with two workers. The sex of the neotenics was determined by their sex-specific morphology as described by Grassé (1982).

RNA preparation

Total RNA from different castes and developmental stages was prepared using the RNAwiz™ solution (Ambion). Poly(A)mRNA was enriched using the MicroPoly(A)Purist™ Kit (Ambion) according to the manufacturer's recommendations. RNA purity and integrity were checked by agarose gel electrophoresis and by UV/Vis spectrometry.

Representational difference analysis

Double-stranded cDNA was prepared by reverse transcription of 2µg poly(A) mRNA using the Universal Riboclone® cDNA Synthesis System (Promega). RDA was performed essentially as described by Heinz et al. (2002). Briefly, the driver representation consisted of cDNA generated from the pooled mRNA of 25 *Cryptotermes secundus* workers. This representation was subtracted from tester cDNA representation of the mRNA repertoire of 11 *C. secundus* female replacement reproductives. After three rounds of subtraction (driver excess: 50x, 400x and 10.000x in successive rounds) and amplification, the entire third difference product was gel-extracted and "shotgun"-cloned into the *BamH* I restriction site of the pZErO-2 vector (Invitrogen) according to the manufacturer's instructions. To check for specificity of the difference product, inserts of randomly picked clones were PCR-amplified from single bacterial colonies utilizing vector-specific primers. The PCR products were denatured with 3 M NaOH for 30 min at room temperature and blotted in duplicates on two separate nylon membranes (Magna NT, 0.22 µm; MSI) in 20x SSC using a vacuum dot blot manifold (Schleicher und Schuell). After UV-cross-linking, one blot was hybridized to driver (worker), the other blot to tester (female neotenics) cDNA representation, which had been labelled radioactively with Klenow fragment (Roche Biochemicals) according to standard protocols. After stringent washing, membranes were exposed to a Molecular Dynamics Storage Phosphor Screen overnight and scanned on a Typhoon 9200 Variable Mode Imager (Amersham Pharmacia). An additional RDA was performed starting with the first difference product of the first RDA. The procedure was

modified by adding *Dpn* II fragments of three genes obtained from the first round to achieve additional *Dpn* II fragments. Here two additional rounds of subtraction (driver excess: 400x and 5.000x in successive rounds) and amplification were performed. Products were cloned and analysed as above.

RNA ligase-mediated 5'- and 3'-Rapid Amplification of c DNA Ends (RACE)-PCR

To obtain complete cDNAs of genes corresponding to the identified RDA fragments, 5'- and 3'-end RACE-PCRs and inter-fragment PCRs were performed. One µg of total RNA from female neotenics was used for cDNA synthesis with the FirstChoice^{TM} RLM-RACE Kit (Ambion). The outer and inner primers for nested PCRs of the genes *Neofem 1-5* and the putative transferrin were derived from gene-specific PCR fragments obtained during the RDA (sequences are given in Additonal Table 1). They were used to amplify 5'- and/or 3'-cDNA fragments. PCR products were cloned into pCR2.1-TOPO vector (TOPO Cloning Kit, Invitrogen) and inserts from several individual plasmid-containing bacterial colonies were sequenced (by GENEART, Regensburg, Germany). Oligonucleotide primers for full-length cDNA amplification were designed according to sequence alignments. PCR products were cloned into pCR2.1-TOPO (TOPO Cloning Kit, Invitrogen) and subsequently sequenced.

Quantitative real-time PCR

Total RNA (1 µg) was reverse transcribed using Superscript II RT (Invitrogen) and Random Decamers (Ambion). qRT-PCR was performed on a Mastercycler® ep *realplex* (Eppendorf) using the QuantiTect SYBR green PCR Kit (Qiagen) according to the manufacturer's instructions. Primers are given in Additional Table 1. Melting curves were analyzed to control for specificity of the PCR reactions. Expression data for genes were normalized for expression of the *18S rRNA*. The relative units were calculated from a standard curve plotting 3 different concentrations of log dilutions against the PCR cycle number (CP) at which the measured fluorescence intensity reached a fixed value. Values represent mean +SD of three independent experiments.

Sequence analysis

Alignments were performed using the software Gene Runner Version 3.05 (Hastings Software Inc.) and BioEdit Version 7.0.1 (Tom Hall Isis Pharmaceuticals, Inc.). BLAST-X

database (http://www.ncbi.nlm.nih.gov/BLAST/) searches were conducted to establish cDNA clone identity.

Authors' contributions

TW performed the study. TW, MR and JK designed the study and drafted the manuscript. MR and JK coordinated the study and acquired funding. All authors read and approved the final manuscript.

Acknowledgements

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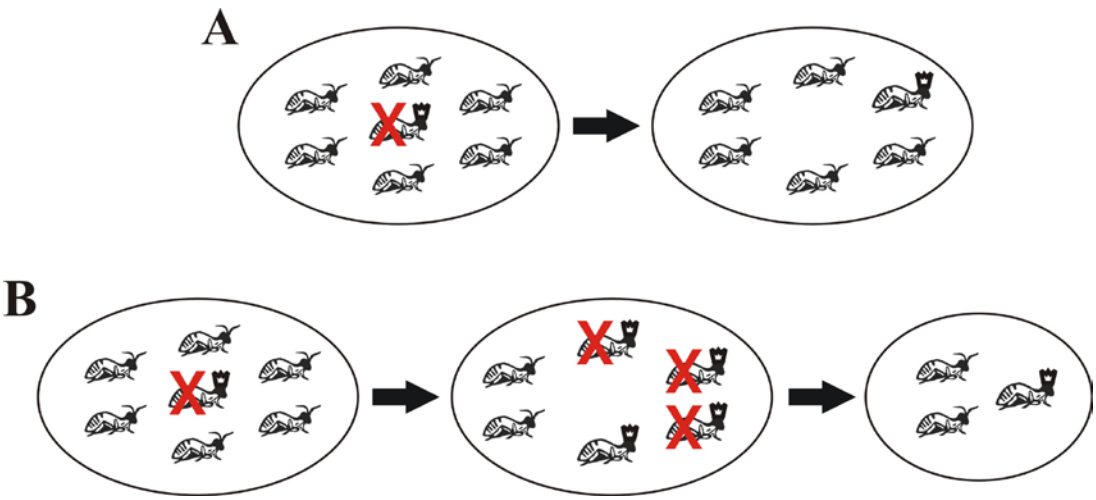
Publication 2

Comparison of queen-specific gene expression in related lower termite species

Tobias Weil, Judith Korb and Michael Rehli



Two alternative modes of reproductive replacment in lower termites: A: One-to-one replacement of a dead reproductive by a neotenic (e.g. in *C. secundus*); B: Excess replacement by neotenics who fight to the death till the vacant breeding position is filled by a new reproductive (e.g. in *C. cynocephalus*)

Publication 2

Tobias Weil, Judith Korb and Michael Rehli

2008

Research Article

Comparison of queen-specific gene expression in related lower termite species

Submitted to Molecular Biology and Evolution

The control of caste determination and reproductive division of labor, the hallmarks of insect societies, are poorly understood on the molecular level. The identification of genes involved in these developmentally important processes will help to understand the molecular mechanisms regulating one of the most impressive examples of polyphenism, the caste structure of eusocial species. Here we applied representational difference analysis (RDA) of cDNAs, to study differential gene expression between female reproductives and workers in the drywood termite *Cryptotermes cynocephalus* and identified thirteen genes that were highly expressed in queens. In addition, we partially cloned several homologous genes of the related termite species *Cryptotermes secundus*, which slightly differs in the mode of reproductive development. Expression profiles of ten homologous genes were compared between both *Cryptotermes* species revealing several genes with specific expression patterns that were not conserved between species and which may be associated with species-specific modes of caste development. Three genes showed a conserved and highly neotenic-specific expression pattern, suggesting an important role of these genes in female sexuals which may be linked with the control and regulation of caste determination and reproductive division of labor.

Keywords: caste determination, gene expression, reproduction, social insects, termites, reproductive division of labor

Introduction

Termites have emerged as model organisms to study various aspects of social behavior. One field that has attracted increasing attention during recent years is the molecular basis for division of labor and caste determination in termites (Miura et al. 1999; Scharf et al. 2003; Hojo et al. 2005; Koshikawa et al. 2005; Miura 2005; Scharf et al. 2005a; Scharf et al. 2005b; Cornette et al. 2006; Lienard et al. 2006; Zhou et al. 2006a; Zhou et al. 2006c; Weil et al. 2007; Zhou et al. 2007). Especially, wood-dwelling termites show a high developmental flexibility of the worker caste (Noirot 1990; Roisin 2000; Korb and Katrantzis 2004; Korb 2007a) and are therefore ideal to study the regulatory mechanisms of caste differentiation in social insects. These termite species, which were also called one-piece nesting termites (Abe 1987), spend their entire colony life in a single piece of wood which serves as both food source and shelter (Korb 2007a). Recent evidence suggests that wood-nesting and flexible development are the ancestral life type in termites evolution, probably inherited from the common ancestor of termites and their sister taxon, the woodroaches (Cryptocercidae) (Inward et al. 2007a; Korb 2007a; Inward et al. 2007b). Thus, the mechanisms underlying caste differentiation and reproductive division of labor in these species might provide insights into the molecular building blocks of termite's social evolution.

During recent years our group has intensively studied the ultimate causes of caste differentiation in the wood-dwelling termite *Cryptotermes secundus* (Kalotermitidae) (Korb and Lenz 2004; Korb and Schmidinger 2004; Korb and Fuchs 2006; Korb and Schneider 2007). As shown for other wood-dwelling termites (reviewed by (Roisin 2000)), the above studies demonstrated that all workers are ontogenetically totipotent immatures that can develop into (i) sterile soldiers, (ii) winged dispersing sexuals that found a new colony, or (iii) neotenic replacement reproductives that inherit the natal breeding position without dispersal (Roisin 2000; Korb and Katrantzis 2004). To distinguish these totipotent individuals from the developmentally less flexible workers of non-wood-dwelling termites (i.e. all other, foraging species) we have suggested the terms 'false workers' and 'true workers' to describe the former or latter, respectively (Korb and Hartfelder 2008).

In contrast to what was recently found for a Reticulitermes species (Hayashi et al. 2007), caste development in *Cryptotermes* is not determined genetically. The 'decision' to stay at the natal nest is influenced by colony size (Lenz 1994; Korb and Lenz 2004; Korb

and Schneider 2007), food availability (Korb and Lenz 2004; Korb and Schmidinger 2004), and parasite pressure (Korb and Fuchs 2006), but not by the number of young offspring present at the nest (Korb 2007b).

So far, research on caste differentiation in termites mainly concentrated on juvenile hormone (JH) induced soldier differentiation (Miura et al. 1999; Miura et al. 2003; Koshikawa et al. 2005; Scharf et al. 2005a; Cornette et al. 2006; Hrdy et al. 2006; Zhou et al. 2006a; Zhou et al. 2006b; Zhou et al. 2006c; Scharf et al. 2007; Zhou et al. 2007). However, the proximate mechanisms that drive the reproductive division of labor between neotenic replacement reproductives and false workers are largely unknown. This is especially important as the evolutionary development of neotenic reproductives is proposed to be a crucial step in termites' evolution of social life (Myles TG 1988; Korb and Hartfelder 2008). To address the mechanisms controlling differentiation of reproductive castes, we recently analyzed transcriptional differences between neotenic replacement reproductives and false workers of *C. secundus* and were able to identify genes that were up-regulated in female replacement reproductives relative to non-reproducing false workers (Weil et al. 2007). Here, we extended our previous work and performed a cross-species comparison of queen specific gene expression in the two closely related species, *Cryptotermes secundus* and *Cryptotermes cynocephalus* (Thompson et al. 2000) which differ in the development of replacement reproductives (Lenz 1994). In *C. secundus*, a dead reproductive is generally replaced by a single false worker of the same sex that develops into a replacement reproductive. By contrast in the pest species *C. cynocephalus*, several false workers become replacement reproductives which fight among each other over the breeding position until one pair of reproductives is left (Lenz 1994). These are the two prototypic forms of the development of replacement reproductives that are common amongst related *Cryptotermes* species (shown in fig. 1).

Comparative studies in closely related species might aid to define important (evolutionary conserved) mechanisms of caste differentiation. However, on the molecular level such analyses are rare in social insects (Sen Sarma et al. 2007). Our study identified several genes that showed a conserved neotenic-specific expression profile in both species, but also a number of genes that were specific for neotenics in only one species. Whereas the former represent candidates that might be involved in reproductive caste determination in general (division of labor), the latter may account for the species-specific differences in the mode of development.

