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Differences between uncapping and removal behaviors in *Apis cerana* from the perspective of long non-coding RNAs

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Abstract

Background Hygienic behavior, a specialized form of immune response evolved in social insects, plays a crucial role in safeguarding colonies from disease spread. In honeybee colonies, such behavior typically entails the dual steps of uncapping and removal of unhealthy and deceased brood. Although in recent years, numerous studies have examined the development of hygienic behavior, the mechanisms underlying the division in the performance of uncapping and removal have yet to be sufficiently elucidated. In this regard, long non-coding RNAs (lncRNAs) have been evidenced to be engaged in regulating the physiological activities of honeybees; however, whether lncRNAs are likewise involved in the uncapping and removal tasks has not been clarified.

Results In this study, the strong hygienic *Apis cerana* worker bees were used and the processes of uncapping and removal behaviors in three colonies were assayed with freeze-killed brood in the field. We then sequenced the antennal RNAs of honeybees to identify differentially expressed lncRNAs and performed lncRNA-mRNA association analysis to establish the differences between uncapping and removal. We detected 1,323 differentially expressed lncRNAs in the antennae, and the findings of lncRNA-mRNA association analyses revealed that the target genes of differentially expressed lncRNAs between uncapping and removal worker bees were predominantly linked to response to stimulus, receptor activity, and synapse. Notably, among the lncRNAs enriched in cellular response to stimulus, XR_001766094.2 was exclusively expressed in the uncapping worker bees. Based on these findings, we hypothesize that XR_001766094.2 plays a key role in distinguishing uncapping from removal behaviors by responding to external stimulus, thereby suggesting that the division of hygienic behaviors is governed by differential thresholds of responsiveness to environmental cues.

Conclusion We characterized differences in the uncapping and removal behaviors of worker bees from a perspective of lncRNAs. Uncapping bees may be equipped with a more rapid stimulatory response and more acute olfactory sensitivity, contributing to the rapid hygienic behavior in honeybee colonies. Our results thus establish a foundation for potential lncRNA-mediated gene expression regulation in hygienic behavior.

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Keywords Honeybee, *Apis cerana*, Antenna, lncRNA, Hygienic behavior, Uncapping, Removal

Background

The honeybee, standing as an indispensable economic insect of our agricultural ecosystem, contributes to over a third of the world's crops productivity through pollination services [1, 2]. However, a marked decline in the numbers of honeybee colonies has been reported in the past decades, fueled by a cocktail of viral, bacterial, and parasitic infections, exacerbated by the harmful effects of widespread insecticides [3]. As highly evolved social insects, honeybees have developed a sophisticated social immune system that fortifies their colonies against diseases [4]. Hygienic behavior is a specialized type of immune response unique to social insects, which effectively thwarts the spread of pathogens within the hive [5]. The hygienic behavior typically involves a meticulous two-step process executed by different worker bees, who first detect and uncap and then remove unhealthy or deceased bee brood from their cells, along with thoroughly cleaning the vacated spaces [6, 7]. Such behavior equips bee colonies with the resilience to withstand a range of potentially devastating diseases such as European foulbrood and chalkbrood [8, 9].

The hygienic behavior in honeybees is a highly heritable trait, rooted in an intricate genetic landscape that encompasses a potentially vast gene network [5, 10, 11]. While inter-subspecific variations in this behavior have been observed among *Apis mellifera* subspecies [12, 13], at the species level, *Apis cerana* is evidenced to be faster at hygienic response, including both uncapping and removal processes, in comparison to *A. mellifera* [14]. This may be attributed to differences in olfactory perception, with the antennae serving as the pivotal sensory organ [15–17]. Honeybees rely heavily on their antennae to detect external olfactory stimulus, and strong hygienic worker bees are regarded to possess acute olfactory sensitivity in discerning between healthy and deceased brood based on specific death pheromones, such as β-octene and oleic acid [18–20]. Therefore, the facilitation of rapid hygienic behavior in honeybees may stem from an enhanced sensitivtiy to detect and respond to stimulatory signals, but the mechanisms underlying the distinction between uncapping and removal behaviors within this context remains unclear.

Long non-coding RNAs (lncRNAs), a class of non-protein-coding transcripts exceeding 200 nt in length and often harboring two or more exons, play crucial yet understated roles in orchestrating an array of complex biological processes across eukaryotes, encompassing physiology, metabolism, immunity, and disease pathogenesis [21–23]. Within the highly organized societies of honeybees, lncRNAs has been established to be engaged in diverse phenomena such as labor partitioning [24], caste differentiation [25], immune defense [26], and reproductive strategies [27]. However, their roles in hygienic behavior have yet to be well characterized. In this study, we performed a comprehensive analysis of lncRNAs and mRNAs in the antennae of *A. cerana* workers undertaking the tasks of uncapping or removal, based upon which, we aimed to provide novel insights into the intricate regulatory mechanisms that govern hygienic behavior in these social insects, thereby advancing our understanding of social immunity and its implications for honeybee health.

