Hemimetabolous genomes reveal molecular basis of termite eusociality

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Around 150 million years ago, eusocial termites evolved from within the cockroaches, 50 million years before eusocial Hymenoptera, such as bees and ants, appeared. Here, we report the first, 2GB genome of a cockroach, *Blattella germanica*, and the 1.3GB genome of the drywood termite, *Cryptotermes secundus*. We show evolutionary signatures of termite eusociality by comparing the genomes and transcriptomes of three termites and the cockroach against the background of 16 other eusocial and non-eusocial insects. Dramatic adaptive changes in genes underlying the production and perception of pheromones confirm the importance of chemical communication in the termites. These are accompanied by major changes in gene regulation and the molecular evolution of caste determination. Many of these results parallel molecular mechanisms of eusocial evolution in Hymenoptera. However, the specific solutions are remarkably different, thus revealing a striking case of convergence in one of the major evolutionary transitions in biological complexity.

Eusociality, the reproductive division of labour with overlapping generations and cooperative brood care, is one of the major evolutionary transitions in biology[1]. Although rare, eusociality has been observed in a diverse range of organisms, including shrimps, mole-rats and several insect lineages[2,3,4]. A particularly striking case of convergent evolution occurred within the holometabolous Hymenoptera and in the hemimetabolous termites (Isoptera), which are separated by ca. 400 my of evolution[5]. Termites evolved within the cockroaches around 150 mya, towards the end of the Jurassic[6,7], about 50 my before the first bees and ants appeared[5]. Therefore, identifying the molecular mechanisms common to both origins of eusociality is crucial to understanding the fundamental signatures of these rare evolutionary transitions. While the availability of genomes from many eusocial and non-eusocial hymenopteran species[8] has allowed extensive research into the origins of eusociality within ants and bees[9,10,11], a paucity of genomic data from cockroaches and termites has precluded large-scale investigations into the evolution of eusociality in this hemimetabolous clade.

The conditions under which eusociality arose from within the cockroaches differ greatly from those present in the non-eusocial ancestors of eusocial Hymenoptera. Termites and cockroaches are hemimetabolous and so show a direct development, while holometabolous hymenopterans complete the adult body plan during metamorphosis. In termites, workers are immatures and only reproductive castes are adults[12], while in Hymenoptera, adult workers and queens represent the primary division of labour. Moreover, termites are diploid and their colonies consist of both male and female workers, and usually a queen and king dominate reproduction. This is in contrast to the haplodiploid system found in Hymenoptera, in which all workers and dominant reproductives are female. It is therefore intriguing that strong similarities have evolved convergently within the termites and the hymenopterans, such as differentiated castes and a nest life with reproductive division of labour. The termites can be subdivided into wood-dwelling and foraging termites. The former belong to the lower termites and produce simple, small

colonies with totipotent workers that can become reproductives. Foraging termites (some lower and all higher termites) form large, complex societies, in which worker castes can be irreversible[12]. Similarly, within ants, bees and wasps, varying levels of eusociality exist.

Here we provide insights into the genomic signatures of eusociality within the termites. We analysed the genomes of three termite species with differing levels of social complexity and compared them to the first cockroach genome, as a closely-related non-eusocial outgroup. Furthermore, differences in expression between nymphs and adults of the cockroach were compared to differences in expression between workers and reproductives of the three termites, in order to gain insights into how expression patterns changed along with the evolution of castes. Using fifteen additional insect genomes to infer background gene family turnover rates, we analysed the evolution of gene families along the transition from non-social cockroaches to eusociality in the termites. In this study we concentrated particularly on two hallmarks of insect eusociality, as previously described for Hymenoptera, with the expectation that similar patterns occurred along with the emergence of termites. These are the evolution of a sophisticated chemical communication, which is essential for the functioning of a eusocial insect colony[3,13,14] and major changes in gene regulation along with the evolution of castes[9,10]. Additionally, we tested the hypothesis that the high levels of transposable elements present in cockroach and termite genomes allowed the evolution of gene families which were essential to the transition to eusociality.

Evolution of genomes and proteomes

We sequenced and assembled the genome of the German cockroach, *Blattella germanica* (Ectobiidae), and of the lower, drywood termite, *Cryptotermes secundus* (Kalotermitidae; for assembly statistics see supplementary table S1). The cockroach genome (2.0 Gb) is considerably larger than all three termite genomes. The genome size of *C. secundus* (1.30 Gb) is comparable to the higher, fungus-growing termite, *Macrotermes natalensis*, (1.31 Gb, Termitidae)[15] but more than twice as large as the lower, dampwood termite, *Zootermopsis nevadensis* (562 Mb, Termopsidae)[16]. The smaller genomes of termites compared to the cockroach are in line with previous size estimations based on C-values[17]. The proteome of *B. germanica* (29,216 proteins) is also much larger than in the termites, where we find the proteome size in *C. secundus* (18,162) to be similar to the other two termites (*M. natalensis*: 16,140; *Z. nevadensis*: 15,459; Fig. 1). In fact, the *B. germanica* proteome was the largest among all 21 arthropod species analysed here (20 insects and the centipede *Strigamia maritima*; Fig. 1). Strong evidential support for over 80% of these proteins in *B. germanica* (see supporting material) and large expansions in many manually annotated gene families offer high confidence in the accuracy of this proteome size. We compared gene expression between nymphs (5th and 6th instars) and female reproductive adults in *B. germanica*, and

between workers, queens and kings in each of the three termites. Gene expression differed significantly ($p < 0.05$) between female reproductives and nymphs in 2457 genes for *B. germanica*. In the termites 3369 (*C. secundus*) to 6756 (*Z. nevadensis*) genes differed significantly between queens and workers, which are arguably analogous to female adults and nymphs in the cockroach (Fig. 2).

