Supporting Online Material for

A Gene Necessary for Reproductive Suppression in Termites

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Movie S1

Supporting Online
Material Materials and Methods
For Korb et al.

Termites. Termite colonies of *Cryptotermes secundus* were collected in mangroves around Darwin (NT, Australia). Colony rearing and the generation of neotenic (queen and king) replacement reproductives were performed, as described (S1, S2).

RNAi. *Neofem2* knockdown was performed on four groups containing eight colonies each. **1** Queens injected with Neofem2 siRNAs **2** Queens injected with control siRNA **3** Queens injected with Ringer's solution alone **4** Untreated queens as reference.

The behavior of queens and a focal worker in each colony was observed for half an hour, 24 hours before the treatment (see Behavioral Assays) then queens were treated with a construct or control, and 23.5 hours later, the queen and focal workers were observed again for half an hour. At 24 hours from the injection time, the queen was immediately collected and qPCR was used to determine the expression level of *Neofem2*. In preliminary experiments, we checked relative gene expression levels after 24h, 36h and 48h . After 1 day (24h) we observed an optimum in gene knockdown that successively decreased thereafter. This is consistent with previous RNAi experiments on termites (S3).

Custom StealthTM RNAi primers (Invitrogen, Carlsbad, CA) were designed from the sequence of *Cryptotermes secundus Neofem2* gene (EF029055) (S1) with the Block-iTTM RNAi Designer tool (Invitrogen) (Table S6), as was the control RNAi. The 25bp double stranded siRNAs (*Neofem2* siRNAs and control siRNA) were dissolved in nuclease-free water to 3.6µg/µl. We used a mixture of *Neofem2*-specific siRNAs in order to increase knockdown efficiency (Table S6). The ds siRNAs were diluted with a10x Ringer solution (15.75g NaCl, 2.35ml 2M KCl, 3.7ml 2M KH2PO4, 0.05g NA2HPO4, 0.37g MgCl2, total volume 100ml, pH 7.4) and a volume of 0.1µl (36ng in 1x Ringer's solution) was injected into the dorsal skin between head and thorax of neotenics with a microinjector and sterile microcapillaries (*Femtotips*® II, Eppendorf, Hamburg, Germany).

Quantification. Total RNA (1 µg) was reverse transcribed with AMV-RT (Promega, Madison, WI) and Random Decamers (Ambion, Austin, TX). qRT-PCR was performed on a Mastercycler® ep realplex (Eppendorf, , Hamburg, Germany) with 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 were normalized for expression of the 18S rRNA gene, as described (S1). 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. Primers are given in Table S6. To confirm that the *Neofem2* knockdown did not affect all genes, the expression of the gene *ß-actin* was also checked in both *Neofem2* siRNA queens and untreated queens.

Behavioral assays. Behavioral observations were performed as previously described (S2). In brief, for each colony an arbitrarily chosen worker was marked and observed

using focal sampling for 30 minutes and we recorded the following behaviors: (i) running, (ii) allogrooming, (iii) proctodeal trophallaxis (anal feeding), (iv) butting, and (v) antennation. The first three behaviors are recorded as the duration spent in the interaction and the last two as the total number of interactions between two individuals. Butting is a distinctive behavior whereby one worker moves repeatedly backwards and forwards and contacts another worker, often causing the recipient worker to pull back (S4). Butting is indicative of reproductive dominance: workers that will develop into a new reproductive perform more butting than other workers (Fig. S2). This was shown in additional experiments where reproductives were removed, all workers were marked and the behavior of all workers was observed as described above. After the development of the new reproductives, the behaviors of the workers that became the new reproductives were compared with those of the remaining workers.

To identify behaviors that characterize the absence of a queen, we used nine colonies in which we observed one worker when the queen was present (queenright) and the same worker again one day after we had removed the queen (queenless). There was no observational bias as the observer was uninformed about the treatments in all experiments. For interactive behaviors (ii-v), 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 (S5). At any one time, a very small minority of workers in each colony initiate the great majority of the interactions but many workers receive them (Fig. S3), such that the latter data provide a much cleaner estimate of the colony mean when following a single worker. Consistent with this, all comparisons based upon behaviors given by the focal workers were non-significant.

