The Scent of Royalty: A P450 Gene Signals Reproductive Status in a Social Insect

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Abstract

Cooperation requires communication; this applies to animals and humans alike. The main communication means differ between taxa and social insects (ants, termites, and some bees and wasps) lack the cognitive abilities of most social vertebrates. Central to the regulation of the reproductive harmony in insect societies is the production of a royalty scent which signals the fertility status of the reproducing queen to the nonreproducing workers. Here, we revealed a central genetic component underlying this hallmark of insect societies in the termite *Cryptotermes secundus*. Communication between queens and workers relied upon the expression of a gene, *Neofem4*, which belongs to the cytochrome P450 genes. We inhibited *Neofem4* in queens by RNA interference. This resulted in the loss of the royalty scent in queens and the workers behaved as though the queen were absent. The queen's behavior was not generally affected by silencing *Neofem4*. This suggests that the lack of the royalty scent lead to workers not recognizing her anymore as queen. P450 genes are known to be involved in the production of chemical signals in cockroaches and their expression has been linked to a major fertility regulator, juvenile hormone. This makes P450 genes, both a suitable and available evolutionary substrate in the face of natural selection for production of a queen substance. Our data suggest that in an organism without elaborate cognitive abilities communication has been achieved by the exploitation of a central gene that links the fertility network with the chemical communication pathway. As termites and social Hymenoptera seem to share the same class of compounds in signaling fertility, this role of P450 genes might be more widespread across social insects.

Key words: communication, sociogenomics, cytochrome P450, social insects.

Introduction

Stable cooperation requires communication; this applies as much to other animals as it does to humans (Boyd et al. 2010; Hauber and Zuk 2010; Janssen et al. 2010). In humans, cooperation is often contingent upon the reputation of a person (Fehr and Fischbacher 2003; Gardner and West 2004; Rockenbach and Milinski 2009) and by this it is linked to the evolution of language and probably the FoxP2 gene circuit (Scharff and Petri 2011). However, reputation requires high cognitive abilities but these are absent in the group of animals that is often regarded as the pinnacle of social evolution, the social insects (termites, ants, and some bees and wasps). Considerable progress has been made in explaining why some individuals (workers, soldiers) forgo their own reproduction in these insect societies (Bourke 2011). Specifically, genetic relatedness (Hamilton 1964) combined with ecological factors (Korb and Heinze 2008), and sometimes policing behaviors (Wenseleers and Ratnieks 2006), appear sufficient to explain the altruism of workers/soldiers at an evolutionary level. But how this is achieved at the molecular level represents a new frontier for sociobiology ("sociogenomics") (Robinson et al. 2005; Smith et al. 2008; Barron and Robinson 2009). The "reproductive harmony" in social insects, expressed in the queen's reproductive monopoly, is often maintained via chemical signals on the queen's cuticle (cuticular hydrocarbons, CHC) that reveal her fertility to the nonproductive workers (fig. 1) (Barron and Robinson 2009; Peeters and Liebig 2009; Liebig 2010; Matsuura et al. 2010; van Zweden 2010; van Oystaeyen et al. 2014). When the queen becomes less fertile or unhealthy her scent changes and then, in species with workers still capable of reproducing, new individuals become queens and/or workers start to lay eggs (Peeters and Liebig 2009; Liebig 2010; van Oystaeyen et al. 2014). The genetic mechanism underlying this royalty scent is central to the reproductive monopoly by the most fertile individual within a colony. In the best studied social insect, the honeybee *Apis mellifera*, where recently the queen inducing substance, royalactin, was identified (Kamakura 2011), the queen mandibular pheromone is thought to act as royalty scent (Kocher et al. 2009), yet its genetic basis in honeybees, as in all other social insects, remains unclear. Here, we used a combination of RNA interference (RNAi), behavioral assays, and chemical analyses to identify a gene involved in the production of the queen's royalty scent.

Article

Fig. 1. *Cryptotermes secundus* neotenic queen (top) and worker (bottom).