The transitions to eusociality in ants[10] and bees[9] have been linked to major changes in gene family sizes. Similarly, we detected significant gene family changes on the branch leading to the termites (7 expansions and 10 contractions; Fig. S1, table S2). The numbers of species-specific, significant expansions and contractions of gene families varied within termites (*Z. nevadensis*: 15/5; *C. secundus*: 27/3; *M. natalensis*: 24/20; tables S3-S5). Interestingly, in *B. germanica* we measured 93 significant gene family expansions but no contractions (table S6), which contributed to the large proteome. The *C. secundus* and *B. germanica* genomes contain similar proportions of repetitive content (both 55%; table S7), which is higher than in both *Z. nevadensis* (28%) and the higher termite, *M. natalensis* (46%)[18]. This is in contrast to the reported negative correlation between repetitive content and the level of eusociality in bees[9]. As also found in *Z. nevadensis* and *M. natalensis*[18], LINEs and especially the subfamily BovB were the most abundant transposable elements (TEs) in the *B. germanica* and *C. secundus* genomes, indicating that a proliferation of LINEs may have occurred in the ancestors of Blattodea (cockroaches and termites). We hypothesised that these high levels of TEs may be driving the high turnover in gene family sizes within the termites and *B. germanica*[19]. Expanded gene families indeed had more repetitive content within 10 kb flanking regions in all three termites ($p < 1.3 \times 10^{-8}$; Wald *t*-test; table S8-S9), in particular in the higher termite *M. natalensis*. In contrast, gene family expansions were not correlated with TE content in flanking regions for *B. germanica*. These results suggest a major expansion of LINEs at the root of the Blattodea clade contributed to the evolution of gene families within termites, likely via unequal crossing-over[19]; however, the expansions in *B. germanica* were not facilitated by TEs. It can therefore be concluded that the large expansion of LINEs within Blattodea allowed the evolution of gene families which ultimately facilitated the transition to eusociality.

Out of 729 non-saturated (synonymous substitution rate: $dS < 3$) 1-to-1 protein orthologs between the termites and the two closest related, available non-eusocial species, *B. germanica* and the orthopteran *Locusta migratoria*, we found 165 (22.6%) to be evolving significantly faster (ratio of nonsynonymous to synonymous nucleotide substitution rates: dN/dS or ω) among the termites. These genes were enriched in functions related to carbohydrate metabolism (table S10), which was also over-represented in genes with higher ω values in eusocial compared to non-eusocial bees[11]. Functions related to oxidation-reduction processes, including a number of mitochondrial genes, were also enriched among genes with a higher ω within termites. This is consistent with the finding that mitochondrial genes were found to be evolving under positive selection during the evolution of ants[20]. One hundred (60.6%) of the genes with a signifi-

cantly higher ω within the termites were evolving even faster on the branch leading to the higher termite, *M. natalensis*. The ten most significant of these genes have functions related to signaling, cell transport, glycogen metabolism, transcription regulation, proteolysis and morphogenesis (table S11). These findings support the notion that changes in gene regulation, diet and developmental pathways have facilitated the transition to higher eusociality and a change from simple wood-dwelling colonies to large, complex, foraging societies.

CHC production

Despite their different ancestry, both termites and eusocial hymenopterans are characterised by the production of caste-specific cuticular hydrocarbons (CHCs)[21,22,23], which are often crucial for regulating reproductive division of labour and chemical communication. Accordingly, we find changes in the termites in three groups of proteins involved in the synthesis of CHCs: desaturases (introduction of double bonds[24]), elongases (extension of C-chain length[25]) and CYP4G1 (last step of CHC biosynthesis[26]).

Desaturases are thought to be important for division of labour and social communication in ants[27]. As previously described for ants[27], Desat B genes are the most abundant desaturase family in the termites and the cockroach (table S12), especially in *M. natalensis* where we found ten gene copies (significant expansion; $p = 0.0024$; table S5; Fig. S7). As in ants, especially the First Desaturases (Desat A - Desat E) vary greatly in their expression between castes and species in the three termites (Fig. 2; table S13)[27]. Both in *Z. nevadensis* and *M. natalensis*, most desaturases are more highly expressed in worker castes than in queens, while these genes are generally more evenly expressed between castes in *C. secundus*. In *B. germanica* 4 out of 7 Desat B genes are over-expressed in nymphs compared to female adults and only one is more highly expressed in female adults (table S13). This pattern has been maintained in *Z. nevadensis* (1 queen, 2 worker genes) and *M. natalensis* (5 worker genes), in which most Desat B genes are worker-specific. In contrast to ants, where these genes are under strong purifying selection[27], we found significant positive selection within the Desat B genes for the highly eusocial termite, *M. natalensis*, (codeml site models 7 & 8; $p = 1.1\text{x}10^{-16}$), indicating a diversification in function, possibly related to their greater diversification of worker castes (major and minor workers, major and minor soldiers). Although desaturases are often discussed in the context of CHC production and chemical communication, their biochemical roles are quite diverse[27], and the positive selection we observe for *M. natalensis* may, at least in part, be related to their rather different ecology of foraging and fungus farming rather than nest mate recognition. Future experimental verification of the function of these genes will help better understand these observed genomic and transcriptomic patterns.

Underlining an increased importance of CHC communication in termites, the expression patterns

of elongases (extension of C-chain length) differ considerably in the termites compared to the cockroach (Fig. 2; table S14). In contrast to *B. germanica*, in which elongases are both nymph- (6 genes) and adult-biased (5 genes), only one or two elongase genes in each termite are queen-biased in their expression, while many are worker-biased. As with the desaturases, a group of *M. natalensis* elongases also reveal significant signals of positive selection (codeml branch-site test; $p = 4x10^{-4}$), further indicating a greater diversification of CHC production in this higher termite.

The last step of CHC biosynthesis, the production of hydrocarbons from long-chain fatty aldehydes, is catalyzed by a P450 gene, CYP4G1, in *Drosophila melanogaster*[26]. We found one copy of CYP4G1 in *B. germanica*, *Z. nevadensis* and *C. secundus*, but three copies in *M. natalensis*, reinforcing the greater importance of CHC synthesis in this higher termite. Such P450 genes have experimentally been shown to be crucial for maintaining reproductive division in the termite *C. secundus*[28]. Corroborating the known importance of maternal CHCs in *B. germanica*[29], CYP4G1 is over-expressed in female adults compared to nymphs (Fig. 2; table S15). In each of the termites, however, CYP4G1 is more highly expressed in workers (or kings in *C. secundus*) compared to queens (Fig. 2; table S15), adding support that, compared to cockroach nymphs, a change in the dynamics and turnover of CHCs in termite workers has taken place.