Methods

Experimental colonies

Honeybee (*A. cerana*) colonies were kept in an experimental apiary, owned by our laboratory, at Yangzhou University (Yangzhou, China). Three queenright colonies (C01, C02, and C03) used in this study were housed in Langstroth hives containing four frames, including numerous healthy capped larvae with ample honey and pollen stores.

Freeze-killed brood assays

To assess the uncapping and removal behaviors of worker bees, we used a standard freeze-killed brood method [18]. In brief, we used a comb with a concentrated capped brood area filled with purple-eyed pupae, from each colony. A circular area (Ø = 75 mm) of the capped pupae on one side of the comb was frozen using liquid nitrogen, and the treated combs were returned to their original colonies after thawing. We recorded the status of the treated cells as uncapping or removal at 3, 6, 24, 27, 30, 48, 51, 54, and 72 h. An occupied cell that was partially or completely uncapped was considered targeted by hygienic behavior and was recorded as uncapping. An empty cell derived from an originally occupied one, which implied that a pupa had been removed by hygienic workers, was recorded as removal.

In order to sample the worker bees that performed uncapping or removal, we repeated the freeze-killed brood assays in the same colonies using observation hives. When an *A. cerana* worker bee performed uncapping or removal behavior targeting the treated cells, it was removed from the comb and placed in liquid nitrogen for snap freezing.

Removal of honeybee antennae

Bees sampled from the colonies C01, C02, and C03 were categorized as C01-uncapping (C01-u), C01-removal (C01-r), C02-uncapping (C02-u), C02-removal (C02-r),