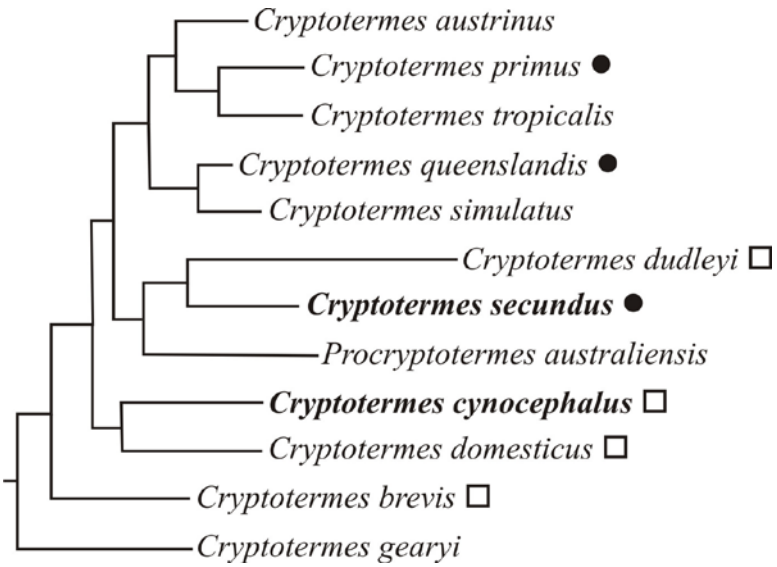


Figure 1

Phylogenetic tree of *Cryptotermes* (modified after Thompson et al. 2000). If known, the different modes of neotenic development are marked: ● single replacement of the reproductive; □ multiple young neotenics attempt to become the next reproductive (for more information see text); *C. primus*, *C. queenslandis*, *C. cynocephalus*, *C. domesticus* and *C. brevis*: described in literature (Lenz et al. 1985; Lenz 1994), *C. secundus*: own research and *C. dudleyi*: Michael Lenz, personal communication.

Materials and Methods

Termites

Termite colonies of the pest species *Cryptotermes cynocephalus* were collected in dry wood of diverse origins (infested furniture, wooden slats and trees) in Bukit Badong and Kuantan (Selangor and Panang, Malaysia). Colonies of *Cryptotermes secundus* were collected in mangroves around Darwin (NT, Australia). Colony rearing and the generation of neotenic replacement reproductives were performed as previously described (Korb and Schmidinger 2004; Weil et al. 2007).

RNA preparation

Total RNA from different castes and developmental stages was prepared using the RNAwizTM solution (Ambion, Ausitn, TX). Poly(A)mRNA was enriched using the MicroPoly(A)PuristTM Kit (Ambion, Austin, TX) according to the manufacturer's recommendations. RNA purity and integrity were checked by agarose gel electrophoresis and by UV/Vis spectrometry.

Representational difference analysis

RDA for *Cryptotermes cynocephalus* was performed essentially as described (Weil et al. 2007). In brief, double-stranded cDNA was prepared by reverse transcription of 1.5-2 µg poly(A) mRNA using the Universal Riboclone® cDNA Synthesis System (Promega, Madison, WI). The driver representation consisted of cDNA generated from the pooled mRNA of 24 *C. cynocephalus* workers. This representation was subtracted from tester cDNA representation of the mRNA repertoire of 19 *C. cynocephalus* female replacement reproductives. After three rounds of subtraction (driver excess: 50x, 400x and 10.000x in successive rounds) and amplification, the entire third difference product was gel-extracted and "shotgun"-cloned into the *BamH*I restriction site of the pZErO-2 vector (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. To check for specificity of the difference product, inserts of randomly picked clones were PCR-amplified from single bacterial colonies utilizing vector-specific primers. The PCR products were denatured and blotted in duplicates on two separate nylon membranes (Magna NT, 0.22 µm; MSI, Westboro, MA) using a vacuum dot blot manifold (Schleicher und Schuell, Dassel, Germany). After UV-crosslinking, one blot was hybridized to the driver (false worker), the other blot to the tester (female neotenics) cDNA representation, which had been labeled radioactively with Klenow fragment (Roche Biochemicals, Mannheim, Germany) according to standard protocols. After stringent washing, membranes were exposed to a Molecular Dynamics Storage Phosphor Screen overnight and scanned on a Typhoon 9200 Variable Mode Imager (Amersham Pharmacia, Piscataway, NJ).

RNA ligase-mediated 3'-Rapid Amplification of c DNA Ends (RACE)-PCR

Corresponding 3'-ends of the identified RDA fragments were obtained using 3'-Rapid Amplification of cDNA ends (FirstChoiceTM RLM-RACE Kit, Ambion, Austin, TX). The outer and inner primers for nested amplification of female neotenic specific (*Neofem*) genes were derived from gene-specific PCR fragments obtained during the RDAs for *C. cynocephalus* (*Neofem2*, *3* and *6-16*) and *C. secundus* (*Neofem1-5*, Weil et al. 2007). Primer sequences are given in supplementary table 1. PCR products were cloned into pCR2.1-TOPO vector (TOPO Cloning Kit, Invitrogen, Carlsbad, CA) and inserts from several individual plasmid-containing bacterial colonies were sequenced (by GENEART, Regensburg, Germany and Macrogen, Seoul, Korea).

Quantitative RT-PCR

Primers were designed using the *PerlPrimer* software (Marshall 2004) according to the obtained sequences (see supplementary table 1). Total RNA (0.5-1 µg) was reverse transcribed using Avian Myeloblastosis Virus Reverse Transcriptase (Promega, Madison, WI) and Random Decamers (Ambion, Austin, TX). qRT-PCR was performed on a Mastercycler® ep *realplex* (Eppendorf, Hamburg, Germany) using the QuantiTect SYBR green PCR Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Melting curves were analyzed to control for specificity of the PCR reactions. Expression data for genes were normalized for expression of the *18S rRNA* gene. The suitability of this reference gene was previously evaluated using the *BestKeeper* software (Pfaffl et al. 2004; Weil et al. 2007). Relative units were calculated from a standard curve plotting three different concentrations of log dilutions against the PCR cycle number (CP) at which the measured fluorescence intensity reached a fixed value. Values represent mean ±SD of three independent experiments.

Sequence analysis

Alignments were performed using the software Gene Runner Version 3.05 (Hastings Software Inc., Hastings on Hudson, NY) and BioEdit Version 7.0.1 (Tom Hall Isis Pharmaceuticals, Inc., Carlsbad, CA). BLAST-X database searches were conducted to establish cDNA clone identity. Pfam searches (Finn et al. 2006) of the corresponding protein sequences were performed to find common protein domains and families. To determine the level of conservation of orthologous nucleotide sequences within the studied species, all indels, transitions and transversions were counted and differences were expressed as a percentage (supplementary table 2). Nucleotide sequences were submitted to GenBank [GeneBank: EU546144-EU546164].

Results

Identification of caste-specific transcripts in female neotenics of C. cynocephalus

To identify genes that are specifically expressed in female neotenics of the drywood termite *C. cynocephalus* (Kalotermitidae), we performed a cDNA-RDA using female neotenics as tester cDNA and false workers of both sexes as driver cDNA. The difference product of the third RDA round was shotgun cloned and 86 randomly picked clones were sequenced and validated using reverse dot blot hybridization. Sequences of 18 fragments

revealed either no significant BLAST X match (10 fragments) or were most likely of non-termite origin (8 fragments) and therefore excluded from further analyses. Sequences of 68 fragments with highly specific signals in reverse dot blot hybridization were most likely derived from termites. Fourty six fragments were homologous to two genes that were previously identified in *C. secundus* using a similar screen (Weil et al. 2007) and represented a gene belonging to the family of glycosyl hydrolases (*Neofem2*) and a vitellogenin homolog (*Neofem3*). In addition, we found 22 fragments belonging to 11 independent sequence units that were named *Neofem6 – Neofem16* (table 1). Additional sequence information for individual fragments was obtained by 3'-RACE-PCR, confirming the initial assignment of the identified fragments to 11 independent and novel genes that were not previously detected in *C. secundus*. The sequence of the *Neofem6* gene showed highest sequence similarities to antennal expressed genes of mosquitoes, in particular to the "takeout" gene sequence of the yellow fever mosquito *Aedes aegypti* (Bohbot and Vogt 2005) and to the putative antennal carrier protein TOL-2 of the African malaria vector *Anopheles gambiae* (Justice et al. 2003). The partial sequence of *Neofem7* showed homologies as well to serine protease inhibitors as to an uncharacterized *Drosophila* gene. *Neofem8* is closely related to the follicle cell protein 3C of *Drosophila melanogaster* and its ortholog of the parasitoid wasp *Nasonia vitripennis*, and to an uncharacterized honeybee (*Apis mellifera*) gene. *Neofem9* showed similarities to genes of the Histone2A family of arthropods (black tiger prawn *Penaeus monodon*, cotton boll weevil *Anthonomus grandis* and red flour beetle *Tribolium castaneum*). *Neofem10* is closely related to the *Apis mellifera* and *Tribolium castaneum* homologs of the *Drosophila* gene CG1962-Pa, whose molecular function is unknown. Similarity searches identified homologies of the *Neofem11* gene product to the gene family of voltage-gated potassium channels of *Tribolium castaneum* and mosquitoes. The partial sequence of *Neofem12* showed homologies to genes with tyrosine transmembrane transporter activity of mosquitoes (*Anopheles gambiae* and *Aedes aegypti*), and the parasitoid wasp *Nasonia vitripennis*. *Neofem13* encodes a putative *Big Brain* (bib, *Drosophila melanogaster*) homolog of the honeybee. The neurogenic gene *Big Brain* appears to be a channel protein (aquaporin homolog; MIP (major intrinsic protein) family signature) (Rao et al. 1990; Rao et al. 1992). The *Neofem14* gene showed homologies to several camp-dependent rap1 guanine-nucleotide exchange factor genes including the *Drosophila melanogaster Epac* gene. The genes *Neofem15* and *16* are related to transcription factors containing zinc finger DNA binding domains. Table 1 summarizes the sequence characteristics of all identified genes.

Table 1

	Size (bp)	No. of clones[a]	Pfam[b]	Identity match by BLASTX [species][c]	GeneBank accession no.	BLASTX *e*-value
Neofem1	657	-	Carboxylesterase; Nucleoporin Nup153-like; Probable molybdopterin binding domain	female neotenic-specific protein 1 [*Cryptotermes secundus*]	ABN05619	3e-63
				carboxylesterase-like protein [*Locusta migratoria manilensis*]	ABY53601	9e-32
				PREDICTED: similar to female neotenic-specific protein 1 [*Nasonia vitripennis*]	XP_001603584	2e-30
Neofem2	939	3	Glycosyl hydrolase family 1	female neotenic-specific protein 2 [*Cryptotermes secundus*]	ABN05620	3e-126
				male-specific beta-glycosidase [*Leucophaea maderae*]	AAL40863	7e-53
				beta-glucosidase [*Neotermes koshunensis*]	BAB91145	1e-51
Neofem3	933	27	DUF1943; von Willebrand factor type D domain; Peptidase family C1 propeptide	female neotenic-specific protein 3 [*Cryptotermes secundus*]	ABN05621	1e-117
				Vitellogenin 1 precursor (Vg-1) [*Periplaneta americana*]	Q9U8M0	1e-52
				vitellogenin-3 [*Plautia stali*]	BAA88077	9e-31
Neofem4	891	-	Cytochrome P450; Human adenovirus early E3A glycoprotein	female neotenic-specific protein 4 [*Cryptotermes secundus*]	ABN05622	4e-105
				family 4 Cytochrome P450 [*Coptotermes acinaciformis*]	AAC03111	3e-65
				cytochrome P450 CYP4 [*Cherax quadricarinatus*]	AAL56662	6e-52
Neofem5	-	-	-	-	-	-
Neofem6	418	-	Odorant binding protein (DUF233)	takeout [*Aedes aegypti*]	AAL60239	2e-15
				conserved hypothetical protein [*Aedes aegypti*]	XP_001655779	1e-14
				Putative antennal carrier protein TOL-2 [*Anopheles gambiae*]	AAO39756	7e-12
Neofem7	910	5	Serpin (serine protease inhibitor)	serpin 2 precursor [*Branchiostoma lanceolatum*]	CAJ38562	2e-21
				GA20188-PA [*Drosophila pseudoobscura*]	XP_001356876	5e-21
				serpin 4B [*Anopheles gambiae*]	ABJ52803	3e-20
Neofem8	288	3	-	PREDICTED: similar to GA17864-PA [*Nasonia vitripennis*]	XP_001606122	4e-34
				PREDICTED: similar to CG14881-PA, isoform A [*Apis mellifera*]	XP_001120760	7e-28
				Fcp3C [*Drosophila melanogaster*]	AAY27504	2e-26