Fig. 2. Relative expression levels of the *Neofem4* gene in different treatments. Relative expression levels of the *Neofem4* gene in 1) untreated queens, and in queens 24 h after injection of 2) *Neofem4* siRNA, 3) control siRNA, and 4) Ringer's solution. Shown are boxplots with median, quartiles, and minimal and maximal values. The relative expression of *Neofem4* was significantly lower after *Neofem4* siRNA treatment compared with individuals treated with control siRNA (*t*-test: $T_{14} = -3.42$, SEM: ± 0.97, after FDR: $P < 0.05$), injection of Ringer's solution (*t*-test: $T_{14} = -7.15$, SEM: ± 0.25, after FDR: $P < 0.05$) and also compared with untreated queens (*t*-test: $T_{14} = -3.97$, SEM: ± 1.58, after FDR: $P < 0.05$). Although the expression levels between control siRNA and Ringer's solution (*t*-test: $T_{14} = -1.55$, SEM: ± 1.00, after FDR: $P > 0.05$) and control siRNA and untreated queens did not differ significantly (*t*-test: $T_{14} = 1.59$, SEM: ± 1.85, after FDR: $P > 0.05$) untreated queens differed significantly from Ringer's solution (*t*-test: $T_{14} = 2.82$, SEM: ± 1.60, after FDR: $P < 0.05$). These results were identical with and without false discovery rate (FDR) correction for multiple testing. Different letters indicate significant differences (see also supplementary fig. S1, Supplementary Material online). For all groups $n = 7$.

By using cDNA representational difference analysis (cDNA-RDA), we previously identified genes that are specifically overexpressed in queens but not in kings and workers of the lower termite *Cryptotermes secundus* (Weil et al. 2007). For one of these genes, *Neofem2*, we could show its functional significance in maintaining the queen's reproductive monopoly (Korb et al. 2009), but follow up studies were unsuccessful in revealing a link to chemical communication. A promising candidate for a royalty scent gene is *Neofem4* which belongs to the cytochrome P450 genes (Weil et al. 2007). Cytochrome P450s are ubiquitous heme-containing oxidative enzymes found in all organisms. In insects, P450s play diverse roles: They are also involved in the biosynthesis of CHCs (Reed et al. 1994; Howard and Blomquist 2005) and several P450s have been linked to Juvenile Hormone (JH) production or degradation (Sutherland et al. 1998; Feyereisen 2005). JH titers in adult insects are important regulators of female fertility (Peeters and Liebig 2009). This has also been shown for termites and their closest relatives, the cockroaches, where JH titers and fertility are positively correlated probably through the effect that increasing JH drives vitellogenesis and other processes related to gamete production (Engelmann 2002; Brent 2009; Korb, Hoffmann, Hartfelder 2009). Thus, we hypothesized that *Neofem4* plays a critical role in queen-worker communication as it might link fertility and royalty scent. This we tested with experiments; we investigated the functional significance of high *Neofem4* expression on the queen's reproductive monopoly first and then its influence on her royalty scent via RNAi.

In lower termites, workers are totipotent and can develop into replacement reproductives when the queen or king of the colony dies (Korb and Hartfelder 2008) (fig. 1). In these species, the presence of the reproductives prevents the final imaginal molt of the immature termite workers that would render the workers reproductive and break down the reproductive division of labor (Matsuura et al. 2010). As it is not possible to induce a worker to molt into a new queen within the short functional period of RNAi (former experiments reve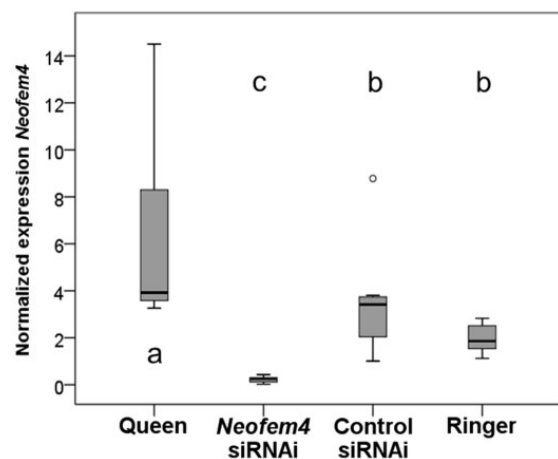aled a decline of gene knockdown after 48 h), we used an established behavioral assay that can function as a proxy for the absence of queens: Queenless colonies are characterized by a specific behavioral change in workers; the frequency of antennation and especially butting interactions received by workers from other workers increases in queenless colonies (Korb et al. 2009; Hoffmann and Korb 2011). Butting is characteristic for workers developing into replacement reproductives in queenless colonies and thus can be used as an indicator of the queen's absence and the eventual succession of the queen by a worker that develops into a replacement reproductive; it is indicative for the break down of the reproductive monopoly (Korb et al. 2009) (see also Materials and Methods).