Perception of chemical cues

Insects perceive chemical cues from toxins, pathogens, food and pheromones with three major families of chemoreceptors, the Odorant (ORs), Gustatory (GRs) and Ionotropic (IRs) Receptors[30]. Especially ORs have been linked to colony communication in eusocial Hymenoptera, where they abound[13,14]. Interestingly, as previously detected for *Z. nevadensis*[16], the OR repertoire is substantially smaller in *B. germanica* and all three termites compared to hymenopterans. IRs, on the other hand, which are less frequent in hymenopterans, are strongly expanded in the cockroach and termite genomes (Fig. 3 & Fig. S6). Intronless IRs, which are known to be particularly divergent[31], show the greatest cockroach- and Blattodea-specific expansions (Fig. 3a, Blattodea-, Cockroach- and Group D-IRs). By far the most IRs among all investigated species were found in *B. germanica* (455 complete gene models), underlining that the capacity for detecting many different kinds of chemosensory cues is crucial for this generalist that thrives in challenging, human environments. In line with a specialisation in diet and habitat, the total number of IRs is lower within the termites (*Z. nevadensis*: 141; *C. secundus*: 135; *M. natalensis*: 75). Nevertheless, IRs are more numerous in termites than in all other analysed species (except *Nasonia vitripennis*: 111). This is strikingly similar to the pattern for ORs in Hymenoptera, which are also highly numerous in non-eusocial outgroups as well as in eusocial species[13,32].

We scanned each IR group for signs of species-specific positive selection. Within the Blattodea-specific

intronless IRs, we found several codon positions under significant positive selection for the higher termite, *M. natalensis* (codeml site models 7 & 8; $p < 1.7x10^{-10}$). The positively evolving codons are situated within the two ligand-binding lobes of the receptors (Fig. 3c), showing a diversification of ligand specificity has occurred along with the transition to higher eusociality and a change from wood-feeding to fungus-farming in this higher termite. In total, only two IRs were differentially expressed between nymphs and adult females in *B. germanica*. Underlining a change in expression along with the evolution of castes, we found 35 IRs to be differentially expressed between workers and queens in *Z. nevadensis*, 12 in *C. secundus* and 11 in *M. natalensis* (Fig. 3, table S16). The possible role of IRs in pheromonal communication has been highlighted both in the cockroach *Periplaneta americana*[33] and in *D. melanogaster*[34], where several IRs show sex-biased expression.

One group of ORs (orange clade in Fig. 3b) is evolving under significant positive selection at codon positions within the second transmembrane domain in *M. natalensis* (codeml site model; $p = 1.1x10^{-11}$) and *C. secundus* ($p = 5.6x10^{-16}$; Fig. 3d). Such a variation in the transmembrane domain can be related to ligand binding specificity, as has been shown for a polymorphism in the third transmembrane domain for an OR in *D. melanogaster*[35,36], adding further support for an adaptive evolution of chemoreceptors, in line with the greater need for a sophisticated colony communication in the termites. Similar to IRs, a higher proportion of ORs were differentially expressed between workers and queens in the three termites than between nymphs and adults in the cockroach (Fig. 2; table S17), highlighting a change in expression and function along with the transition to eusociality. The evolution of chemoreceptors along with the emergence of the termites can also be related to adaptation processes and changes in diet compared to the cockroach. Experimental verification will help pinpoint which receptors are particularly important for communication.

Changes in gene regulation in termites

The development of distinct castes underlying division of labour is achieved via differential gene expression. Major changes in gene regulation have been reported as being central to the transition to eusociality in bees[9] and ants[10]. Accordingly, we found major changes in DNA methylation patterns (levels per 1-to-1 ortholog) among the termites compared to four other hemimetabolous insect species (Fig. 4a). This is revealed by CpG depletion patterns ($CpG_{o/e}$), a reliable predictor of DNA methylation[37,38], correlating more strongly between the termites than among any of the other analysed hemimetabolous insects (Fig. 4). In other words, within orthologous genes, DNA methylation levels differ greatly between termites and other hemimetabolous species but remain conserved among termite species. Furthermore, a higher proportion of genes were putatively DNA methylated ($CpG_{o/e} < 0.5$) within the termites (40.7% to 50.6%)

compared to other hemimetabolous species (11.5% to 34.0%), as also described for eusocial compared to solitary bees[9].

Levels of DNA methylation correlated negatively with caste-specificity of expression for each of the termites. This is confirmed by a positive correlation between $CpG_{o/e}$ (negative association with level of DNA methylation) and $\log_2$-fold change of expression between queens and workers (Pearson's r = 0.32 to 0.36; $p < 2.2x10^{-16}$). The caste-specific expression of unmethylated genes in termites is reflected in the enrichment of GO terms related to sensory perception, regulation of transcription, signalling and development, whereas methylated genes are mainly related to general metabolic processes (Fig. 4b, tables S18). These results show strong parallels to findings for eusocial Hymenoptera[39,40,41,42]. This is in stark contrast to the non-eusocial cockroach, *B. germanica*, where there was only a very weak relationship between $CpG_{o/e}$ and differential expression between nymphs and adult females (r = 0.14), nor were any large differences apparent in enriched GO terms between methylated and non-methylated genes (Fig. 4b).

Our results argue in favour of a diminished role of DNA methylation in caste-specific expression within eusocial insects, as recently shown[37,43]. In fact, DNA methylation appears to be important for the regulation of house-keeping genes because methylated genes are related to general biological processes (further supported by lower $CpG_{o/e}$ within 1-to-1 orthologs than in non-conserved genes)[44], while caste-specific genes are 'released' from this type of gene regulation. However, a recent study linked caste-specific DNA methylation to alternative splicing in *Z. nevadensis*[45].

Major biological transitions are often accompanied by expansions of transcription factor (TF) families, such as genes containing zinc-finger (ZF) domains[46]. We also observed large differences in ZF families within the termites compared to *B. germanica*. Many ZF families were reduced or absent in termites, while different, unrelated ZF gene families were significantly expanded (tables S2-S5). Queen-biased genes were significantly over-represented among ZF genes for termites ($p < 2 \times 10^{-10}$;χ^2 test; table S19), indicating an important role of ZF genes in the regulation of genes related to caste-specific tasks and colony organisation in the termites. This is in contrast to the significant under-representation of differentially expressed ZF genes within *B. germanica* ($p = 1.42 \times 10^{-5}$; χ^2-test). Interestingly, two other important TF families (bHLH and bZIP)[46], which were not expanded in the termites, showed no caste-specific expression pattern ($p > 0.05$). These major upheavals in ZF gene families and their caste-specific expression show that major changes in TFs accompanied the evolution of termites, strikingly similar to the evolution of ants[10].