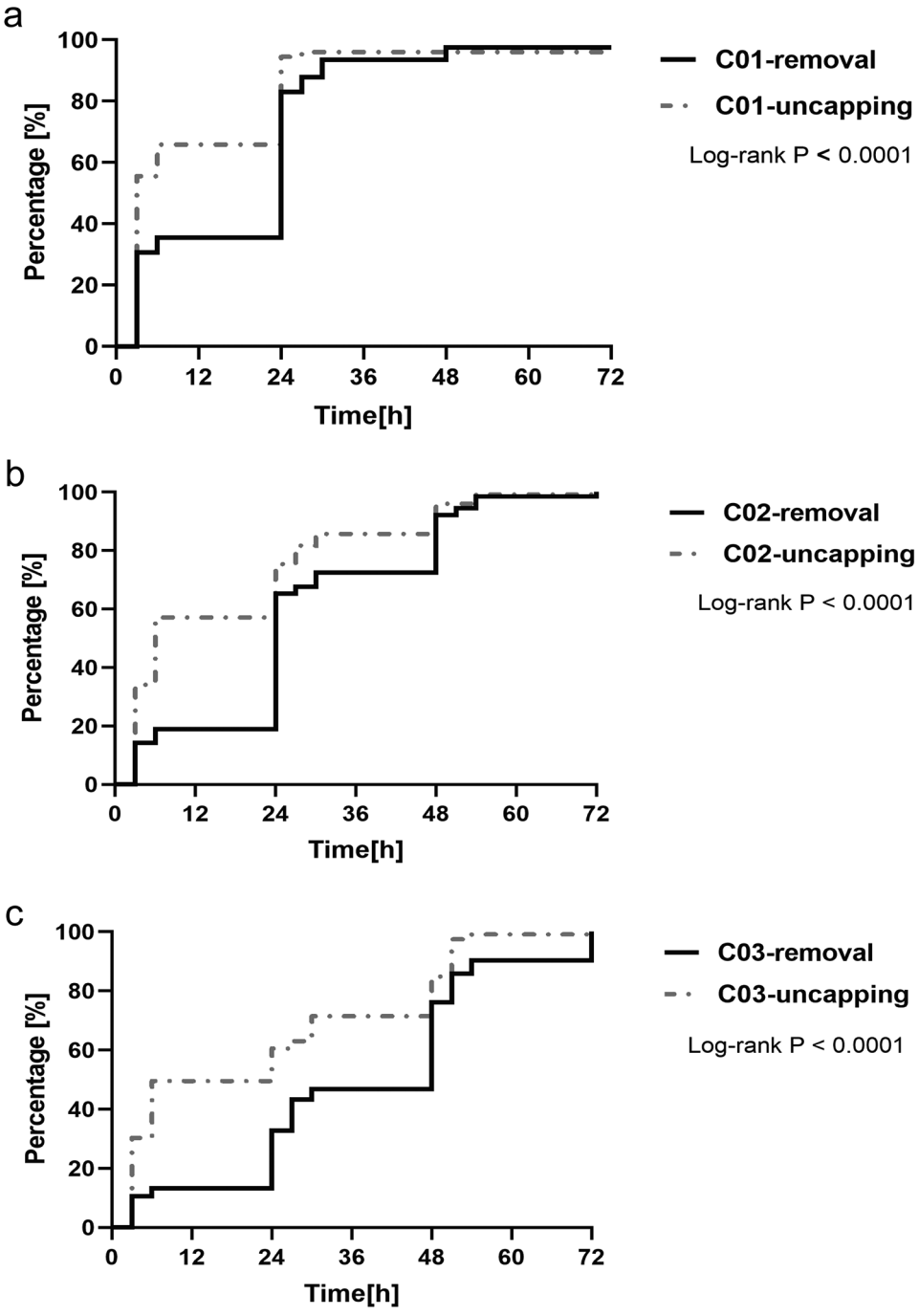

Fig. 1 The process of uncapping and subsequent removal of freeze-killed broods over time in three different *Apis cerana* colonies. Kaplan-Meier plots are shown for cell uncapping and brood removal in colonies C01 (**a**), C02 (**b**), and C03 (**c**). The percentage values indicate the proportion of uncapping and removal behaviors in each of the three colonies. Throughout the 72-h observation period, the increase in the proportions of uncapping and removal behaviors is represented by line segments connecting the time points at 3, 6, 24, 27, 30, 48, 51, 54, and 72 h. Log-rank P: the P value obtained from the log-rank test

C03-uncapping (C03-u), and C03-removal (C03-r), respectively. From each of the six groups, we randomly selected approximately 50 ($n=50\pm5$) workers which were stored in liquid nitrogen. Both antennae of each worker were detached using sterilized forceps and were placed in enzyme-free tubes and stored at -80℃.

RNA sequencing of honeybee antennae

Total RNA was extracted from antennae collected from the six groups of bees using a TRIzol reagent kit (Invitrogen, Carlsbad, CA, US) according to the manufacturer's protocol, the quality of which was assessed using

an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, US). After total RNA was extracted, rRNA was removed to retain mRNAs and ncRNAs. The enriched mRNAs and ncRNAs were fragmented into short fragments, and random hexamers, DNA polymerase I, RNase H, and dNTP were used to obtained first- and second-strand cDNAs. The cDNA fragments were purified using a QiaQuick PCR extraction kit (Qiagen, Venlo, Netherlands) and were ligated with Illumina sequencing adapters (Illumina, San Diego, CA, USA). Sequencing was performed using an Illumina HiSeqTM 4000 platform.

Bioinformatic analysis of the RNA sequencing data

RNA sequencing of antennae from uncapping and removal worker bees yielded approximately 400 million raw reads from the six assessed worker bee groups (Table S1). Reads containing adapters or more than 10% unknown nucleotides and low-quality reads were eliminated using fastp (version 0.18.0) [28] to retain only clean high-quality reads. QC calculation (Q20 and Q30) was performed to assess the bases quality, and the high-quality reads were mapped to the ribosomal RNA database, using the uncapping reads for subsequent analysis. First, all high-quality clean reads were mapped to the reference genome, and paired-end clean reads were aligned to the genome. Reference genome-based comparative analyses were performed using HISAT2 software. The reconstruction of transcripts was carried out using Stringtie software (version 1.3.4) [29, 30], and we used Cuffcompare to categorize the reconstructed transcripts. Transcript abundance was quantified using Stringtie, and for each transcription region, an FPKM (fragment per kilobase of transcript per million mapped reads) value was calculated to quantify its expression abundance and variations so that the calculated transcripts expression can be directly used for comparing the difference of transcripts expression among samples. RNAs and lncRNAs differential expression analysis was performed using DESeq2 (version 1.42.1) [31]. We defined the differentially expressed lncRNAs (DElncRNAs) among the uncapping and removal groups, with the differential expression of genes being defined based on thresholds of a false discovery rate (FDR) < 0.05 and an absolute fold change ≥ 2.

Target gene prediction

To examine the function of lncRNAs in "uncapping" and "removal", we used antisense, *cis*, and *trans* to predict the putative targets of lncRNAs, as follows.

(1) Antisense lncRNA analysis: Using RNAplex, we defined the putative target genes by analyzing the binding of antisense DElncRNAs and mRNAs [32], and determined correlations between the antisense DElncRNAs and mRNAs.
(2) LncRNA *cis*-regulation analysis: The basic principle underlying *cis*-regulation target gene prediction is that the functions of lncRNAs are associated with neighboring protein-coding genes, and that the target genes are located within a 10-kb region upstream or downstream of the DNA.
(3) LncRNA *trans*-regulation analysis: The basic principle underlying *trans*-acting target gene prediction is that the function of a lncRNA is unrelated to the location of a target coding gene, but is instead associated with that of the protein-coding gene with which it co-expresses.

We performed GO and KEGG enrichment analyses for differentially expressed mRNAs or target genes of the lncRNAs.