Results

After Silencing *Neofem4* Workers Behave as in Queenless Colonies

In an experiment involving a total of 28 queen-right colonies (see also Materials and Methods), we successfully silenced *Neofem4* in queens with RNAi (fig. 2) (the expression of the control gene *β-actin* was not affected by the treatment; supplementary fig. S1, Supplementary Material online) and
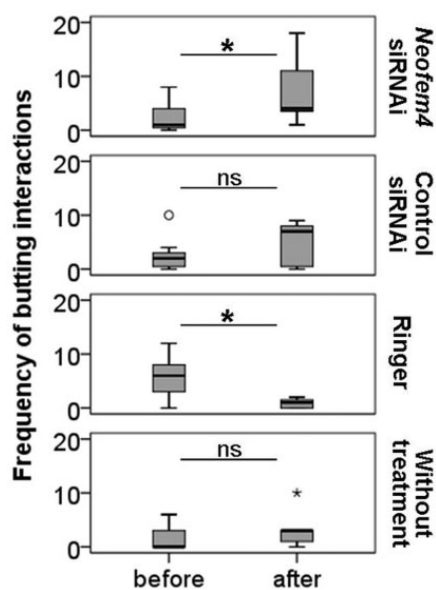
FIG. 3. Frequency of butting among workers before and after queen treatments. Frequency of butting interactions among workers before and after treatment of the queen with *Neofem4* siRNA, control siRNA, Ringer's solution, and without treatment. Shown are boxplots with median, quartiles, and minimal and maximal values. The frequency of butting by workers increased significantly only after treatment of the queen with *Neofem4* siRNA indicated by a large asterisk (see also supplementary tables S1 and S2 and fig. S3, Supplementary Material online).

recorded the behavioral repertoire 1 day before and 1 day after silencing. Silencing had no observable effect on queen behavior with one exception that she antennated more (Wilcoxon paired rank tests: Always $P > 0.05$, except antennation: $Z = -2.21$, $n = 7$ pairs, $P = 0.027$; supplementary table S1 and fig. S2 and S2E, Supplementary Material online) which was probably a response to the increased antennation she received by workers (see below). Hence, the queen did not change her behavior, which would have happened if she had been sickened by the treatment (Korb and Fuchs 2006). In contrast, silencing *Neofem4* had a distinctive effect on worker behavior. Exactly as is typical for queenless colonies (Korb et al. 2009; Hoffmann and Korb 2011), the frequency of butting behavior, and to a lesser extent antennation, increased after gene silencing (Wilcoxon paired rank test: Butting: $Z = -2.37$, $n = 7$ pairs, $P = 0.018$; antennation: $Z = -1.87$, $n = 7$ pairs, $P = 0.062$; fig. 3 and supplementary fig. S3, Supplementary Material online) while no other behavior was significantly affected (Wilcoxon paired rank tests: Always $P > 0.600$; supplementary table S2, Supplementary Material online). Importantly, injecting control siRNA ("nonsense" siRNA not targeted at *Neofem4*; see Materials and Methods) had no observable effect on worker behavior (Wilcoxon paired rank tests: Always $P > 0.100$; supplementary table S3 and fig. S4, Supplementary Material online) which shows that the change in worker behavior observed with *Neofem4* siRNA was a specific response to silencing *Neofem4* and not to injecting siRNA. Treating queens with Ringer's solution changed the worker's behavior but in a

manner untypical for queenless colonies: Workers decreased butting and antennation after treatment with Ringer's solution (Wilcoxon paired rank tests: Butting: $Z = -2.20$, $n = 7$ pairs, $P = 0.028$; fig. 3; antennation: $Z = -2.03$, $n = 7$ pairs, $P = 0.042$; supplementary table S3 and fig. S5, Supplementary Material online), which is in the opposite direction of the observed *Neofem4* effect (supplementary fig. S3, Supplementary Material online).