Neofem9	255	1	Core histone H2A/H2B/H3/H4 Chitin synthase N-terminal	histone H2A [*Penaeus monodon*]	ABX84387	1e-31
				histone H2A [*Anthonomus grandis*]	ABW86651	1e-31
				PREDICTED: similar to CG31618-PA [*Tribolium castaneum*]	XP_974617	1e-31
Neofem10	357	1	Spc24 subunit of Ndc80; Bacterial RNA polymerase, alpha chain C terminal domain	PREDICTED: similar to CG1962-PA, isoform A [*Tribolium castaneum*]	XP_969297	2e-25
				PREDICTED: similar to CG1962-PA, isoform A [*Apis mellifera*]	XP_392258	3e-25
				PREDICTED: hypothetical protein [*Nasonia vitripennis*]	XP_001600204	8e-24
Neofem11	706	3	Ion Channel (Ion trans 2); PetM family of cytochrome b6f complex subunit 7; IQ calmodulin-binding motif	PREDICTED: similar to Potassium voltage-gated channel subfamily KQT member [*Tribolium castaneum*]	XP_974805	1e-95
				voltage-gated potassium channel [*Aedes aegypti*]	XP_001661979	3e-87
				AGAP011113-PA [*Anopheles gambiae str.* PEST]	XP_309533	5e-87
Neofem12	398	1	Integral membrane protein DUF6	AGAP009284-PA [*Anopheles gambiae str.* PEST]	XP_320080	1e-28
				PREDICTED: similar to tyrosine transporter [*Nasonia vitripennis*]	XP_001601473	1e-25
				tyrosine transporter [*Aedes aegypti*]	XP_001658764	1e-25
				PREDICTED: similar to hoepel1 CG12787-PA, isoform A isoform2 [*Apis mellifera*]	XP_624260	9e-25
Neofem13	304	1	-	PREDICTED: similar to big brain CG4722-PA [*Apis mellifera*	XP_396705	3e-08
Neofem14	262	1	Domain found in Dishevelled, Egl-10, and Pleckstrin (DEP)	PREDICTED: similar to CG3427-PA [*Tribolium castaneum*]	XP_972857	2e-24
				camp-dependent rap1 guanine-nucleotide exchange factor [*Aedes aegypti*]	XP_001660814	4e-20
				AGAP007307-PA [*Anopheles gambiae str.* PEST]	XP_308514	1e-19
				Epac CG34392-PC, isoform C [*Drosophila melanogaster*	NP_001097202	3e-19
Neofem15	558	3	2x Zinc finger, C2H2 type	PREDICTED: similar to zinc finger protein	-	-
Neofem16	341	1	2x Zinc finger, C2H2 type; Ribosomal protein L19e	PREDICTED: similar to Kruppel-like factor 5 (intestinal), [*Danio rerio*]	XP_688525	2e-12
				unnamed protein product [*Tetradon nigroviridis*]	CAF96957	2e-12
				rCG26549 [*Rattus norvegicus*]	EDL79132	4e-12

Table 1 (previous page)

[a]Number of clones obtained from the RDA approach
[b]Pfam matches (http://pfam.sanger.ac.uk) of the corresponding protein sequence
[c]BLASTX results show best hits against the non-redundant NCBI database (http://ncbi.nlm.nih.gov/blast/Blast.cgi)

Species specific analyses of the relative expression of Neofem genes

The drywood termite species *C. cynocephalus* and *C. secundus* are closely related (see fig. 1). It was therefore surprising that only two of the five previously identified neotenic-specific *C. secundus* genes were also discovered in our *C. cynocephalus* screen. To validate our RDA approach and to compare the relative expression of *Neofem1-5* genes in both *Cryptotermes* species, we used PCR-based cloning to obtain sequences for orthologous genes and performed mRNA expression analyses using quantitative RT-PCR for all successfully cloned genes (*Neofem1-4*) in RNA samples derived from different termite castes (female neotenics, male neotenics and false workers of both sexes). As shown in figure 2, expression profiles of the analyzed *Neofem* genes were largely consistent with the RDA results. *Neofem1*, *2* and *3* genes were highly specific for neotenics in *C. cynocephalus* and *C. secundus*. Reasons for the absence of *Neofem1* fragments in our RDA-screen are unclear but may be explained by a possible absence of *Dpn*II restriction sites in the *C. cynocephalus* sequence. In contrast to *Neofem1*, *2* and *3* genes, *Neofem4* which was identified as neotenic-specific in *C. secundus* was expressed at equal levels in false workers and neotenics of *C. cynocephalus.*

For the remaining 11 novel *C. cynocephalus* genes, we also made efforts to clone corresponding 3'-ends of *C. secundus* genes to be able to compare their relative expression in false workers and neotenics. For six Neofem genes (*Neofem6-11*), orthologous sequences were obtained for the sibling species. As expected, transcript sequences of both species generally showed a high level of conservation (see supplementary table 2). Although we tested a number of primer combinations, we were unable to obtain orthologous sequence information for *Neofem12-1*6. It is possible that the corresponding orthologues are missing in *C. secundus;* however, it is also possible that the PCR-based cloning strategy did not succeed because the degree of homology in corresponding *C. secundus* sequences is too low or because multiple homologous sequences exist (e.g. in the case of zinc finger containing *Neofem15* and *16*). Quantitative RT-PCR revealed that all novel *C. cynocephalus Neofem* genes met the selection criteria of their initial RDA – their

expression was generally higher in female neotenics as compared with false workers (fig. 2, 3 and 4). The neotenic-specific expression profile for *Neofem7-10* genes, however, was exclusively detected in *C. cynocephalus* but not in *C. secundus* (see fig. 3). Only *Neofem11* showed a slightly stronger expression in neotenics of both species. In male neotenics, expression levels of orthologous genes appeared largely conserved with two exceptions.

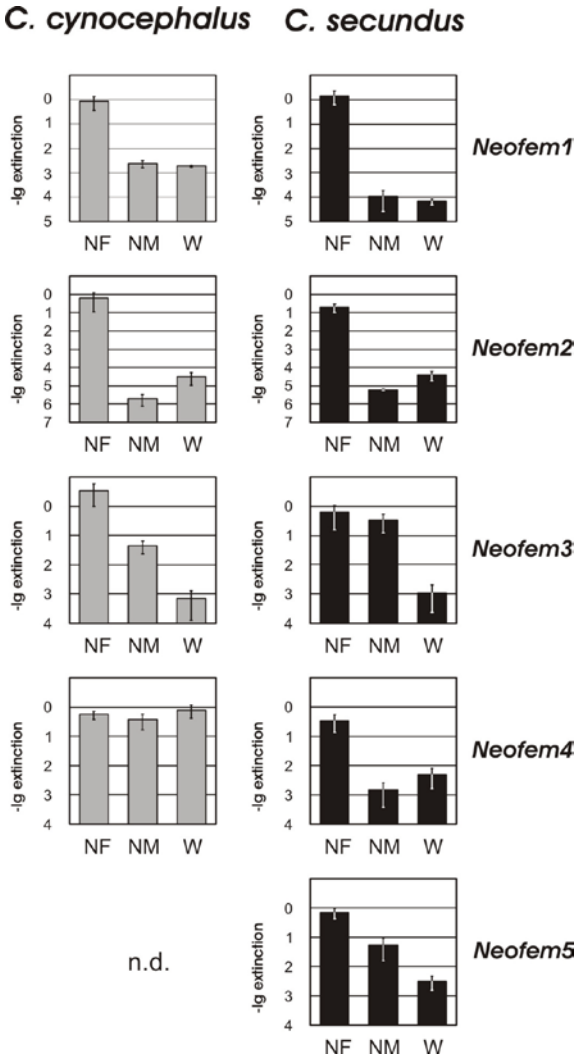


Figure 2

Relative expression levels of *Neofem 1-5* genes in different castes measured by qRT-PCR. The Y-axis is on negative log10 scale indicating the gene expression levels and the calculated errors (±SD), for female neotenics (NF), male neotenics (NM), and false workers (W) of both sexes. n.d.: not detected.

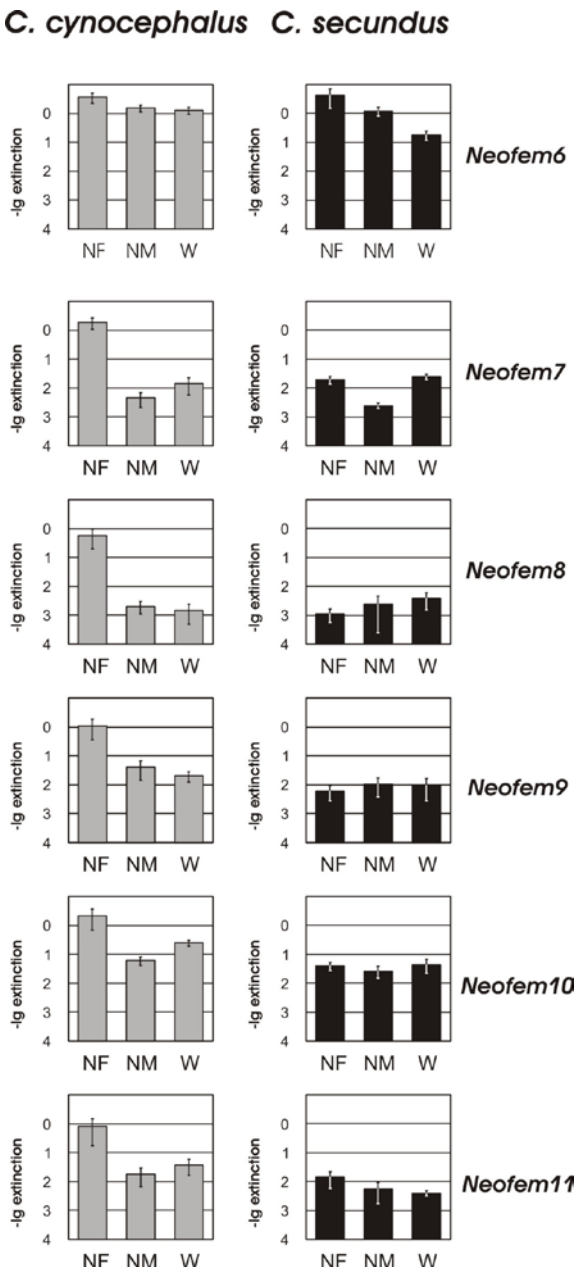


Figure 3

Relative expression levels of *Neofem 6-11* genes in different castes measured by qRT-PCR. The Y-axis is on negative log10 scale indicating the gene expression levels and the calculated errors (±SD), for female neotenics (NF), male neotenics (NM), and false workers (W) of both sexes.

Neofem4 gene (see fig. 2) was expressed at similar levels in male and female neotenics of *C. cynocephalus*, whereas it was down-regulated in male neotenics of *C. secundus.* The vitellogenin-homolog was expressed at lower levels in male neotenics of *C. cynocephalus* than *C. secundus*

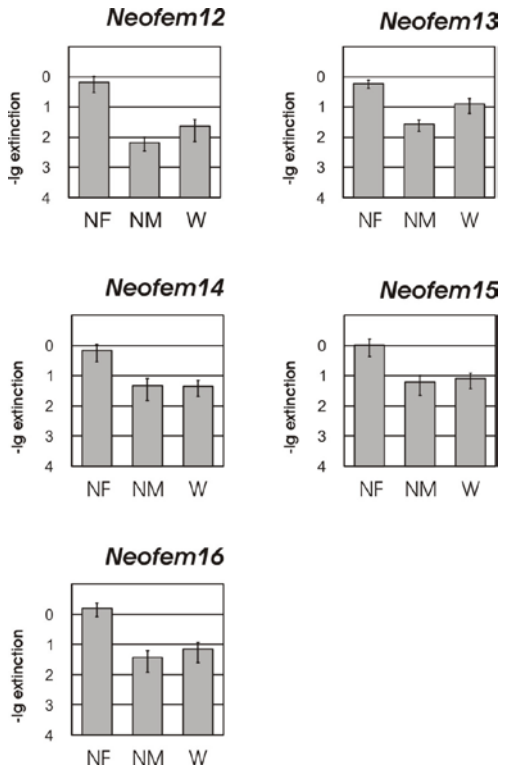


Figure 4

Relative expression levels of *Neofem* 12-16 genes in different castes measured by qRT-PCR. The Y-axis is on negative log10 scale indicating the gene expression levels and the calculated errors (±SD), for female neotenics (NF), male neotenics (NM), and false workers (W) of both sexes. n.d.: not detected.