Endocrine regulation

Hemimetabolous eusociality is characterised by differentiated castes, which represent different developmental stages. This is in contrast to eusocial Hymenoptera, in which workers and reproductives are adults. While cockroaches develop directly through several nymphal stages before becoming reproductive adults, termite development is more phenotypically plastic, and workers are essentially immatures (Fig. 2). In wood-dwelling termites, such as *C. secundus* and *Z. nevadensis*, worker castes are non-reproductive immatures that are totipotent to develop into other castes, while in the higher termite, *M. natalensis*, workers can be irreversibly defined instars. It is therefore clear that a major change during the evolution of termites occurred within developmental pathways. Accordingly, we found changes in expression and gene family size of several genes related both to molting and metamorphosis.

In the synthesis of the molting hormone, 20-hydroxyecdysone, the six Halloween genes (5 Cytochrome P450s and a Rieske-domain oxygenase) play a key role[47,48]. Only one Halloween gene, Shade (Shd; CYP314A1), which mediates the final step of 20-hydroxyecdysone synthesis, is differentially expressed between the final nymphal stages and adults females in *B. germanica* (Fig. 2; table S20), consistent with its role in the nymphal or imaginal molt. In the three termites, the Halloween genes show varying caste-specific expression (Fig. 2; table S20), showing that ecdysone plays a significant role in the regulation of caste differences. Ecdysteroid kinase genes (EcK), which convert the insect molting hormone into its inactive state, ecdysone 22-phosphate, for storage[49], are only over-expressed in female adults compared to nymphs in *B. germanica* (16/51 genes, Fig.2, table S21). In termites, however, where the gene copy number is reduced (18 to 20 per species), these important molting genes appear to have evolved worker-specific functions (Fig. 2; table S21).

Whereas 20-hydroxyecdysone promotes molting, juvenile hormone (JH) represses imaginal development in pre-adult instars[50]. JH is important in caste differentiation in eusocial insects, including termites[12,51]. Hemolymph juvenile hormone binding proteins (JHBP), which transport JH to its target tissues[52], are reduced within the termites (21 to 33 genes) but significantly expanded in *B. germanica* (51 copies). Thirteen of the JHBP genes are over-expressed in adult females and only 8 in nymphs in *B. germanica*. In both *Z. nevadensis* (15 worker-specific and 1 queen-specific) and *M. natalensis* (11 worker-specific and 4 queen-specific), on the other hand, JHBPs are significantly more worker-biased ($p < 0.01$, χ^2 test; table S22; Fig. 2). In *C. secundus*, expression is more varied, with 5 worker-biased, 8 king-biased and 3 queen-biased genes (Fig. 2; table S22).

These changes in copy number and caste-specific expression of genes involved in metamorphosis and molting within termites compared to the German cockroach demonstrate that changes occurred in the control of the developmental pathway along with the evolution of castes. However, this interpretation

needs to be experimentally verified.

Conclusions

These results, considered alongside many studies on eusociality in Hymenoptera[9,10,13,27], provide evidence that major changes in gene regulation and the evolution of sophisticated chemical communication are fundamental to the transition to eusociality in insects. Strong changes in DNA methylation patterns correlated with broad-scale modifications of expression patterns. Many of these modified expression patterns remained consistent among the three studied termite species and occurred within protein pathways essential for eusocial life, such as CHC production, chemoperception, ecdysteroid synthesis and JH transport. Many of the mechanisms implicated in the evolution of eusociality in the termites occurred convergently around 50 my later in the phylogenetically distant Hymenoptera. However, several details are unique due to the distinct conditions within which eusociality arose. One important difference is the higher TE content within cockroaches and termites, which likely facilitated changes in gene family sizes, supporting the transition to eusociality. However, the most striking difference is the apparent importance of IRs for chemical communication in the termites, compared to ORs in Hymenoptera. According to our results, the non-eusocial ancestors of termites possessed a broad repertoire of IRs, which favoured the evolution of important functions for colony communication in these chemoreceptors within the termites, whereas in the solitary ancestors of eusocial hymenopterans ORs were most abundant[13,32]. The parallel expansions of different chemoreceptor families in these two independent origins of eusociality indicate that convergent selection pressures existed during the evolution of colony communication in both lineages.

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MATERIALS AND METHODS

Genome sequencing and assembly

Genomic DNA from a single *Blattella germanica* male from an inbred line (strain: American Cyanamid = Orlando Normal) was used to construct two paired-end (180 bp and 500 bp inserts) and one of the two mate pair libraries (2 kb inserts). An 8kb mate pair library was constructed from a single female. The libraries were sequenced on an Illumina HiSeq2000 sequencing platform. The 413 Gb of raw sequence data were assembled with Allpaths LG[1], then scaffolded and gap-filled using the in-house tools Atlas-Link v.1.0 (https://www.hgsc.bcm.edu/software/atlas-link) and Atlas gap-fill v.2.2. For *Cryptotermes secundus*, three paired-end libraries (250 bp, 500 bp and 800 bp inserts) and three mate pair libraries (2 kb, 5 kb and 10 kb inserts) were constructed from genomic DNA that was extracted from the head and thorax of 1 000 individuals, originating from a single, inbred field colony. The libraries were sequenced on an Illumina HiSeq2000 sequencing platform. The *C. secundus* genome was assembled using SOAPdenovo (v.2.04)[2] with optimised parameters, followed by gapcloser (v1.10, released with SOAPdenovo) and kgf (v1.18, released with SOAPdenovo).

Transcriptome sequencing and assembly

For annotation purposes, twenty-two whole body RNAseq samples from various developmental stages were obtained for *B. germanica*. For *C. secundus* RNAseq libraries were obtained for three workers, four queens and four kings, based on degutted, whole body extracts. In addition, we sequenced 10 *M. natalensis* RNAseq libraries from three queens, one king and six pools of workers. All libraries were constructed using the Illumina (TruSeq) RNA-Seq kit.