Results

Data recording and statistical analyses of freeze-killed broods

For all of the assessed colonies, the results revealed significant differences in task performances of uncapping and removal worker bees (Fig. 1; $P < 0.0001$). In all three colonies, the percentage of worker bees performing "uncapping" was higher than that of bees performing "removal" in the 3 h, and this trend continued until the 24 h. At 24 h, the percentage of "removal" worker bees began to increase and was more pronounced in colonies C01 and C02, where the percentage of "removal" individuals exceeded 60%.

Quality control and evaluation of RNA sequencing results

After filtering low-quality data, QC calculation (Q20 and Q30) was performed to assess the bases quality, and among the six groups, the Q20 and Q30 values ranged from 96.99% to 98.44% and 91.46% to 94.47%, respectively (Table S2). The percentage of clean high-quality reads mapped to the ribosomal RNA database ranged from 7.3% to 29.01%, and after eliminating these (Table S3), we used the remaining unmapped reads for subsequent analyses.

Differentially expressed transcripts between "uncapping" and "removal"

In accordance with RNA-seq analysis, we detected 4,957 lncRNAs in the six assessed honeybee groups, among which 3,341 and 1,616 were identified as known and novel lncRNAs, respectively (Table S4). In addition, by reconstructing transcripts using Stringtie, we defined 1,616 novel lncRNA commonly identified using CPC2 and CNCI (Fig. S1).

DElncRNAs were detected in all six sample groups, and in total, we identified 1,323 lncRNAs that were differently expressed in the three comparison groups

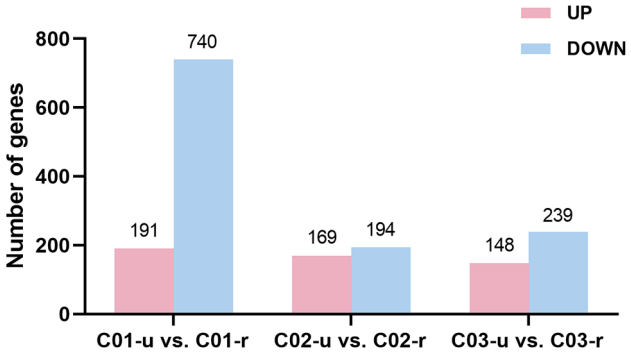

Fig. 2 The number of differentially expressed transcripts between honeybee workers performing uncapping (u) and removal (r) behaviors in the three *Apis. cerana* colonies C01, C02, and C03, respectively

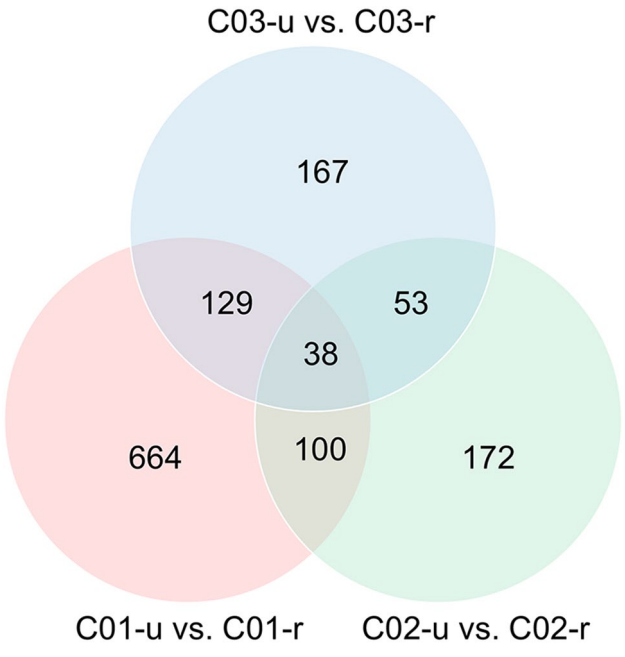

Fig. 3 Venn diagram showing commonly differentially expressed lncRNAs between honeybees performing uncapping (u) and removal (r) behaviors. Thirty-eight lncRNAs were differentially expressed in all the three comparison groups (C01-u vs. C01-r, C02-u vs. C02-r, and C03-u vs. C03-r)

corresponding to the three assessed colonies (Table S5). Specifically, we detected 931 DElncRNAs for the C01-u vs. C01-r comparison, among which 191 and 740 were up- and down-regulated, respectively; 363 DElncRNAs for the C02-u vs. C02-r comparison, among which 169 and 194 were up- and down-regulated, respectively; and 387 DElncRNAs for the C03-u vs. C03-r comparison, among which 148 and 239 were up- and down-regulated, respectively (Fig. 2). Among these, we identified the 38 lncRNAs (Fig. 3; Table S6) that were commonly differentially expressed in all three comparison groups.