Discussion

The identification of genes that are differentially expressed between reproducing and nonreproducing castes is an established first step towards uncovering the molecular mechanisms controlling caste differentiation and reproductive division of labor in social insects. Castespecific genes in queens and workers have been characterized in several social insect species, e.g. including bees (Corona et al. 1999; Evans and Wheeler 2001b; Evans and Wheeler 2001a; Kamikouchi et al. 2004; Pereboom et al. 2005; Cristino et al. 2006; Judice et al. 2006; Wheeler et al. 2006; Barchuk et al. 2007; Grozinger et al. 2007; Thompson et al. 2007), wasps (Sumner et al. 2006; Hoffman and Goodisman 2007; Toth et al. 2007), ants (Gräff et al. 2007) and termites (Weil et al. 2007). Compared with social Hymenoptera (ants, some bees and wasps) termites differ in the mode of caste differentiation. Social Hymenoptera are holometabolous insects and their castes are the result of adult polyphenism, due to complete metamorphosis from a larval stage, via a pupa

directly into adults. By contrast termites are hemimetabolous insects (incomplete metamorphosis), with multiple immature stages that stepwise develop into adults.

Our group has focused on identifying queen-specific genes in drywood termites. In a recent study we successfully identified a number of neotenic-specific transcripts in *C. secundus* using representational difference analysis (RDA) of cDNAs (Weil et al. 2007). To further extend our knowledge on caste differentiation and to identify genes characterized by an evolutionary conserved caste-specific expression, we now applied the same approach to the closely related drywood species *C. cynocephalus*, which slightly differs in its development of neotenic replacement reproductives. Here, we isolated an additional set of genes that were over-expressed in female neotenics and performed a cross-species comparison of castespecific gene expression between both drywood termites.

Genes up regulated in female neotenics of both species

Good candidate genes that play a crucial role in the development of neotenics should be conserved, and therefore, expressed in a similar manner in female neotenics of two closely related species. We identified four genes with such an expression pattern. One gene, *Neofem6,* was initially detected in *C. cynocephalus*, however, its differential expression in neotenic (Weil et al. 2007) females was even more pronounced in *C. secundus.* This gene is related to antennal expressed genes of mosquitoes that are homologs of the *Drosophila melanogaster* protein takeout. Takeout was first described in *Drosophila melanogaster* where it is expressed in the brain, antennae and gut. It is circadian regulated, induced by starvation and thought to be involved in the regulation of feeding behavior, male courtship and mating behavior (Sarov-Blat et al. 2000; So et al. 2000; Dauwalder et al. 2002). Takeout-like proteins have been suggested to be involved in chemoreception by regulating the antennal response to pheromones, host or food (Justice et al. 2003; Bohbot and Vogt 2005). Proteins of this family were previously described for social insects (Hojo et al. 2005; Hagai et al. 2007). For example, the takeout-like protein *Ntsp-1* is specifically expressed in *Nasutitermes takasagoensis* soldiers and suggested to be constantly synthesized after soldier differentiation (Hojo et al. 2005). Three genes, designated as *Neofem 1-3,* were previously described for *C. secundus* (Weil et al. 2007). The genes *Neofem1* and *Neofem2,* putative esterase-lipase and β-glycosidase homologs, respectively, are highly over-expressed in female neotenics and are likely to be important female reproductive specific genes in wood-dwelling, lower termites. The *Neofem3* gene, a vitellogenin, shows similar differential expression levels in female neotenics and workers

of both species, but the expression slightly differed in male neotenics of both species (see fig. 2). Vitellogenin is known to be an important developmental protein throughout all social insects that fulfill different functions in different castes (Wheeler 1996; Amdam et al. 2003; Nelson et al. 2007). For *Neofem1* and *Neofem2* a possible involvement in pheromone processing was discussed (Weil et al. 2007). Here, the close homology of *Neofem2* to a sex specific expressed surface protein of male cockroaches, which seems to attract females (Cornette et al. 2003), is of interest, especially as termites are now nested within the cockroaches (Inward et al. 2007a; Inward et al. 2007b).

Neofem genes up regulated in female neotenics of either C. cynocephalus or C. secundus

The gene *Neofem4*, a cytochrome P450 homolog, was only differentially expressed in *C. secundus* (Weil et al. 2007), whereas the expression of its homolog in *C. cynocephalus* was similar in all neotenics and false workers. Orthologous sequence information for *Neofem7-11* was also obtained for *C. secundus.* However all four genes showed a female specific expression in *C. cynocephalus* only, while no differential expression was detected in *C. secundus.* This is in line with the results of our present and previous RDA screenings. *Neofem7*, a serine protease inhibitor (serpin) homolog, seems to be involved in innate immunity of insects (Kanost 1999; Zou et al. 2006; Zou et al. 2007). The insect haemolymph generally contains high concentrations of serpin family members. They remove microbial proteases and regulate haemolymph coagulation, antimicrobial protein synthesis as well as melanization after infection (Kanost 1999). In a recent study on differential gene expression between queens and workers in the black garden ant *Lasius niger*, the serine protease inhibitor homolog Ln252_3 was found to be over-expressed in queens. Here the authors discussed that queens invest more in their immune defense than workers (Gräff et al. 2007).

The *Neofem8* gene product showed close homologies to the follicle cell protein 3C (*Fcp3C*) of *Drosophila melanogaster* and its ortholog in *Nasonia vitripennis*. *Fcp3C* is expressed during the formation of the follicle cuticle (DuMont and Aquadro 2005), however, its molecular function is unknown. Its specific expression in reproductive females of *C. cynocephalus* is in line with above observations but apparently not conserved in *C. secundus*. Sequence comparison of *Neofem9* revealed homologies to arthropod's histone 2A (H2A), a member of the core histones that assemble nucleosomes. Histones were previously described to be differentially expressed between queens and workers in

social Hymenoptera (Barchuk et al. 2007; Gräff et al. 2007). The overexpression of H2A in queens could reflect a higher rate of cell division in reproducing castes.

The genes *Neofem11* to *13* all show homologies to channel proteins. *Neofem13* encodes a homolog of the neurogenic gene *Big Brain* that is known to be involved in cell fate determination of ectodermal cell during *Drosophila* neurogenesis (Rao et al. 1990; Rao et al. 1992; Doherty et al. 1997). *Big Brain* is also expressed in oocytes and is discussed to play a role in the follicle cell morphogenesis (Larkin et al. 1996; Dobens and Raftery 2000; Yanochko and Yool 2002). Furthermore, gene expression analysis during the life cycle of *Drosophila melanogaster* revealed that *Big Brain* expression is higher in adult females as compared to males (Arbeitman et al. 2002). The same study also demonstrated a higher expression of CG3472 in adult females (Arbeitman et al. 2002), which is a homolog of the *Neofem14* gene. The *Neofem14* gene is close related to cAMP dependent effectors, mainly to the *Epac* (exchange protein directly activated by cAMP) gene of *Drosophila melanogaster*.

Conclusion

The comparative expression analysis of two closely related lower drywood termite species revealed several genes that are specifically expressed in female neotenics of both species. A putative esterase-lipase (*Neofem1*) and a β-glycosidase homolog (*Neofem2*) are highly and exclusively expressed in female reproductives of wood-dwelling lower termites, suggesting an important developmental or functional role for both genes in female reproductives.

Acknowledgements

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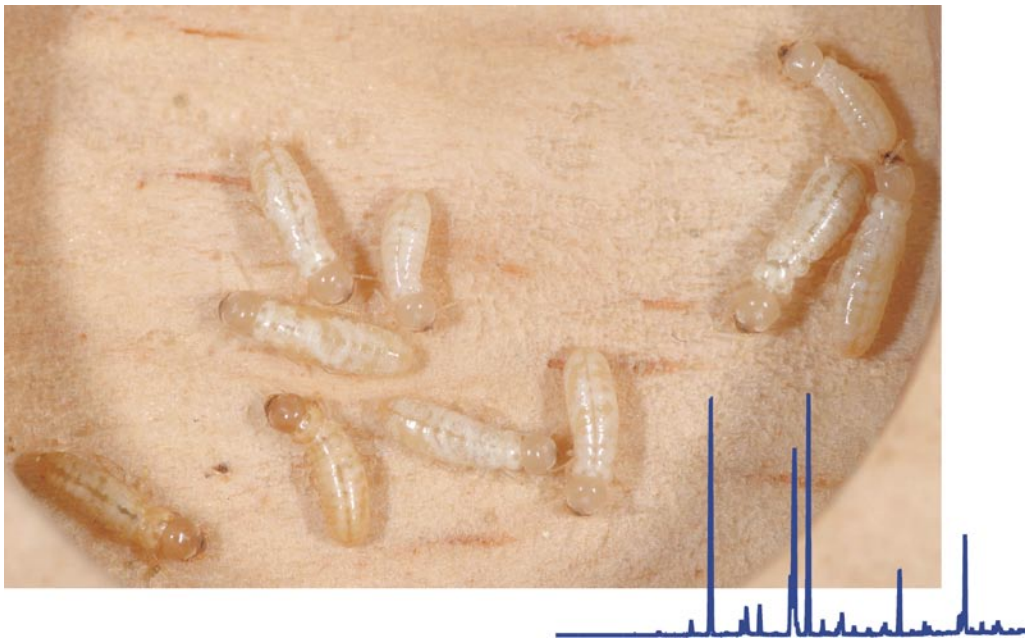
Publication 3

Scent of a queen – cuticular hydrocarbons specific for female reproductives in lower termites

Tobias Weil, Katharina Hoffmann, Johannes Kroiss,
Erhard Strohm and Judith Korb



Neotenic reproductives (more sclerotised, on the left, below) and false workers of *C. secundus*. Workers performing proctodeal trophallaxis (on the right) and antennation (in the center) .

Publication 3

Tobias Weil, Katharina Hoffmann, Johannes Kroiss, Erhard Strohm and Judith Korb

2008

Short Communication

Scent of a queen – cuticular hydrocarbons specific for female reproductives in lower termites

Submitted to Naturwissenschaften

In social insects it is assumed that signals of the queen inform nestmates about her reproductive status. Thus workers forego their own reproduction if the queen signals high fertility. In hemimetabolous termites little is known about reproductive inhibition but evidence exists for a royal-pair control. Workers of lower termites exhibit a high developmental flexibility and are potentially able to become reproductives, but the presence of a fertile reproductive restrains them from reaching sexual maturity. The nature of this control, however, remains unknown. By analyzing cuticular hydrocarbons (CHCs) of queens and workers of the basal drywood termite *Cryptotermes secundus* we found that both castes differed qualitatively in their CHC profiles. Queens were characterized by a shift to long-chained and branched hydrocarbons. Most remarkably, similar chemical patterns are regarded as fertility cues of reproductives in social Hymenoptera. This might suggest that both groups of social insects convergently evolved similar chemical signatures. We discuss how termites might have socially exploited these signatures from sexual communication in their cockroach-like ancestor.

Keywords: termites, cuticular hydrocarbons, fertility signals, chemical communication, queen signal

Introduction

Reproductive division of labor is a key characteristic of social insects. In colonies of basal termites all ontogenetically totipotent immatures (false workers, formerly also called pseudergates) have the possibility to develop via a single molt into neotenic replacement reproductives (Korb and Hartfelder 2008). Nevertheless, the queen is the sole egg-layer within the colony and the development of a false worker into a neotenic reproductive only occurs, when a colony's reproductive of the same sex dies or becomes unhealthy. The mechanism that prevents colony members from becoming a reproductive is poorly understood. In eusocial insects the maintenance of the reproductive hierarchy is generally assured by the recognition of a fecund queen due to her characteristic chemical signature, that indicates her ovarian activity (e.g. Peeters et al. 1999; Cuvillier-Hot et al. 2001; Heinze et al. 2002; Dietemann et al. 2003; Sledge et al. 2004; Cuvillier-Hot et al. 2005; Hartmann et al. 2005; Lommelen et al. 2006; Sramkova et al. 2008). Such chemical compounds of the queen’s cuticular are often regarded as honest signals that inform nestmates of the presence of a fertile and healthy queen and therefore ensure an evolutionary stable regulation of worker sterility (Keller and Nonacs 1993).

Wood-dwelling termites spend their entire life in the darkness of a single piece of wood and most colony members are blind, thus communication via scent is conceivable. It was shown that chemical communication occurs in termite colonies (e.g. Howard et al. 1982; Haverty et al. 1996; Haverty et al. 1997; Bagneres et al. 1998; Sevala et al. 2000; Dronnet et al. 2006). However, the relationship between cuticular hydrocarbon (CHC) profiles and fertility was not investigated in termites so far.