For protein coding gene annotation, *B. germanica* reads were assembled with *de novo* Trinity (version r2014-04-13)[3]. The *C. secundus* reads were assembled using i) Cufflinks on reads mapped with TopHat (version2.2.1)[4,5], ii) *de novo* Trinity[3]; and iii) genome-guided Trinity on reads mapped with TopHat.

Repeat annotation

A custom *C. secundus* and *B. germanica* repeat library was constructed using a combination of homology-based and *de novo* approaches, including RepeatModeler/RepeatClassifier (http://www.repeatmasker.org/RepeatModeler.html), LTRharvest/LTRdigest[6] and TransposonPSI (http://transposonpsi.sourceforge.net/). The *ab initio* repeat library was complemented with the RepBase (update 29-

08-2016)[7] and SINE repeat databases, filtered for redundancy with CD-hit and classified with RepeatClassifier. RepeatMasker (version open-4.0.6, http://www.repeatmasker.org) was used to mask the *C. secundus* and *B. germanica* genome. Repeat content for the other studied species (Fig. 1) was obtained from the literature[8,9,10,11,12,13,14].

Protein-coding gene annotation

The *B. germanica* genome was annotated with Maker (version 2.31.8)[15], using (i) the species-specific repeat library, (ii) *B. germanica* transcriptome data (22 whole body RNAseq samples), and (iii) the swissprot/uniprot database (last accessed: 21-01-2016) plus the *C. secundus* and *Zootermopsis nevadensis* protein sequences for evidence-based gene model predictions. AUGUSTUS (version 3.2)[16], GeneMark-ES Suite (version 4.21)[17] and SNAP[18] were used for *ab initio* predictions. *Cryptotermes secundus* protein-coding genes were predicted using homology-based, *ab initio* and expression-based methods, and integrated into a final gene set (see supplementary information). Gene structures were predicted by GeneWise[19]. The *ab initio* annotations were predicted with AUGUSTUS[20] and SNAP[18], retained if supported by both methods and integrated with the homology-based predictions using GLEAN[21]. Transcriptome-based gene models were merged with PASA[22] and tested for coding potential with CPC[23] and OrfPredictor[24]. PASA gene models were merged with the homology-based and *ab initio* gene set, retaining the PASA models in case of overlap. Desaturases, elongases, chemosensory receptors, Cytochrome P450's and genes involved in the juvenile hormone pathway were manually curated in Blattodea.

Differential gene expression

The *C. secundus* and *M. natalensis* RNAseq libraries, were complemented with nine published *Z. nevadensis* libraries, yielding 2 to 6 libraries from workers, queens and kings for each termite. These were compared to six of the *B. germanica* libraries: two from 5^{th} instar nymphs, two from 6^{th} instar nymphs and two from adult females. Reads were mapped to the genome using HiSat2[25]. Read counts per gene where obtained using htseq-count and DESeq2[26] was used for differential expression analysis.

Protein orthology

In addition to *B. germanica*, *C. secundus*, *Z. nevadensis* and *M. natalensis*, 18 other insect proteomes were included in our analyses; *L. migratoria*, *R. prolixus*, *E. danica*, *D. melanogaster*, *A. aegypti*, *T. castaneum*, *N. vitripennis*, *P. canadensis*, *A. mellifera*, *H. saltator*, *L. humile*, *C. floridanus*, *P. barbatus*,

S. invicta, *A. echinatior* and *A. cephalotes*; as well as for the centipede, *S. maritima*, as an outgroup (for sources see Table S23). These proteomes were grouped in to orthologous clusters with OrthoMCL[27], with a granularity of 1.5.

IR and OR identification, phylogeny and structure

Ionotropic receptors (IRs) were identified using two custom Hidden Markov Models (HMMs) obtained with `hmmbuild` and `hmmpress` of the HMMER suite[28]. The first HMM comprises the IR's ion channel and ligand-binding domain based on a MAFFT[29] protein alignment of 76 IRs from 15 species (Table S24). The second HMM was built to distinguish IRs from iGluRs, IR8a and IR25a, which have an additional amino-terminal domain (ATD)[30]. For this we built an HMM from 48 protein sequences (Table S24). The proteomes were scanned with `pfam_scan` and the two custom HMMs, where proteins that matched the IR HMM, but not the ATD HMM were annotated as IRs. ORs were identified based on the Pfam domain `PF02949` (7tm Odorant receptor).

Multiple sequence alignments of IRs and ORs were obtained with `hmmalign`[28], using the Pfam OR HMM `PF02949` and custom IR HMM to guide the alignment. Gene trees were computed with FastTree[31] (options: `-pseudo -spr 4 -mlacc 2 -slownni`) and visualised with iTOL v3[32]. Putative IR ligand-binding residues and structural regions were identified based on the alignments with *D. melanogaster* IRs and iGluRs of known structure[33].

Gene family expansions and contractions

For the analyses of gene family expansions and contractions, the hierarchical clustering algorithm MC-UPGMA[34] was used, with a ProtoLevel cutoff of 80[35]. Protein families were further divided into sub-families if they contained more than 100 proteins in a single species, or more than an average of 35 proteins per species. Proteins were blasted against the RepeatMasker TE database (E-value $< 10^{-5}$) and clusters where $> 50\%$ of the proteins were identified as transposable elements were discarded. Clade- and species-specific protein family expansions and contractions, were identified with CAFE v3.0[36] using the same protocol as[37,38] (see also Supplementary material).

TE-facilitated expansions

The repeat content in the 10 kb flanking regions of *B. germanica*, *C. secundus*, *Z. nevadensis* and *M. natalensis* genes was calculated using bedtools[39]. CDS' from neighbouring genes were removed and

the repeat content was analysed using Generalized Linear Mixed Models (glmmPQL implemented in the R[40] package MASS[41]) with binomial error distribution. Fixed predictors included gene family expansion, species ID and their interaction. Cluster ID was fitted as random factor to avoid pseudo-replication. Significance was assessed based on the Wald-t test (R package aod[42]) at $\alpha < 0.05$. Main and interaction effects for each of the genomic regions are listed in table S8. Model parameters are listed in table S9.