GO and KEGG analysis of DElncRNAs via antisense regulatory mechanisms

Based on our antisense regulation analysis, we identified a total of 82 DElncRNAs targeting 62 genes via an antisense regulatory mechanism. GO enrichment results indicated that 10 of these genes were enriched in the biological process category, a majority of which were associated with the response to stimulus, sensory perception, and ion homeostasis. A further five genes were enriched in the molecular function category, of which three were annotated to receptor activity, while another three genes were enriched in the cellular component category and associated with synapse (Fig. 4; Table S7). The findings of KEGG pathway enrichment analysis indicated that the putative target genes are primarily associated with nerve excitation conduction, including neuroactive ligand-receptor interactions, calcium signaling pathways, dopaminergic synapses, and glutamatergic synapses (Fig. 5).

GO and KEGG analyses of DElncRNAs via *cis*regulatory mechanisms

We predicted that a total of 388 DElncRNAs would target 244 genes via a *cis* regulatory mechanism. GO enrichment results revealed that the predicted targets are mainly associated with response to stimulus (Fig. 6), whereas KEGG annotations indicated that the putative target genes were primarily enriched with respect to thermogenesis and signaling pathways, including the relaxin signaling pathways and GnRH signaling pathways (Fig. S2; Table S8).

Analysis of the core target genes regulated by common DElncRNAs between "uncapping" and "removal" bees

Among the 38 lncRNAs that were commonly differentially expressed among the three comparison groups, we identified 22 lncRNAs that target 47 genes via a *cis* regulatory mechanism. GO enrichment results indicated that five putative target genes were annotated to four terms in the cellular components category; seven putative target genes were annotated to five terms in the molecular function category; and eight putative target genes were annotated to seven terms in the biological processes category (Fig. S3). The top 20 GO enrichment terms were found to be primarily associated with biosynthesis processes and the response to stimulus (Fig. 7). Meanwhile, the KEGG pathway enrichment analysis revealed that the putative target genes were primarily enriched in signaling pathways, such as the relaxin signaling pathway, cAMP signaling pathway, and GnRH signaling pathway (Fig. 8; Table S9).

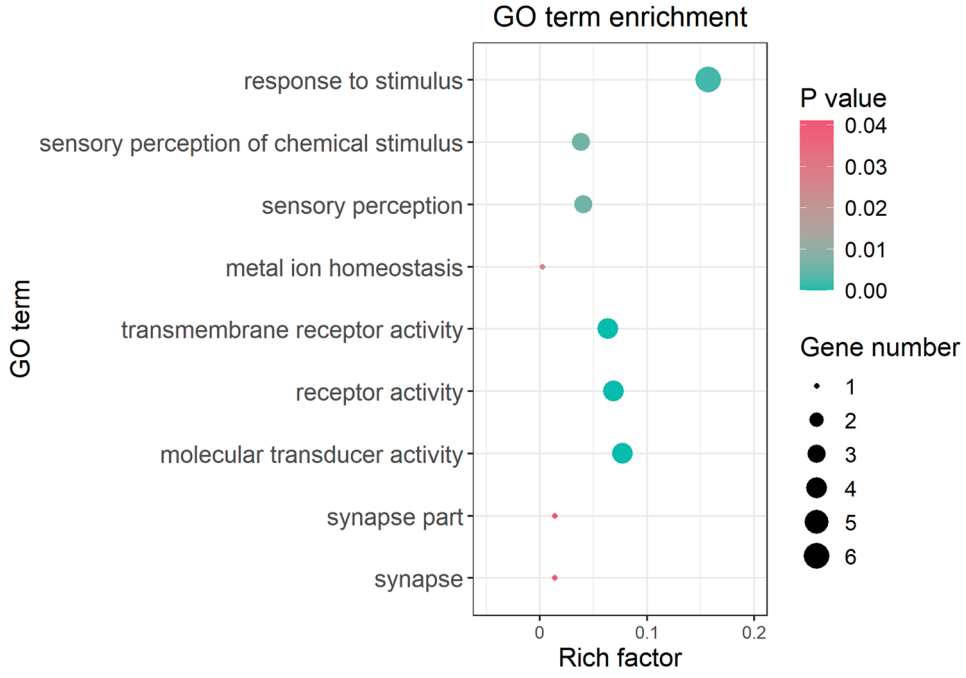

Fig. 4 GO enrichment analysis of differentially expressed lncRNAs (DElncRNAs). Enrichment is shown for genes regulated by DElncRNAs in C01-u vs. C01-r, C02-u vs. C02-r, and C03-u vs. C03-r comparisons via antisense regulatory mechanisms