Statistics. All data were checked for normal distribution by visual inspections and one-sample Kolmogorov –Smirnov tests. Data differed from normal distribution and thus were analyzed with non-parametric statistics. Mean and standard error for the transcription data were determined by averaging relative expression levels across eight independent experiments per group, each determined in triplicates (see above). For the gene expression analyses, the same data sets were also subjected to multiple testing. Here, the step-up false discovery rate (FDR) approach was used to correct P-values, which overcomes problems of the Bonferroni correction (S6). All statistical analyses were conducted with SPSS 15.0 (SPSS Inc., Chicago, IL). All tests were two-tailed. We evaluated the possibility of multiple testing artifacts in the behavioral data by calculating the probability that the one of the five measured behaviors would be found to be significantly different both in the queenless and *Neofem2* siRNA treated colonies but not in the two controls as: $[(0.954\times0.05) \times (0.954\times0.05) \times0.955\times0.955] \times5 = 0.005$.

Movie 1 Movie of butting behavior in a colony of *Cryptotermes secundus*.

Figure S1.

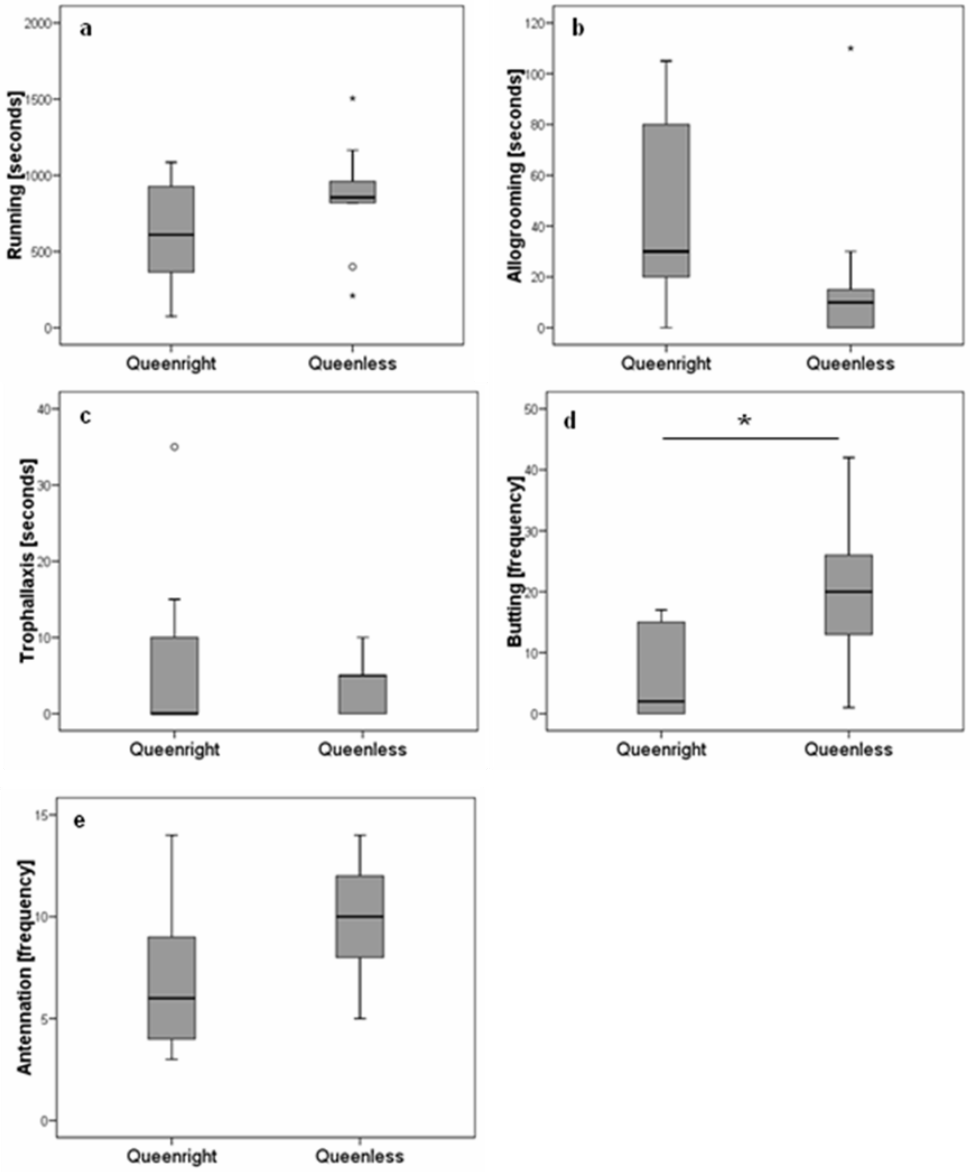