Evolutionary rates

The rate of protein evolution (ω; ratio of non-synonymous to synonymous substitutions) was estimated for the OrthoMCL 1-to-1 orthologs in *L. migratoria*, *B. germanica*, *Z. nevadensis*, *C. secundus* and *M. natalensis*. Protein sequences were aligned with t-coffee[43]. CDS alignments were obtained with pal2nal.pl[44] and trimmed with Gblocks[45]. To identify genes with different rates of protein evolution within the termites compared to outgroups, a set of codeml branch models was used (model = 2; NSsites = 0; PAML suite[46]). Specifically, we compared the null model (H_0: one ω across all branches) to i) H_{A1}: allowing for termite-specific ω (`((Lmig,Bger,(Znev#1,(Csec#1, Mnat#1)#1))`; and ii) H_{A2}: allowing for different ω for different levels of eusocial complexity `(Lmig,Bger,(Znev#1,(Csec#1, Mnat#2)#1))`. LR-tests were performed on unsaturated models (dS < 3) and p-values were Bonferroni-corrected. Gene ontology enrichment of genes with significantly higher rates of protein evolution in termites was performed with the TopGo[47] package in R.

To test for positive selection within gene families of interest, i) site model tests 7 and 8 were performed (model = 0; NSsites = 7 8) on species-specific CDS alignments or ii) branch-site test (model = 2; NSsites = 2; fix_omega = 1 for null model and 0 for alternative model) on multi-species alignments. Protein sequences were aligned using MAFFT[29] with the E-INS-i strategy, and CDS alignments were created using pal2nal.pl[44]. Phylogenetic trees were created with FastTree[31]. Alignments were trimmed using Gblocks (settings: -b2 = 21; -b3 = 20; -b4 = 5; -b5 = a). Models were compared using LR test and where $p < 0.05$, Bayes Empirical Bayes (BEB) results were consulted for codon positions under positive selection.

CpG depletion patterns and GO enrichment

To estimate DNA methylation we compared observed to expected CpG counts within CDS sequences[48,49]. A low $CpG_{o/e}$ indicates a high level of DNA methylation, as the cytosine of methylated CpGs often mutate to thymines. Expected CpG counts were calculated by dividing the product of cytosine and guanine counts by the sequence length. The PCA in figure 3 was created using the R function prcomp

on log transformed $CpG_{o/e}$ values for all 1-to-1 orthologs for the seven hemimetabolous species. These orthologs were extracted from the OrthoMCL results. The 3D plot was created with the plot3d command from the R package rgl.

CpG depleted (first quartile) and enriched genes (fourth quartile) were tested for enrichment of Gene Ontology terms. Pfam protein domains were obtained for *B. germanica*, *Z. nevadensis*, *C. secundus* and *M. natalensis* protein sequences using PfamScan[50]. Corresponding GO terms were obtained with Pfam2GO. GO-term over-representation was assessed using TopGO[47] package in R. Enrichment analysis was performed using the weight algorithm selecting nodesize=10 to remove terms with less than 10 annotated GO terms. After that GO terms classified as significant (topGOFisher¡0.01) were visualized using R package tagcloud (https://cran.r-project.org/web/packages/tagcloud/).

Data availability

The data reported in this study are archived at the following databases: NCBI (genomes sequences), SRA (genomic and transcriptomic reads), i5k Workspace@NAL & Dryad (annotations). Detailed accession information is tabulated in the Supplementary Materials (table S26).

Scripts and output files are available on request to E.B.B.

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Figures

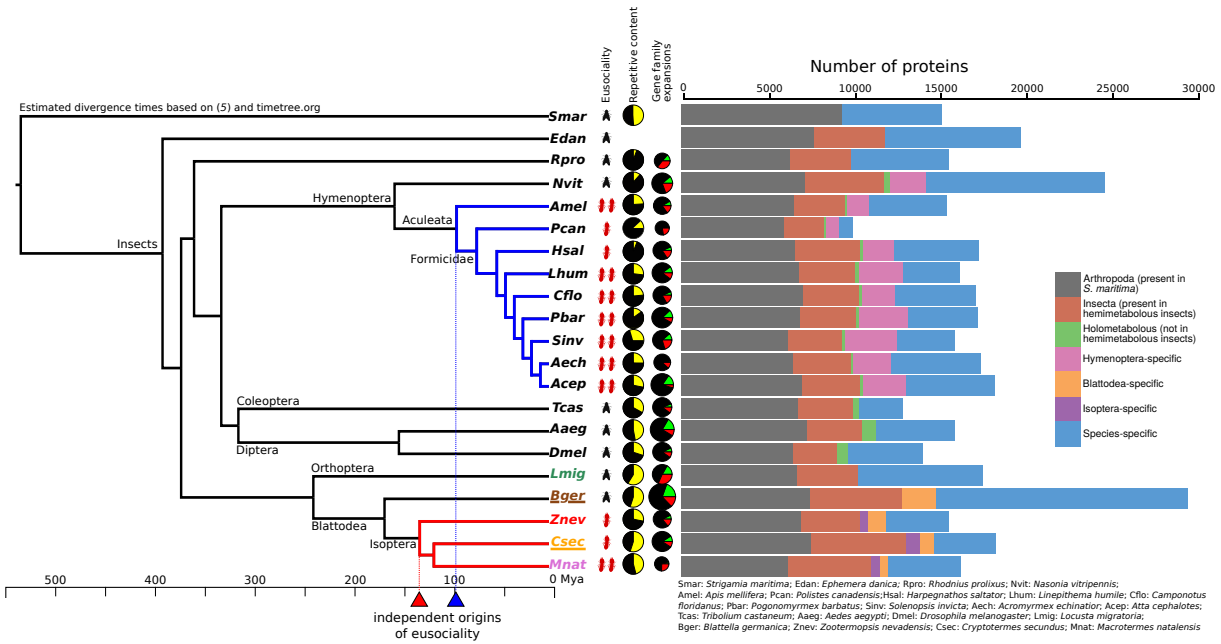

Figure 1: **Phylogenetic, genomic and proteomic comparisons of 20 insect species.** From left to right: Phylogenetic tree of 20 insect species with *Strigamia maritima* (centipede) as outgroup; level of eusociality (one red insect: simple eusociality; two red insects: advanced eusociality; black fly: non-eusocial); fractions of repetitive content (yellow) within genomes of selected species (for sources see supplementary material); proportions of species-specific gene family expansions (green), contractions (red) and stable gene families (black), size of pies represents relative size of gene family change (based on total numbers). Bar chart showing protein orthology across taxonomic groups within each genome.