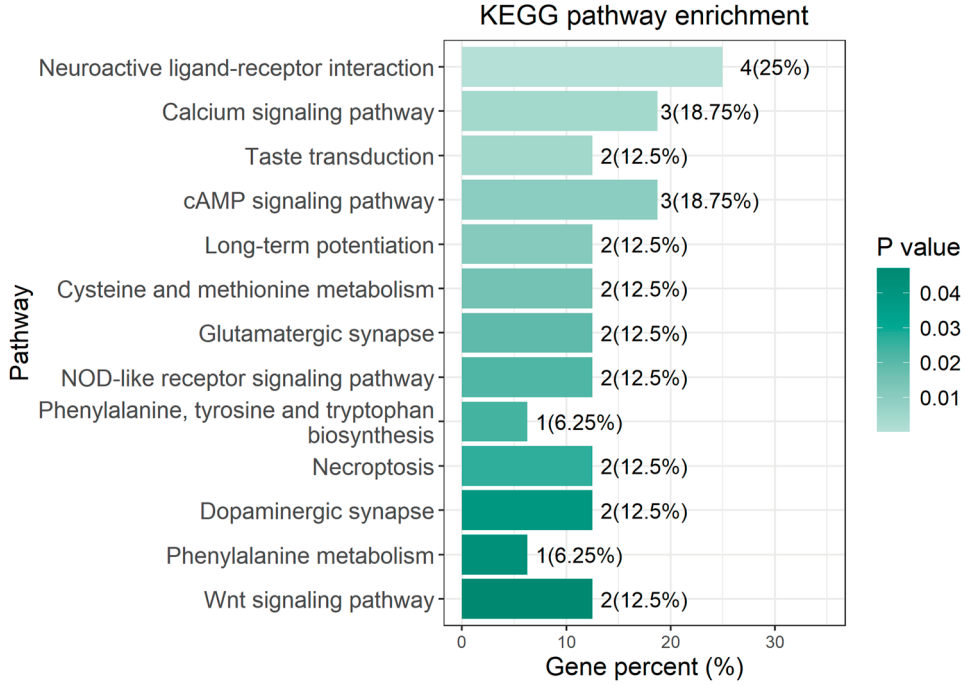

Fig. 5 KEGG enrichment analysis of differentially expressed lncRNAs (DElncRNAs). Enrichment is shown for genes regulated by DElncRNAs via antisense regulatory mechanisms in C01-u vs. C01-r, C02-u vs. C02-r, and C03-u vs. C03-r comparisons

Discussion

Despite the crucial role of hygienic behavior in fortifying the social immunity in honeybee colonies, research endeavors are still insufficient. In this study, we compared the uncapping and removal behaviors exhibited by different worker bees in three *A. cerana* colonies, employing a lncRNA-centric approach. The rapid uncapping behavior was observed, followed by cell removal, in all the colonies. On the basis of our sequencing of antennal RNAs, we detected 4,957 lncRNAs and 10,845

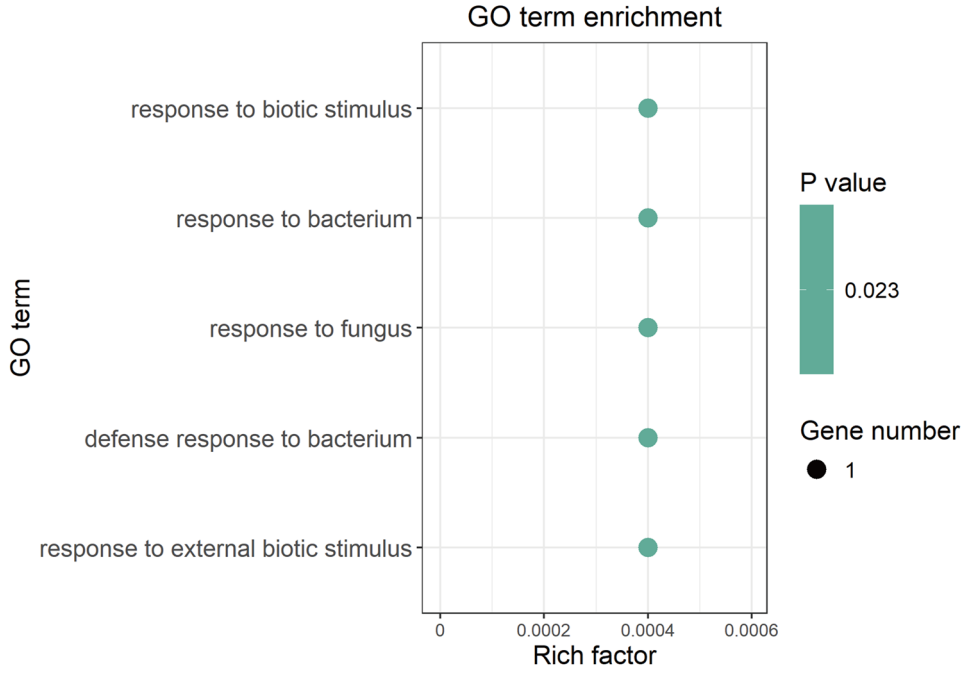

Fig. 6 GO enrichment analysis of differentially expressed lncRNAs (DElncRNAs). Enrichment is shown for genes regulated by DElncRNAs via *cis* regulatory mechanisms in C01-u vs. C01-r, C02-u vs. C02-r, and C03-u vs. C03-r comparisons