Fig. S1. Comparison of worker behaviors in queenright and queenless colonies. Shown are boxplots with median, quartiles and minimal and maximal values (n = 9 pairs). Only the number of butting interactions increased significantly when the queen was absent (see also Table S2). (A) running, (B) allogrooming, (C) proctodeal trophallaxis, (D) butting, and (E) antennation.

Fig. S2.

Fig. S2. Comparison of butting behavior among workers in colonies where the queen and king have been removed. Individuals that become the next reproductives do significantly more butting (future reproductives) than individuals that do not become new reproductives (workers) (t-test for unequal variances: $t_{1,\ 151} = 3.91$; $P < 0.001$). Data are based on experiments with 14 colonies. Data were normally distributed, which allowed parametric analyses. Shown are means ± 1 S.E.

Fig. S3.

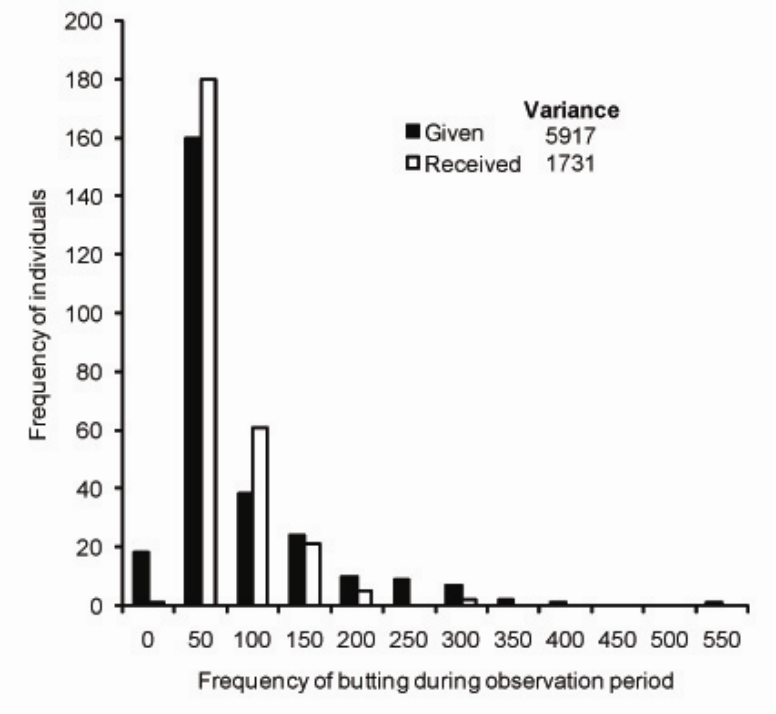

Fig. S3. Frequency distribution of butting behavior within a colony across individuals as a function of whether the behavior is given (black bars) or received (white bars). Within a colony, a few individuals perform a lot of butting behavior. By contrast, the number of butts received by any given individual is less variable.

Fig. S4.