Silencing of *Neofem4* Affects Royalty Scent

To test for an effect of *Neofem4* on the royalty scent, we studied the CHC profile of the same queens and workers before and after RNAi treatment. Like in many social Hymenoptera (van Oystaeyen et al. 2014), the queen scent in *C. secundus* is characterized by a number of queen-specific long-chained CHCs that are absent in workers (supplementary tables S4 and S5, Supplementary Material online; fig. 4) (Weil et al. 2009). After silencing *Neofem4*, the CHC profile of queens changed. On the basis of one discriminant function that accounted for 100% of the variance (Wilks' $\lambda = 0.08$, $\chi^2_{10} = 17.40$, $P = 0.066$), 100% of the queens were assigned correctly to before and after *Neofem4* silencing (figs. 5 and Supplementary fig. S6C, Supplementary Material online). The control treatments did not affect the CHC profiles (discriminant analysis [DA]: Wilks' $\lambda = 0.63$, $\chi^2_{10} = 15.93$, $P = 0.101$; fig. 5 and supplementary fig. S6, Supplementary Material online). The CHC profile of *Neofem4* silenced queens became significantly less queen-like with a reduction in queen-specific substances whereas worker-like substances increased (supplementary table S6, Supplementary Material online and fig. 4). Importantly, injecting Ringer's solution or control siRNA did not change the CHC profile of queens (DAs: Ringer's solution: Wilk's $\lambda = 0.58$; $\chi^2_8 = 4.30$; $P = 0.829$; Control siRNA: Wilk's $\lambda = 0.51$; $\chi^2_{10} = 4.78$; $P = 0.905$; supplementary fig. S6D, Supplementary Material online) nor did the worker's CHC profile change after silencing (DA: Wilks' $\lambda = 0.81$, $\chi^2_{13} = 10.28$, $P = 0.671$; supplementary fig. S6B, Supplementary Material online). This strongly implies that the specific silencing of *Neofem4* changed the queen scent by a loss of queen-specific substances and that *Neofem4* is linked to the production of the queen scent.