To study whether chemical cues exist that signal the presence of an established and fecund queen we compared the CHC profiles of female neotenics and false workers of the drywood termite *Cryptotermes secundus*.

Materials and Methods

Termites

Colonies of *C. secundus* were collected in mangroves near Darwin (NT, Australia). Colony rearing and the generation of neotenic queens were performed as previously described (Weil et al. 2007).

Chemical analyses

CHCs were obtained by solid phase microextraction (SPME) of 80 *C. secundus* termites, 40 false workers and neotenic queens respectively. The cuticular surface of an individual was gently rubbed for 5 minutes with a Supleco (Deisenhofen, Germany) Polydimethylsiloxane fiber for SPME (df 7µm for gas chromatography (GC) and df 100µm for GC-mass spectrometry (GC-MS)), and then immediately placed in the injection port of a GC.

GC-analysis was performed with an Agilent Technologies 6890N GC equipped with a *split-splitless* injector (280°C; purge valve opened after 5 minutes), a flame-ionisation detector (FID) and a HP-5 fused silica capillary column (30 m x 0.32 mm ID; df 0.25 µm, J&W Scientific) using Helium as carrier gas (constant flow, 1ml min^{-1}). The temperature programming was as follows: 120-150°C at 30°C min^{-1}, 150- 280°C at 4°C min^{-1} and 280-300°C at 10°C min^{-1}.

GC-MS was performed with an Agilent Technologies 6890N GC coupled to an Agilent 5973 inert mass selective detector (70 eV ionization voltage). The GC was equipped with a RH- 5ms+ fused silica capillary column (30 m × 0.25 mm ID; df 0.25 µm, J&W Scientific). GC conditions were set as mentioned above but the *split-splitless* injector was set 250°C with the purge valve opened after 60 sec. For GC-MS analyses we used both SPME and hexane extractions. For the latter, 10 false workers or 3 queens were pooled and extracted in 400µl of distilled hexane for 5 minutes. The extracts were reduced to a final volume of 50µl by a gentle stream of nitrogen at room temperature.

MSD ChemStation software (Agilent Technologies) was used for data acquisition. Linear compounds were identified by retention times, NIST MS library (Gaithersburg, MD, USA) and fragmentation patterns. Methyl alkanes were identified by diagnostic ions, standard MS databases (see above), and by determining Kovats indices using the method of Carlson et al. (1998).

Statistical analyses

In total we found 55 peaks. However, there were large qualitative differences in the chemical profiles between both castes with several peaks being only present in one caste. To analyse these qualitative differences between false workers and neotenic queens a multiple correspondence analysis (MCA, XLSTAT 2008, Addinsoft, Andernach, Germany) was performed. Peaks that contributed highly to the difference between the studied castes were selected. Differences between castes for the detected compounds were

compared with Mann-Whitney *U*-tests using SPSS 15.0 (SPSS Inc., Chicago, IL, USA). All tests were two-tailed. The significance level α was corrected for the number of tests using Bonferroni.

Results

C. secundus neotenic queens and false workers exhibit different CHC profiles as shown in Figure 1. For the 80 analyzed individuals, 55 peaks were obtained by GC analysis (FID). Using SPME and hexane extractions 40 substances could be identified with GC-MS according to their characteristic mass spectral fragmentation patterns. The general CHC profile of *C. secundus* consisted of linear alkanes, monomethyl-branched alkanes and linear alkenes with chain lengths from C21 to C35 (Fig 1). It was dominated by *n*-C25, *n*-C27, C27:1 and *n*-C29 (Fig 1).

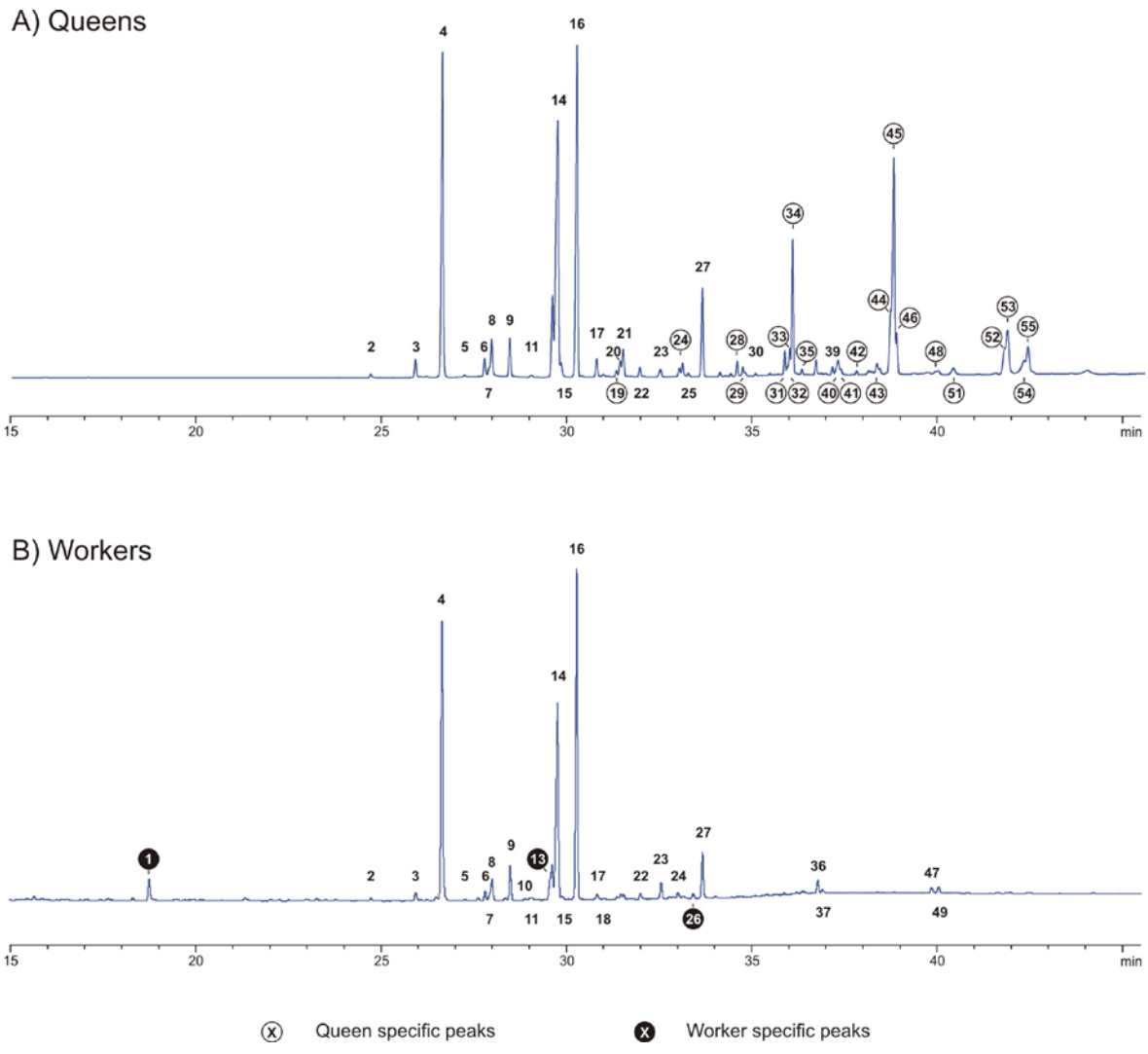


Figure 1 (previous page)

Representative chromatograms of cuticular hydrocarbons extracted by SPME from a neotenic queen (A) and a false worker (B) of *C. secundus*. Peaks 12, 38 and 50 are not labeled as they are not visible in these specific chromatograms. Only identified peaks are listed: **1** *n*-C21; **2** *n*-C24; **3** 4meC24; **4** *n*-C25; **5** 13meC25; **6** 4meC25; **7** C26:1; **8** 3meC25; **9** *n*-C26; **13** 4meC26; **14** C27:1; **15** C27:1 **16** *n*-C27; **17** 13meC27; **19** 4meC27; **20** C28:1; **21** 3meC27; **22** *n*-C28; **24** 4meC28 + C29:1; **25** C29:1; **27** *n*-C29; **28** 4meC29; **29** 3meC29; **30** *n*-C30; **31** 4meC30; **33** C31:1; **34** C31:1; **35** *n*-C31; **36** 13meC31; **39** 4meC31; **41** 3meC31; **44** C33:1; **45** C33:1; **46** C33:1; **52** C35:2; **53** C35:2; **54** C35:1; **55** C35:1.

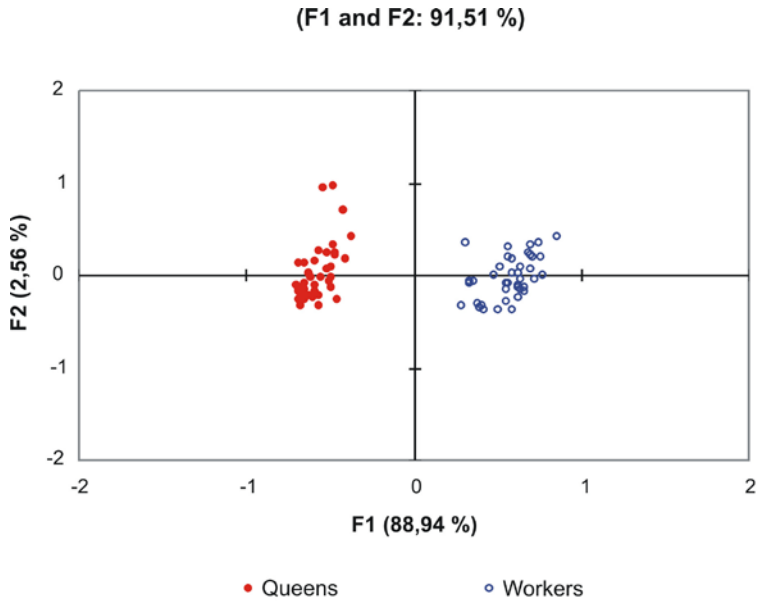


Figure 2

Correspondence map depicting MCA results. Neotenic queens are completely separated from false workers on the basis of function 1 (F1), which explains 88.94% of the variance.

According to the MCA analysis of the 55 compounds, false workers were clearly separated from neotenic queens by their CHCs (Fig. 2). There was no misclassification; all individuals of one caste clustered together (Fig. 2). The female neotenic queens were separated from false workers on the basis of function 1 (F1), which explained 88.9% of the total variance (Fig. 2). According to the contributions and cosine^2 values of the variables to function 1, 25 peaks were selected. Twentytwo of these peaks were queen specific, while 3 peaks were specific for false workers. Besides long-chained alkenes and *n*-C31, mainly monomethyl-branched alkanes were characteristic for neotenic queens (Fig. 1A). Six queen specific compounds could not be identified due to small amounts (peaks: 32, 40, 42, 43,

48, 51; Fig 1A). *n*-C21, 4meC26 and an unknown compound (peak 26) were specific for false workers (Fig 1B). All 25 compounds differed significantly between the tested castes (Mann-Whitney *U*-test $P \leq 0.0001$; < corrected $\alpha = 0.002$).

Discussion

Our analyses revealed distinct, qualitative differences in the CHC profiles of non-reproducing false workers and neotenic queens of *C. secundus*. Especially branched hydrocarbons were characteristic for the queen's CHCs. We observed a shift to hydrocarbons with longer chain lengths in neotenic queens. These two characteristic chemical patterns have been discussed as fertility cues of reproductives in social Hymenoptera (Cuvillier-Hot et al. 2001; Heinze et al. 2002; Dietemann et al. 2003; Hartmann et al. 2005; Lommelen et al. 2006). The similarity may indicate that this CHC pattern also functions as a fertility signal in *C. secundus*. Possibly, a similar "royal" signature independently evolved in hemimetabolous termites and holometabolous social Hymenoptera despite their different ancestry.

In termites, caste-specific variations in CHCs were described mainly for non-reproductives (workers, soldiers and nymphs) and "hopeful" reproductives (alates before colony foundation) (Howard et al. 1982; Haverty et al. 1996; Haverty et al. 1997; Bagneres et al. 1998; Sevala et al. 2000; Dronnet et al. 2006). Former studies also included unsexed neotenic reproductives in their analyses, but did not achieve good discrimination between reproductive and non-reproductive castes, likely due to the small sample sizes used (Howard et al. 1982; Bagneres et al. 1998).