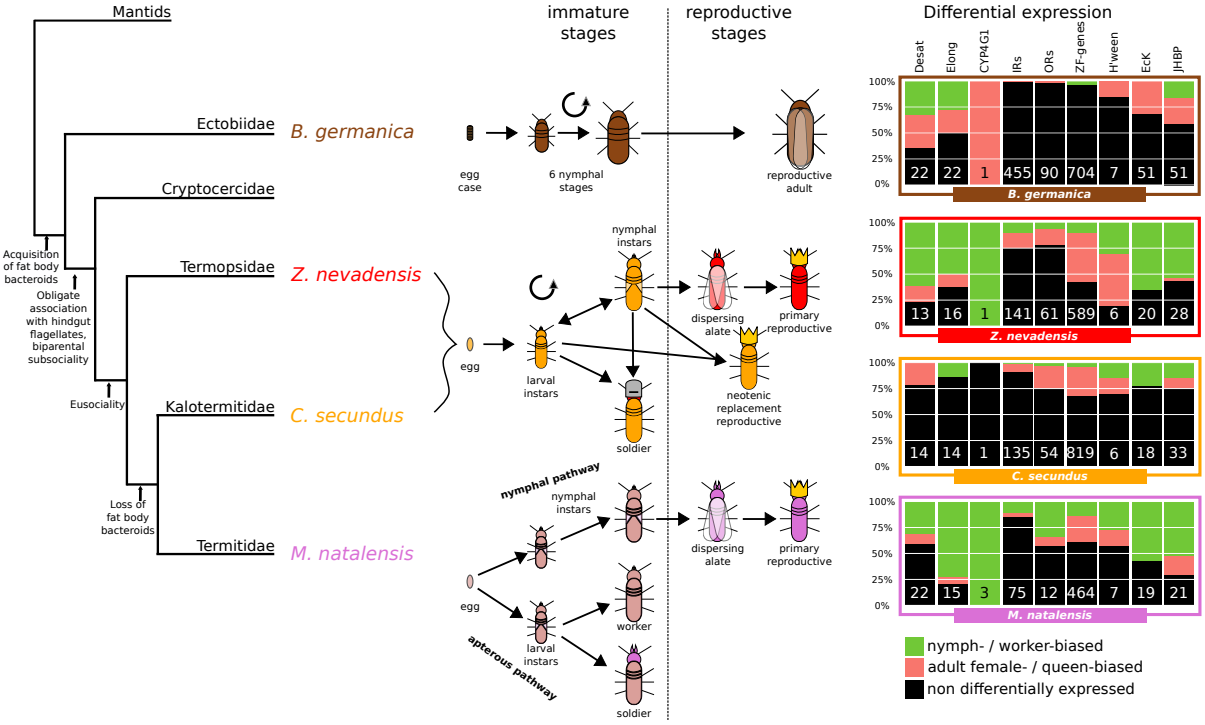

Figure 2: **Comparison of developmental pathways between *B. germanica*, the lower termites, *Z. nevadensis* and *C. secundus*, and the higher termite, *M. natalensis*.** Shown from left to right are: a simple phylogeny[51] describing important novelties along the evolutionary trajectory to termites; life cycles; differential expression between workers and queens (between nymphs and adult females in *B. germanica*) of selected gene families (Desat = desaturases, Elong = elongases, H'ween = Halloween genes; numbers denote total numbers of genes in each gene family).