Discussion

When the queen's *Neofem4* was knocked down, her royal scent changed to a less queen-like scent (fig. 5), and workers in colonies with such a queen behaved as if they were in a queenless colony (fig. 3). This strongly suggests that *Neofem4* is required for producing the royalty scent which maintains the reproductive monopoly and social harmony in these societies. In our former study we identified a gene, *Neofem2*, which is necessary for maintaining the queen status (Korb et al. 2009). Now, we characterized a second queen gene involved in regulating reproductive division of labor and this time we were also able to show its functioning mechanism: Namely its involvement in the production of the royalty scent, a hallmark of insect societies.
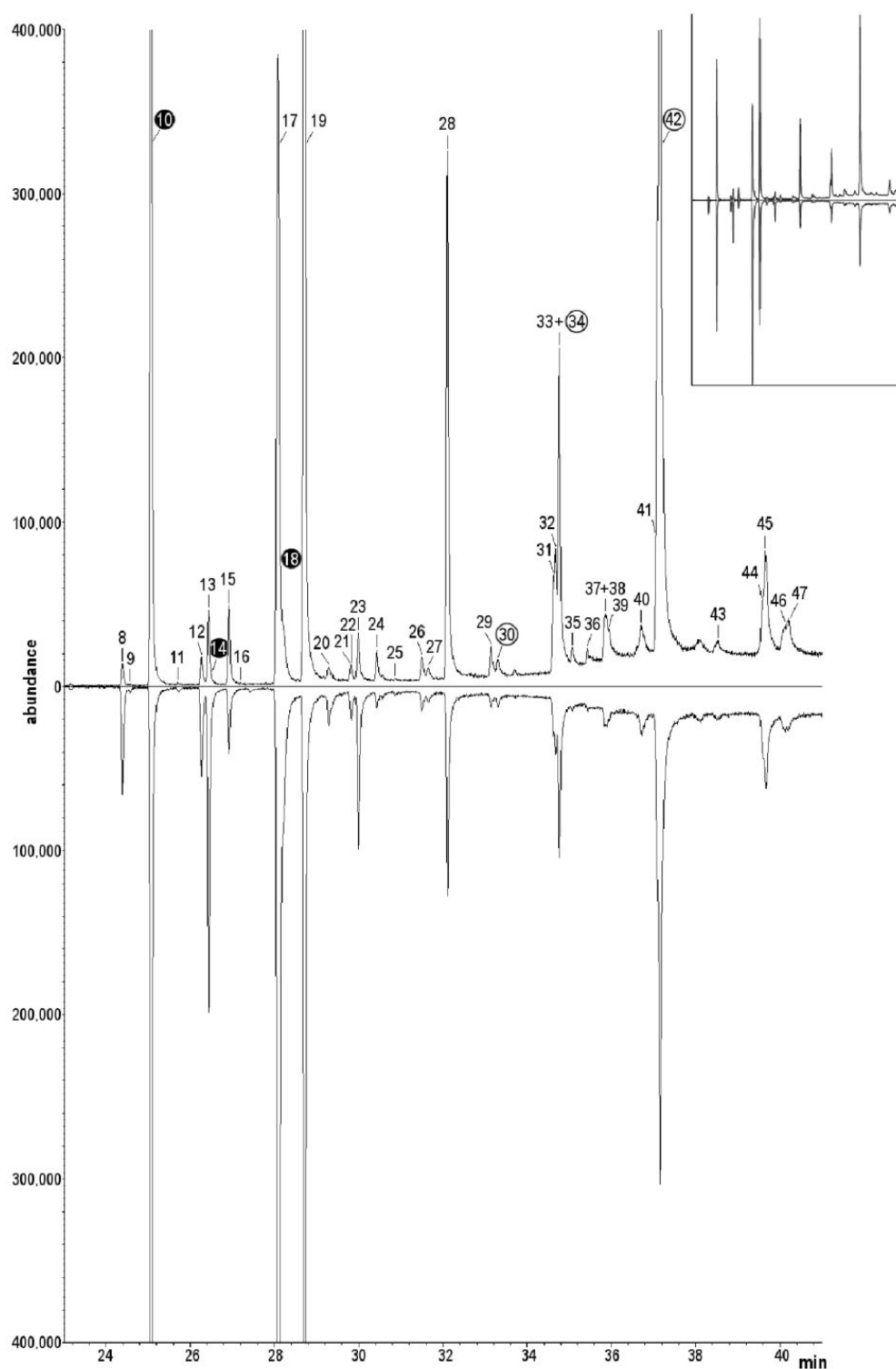
FIG. 4. Chromatograms of a queen before and after *Neofem4* treatment. Silencing *Neofem4* in the queen causes her scent to shift from a typical queen- to a more worker-like scent. Shown are representative chromatograms of a queen before (top) and after (below) treatment with *Neofem4* siRNA. Peaks 1–7, 16, 25, 41, and 44 are not labeled as they are not visible in these specific chromatograms. Only visible peaks are listed: 8 4meC24; 9 3meC24; 10 n-C25; 11 13meC25; 12 4meC25; 13 C26:1; 14 3meC25; 15 n-C26; 17 4meC26; 18 C27:1; 19 n-C27; 20 13meC27; 21 4meC27; 22 C28:1; 23 3meC27; 24 n-C28; 26 4meC28 + C29:1; 27 C29:1; 28 n-C29; 29 4meC29; 30 3meC29; 31 4meC30; 32 C31:1; 33 3meC30; 34 C31:1; 35 n-C31; 36 15meC31; 37 4meC31; 38 C32:1; 39 3meC31; 40 C33:2; 42 C33:1; 43 C34:1; 45 C35:2, 46 C35:1; 47 C35:1. Circles indicate peaks that were indicative for the changing profile from queen into worker: Open and filled circles indicate a decrease, respectively increase, of this peak in the queen after silencing *Neofem4*. In the upper right corner an overview of the same chromatogram is given to show the full range of peaks (note the abundance in the main figure was reduced to 400 000) (see also supplementary table S4, Supplementary Material online).
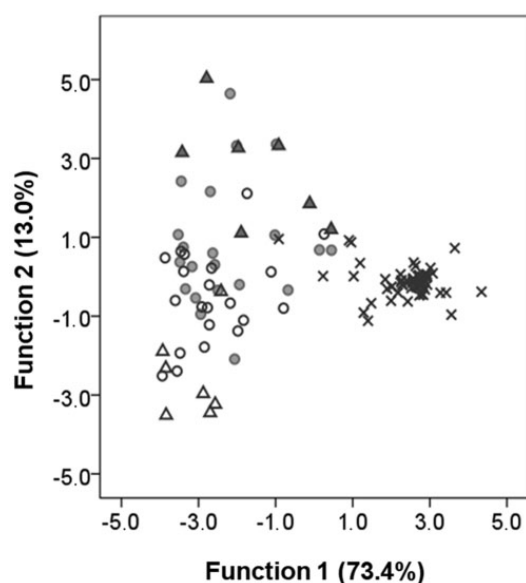
FIG. 5. Scent of queens before and after *Neofem4* silencing compared with controls and workers. Scent of queens before and after *Neofem4* siRNA treatment in comparison to control queens before and after treatments (Ringer's solution, control siRNA, no treatment) and workers. Shown are the results of a DA based on the relative amounts of 34 cuticular compounds. Four discriminant functions were calculated: F1 accounted for 73.4% of the total variance (Wilk's $\lambda = 0.03$, $\chi^2_{92} = 349.89$, $P < 0.0001$) and separated the queens from the worker caste; F2: 13.0% (Wilk's $\lambda = 0.19$, $\chi^2_{66} = 159.12$, $P < 0.0001$) separated queens before and after *Neofem4* siRNA treatment; F3: 7.2% (Wilk's $\lambda = 0.41$, $\chi^2_{42} = 87.77$, $P < 0.0001$); F4: 6.5% (Wilk's $\lambda = 0.65$, $\chi^2_{20} = 41.96$, $P = 0.003$). Approximately 84.8% of the samples were assigned correctly (for a detailed analysis of the different groups see supplementary fig. S4, Supplementary Material online). X: Worker ($n = 56$); open triangles: Queens before *Neofem4* treatment ($n = 7$); open circles: Queens before control treatments ($n = 28$); filled triangles: Queens after *Neofem4* treatment ($n = 7$); filled circles: Queens after control treatments ($n = 28$).