It is unknown, whether the queen's scent is sufficient to maintain her reproductive primacy in termites. Similar to Lüscher (1974), Brent et al. (2005) suggested that the termite queen releases inhibitory stimuli which result in a reversible endocrine inhibition of the sexual development in nestmates and thus maintains the queen's reproductive dominance (Lüscher 1974; Brent et al. 2005). So far, clear evidence is lacking whether the inhibitory signal is spread throughout the termite colony via proctodeal trophallaxis or whether it has a olfactory basis (Bordereau 1985; Noirot 1990; Korb 2005). In cockroaches (Blattodea), to which the termites belong (Inward et al. 2007a), the epicuticle of sexual mature females contains contact pheromones which elicit courtship response upon contact with the male's antennae. Interestingly, methylbranched C27 and C29 carbons are active components of the female contact sex pheromone (Eliyahu et al. 2008). Stimulatory tergal compounds are also discussed for termites as sex-specific cues (Park et al. 2004). This

might suggest that similar sex-specific contact pheromones were exploited by termites during social evolution.

In summary, our results demonstrate that CHCs differed significantly among neotenic queens and false workers of *C. secundus* and therefore might provide information about the presence of a reproductive. Interestingly, among holo- and hemimetabolous social insects, female reproductives reveal similarities in the signature of chemical compounds which might reflect common mechanisms in maintaining and/or signaling reproductive status.

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General Discussion

Genetical techniques have enabled the identification of conserved molecular pathways that regulate caste development in insect societies by generating morphological, physiological and behavioral diversity. Differential gene expression between queens and workers has been studied previously in several social insect species including bees (Corona et al. 1999; Evans and Wheeler 2001b; Pereboom et al. 2005; Cristino et al. 2006; Judice et al. 2006; Wheeler et al. 2006; Barchuk et al. 2007; Grozinger et al. 2007), wasps (Sumner et al. 2006; Hoffman and Goodisman 2007; Toth et al. 2007) and one ant species (Gräff et al. 2007). However, at the beginning of this thesis no studies on the molecular basis of reproductive division of labor in the order Isoptera were available. Termites are hemimetabolous insects and both sexes are diploid, while social Hymenoptera have a holometabolous mode of development with haplodiploid sex determination. The comparison of common regulatory mechanisms of caste determination and reproductive division of labor in both groups might help to gain insights into the evolution of insects' sociality.

Publications 1 and 2 present the first data on the relationship between queen/worker castes and gene expression in termites. By using a highly sensitive subtractive hybridization method (Representational Difference Analysis) I was able to identify sixteen genes (*Neofem1-16*) whose differential expression pattern is associated with the queen phenotype in two closely related drywood termite species. In a first approach, five genes (*Neofem1-5*) were identified which were up-regulated in neotenic queens relative to false workers of *Cryptotermes secundus* (Publication 1). The follow-up study yielded two known (*Neofem2* and *3*), and eleven novel neotenic queen-specific transcripts (*Neofem6-16*) in the closely related drywood termite species *C. cynocephalus* (Publication 2). It is generally assumed that molecular functions of genes are conserved across closely related species. To investigate this issue I compared caste specific gene expression of *Neofem* genes between the studied *Cryptotermes* species (Publication 2). An intriguing finding of this study was that two termite species in the same genus are rather dissimilar in the genes that are differentially expressed between neotenic queens and false workers (Publication 2). This might be due to the different modes of replacing an absent reproductive (Publication 2), which are either a one-to-one replacement of a dead reproductive by a neotenic or an excess replacement by neotenics with subsequent fighting to the death till

the vacant breeding position is filled by a new reproductive. As life in a society is often complex with a strong environmental influence, I expanded the genetic approach and included chemical analyses (Publication 3) to ameliorate our understanding of reproductive caste determination. By comparing cuticular hydrocarbon profiles of queens and false workers, chemical patterns were detected that are discussed as fertility cues in social Hymenoptera and in sexually mature cockroach females (Publication 3).

The detected *Neofem* genes show close homologies to genes associated with metabolism (*Neofem4* and *9*), transport and storage (*Neofem3, 6, 11, 12, 13* and *14*), immune response (*Neofem4, 7*), reproduction (*Neofem1, 2, 3* and *8*), development and neurogenesis (*Neofem1, 3, 6, 13* and *14*), transcription (*Neofem15, 16*), cell communication (*Neofem11, 13* and *14*), circadian processes (*Neofem6*), and pheromone processing (*Neofem1, 2, 4* and 6) (see Publications 1 and 2). Earlier studies on differential expression between queens and workers of social insects identified genes operating in similar biological processes (Cristino et al. 2006; Sumner et al. 2006; Gräff et al. 2007; Grozinger et al. 2007; Hoffman and Goodisman 2007). Especially genes involved in metabolism, storage, immunity and transcription were repeatedly found to be up-regulated in queens (Cristino et al. 2006; Grozinger et al. 2007; Hoffman and Goodisman 2007). In social Hymenoptera, marked differences in gene expression between workers and queens are expected due to the focus of queens to exclusive maternal behavior, whereas neotenic queens of wood-dwelling termites retain their worker-like appearance (Weesner 1969) and continue performing worker related tasks. This limits the possible number of differentially expressed genes mainly to those genes associated with apparent modulatory mechanisms of egg-laying and their underlying regulatory networks as well as those responsible for maintaining the queens' reproductive dominance within the colony.

Three of sixteen detected genes (*Neofem1, 2* and 6) showed a conserved queen-specific expression profile in the two studied species *C. secundus and C. cynocephalus* and thus might be candidate genes involved in reproductive caste determination. Especially *Neofem1* and *2* were highly overexpressed in queens and the tissue specific localization of both genes revealed a head-specific expression (Publication 1). Similarity searches identified homologies of the *Neofem1* and *2* gene products to members of the esterase-lipase and ß-glucosidase family, respectively. The third common candidate gene *Neofem6,* whose overexpression in queens was less pronounced compared to *Neofem1* and *2*, was first detected in *C. cynocephalus* during the second screen and is closely related to antennal

expressed genes of mosquitoes (Publication 2). Strikingly, all three genes share homologies to genes involved in pheromone processing (Ishida and Leal 2002; Cornette et al. 2003; Justice et al. 2003; Bohbot and Vogt 2005; Claudianos et al. 2006). The close homology of *Neofem2* to a sex specific expressed ß-glycosidase of male cockroaches, which seems to function in the processing of the cockroach sex pheromone (Cornette et al. 2003), indicates a pheromonal function (Publication 1).

Homologs of *Neofem1* and *6* are described to function in antennal responses to pheromones (Publications 1 and 2). The biochemistry of insects' odor detection requires, amongst others, the involvement of odorant binding proteins and odorant degrading enzymes. Whereas the former are regarded to carry the odors from the cuticular surface to the odor receptors, the latter are subsequently degrading the odor (Ishida and Leal 2002; Bohbot and Vogt 2005). A head-expressed honeybee homolog of *Neofem1* is closely related to a putative odorant degrading esterase of the silk moth antenna (Publication 1). *Neofem6* revealed close homologies to Takeout-like proteins of mosquito antennas which are suggested to function in odorant molecule binding (Justice et al. 2003) or in the regulation of the antennal response to odors in a juvenile hormone (JH) sensitive manner (Bohbot and Vogt 2005) (see Publication 2).

Some Takeout-like transcripts are capable to bind the sesquiterpenoid JH, or its precursors (Noriega et al. 2006). The morphogenetic hormone JH plays important roles in the regulation of caste development (Nijhout and Wheeler 1982). Moreover, JH is thought to mediate honeybee behavioral maturation by interacting with the yolk precursor and storage protein vitellogenin (*Neofem3* homolog; see Publication 1). In this case JH and vitellogenin reciprocally inhibit each other and are regarded to function in sensory responsiveness and the onset of foraging behavior (Guidugli et al 2005; Amdam et al 2006; Nelson et al 2007).

In termites JH is known for its ability to induce soldier differentiation (Lüscher 1974; Okot-Kober 1983; Miura et al. 2003; Hrdy et al. 2006) and some indications exist that JH is involved in termite reproduction (Lüscher 1972; Greenberg and Tobe 1985; Brent et al. 2005). Recently, Zhou et al discussed that two hexamerins (insect storage proteins that are capable of binding JH) regulate termite caste development by modulating JH availability (Zhou et al. 2006a; Zhou et al. 2006c; Zhou et al. 2007). Based on their sequence homology to an *Aedes aegypti* JH esterase (*Neofem1*) and to putative JH binding proteins (*Neofem6*) (see Publications 1 and 2), *Neofem1* and *6* could also be discussed as regulators of JH and thus of caste development or reproduction as shown for the previous

mentioned termite studies. In *C. secundus*, however, neotenic reproductives have a ten times higher JH titer compared to all other castes (Korb et al unpublished data), which presumably exclude gene functions involved in lowering JH titer. The close homologies of *Neofem1* and *6* to antennal expressed genes lead to the assumption that both genes might be involved in pheromone processing or behavioral maturation.

In social insects the shift among tasks due to caste development is accompanied by a shift of individuals' receptiveness to certain signals (Wilson 2006). The repeated detection of caste-specific odorant-binding proteins implicates an important role as key regulators of social behavior (Krieger and Ross 2002; Gräff et al. 2007; Hoffman and Goodisman 2007). While these proteins are discussed for the recognition of queens in social Hymenoptera, in *C. secundus* they may be associated with the detection of mating-pheromones (see Publication 3).

The possible involvment of four *Neofem* genes (*Neofem1*, *2*, *4* and 6) in chemical communication (Publications 1 and 2) and the fact that ovarian activity correlates with changes in the cuticular hydrocarbon profile in many eusocial insects, led to the investigation of cuticular odor profiles in *C. secundus* (Publication 3). The comparison of CHCs between neotenic queens and false workers of *C. secundus* resulted in several qualitative differences in their odor profiles. In general the odor profile of neotenic queens appears to be more complex. This is reflected in the high number of queen-specific- (22) compared to false worker-specific compounds (3) (Publication 3). Furthermore, chemical signatures, which have been reported as characteristic cues for fecund egg-layer recognition in holometabolous ants, were also found in neotenic queens of *C. secundus* and thus seem to have a similar function in hemimetabolous termites (Publication 3). It is generally thought that these chemical patterns serve as fertility signals causing totipotent workers to refrain from reproduction in the presence of a fecund and healthy queen (Cuvillier-Hot et al. 2001; Heinze et al. 2002; Dietemann et al. 2003; Hartmann et al. 2005; Lommelen et al. 2006). Besides its similarities to holometabolous social Hymenoptera, the odor profile of neotenic queens resemble those of sexual mature females of hemimetabolous cockroaches (Publication 3). The cockroach female sex pheromone is a blend of long-chained methyl-branched carbons which elicits courtship behavior upon contact with the male's antenna. It has been suggested that this blend of female sex pheromones might be produced by a cytochrome P450 enzyme system (Eliyahu et al. 2008). Female sex-specific compounds and males' courtship behavior between termites

and cockroaches show marked similarities (Park et al. 2004). This is in line with the close relationship of these two orders, and these characteristica may have developed during social evolution of termites and cockroaches. Notably, the close homologies of the candidate gene *Neofem2* and the *Neofem4* gene to a sex specific expressed surface protein of cockroaches and a cytochrome P450, respectively imply a commonality between termite and cockroach courtship behavior.

Whether the scent of the *C. secundus* queen functions as an honest signal (Keller and Nonacs 1993) like in social Hymenoptera, as a contact sex pheromone like in cockroaches, as direct inhibitor of worker reproduction, or as a combination of these possibilities remains unclear. Lüscher's studies on the inhibition effect of wood-dwelling termite reproductives postulate an inhibition of worker reproduction via proctodeal trophallaxis (Lüscher 1956). His assumptions are the result of experiments which allowed nestmates to get in contact with the reproductive via antennas, stomodeal or proctodeal trophallxis. He concluded that the recognition of a fecund queen via antennas does not inhibit the production of same-sex neotenics, but leads to the elimination of the newly developed neotenics, as long as an established reproductive is present. Only the contact to the abdomen of the established reproductive resulted in reproductive inhibition of the nestmates (Lüscher 1956). Lüscher's observations provide the hint that termites might be able to detect reproductives according to their scent. Unfortunately the sample size of his experiments was too small to draw statistically meaningful conclusions. The significant differences in odor profiles suggest that workers could be able to recognize the presence of a fertile queen in *C. secundus*.

Outlook

To narrow down the possible functions of the *Neofem* genes for reproductive division of labor, I have started to established RNA interference (RNAi) for *C. secundus* (unpublished data). Gene silencing by RNAi offers the possibility to manipulate genetic pathways underlying important biological processes and thus is a powerful tool for the rapid and efficient probing of gene function. According to its high overexpression in female reproductives and its proposed functions, *Neofem2* was chosen as a knock-down target and the RNAi approach was combined with behavioral observations.