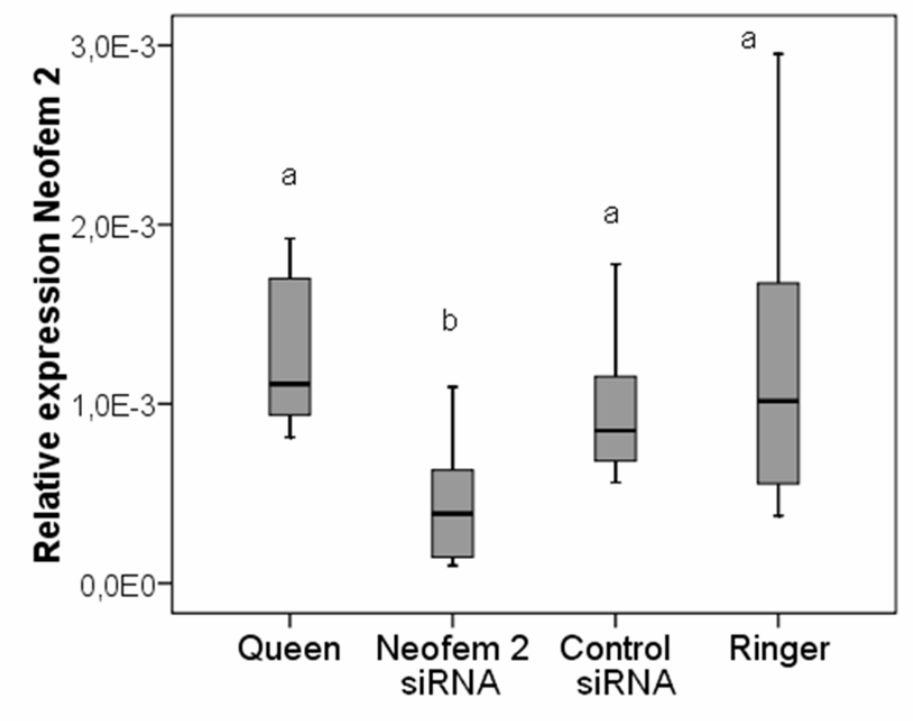

Fig. S4.Relative expression levels of the *Neofem2* gene in (i) untreated queens, and in queens 24 hours after injection of (ii) *Neofem2* siRNA, (iii) control siRNA, and (iv) Ringer's solution. The relative expression of *Neofem2* was significantly down regulated compared to untreated queens only after *Neofem2* siRNA treatment (Mann Whitney rank tests: always $n = 13$; *Neofem2* siRNA: $Z = -2.64$, after step-up FDR: $P < 0.05$; control siRNA: $Z = -1.31$, $P = 0.188$; Ringer' solution: $Z = -0.59$, $P = 0.558$). The relative expression of *Neofem2* was also significantly lower after *Neofem2* siRNA treatment compared to control siRNA treatment (Mann Whitney rank test: $Z = -2.21$, $n = 16$, after step-up FDR: $P < 0.05$) and compared to the injection of Ringer's solution (Mann Whitney rank test: $Z = -2.1$, $n = 16$, after step-up FDR: $P < 0.05$). While the expression levels between control siRNA and Ringer's solution did not differ significantly (Mann Whitney rank test: $Z = -0.31$, $n = 8$ pairs, $P = 0.753$). Different letters indicate data that differ significantly at $P < 0.05$ and, conversely, data indicated by the same letter are not significantly different.

Fig. S5.