A common theme of recent results in the field of evo-devo and sociogenomics is that molecular components of behavior are often conserved between invertebrates and vertebrates, including humans (Reaume and Sokolowski 2011; Lian et al. 2012). This is impossible in the case of communication for cooperation; social insect communication is basically chemical, while other senses are more important in most vertebrates (visual and auditory signals, including language and reputation in human cooperation). Yet, our data fit nicely with results recently obtained mainly on social Hymenoptera (van Oystaeyen et al. 2014); in a comparative analysis of 64 social insect species long-chained, nonvolatile, saturated hydrocarbons were identified to advertise fecundity and/or suppress worker reproduction. In line, the queen-specific compounds in *C. secundus* are long-chained, sometimes methylated hydrocarbons including a few alkenes (see supplementary table S4 and S5, Supplementary Material online). Silencing *Neofem4* reduced the amount of these long-chained compounds and made the queens less queen-like (supplementary table S6, Supplementary Material online). This implies that across all social insects (including social

Hymenoptera and termites) similar compounds signal queen fertility which might indicate to common underlying molecular mechanisms. *Neofem4* belongs to the P450 family #4 (CYP4) which is the most highly represented insect P450 subfamily (a notable exception is the honey bee with only four members) (Honeybee Genome Consortium 2006). CYP4-P450s play diverse roles in the oxidation of xenobiotic substrates that include secondary plant compounds and insecticides; others have been linked to odorant or pheromone metabolism (Feyereisen 2005). In termites, besides a CYP6 gene in *Hodotermopsis sjostedti* (Cornette et al. 2006), CYP4 genes have also been identified in *Mastotermes darwiniensis* (Falckh et al. 1997) and *Reticulitermes flavipes* (Zhou et al. 2006). Strikingly, in *R. flavipes* several CYP4 genes are differentially expressed during JH-induced soldier caste differentiation (Zhou et al. 2006). The *C. secundus Neofem4* gene is similar to CYP4 genes identified in *R. flavipes,* but not at a level where they could be considered orthologous (Miura and Scharf 2011). This could imply that there were several CYP4/P450 gene duplications in the ancestors of recent termites and that these genes were co-opted for different functions during termite social evolution. The expression of P450 genes can be regulated by JH and vice versa (Lu et al. 1999) and this makes them a suitable substrate for cooption during the social evolution of termites; if P450 expression interacts with JH, a linkage betwen fertility and royalty scent exists. This makes P450 genes good candidates to act as signalers of the fertility status of individuals. In addition, a P450 gene has been found to be involved in the synthesis of CHCs in cockroaches but not necessarily in other insects (Howard and Blomquist 1982). Hence, P450 genes are both a suitable and available evolutionary substrate in the face of natural selection for production of a queen substance. Our data suggest that in an organism without elaborate cognitive abilities communication, which is the basis of evolutionarily stable cooperation, has been achieved by the exploitation of a gene at a central position that might link the fertility network with the chemical communication pathway. The similarity in fertility signals across termites and social Hymenoptera makes it worthwhile testing whether P450 genes serve a common role in fertility signaling across taxa (van Oystaeyen et al. 2014).