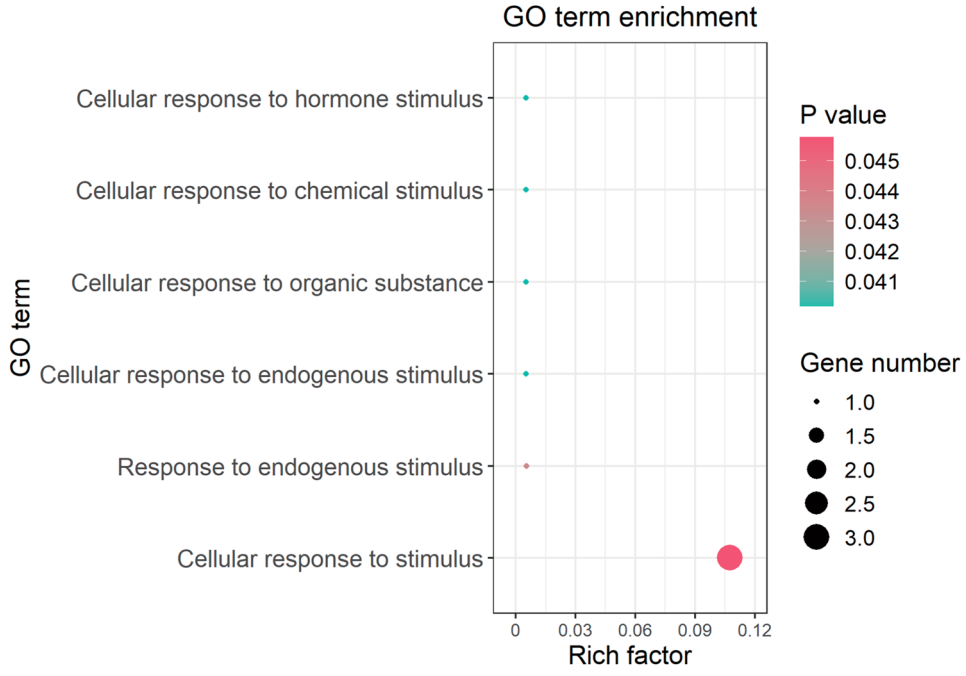

Fig. 7 GO enrichment analysis of target genes regulated by differentially expressed lncRNAs (DElncRNAs) based on colony intersection. Enrichment is shown for DElncRNAs-regulated genes via *cis* regulatory mechanisms for intersections among C01-u vs. C01-r, C02-u vs. C02-r, and C03-u vs. C03-r

mRNAs. Among the former, we identified 1,323 DElncRNAs, of which 38 were found to be commonly differentially expressed across all the three comparative groups, suggesting a key role of lncRNAs in modulating the uncapping and removal behaviors. The target genes of DElncRNAs, predicted via antisense and *cis*-regulation analyses, are mainly annotated to stimulus responses. Moreover, GO enrichment analysis of the 38 common DElncRNAs unveiled a pronounced enrichment of targets associated with stimulus responsiveness, reinforcing the significance of these lncRNAs in regulating honeybees' hygienic division.