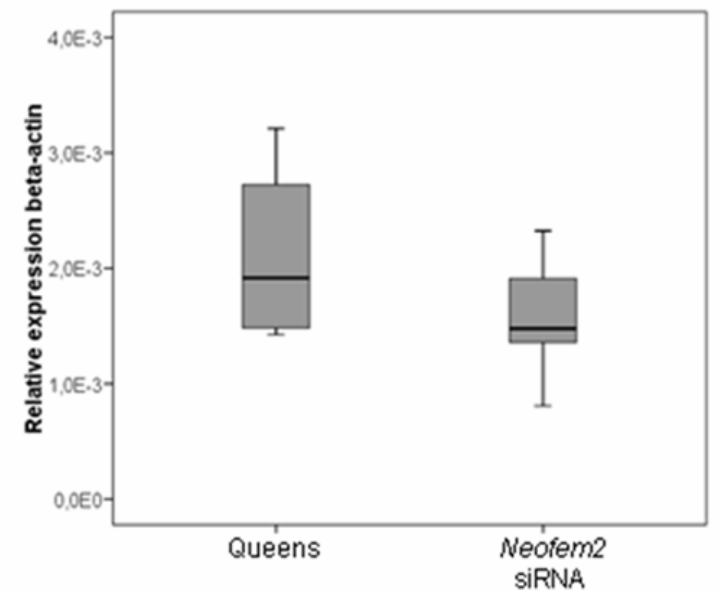

Fig. S5. Relative expression levels of the gene *β- actin* in untreated queens and 24 hours after injection of *Neofem2* siRNA. The relative expression of *β- actin* did not differ significantly between untreated queens and after injection of *Neofem2* siRNA (Mann Whitney rank test: $n = 13$, $U = 10.0$, $P = 0.171$).

Fig. S6.

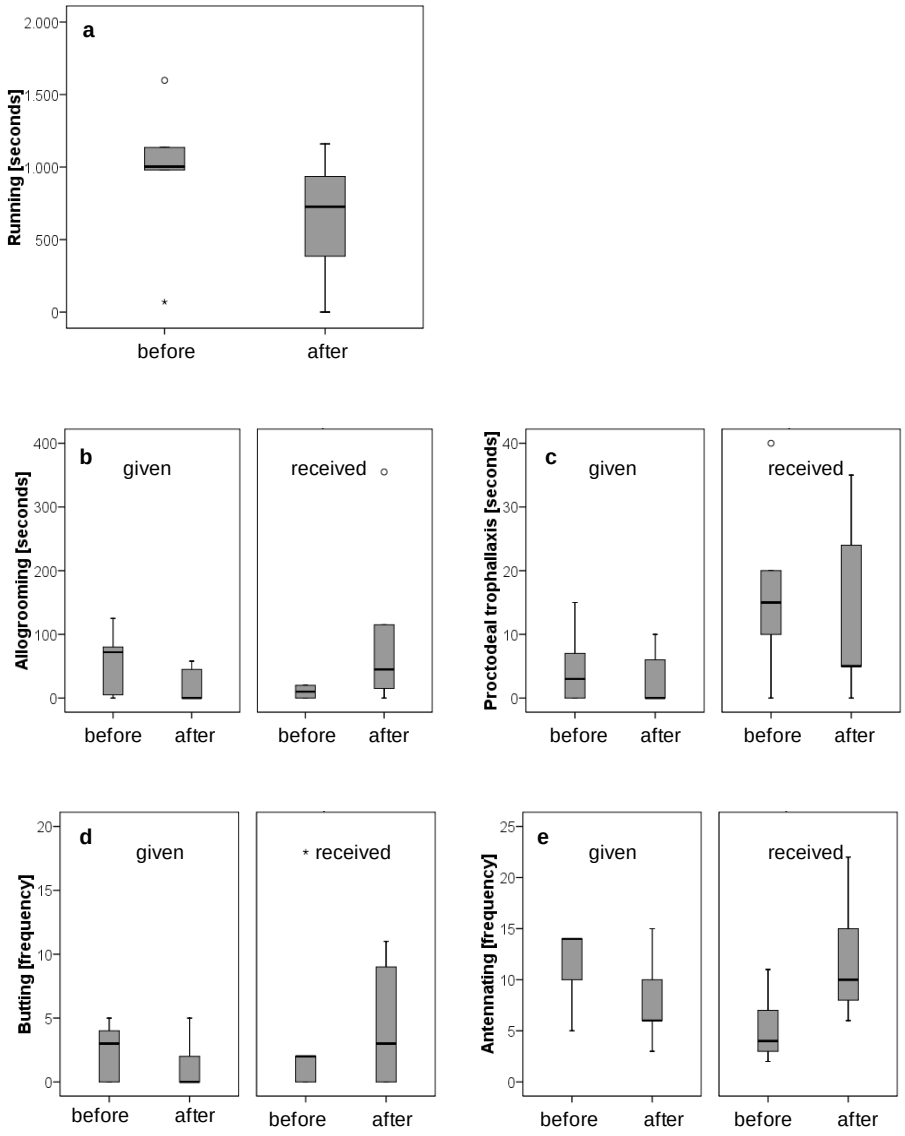

Fig. S6. Comparison of queen behaviors before and after treatment with *Neofem2* siRNA. Shown are boxplots with median, quartiles and minimal and maximal values (n = 5 pairs). In queens, none of the observed behaviors changed significantly before and after treatments with *Neofem2* siRNA (Table S3). (A) running, (B) allogrooming, (C) proctodeal trophallaxis, (D) butting, and (E) antennation.