Materials and Methods

Termites

Termite colonies of *C. secundus* were collected in mangroves around Darwin (NT, Australia). Colony rearing and the generation of neotenic replacement reproductives were performed as described elsewhere (Korb and Schmidinger 2004; Weil et al. 2007). Across all colonies the queens were young, reproducing sexuals.

RNAi Experiment

Neofem4 knockdown was performed on four groups containing seven colonies each: Queens injected with 1) *Neofem4* siRNAs, 2) control siRNA, 3) Ringer's solution alone, and 4) untreated queens as reference.

The behavior of queens and a focal worker per colony was observed (see Behavioral Assays) and their CHC profile determined (see Chemical Scent Analysis) 24 h before and 23 h after treatment. At 24 h from the injection time, queens were killed and the expression level of *Neofem4* determined using quantitative real-time PCR (qPCR). In preliminary experiments, we observed an optimum in gene knockdown after 24 h that successively decreased thereafter, consistent with previous RNAi experiments on termites (Scharf et al. 2008; Korb et al. 2009). Custom Stealth RNAi primers (Invitrogen) and control RNAi (supplementary table S7, Supplementary Material online) were designed from the sequence of *C. secundus Neofem4* (EF029057) with the BLOCK-iT RNAi designer online tool which also randomly determines the control siRNA (Invitrogen) and dissolved in nuclease-free water to 3.6 µg/µl. We used a 1:1 mixture of two *Neofem4*-specific siRNAs to increase knockdown efficiency. Then siRNAs were diluted with a10 × Ringer's solution (15.75 g NaCl, 2.35 ml 2 M KCl, 3.7 ml 2 M KH_2PO_4, 0.05 g NA_2HPO_4, 0.37 g $MgCl_2$, total volume 100 ml, pH 7.4) and 0.1 µl (36 ng in 1 × Ringer's solution) were injected into the dorsal skin between head and thorax with a microinjector (InjectMan NI2 with FemtoJet; Eppendorf).

Quantification

The total RNA (1 µg) was extracted from the living termites using TRI Reagent (Ambion) and remaining DNA removed using the Turbo DNA-free kit (Ambion). The RNA concentration and purity was measured (Nanodrop ND-1000; peqLab) and set to 25 ng/µl with nuclease-free water (Qiagen). Reverse transcription and qPCR were performed on a Biorad C1000 Thermal Cycler with CFX96 Real-Time System (Biorad) using the QuantiTect SYBR Green RT-PCR Kit (Qiagen) according to manufacturer's instructions (primers see supplementary table S8, Supplementary Material online). Three technical replicates were made per sample and gene. Melting curves were analyzed to control for PCR specificity. Expression data were normalized for expression of 18S rRNA (Weil et al. 2007). In *C. secundus*, this gene shows the most stable expression between castes and body parts as evaluated, tested and established in Weil et al. (2007). The expression of 18S did not differ significantly between treatment (ANOVA: $P = 0.630$). To confirm that the *Neofem4* knockdown did not affect all genes, the expression of *ß-actin* was also checked in both *Neofem4* siRNA and untreated queens.