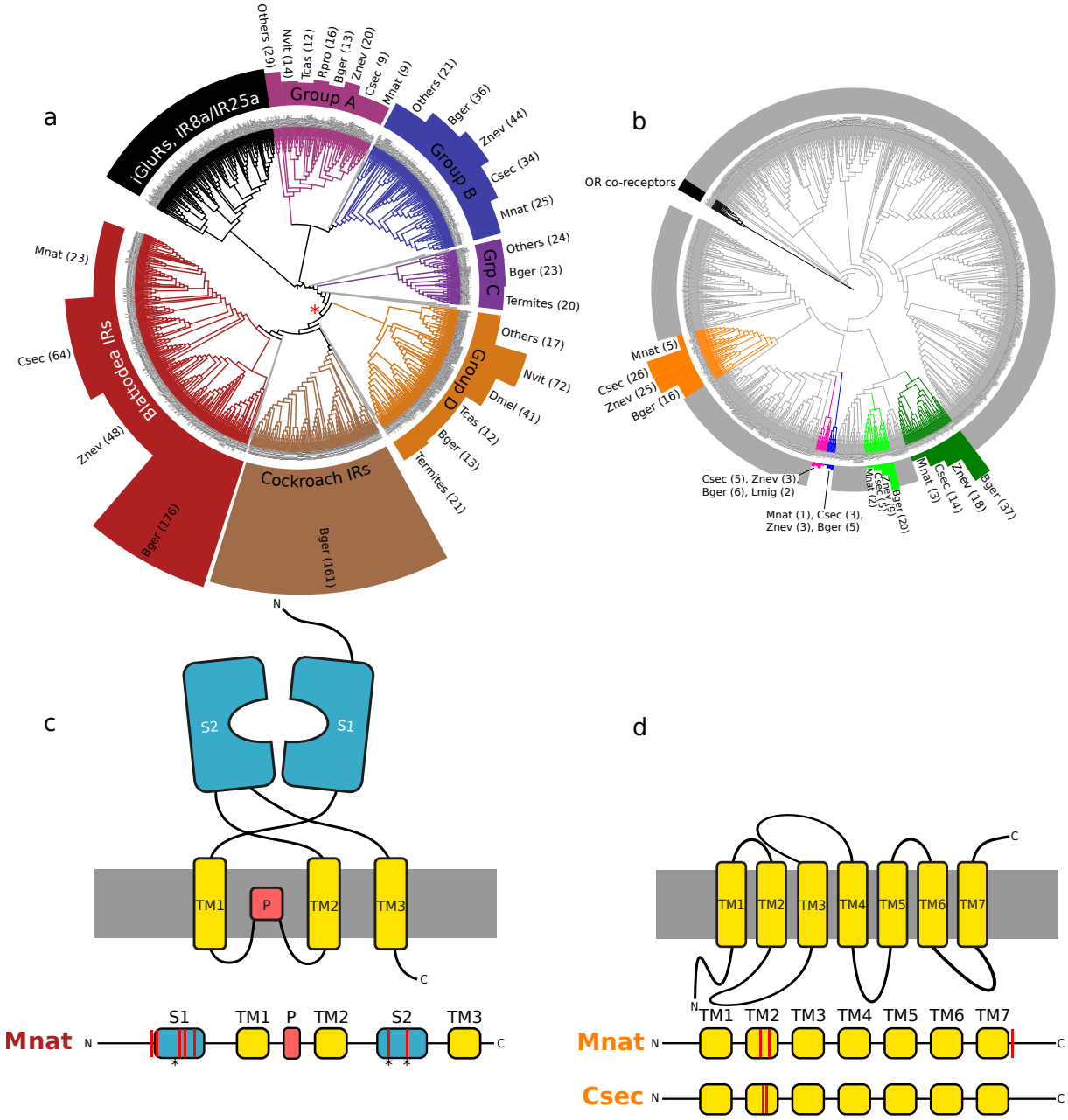

Figure 3: **Expansions, contractions and positive selection within IRs and ORs in termites.** IR (a) and OR (b) gene trees of 13 insect species. Only well supported clades (support values > 85) that include *B. germanica* or termite genes are highlighted within the gene trees. Lengths of coloured bars represent number of genes per species within each of these clades. Red asterisk in (a) denotes putative root of intronless IRs. 2D structure and sites under positive selection (red bars; codeml site models 7 & 8) for Blattodea-IR genes in *M. natalensis* ($p < 1.7x10^{-10}$) (c) (asterisks denote putative ligand binding sites[33]) and orange OR genes in *M. natalensis* ($p = 1.1x10^{-11}$) and *C. secundus* ($p = 5.6x10^{-16}$) (d).

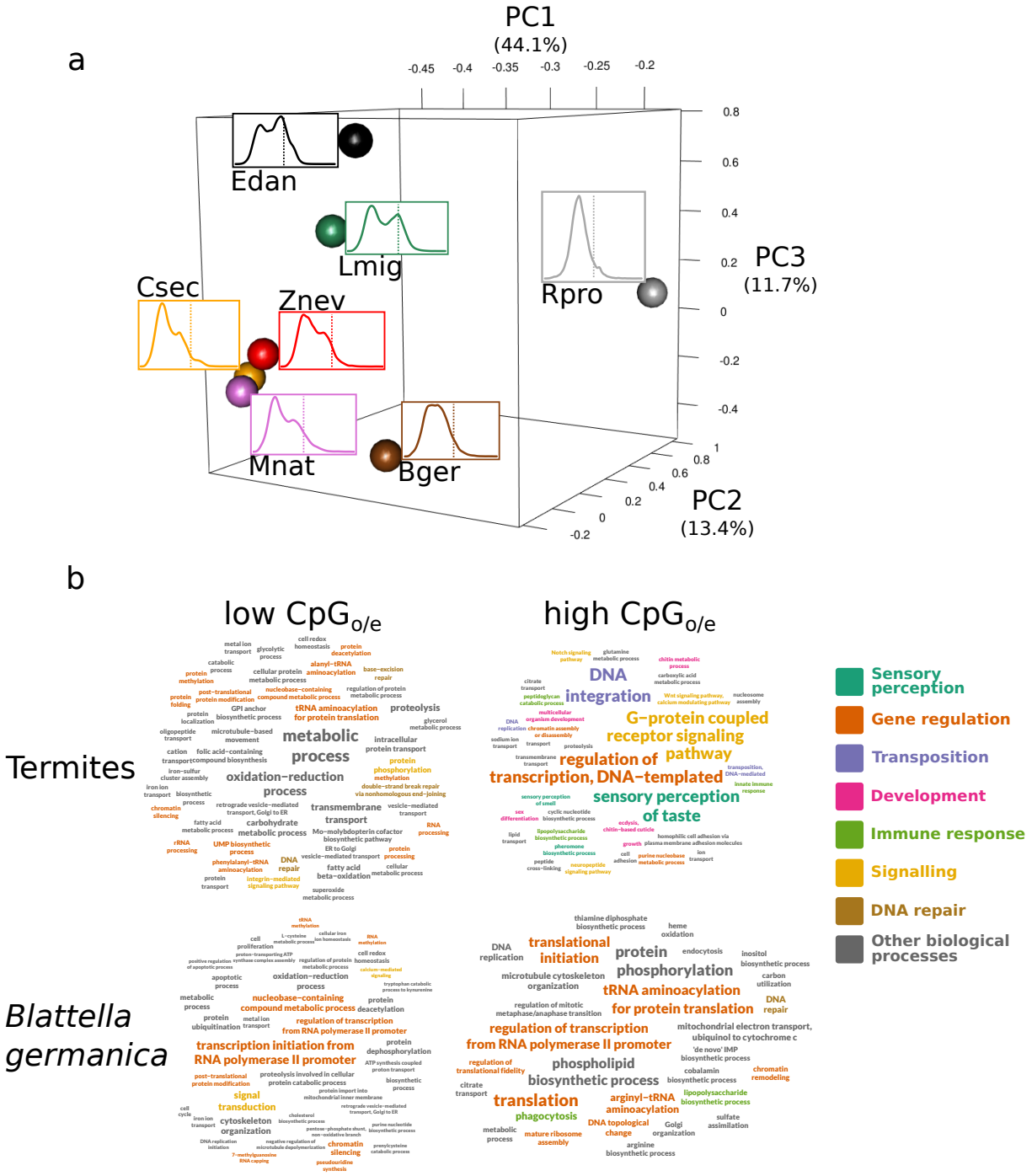

Figure 4: **CpG$_{o/e}$ of seven hemimetabolous insects.** a) PCA of DNA methylation patterns among 2664 1-to-1 orthologs, estimated via CpG$_{o/e}$. Spheres represent positions of species within 3D PCA; curves are distribution of CpG$_{o/e}$ with dotted line showing CpG$_{o/e}$ = 1. b) Tag clouds of enriched ($p < 0.05$) GO terms (biological processes) among lower (left) and higher quartile (right) of CpG$_{o/e}$ within termites (top) and *B. germanica* (bottom). For termites, genes were merged from all three species for analysing GO term enrichment.
High CpG$_{o/e}$ indicates low level of DNA methylation and vice versa.