Fig. S7.

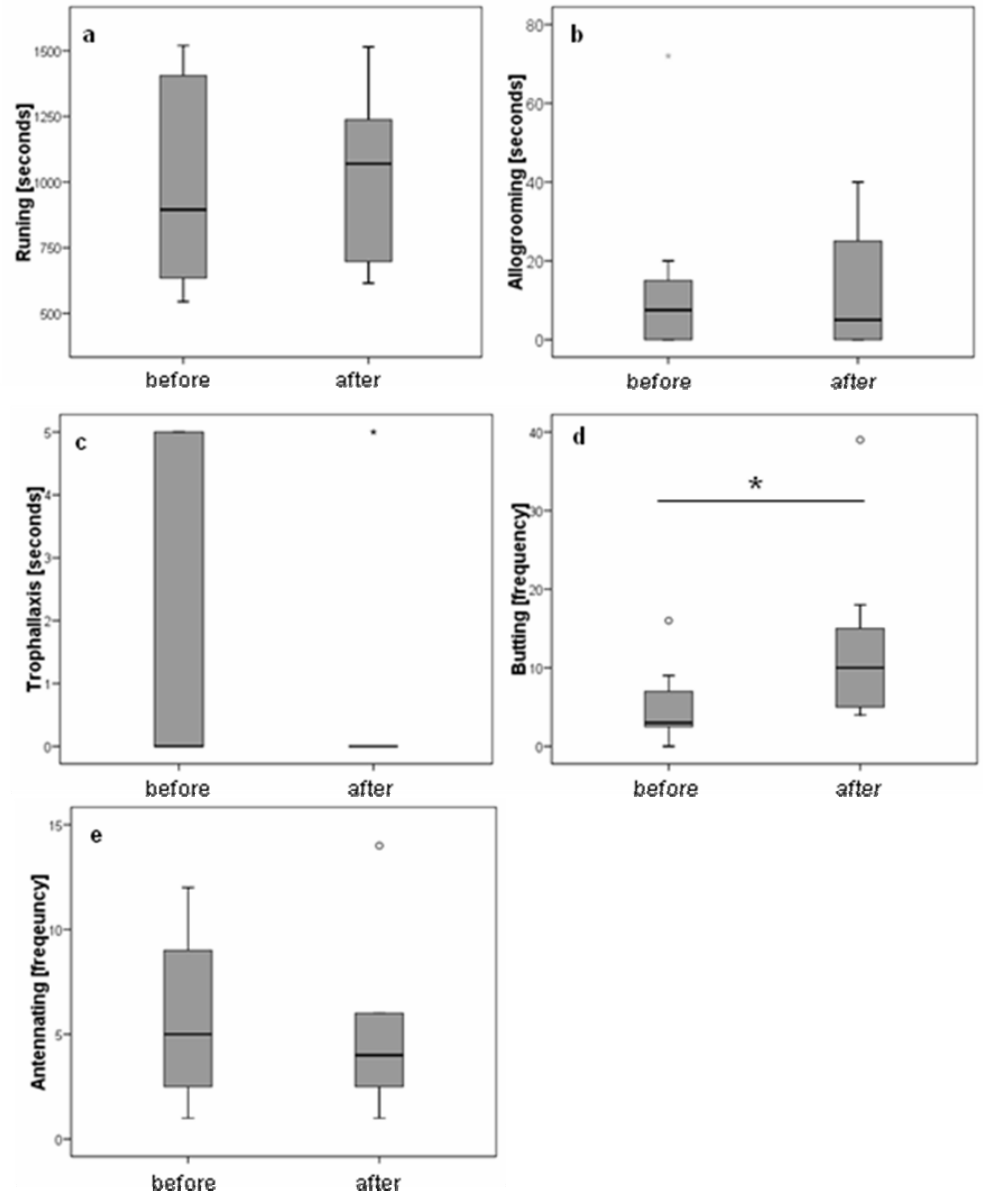

Fig. S7. Comparison of worker behaviors before and after treatment with *Neofem2* siRNA. Shown are boxplots with median, quartiles and minimal and maximal values (n = 8 pairs). Only the number of butting interactions increased significantly after treatment with *Neofem2* siRNA, while none of the other behaviors changed significantly (see also Table S4). (A) running, (B) allogrooming, (C) proctodeal trophallaxis, (D) butting, and (E) antennation.