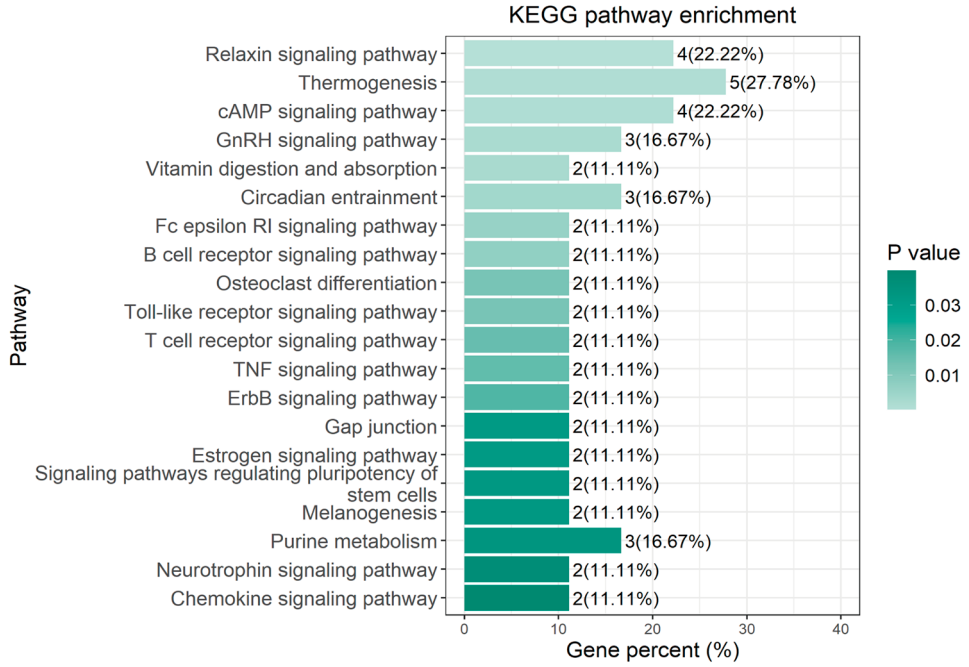

Fig. 8 Top 20 KEGG terms from enrichment analysis of target genes regulated by differentially expressed lncRNAs (DElncRNAs). Enrichment is shown for DElncRNAs-regulated genes via *cis* regulatory mechanisms for the intersections among C01-u vs. C01-r, C02-u vs. C02-r, and C03-u vs. C03-r

As a social insect, the survive and reproduction of honeybee colonies are particularly dependent on the effective communication among individuals [33–35]. Beyond the renowned dance language, auditory cues, physical contact, and a sophisticated array of chemical signals have been established in regulating colony activities. As olfactory signals, the detection of pheromones by insects is chiefly orchestrated by the antennae [36]. Strikingly, oleic acid and β-octene have been identified as death-associated pheromones that trigger hygienic behavior in honeybees [18]. At elevated temperatures, oleic acid has been demonstrated to stimulate nerve depolarization in the antennae of worker bees [19]. Hence, our annotation results, indicating an enrichment of lncRNAs related to sensory perception, synapse part, and synapse, align with the hypothesis that these non-coding RNAs may be involved in the antennal sensing of oleic acid and the subsequent transmission of this excitation signal via the synapses. In line with this notion, KEGG analysis highlighted the enrichment of pathways such as calcium signaling pathway, long-term potentiation, glutamatergic synapse, and dopaminergic synapse, all of which are crucial for excitatory signal conduction. Glutamic acid is a neurotransmitter involved in the neuronal communication of insects [37], in which glutamatergic synapses play excitatory roles. In addition, among the 38 lncRNAs that we identified as being commonly differentially expressed between uncapping and removal worker bees, we detected XR_001766094.2, which was enriched with respect to cellular response to hormones (chemical and endogenous stimulus). Notably, whereas this lncRNA was expressed in uncapping worker bees, its expression was not detected in removal workers (Table S10). Based on these findings, we thus speculate that oleic acid and β-octene may be more effectively detected by hygienic worker bees engaged in the uncapping behavior.

Throughout the world, breeding efforts are mainly aiming at selecting honeybee lineages to resist the primary biotic threat *Varroa destructor* due to the suboptimal performance and negative side effects of medication-based control strategies [38]. Despite established findings that *Varroa* Sensitive Hygiene (VSH) does not directly equate to the removal of freeze-killed brood, a commonly employed proxy for evaluating hygienic capabilities in honeybees [39–41], a consistent tendency of rapid uncapping behavior towards such brood has been observed in *Varroa*-resistant colonies [14, 40, 42], which underscores the strategic importance of selecting colonies from those exhibiting robust rapid uncapping behavior, with XR_001766094.2 emerging as a promising molecular genetic marker that can assist this targeted breeding endeavor. Meanwhile, it is crucial to acknowledge the complex social structure within colonies where worker families coexist as a result of the queen's multiple mating [43] and the inheritance pattern of hygienic trait remains controversial-maternally [44, 45] or paternally influenced [46], which necessitates further research to unravel its intricacies and ensures that breeding programs are grounded in a comprehensive understanding of these essential factors.

Conclusion

By sequencing the antennal RNAs of honeybees engaged in uncapping and removal behaviors, we identified 1,323 lncRNAs that exhibited distinct expression patterns between these two hygienic behavioral cohorts. Subsequent target gene prediction and enrichment analyses revealed that these DElncRNAs were primarily associated with crucial biological functions, including the response to stimulus, receptor activity modulation, and synaptic regulation. We thus speculated that the division of uncapping and removal behaviors could stem from subtle yet significant differences in their responsiveness to environmental stimulus. Notably, a specific lncRNA, XR_001766094.2, was exclusively expressed in uncapping worker bees, suggesting it might play a pivotal role in underpinning the behavioral dichotomy between uncapping and removal tasks.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-024-10817-x.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Acknowledgements
Not applicable.

Author contributions
ZL, ZW, TJ, and QN conceived, initiated, and coordinated the study. XY, FY, CM, ML, and KW performed the experiments. XL, ZL, and ZW conducted the analyses. XL and ZL wrote the first draft and all authors contributed critically to and approved the manuscript.

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Data availability
Sequence data that support the findings of this study have been deposited in the National Center for Biotechnology Information with the BioProject accession code PRJNA1105047.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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