Behavioral Assays

For each colony an arbitrarily chosen worker among the third larval and first nymphal instars that are capable and likely to become replacement reproductives (Korb and Katrantzis 2004) was marked and observed using focal sampling for 30 min. We recorded 1) running, 2) allogrooming, 3) proctodeal trophallaxis (anal feeding), 4) butting, and 5) antennation, measured as duration spent in the interaction (1–3) or as the total number of interactions between two individuals (4–5) (Korb and Schmidinger 2004; Korb et al. 2009).

Butting is a distinctive behavior whereby one individual moves repeatedly back- and forwards, often causing the recipient to pull back (Korb et al. 2009). Butting is indicative in queenless colonies: Workers that will develop into a new reproductive perform more butting than others (Korb et al. 2009; Hoffmann and Korb 2011). To a lesser extent also antennation increases in queenless colonies (Hoffmann and Korb 2011). For interactive behaviors (2–5), all colony estimates for worker behavior are based upon behaviors that the focal worker received, not those that it gave to others, because received behaviors have a much better signal to noise ratio (Korb et al. 2009). By concentrating on passive behavior (i.e., all interactions that our observed focal workers continuously receive) we obtain a good estimate of the interactions (Korb et al. 2009).

Chemical Scent Analysis

The CHC profiles of queens and workers were analyzed as previously described (Weil et al. 2009). In brief, the CHC profiles were obtained by gently rubbing the individuals' surface using a polydimethylsiloxane fibre for solid phase microextraction (SPME; 7 µm; Supelco). Gas chromatography–mass spectroscopy (GC-MS) analysis was performed with an Agilent Technologies 7890A-GC coupled with an Agilent 5975C MS (Agilent), operated in splitless injection mode with helium carrier gas at 1 ml/min flow rate, equipped with a 3 m × 250 µm × 0.25 µm J & W dimethylsiloxane column (Agilent). Oven temperature rose from 120 °C to 150 °C at 30 °C/min continuing with 4 °C/min up to 280 °C and finally with 10 °C/min up to 300 °C (Inlet temperature 250 °C, MS quad 150 °C, MS source 230 °C, auxiliary temperature 300 °C). MSD-ChemStation software (Agilent) was used for data acquisition. Compounds were identified by characteristic mass spectral fragmentation patterns.

Statistics

All data were checked for normal distribution by Kolmogorov–Smirnov tests, statistical tests were chosen accordingly. All analyses were two-tailed and conducted with SPSS PASW Statistics 18.0 (SPSS Inc.).

Gene Expression

Mean and standard error were determined by averaging relative expression levels across seven independent experiments per group, each determined in triplicates (see above). The same data set was subject to multiple testing so the step-up false discovery rate (FDR) approach was used to correct *P* values (Benjamini and Hochberg 1995).

Chemical Scent Analysis

In total 47 compounds were found in workers and queens. As in Liebig et al. (2010) only peaks with a relative peak area of >0.1% occurring in >3% of all samples were used (= 34 compounds; supplementary table S3, Supplementary Material online). The peak areas of these peaks were restandardized to 100% and transformed according to Aitchison's formula (Aitchison 1986). To allow the transformation of profiles with nondetectable components, the constant 0.01

was added to all peak areas (Steiger et al. 2007). The transformed areas were used as variables in a principal component analysis. This extracted only one factor with an eigenvalue >1 which explained 99.0% of the total variance (KMO = 0.973, df = 561, $P < 0.0001$). All 34 compounds had high loadings on this factor (between 0.984 and 0.998) (supplementary table S9, Supplementary Material online). Hence, the transformed peak areas were used as variables in a DA to determine whether caste and treatment can be distinguished on the basis of CHC profiles. Relative proportions of compounds between castes were compared with Mann–Whitney U tests and those between queens before and after siRNA treatment with Wilcoxon paired rank tests.

Behavioral Assay

Behaviors were compared using Wilcoxon paired rank tests. We evaluated the possibility of multiple testing artefacts in the behavioral data by calculating the probability that the same two (butting and antennation) of the five measured behaviors would be found to be significantly different both in queenless colonies (Hoffmann and Korb 2011) and *Neofem4* siRNA treated colonies but not in the two controls as: $[(0.954 \times 0.05) \times (0.954 \times 0.05) \times 0.955 \times 0.955]^2 \times 5 = 0.00002$.

Supplementary Material

Supplementary figures S1–S6 and tables S1–S9 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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