Table S1. Comparison of worker behaviors in queenright and queenless colonies.

Behavior	**Z**	**P**
Running [in seconds]	-1.36	0.173
Allogrooming [in seconds]	-1.35	0.176
Proctodeal trophallaxis [in seconds]	-0.74	0.457
Butting [frequency]	-2.43	**0.015**
Antennation [frequency]	-1.61	0.108

Shown are the results of Wilcoxon paired rank-tests comparing the behavior of workers in nine *Cryptotermes secundus* colonies when the queen was present and after the queen had been removed (n = 9 pairs).

Table S2. Comparison of queen behaviors before and after treatment with *Neofem2* siRNA.

Behavior		**Z**	**P**
Running [in seconds]		-0.67	0.500
Allogrooming [in seconds]	Given	-0.40	0.686
	Received	-1.21	0.225
Proctodeal trophallaxis [in	Given	-0.55	0.581
seconds]	Received	-0.27	0.786
Butting [frequency]	Given	-0.74	0.461
	Received	-0.18	0.854
Antennation [frequency]	Given	-1.22	0.223
	Received	-0.36	0.715

Shown are the results of Wilcoxon paired rank-tests comparing the behavior of queens in five *Cryptotermes secundus* colonies before and after treatment with *Neofem2* siRNA(*n* = 5 pairs).

Table S3. Comparison of worker behaviors before and after treatment with *Neofem2* siRNA.

Behavior	**Z**	**P**
Running [in seconds]	-0.14	0.889
Allogrooming [in seconds]	-0.27	0.786
Proctodeal trophallaxis [in seconds]	-1.41	0.157
Butting [frequency]	-2.54	**0.012**
Antennation [frequency]	-0.21	0.833

Shown are the results of Wilcoxon paired rank-tests comparing the behavior of workers in eight *Cryptotermes secundus* colonies before and after treatment with *Neofem2* siRNA(n = 8 pairs).

Table S4. Comparison of worker behaviors before and after treatment with control siRNA and Ringer solution.

Behavior	**Z**	**P**
Treatment with control siRNA		
Running [in seconds]	-1.26	0.208
Allogrooming [in seconds]	-0.73	0.465
Proctodeal trophallaxis [in seconds]	-0.37	0.715
Butting [frequency]	-0.09	0.933
Antennation [frequency]	-1.55	0.121
Treatment with ringer		
Running [in seconds]	-0.56	0.575
Allogrooming [in seconds]	-1.21	0.225
Proctodeal trophallaxis [in seconds]	-1.10	0.273
Butting [frequency]	-1.01	0.310
Antennation [frequency]	-0.93	0.351

Shown are the results of Wilcoxon paired rank-tests comparing the behavior of workers in eight *Cryptotermes secundus* colonies before and after treatment with control siRNA or Ringer solution (n = 9 pairs).

Table S5. BLASTX results for the *Neofem* 2 gene that show best hits against the non-redundant NCBI database; species are in square brackets.

Gene	Size (bp)	Identity match by BLASTX [species]	Accession no.	Local identity (%)	Score (bits)	*e*-value
Neofem2	1918	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

GenBank accession number, local identity (%), score (bits) and E-score value [taken from (S1)].

Table S6. Stealth primers used in this study.

Stealth™ RNAi primer	Sequence 5' - 3'
Neofem2 siRNA – 1	AAGTGATCATGAGGCAGCACTTCAA
Neofem2 siRNA – 2	GGAGTGAGAGTTGTTGGCTACATGA
control siRNA	GGAGAGTGATGTGGTAT CACGTTGA

Supplemental References

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