The analysis of false worker behavior yielded that one specific behavior had significantly changed 24 hours after queen removal. False workers received significantly more body shaking (back and forward movement of an individual) by nestmates. After

specific knockdown of the target *Neofem2* in the queen, false workers showed exactly the same behavior. Interestingly there was no behavioral switch in false workers of control colonies where the queen received either Ringer solution or control RNAi (unpublished data). These data provide preliminary evidence that silencing of the *Neofem2* gene induces false worker behavior like in a queen-less colony, which might suggest that false workers fail to recognize the queen and/or that the inhibitory stimulus of the queen was affected by RNAi treatment. Further integrative studies that include also investigations on CHC profile changes of the queen following RNAi treatment are currently under way (unpublished data). These studies will help to reveal the functions of the queen-specific *Neofem* genes and further our understanding of mechanisms underlying the evolution of sociality in termites.

Summary

Social insects (termites, ants, some bees and wasps) are prominent model organisms of evolutionary biology. Their castes are an example of phenotypic plasticity and differential gene expression produces strong differences among their traits. The highly structured life of social insects is characterized by reproductive division of labor and complexity of communication. Within a colony, nestmates mutually influence colony members in their development. This social regulation affects via hormonal regulation an individual's gene expression. The latter controls the development of an individual, whereby this individual in turn influences nestmates with pheromones or social regulation. For instance, in social Hymenoptera or termites the presence of a reproductive inhibits the development of other reproductives within the nest. These feedback mechanisms ensure an adapted and self-regulating caste differentiation within an insect society. While substantial progress has been achieved in explaining ultimate causes of social behavior, the proximate mechanisms behind the division into reproducing and non-reproducing castes are poorly understood in molecular terms. Especially, the maintenance of reproductive primacy by fertile individuals (in termites: king and queen) remains a mystery.

In this thesis the proximate basis of reproductive division of labor in lower wood-dwelling termites was investigated on the molecular (differential gene expression) and chemical (cuticular hydrocarbon profiles) level.

To identify genes involved in this important process, I studied differential gene expression between neotenic queens and false workers by using a highly sensitive subtractive hybridization method (Representational Difference Analysis; RDA). Major advantages of RDA are its independence of prior sequence knowledge (in termites genomic information is scarce) and high enrichment of genes with a strong differential expression profile in the target population. Queen specificity of identified transcripts was confirmed by quantitative realtime PCR (qRT-PCR) analyzing different castes.

In a first screen five genes (called *Neofem1-5*) were found to be overexpressed in neotenic queens of the Australian drywood termite *Cryptotermes secundus*. Additionally, an approximate localization of *Neofem* expression was obtained by analyzing their relative mRNA levels in different body parts (head, thorax, and abdomen) of neotenic queens. Four *Neofem* genes were over expressed in the queen's head while one was almost equally expressed in all body parts. Especially, three genes (a putative esterase-lipase, a ß-

glucosidase and a cytochrom P450 homolog) were up-regulated in heads of *C. secundus* neotenic queens and were likely to be important female reproductive specific genes.

It is generally assumed that molecular functions of genes playing a prominent role in caste differentiation are conserved across closely related species. To investigate this issue I performed a second RDA of cDNAs with the closely related drywood termite *Cryptotermes cynocephalus*. Both species, *C. cynocephalus* and *C. secundus*, differ in the mode of replacing an absent reproductive. This follow-up study yielded known, as well as eleven novel neotenic queen specific genes. Homologs of the sixteen *Neofem* genes are associated with diverse biological processes such as metabolism, transport and storage, immune response, reproduction, development and neurogenesis, transcription, cell communication, circardian processes and pheromone processing. The comparison of caste specific gene expression of *Neofem* genes between the studied *Cryptotermes* species revealed that two termite species of the same genus are rather dissimilar in the genes that are differentially expressed between neotenic queens and false workers. Only three genes showed a conserved queen-specific expression profile in both species and were discussed to be candidate genes involved in reproductive caste determination. Interestingly, all three genes are suggested to be involved in chemical communication.

Chemical communication plays a crucial role in coordinating and regulating the highly structured life in complex insect societies. In many eusocial insects the ovarian activity correlates with changes in the cuticular hydrocarbon (CHC) profile and it is assumed that the recognition of a fertile queen on account of her characteristic odor profile prevents capable workers from own reproduction. For lower termites a royal control of worker reproduction was suggested, but so far the nature of this control remains unknown. To study whether chemical cues signaling the presence of a fertile termite queen exist, I analyzed CHC profiles in correlation to the reproductive status of individuals in *C. secundus*. By using gas chromatography (GC) and GC-mass spectrometry analysis, significant qualitative differences between neotenic queens and false workers were detected in their CHC profiles. Characteristic chemical patterns of the termite queen's odor bouquet are discussed as fertility cues in social Hymenoptera and in sexually mature cockroach females.

Interestingly, molecular and chemical analysis revealed similarities between hemimetabolous and holometabolus insects, which might have been evolved in both insect groups despite the different ancestry.

To investigate possible functions of the identified genes over expressed in neotenic queens I started to establish RNA interference (RNAi) for *C. secundus*. This RNAi approach might help to gain further insights into the reproductive division of labor by probing gene function of *Neofem* genes.

This thesis presents the first data on the relationship between queen/worker castes and genes expression as well as on differences in chemical odor profiles associated with fertility in termites. It contributes to the emerging field of sociogenomics and provides a step forward to understand the proximate basis of a key characteristic of insects' sociality - the reproductive division of labor.

Zusammenfassung

Soziale Insekten (Termiten, Ameisen, einige Bienen und Wespen) sind bedeutende Modellorganismen der Evolutionsbiologie. Ihre Kasten sind ein Beispiel phänotypischer Plastizität, wobei die großen Unterschiede zwischen den Kasten auf differentieller Genexpression beruhen. Die hochkomplexen Strukturen sozialen Lebens dieser Insekten sind durch eine reproduktive Arbeitsteilung und vielfältige Kommunikationsmechanismen gekennzeichnet. Innerhalb einer Kolonie beeinflussen sich Nestmitglieder gegenseitig in ihrer Entwicklung. Diese soziale Regulation wirkt sich über die Ebene der hormonellen Regulation auf die Genexpression einzelner Individuen aus. Die Genexpression steuert wiederum die Entwicklung eines Individuums, wobei dieses Individuum seinerseits Nestmitglieder über Pheromone oder durch soziale Regulation beeinflußt. Bei sozialen Hymenopteren oder Termiten hemmt beispielsweise die Anwesenheit von Geschlechtstieren die Entwicklung weiterer Geschlechtstiere im Nest. Durch diesen Feedback-Mechanismus wird innerhalb einer Insektengemeinschaft eine angepaßte und sich selbst regulierende Kastendifferenzierung gewährleistet.

Während bei der Untersuchung der ultimativen Ursachen sozialen Verhaltens große Fortschritte erzielt werden konnten, sind die unmittelbaren Mechanismen, welche hinter der Aufspaltung in reproduzierende und nicht reproduzierende Kasten stehen, vor allem auf molekularer Ebene noch unzureichend verstanden. Ein besonderes Mysterium hierbei ist die Aufrechterhaltung der reproduktiven Vorrangstellung fruchtbarer Individuen.

Die unmittelbaren Grundlagen reproduktiver Arbeitsteilung auf molekularer (differentielle Genexpression) und chemischer (kutikuläre Kohlenwasserstoffe) Ebene wurden während dieser Doktorarbeit an niederen, holzbewohnenden Termiten eingehend untersucht.

Um Gene zu identifizieren, die in diesem wichtigen Prozess der Sozialität von Insekten involviert sind, studierte ich mittels einer hochsensitiven subtraktiven Hybridisierungsmethode (Repräsentative Differenzanalyse; RDA) die differentielle Genexpression zwischen neotenen Königinnen und falschen Arbeitern. Bedeutende Vorteile dieser Methode sind die Unabhängigkeit von Sequenzkenntnissen (da nur wenig Information über das Termitengenom existiert) und die hohe Anreicherung von Genen mit sehr stark differentiellem Expressionsmuster in der Zielpopulation. Um zu bestätigen, dass die so identifizierten Transkripte spezifisch für neotene Königinnen sind wurde ihre

Expression in verschiedenen Kasten mittels quantitativer „realtime-PCR“ (qRT-PCR) analysiert.

In einem ersten Screen wurden fünf Gene (*Neofem1-5* benannt) identifiziert, die in neotenen Königinnen der australischen Trockenholztermite *Cryptotermes secundus* überexprimiert waren. Für eine annähernde Lokalisierung der *Neofem* Geneexpression wurden zusätzlich ihre relativen mRNA-Spiegel in verschiedenen Körperregionen (Kopf, Thorax und Abdomen) analysiert. Vier *Neofem* Gene waren im Kopf der Königin hochreguliert, während eines gleichmäßig in allen Körperregionen exprimiert wurde. Besonders die Expression dreier Gene (eine putative Esterase-Lipase, eine ß-Glukosidase und ein Cytochrom P450 Homolog) war in den Köpfen der neotenen Königinnen hochreguliert, wobei es sich sehr wahrscheinlich um wichtige Gene weiblicher Reproduktiver handelt.

Man geht generell davon aus, dass Gene, welche eine wichtige Rolle während der Kastendifferenzierung spielen, in nah verwandten Arten konserviert sind. Um dies zu überprüfen, habe ich eine zweite RDA mit der nah verwandten Trockenholztermite *Cryptotermes cynocephalus* durchgeführt. Beide Arten, *C. cynocephalus* und *C. secundus*, unterscheiden sich in der Art und Weise, wie ein fehlender Reproduktiver ersetzt wird. Sowohl bekannte als auch neue königinnenspezifische Gene konnten in dieser Anschlussstudie identifiziert werden. Homologe dieser 16 *Neofem* Gene sind mit verschiedenen wichtigen biologischen Prozessen assoziiert wie zum Beispiel Metabolismus, Transport und Lagerung, Zellkommunikation, Immunantwort, Reproduktion, Entwicklung und Neurogenese, Transkription, zirkadiane Prozesse und Pheromonprozessierung. Der kastenspezifische Vergleich der Genexpression dieser *Neofem* Gene zwischen den untersuchten *Cryptotermes* Arten ergab, dass zwei Termiten der gleichen Gattung deutliche Unterschiede in differentiell exprimierten Genen zwischen neotenen Königinnen und falschen Arbeitern aufweisen. Nur drei Gene wiesen ein konserviertes Königinnen-spezifisches Expressionsmuster in beiden Arten auf und wurden deshalb als Kandidatengene, die in der reproduktiven Kastendetermination involviert sind, diskutiert. Interessanterweise sind scheinbar alle drei Gene an der chemischen Kommunikation beteiligt.

Die chemische Kommunikation spielt eine tragende Rolle in der Koordination und Regulation des hochstrukturierten Lebens komplexer Insekten Sozietäten. In vielen eusozialen Insekten korreliert die Ovarienaktivität mit Änderungen des kutikulären Kohlenwasserstoffprofils (CHC-Profil) und man nimmt an, dass die Erkennung einer

fruchtbaren Königin, auf Grund ihres charakteristischen Geruchsprofils, totipotente Arbeiter davon abhält sich zu reproduzieren. Bei niederen Termiten wird davon ausgegangen, dass das Königspaar die Reproduktion von Arbeitern kontrolliert, jedoch ist die Art und Weise dieser Kontrolle unbekannt. Um zu erfahren, ob chemische Stimuli existieren, welche die Anwesenheit einer fruchtbaren Termitenkönigin signalisieren, habe ich bei *C. secundus* CHC-Profile im Zusammenhang mit dem reproduktiven Status von einzelnen Individuen untersucht. Mit Hilfe gaschromatographischer (GC) sowie GC-massenspektrometrischer Analysen wurden signifikante qualitative Unterschiede zwischen neotenen Königinnen und falschen Arbeitern in deren CHC-Profilen entdeckt. Charakteristische chemische Muster des Duftgemischs der Termitenkönigin werden auch als Fertilitätssignale für soziale Hymenopteren und sexuell reife weibliche Schaben diskutiert.

Interessanterweise wurden, sowohl bei den molekularen als auch bei den chemischen Analysen, Gemeinsamkeiten zu hemi- und holometabolen Insekten gefunden, die möglicherweise in beiden Insektengruppen trotz ihrer unterschiedlichen Herkunft entwickelt wurden.

Um mögliche Funktionen der in neotenen Königinnen überexprimierten Gene zu untersuchen, habe ich damit begonnen RNA Interferenz (RNAi) für *C. secundus* zu etablieren. Dieser RNAi Ansatz könnte die Erforschung der Funktionen der *Neofem* Gene ermöglichen und helfen, einen tieferen Einblick in die molekularen Mechanismen der reproduktiven Arbeitsteilung zu erlangen.

Diese Doktorarbeit präsentiert die ersten Daten zur differentiellen Genexpression von Königin- und Arbeiterkaste sowie Unterschiede in chemischen Geruchsprofilen, die mit der Fruchtbarkeit bei Termiten zusammenhängen.

Sie trägt somit zu dem immer wichtiger werdenden Bereich der Soziogenomik bei und ermöglicht einen Einblick in die unmittelbaren Grundlagen eines der Schlüsselkriterien der Sozialität bei Insekten - die reproduktive Arbeitsteilung.

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Appendix

Additional material of Publication 1

Additional file 1

List of used Oligonucleotides

Oligonucleotides used in this study for detection of Neofem expression in qRT-PCR, RACE PCR, RDA and standard applications.

Oligonucleotides	**Sequence (5' – 3')**
Real time-Oligonucleotides	
LC-Neofem1-S	CTA TTC TTC ATA CCC ACA CTA GCC CC
LC-Neofem1-AS	CTT GGG AGT TCG CTG TTG CC
LC-Neofem2-S	GAT TAC CAC AAT TTT CCA CGG AGG
LC-Neofem2-AS	CAC GAG AGT CAC TCC TGT GTC CC
LC-Neofem3-S	CTC TGA ATC TGA CGA AAC AGC G
LC-Neofem3-AS	GCT TCT TTC CAT ATG CAG AGG G
LC-Neofem4-S	CAA TCA GCG CTT CAT CCC AC
LC-Neofem4-AS	TAT CTC GCT TGT TGC CTC CC
LC-Neofem5-S	TCT CCG ACA TCT ATC TGT GCC G
LC-Neofem5-AS	TGC AAC TAC CAC GAC GAC GG
LC-trans-S	GAC TTC GAG CTC CTG TGT CCT G
LC-trans-AS	ACA GTC TGA AGA GGT CCG GTC G
LC-18-S	AGG TGA AAT TCT TGG ATCGTC GC
LC-18-AS	AGT CAT CGG AGG AAC TTC GGC
RACE-Oigonucleotides	
5' RACE	
outer-Neo1-5'	TGT AGT AAT AGA GAG GTG CAC CGC
inner-Neo1-5'	TCA TAG AAG AAT GCT ATT GGA GCC

outer2-Neo1-5’	AGA AAC GAA GAA CCA CTC ATG GC
inner2-Neo1-5’	TAA TAG AGC CAA CAC TGT AGC CTG C
outer-Neo2-5’	TTT GTT GTG TAT TGA TTG AGA CCG
inner-Neo2-5’	GTA ATC GCG ATT TTA GGT AGC
outer-Neo3-5’	ATT TCT GTT CGA AGT AGT TAA GCG C
inner-Neo3-5’	TAC GAG TTA CAG ATG CAA ACA GGG
outer-Neo4-5’	TAT CTC GCT TGT TGC CTC CC
inner-Neo4-5’	TAC ATG CAG TGG GAT GAA GCG
outer-Neo5-5’	TGC AAC TAC CAC GAC GAC GG
inner-Neo5-5'	ACA GAT AGA TGT CGG AGA GGC G

3’ RACE

outer-Neo1-3'	TTA TAT CTC ATC TGA GCG GTG CAC
inner-Neo1-3'	ACT ACA AGT TCT CAT ACC AAG GCC G
outer- Neo2-3'	GGA GTC TGC TGG ACA ACA TGG
inner-Neo2-3'	GGG AAA TTG TCA TCA CTG AAA ATG
outer-Neo3-3'	TAC ATT GGA AGC AGC GAC AGC
inner-Neo3-3'	TGG TCC AGT GTG AAA GAA GCG
outer-Neo4-3'	CAA TCA GCG CTT CAT CCC AC
inner-Neo4-3'	CAA CAA GCG AGA TAC CTG GCC
outer-Neo5-3'	TCT CCG ACA TCT ATC TGT GCC G
inner-Neo5-3’	TGG ACG ACG TGA TGG TAG GG
outer-trans-3'	ACA GTC TGA AGA GGT CCG GTC G
inner-trans-3'	AAC TCT ACA GCA AGC GAC CGG

Standard - Oligonucleotides

R-Bgl-24 [1]	AGC ACT CTC CAG CCT CTC ACC GCA
R-Bgl-12 [1]	GAT CTG CGG TGA
J-Bgl-24 [1]	ACC GAC GTC GAC TAT CCA TGA ACA
J-Bgl-12 [1]	GAT CTG TTC ATG

N-Bgl-24 [1]	AGG CAA CTG TGC TAT CCG AGG GAA
N-Bgl-12 [1]	GAT CTT CCC TCG
Neo1-S1	TAA TGT TGC ACA TGG TGT CAC C
Neo1-S2	AAG CAC TGT CTG TCA TTC TAA ACT CCT
Neo1-S3	TTA TAT CTC ATC TGA GCG GTG CAC
Neo1-S4	ACT ACA AGT TCT CAT ACC AAG GCC G
Neo1-AS1	GAC AGA CAG TGC TTT ATG TGA AAC TCC
Neo1-AS2	TAG AAG AAT GCT ATT GGA GCC ACA G
Neo1-AS3	TTG GGA GTT CGC TGT TGC C
Neo2-S1	GAG GGT GAC TAT CCG ACG GTC
Neo2-AS1	GGG AAA TTG TCA TCA CTG AAA ATG
Neo2-AS2	GGA GTC TGC TGG ACA ACA TGG
Neo3-S1	TAC ATT GGA AGC AGC GAC AGC
Neo3-S2	TGG TCC AGT GTG AAA GAA GCG
Neo3-S3	TAA GAT TGA CTC TGG CCT TCC C
Neo3-S4	ATC AAA TTC ACC GAC ATG AGC G
Neo3-S5	AGA TCT CGT GTC CCT CAG CC
Neo3-AS1	TCG TGT CTG GTA GTA TAA GGC AGC
Neo3-AS2	ATA GCC TTC TTC TGT TTA TCC GC
Neo3-AS3	CCT TCT TGC TGA AGT CGG GG

1. Hubank M, Schatz DG: **cDNA representational difference analysis: a sensitive and flexible method for identification of differentially expressed genes**. *Methods Enzymol.* 1999, **303**:325-349.

Supplementary materials of Publication 2

Supplementary Table 1

List of used oligonucleotides for PCR

Oligonucleotides	Sequence (5' – 3')
RACE-Oigonucleotides[a]	
outer3'-Neofem1	ACT ACA AGT TCT CAT ACC AAG GCC G
inner3'-Neofem1	CTA TTC TTC ATA CCC ACA CTA GCC CC
outer3'-Neofem2	GAG GGT GAC TAT CCG ACG GTC
inner3'-Neofem2	GAT TAC CAC AAT TTT CCA CGG AGG
outer3'-Neofem4	CAA TCA GCG CTT CAT CCC AC
inner3'-Neofem4	CAA CAA GCG AGA TAC CTG GCC
outer3'-Neofem5	TCT CCG ACA TCT ATC TGT GCC G
inner3'-Neofem5	TGG ACG ACG TGA TGG TAG GG
inner3'-Neofem6	CAT CTG TCC AAC TTG TTC AAC G
inner3'-Neofem7	CCG AGT TCC ATC AGC ATC TG
inner3'-Neofem8	GCT ATT GAC AGA GAC TGC CA
inner3'-Neofem9	GGT TTA TTT GGC TGC TGT TAT GG
inner3'-Neofem10	CCT AAC CTC ATC CTC CTT CAG AC
inner3'-Neofem11	GCG GAG AAG GAT GAT AAT GG
inner3'-Neofem12	GCT GTT GAA GAA AGT CCG CA
inner3'-Neofem13	TGG TGT TTA TGG TGT TAG CC
inner3'-Neofem14	GAA GGA GTG ATA GTT CAT GTT ACA G
inner3'-Neofem15	ACA GTC GAT AGG ATG AAG AAA CG
inner3'-Neofem16	CGA GGA ACA GAG TAC CTG GG

Real time-Oligonucleotides

C. cynocephalus

ccNeofem1-S	GGG CAA ACT TCA TCC AAA CAG
ccNeofem1-AS	TCC AGA TAG GCA AGG TTC TCA G
ccNeofem2-S	TAC ATG ATC TGG AGT CTG CTG G
ccNeofem2-AS	GCG ATG GTC TTC ACT AAC TCT G
ccNeofem3-S	TGC ACA ACA CAC AGG ACC AAG
ccNeofem3-AS	GCC TTC TTC TGT TTA TCC GCG
ccNeofem4-S	GAA AGT GCTG ATG GAA CAG AAG G
ccNeofem4-AS	GAT GGG TAG AGT CGG ATG GT
ccNeofem6-S	AGAGAAACGGCAACAGATACCAC
ccNeofem6-AS	AGT TCC CAA CAG TTT GTC GCC
ccNeofem7-S	GAT CCC GAG AAG CCA AAT CCT G
ccNeofem7-AS	CGC TGG TCT TCA CGT TCC TC
ccNeofem8-S	GCA CTT CTC CAT GAG CAT CCA G
ccNeofem8-AS	GCA GTC TCT GTC AAT AGC ACC AC
ccNeofem9-S	TGT GGG TCG TAT TCA TCG GCT
ccNeofem9-AS	CCA ACT CCA GAA CTT CGG CAG
ccNeofem10-S	GAG GGC GTT GGA TGA GAT GAC
ccNeofem10-AS	TTG TCT GAA GGA GGA TGA GGT TAG G
ccNeofem11-S	TCA TTC ACA GAC AGG AAC TCA TTA CGA C
ccNeofem11-AS	TTC CAT TAT CAT CCT TCT CCG CCA G
ccNeofem12-S	GCT TCG TTG TCT TCA TAC TCG CA
ccNeofem12-AS	AAC CAG CTT TCT CTT CAG TTC CAG
ccNeofem13-S	CCA ACT TCA GCA GCG GTC AG

ccNeofem13-AS	GGC TAA CAC CAT AAA CAC CAC CAG
ccNeofem14-S	CAG CAT CCA CTC CAG ACA CC
ccNeofem14-AS	TGT AAC ATG AAC TAT CAC TCC TTC CTC C
ccNeofem15-S	GAC AGG TCC TTC AGC GAC ATC AG
ccNeofem15-AS	TCG ACT GTG AAT ATT CTT GTG TCT CGT G
ccNeofem16-S	CTC CCA ATT AGA AAC TCT CAA GGA ACA C
ccNeofem16-AS	AGT GTA TCG CTC CCA GGT ACT C

C. secundus

csNeofem6-S	CAT CTG TCC AAC TTG TTC AAC G
csNeofem6-AS	ACG ATG TTG CCT ACG ACC TG
csNeofem7-S	CGA CAA CTC GTA CTA TCT CCA C
csNeofem7-AS	ACA GAT CCA TTG CAT ACA GTT CAG
csNeofem8-S	GCT ATT GAC AGA GAC TGC CAC
csNeofem8-AS	ATC CTT GCA GCA GTA TTC CC
csNeofem9-S	CAT AAT TAC CCT TAC GCA GAA GCC
csNeofem9-AS	AGT TTG TTC AAT TCC TCG TCG T
csNeofem10-S	CCT TCA GAC AAT AGG GAT TCC A
csNeofem10-AS	GCT CTT CTA GTA ACC TAA TCA CTT CC
csNeofem11-S	GAA GGA TGA TAA TGG AAA GAC TGA C
csNeofem11-AS	ACA CTG AAA CAT GAA GCC AC
csNeofem12-S	CGG TGA TGT TGG TCA GTG TC
csNeofem12-AS	AAA CAG CTG CAC AAT AAC TAC G

[a] For *Neofem6-16* sense "S" real time oligonucleotides of *C. cynocephalus* were used as outer RACE oligonucleotides

Supplementary Table 2

Sequence comparison of Neofem1-11 genes

Neofem	**% Identity**	**Overlap (bp)**
1	93,5	661
2	94,5	934
3	93	942
4	92,8	697
6	95,2	375
7	93,4	483
8	90,9	144
9	74,6	122
10	97,2	72
11	99,5	222

Eidesstattliche Erklärung

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe eines Literaturzitats gekennzeichnet.

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(Ort, Datum) (Tobias Weil)