Transcriptomic analysis combined with RNAi reveal defense strategies and molecular mechanisms in Western and Eastern honeybees against attacks by the yellow-legged hornet

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ABSTRACT

Attack and defense are critical to animal survival, yet the defensive strategies of Western honeybees (*Apis mellifera*) and Eastern honeybees (*Apis cerana*) against the yellow-legged hornet (*Vespa velutina*), as well as the molecular basis for their differences, remain poorly understood. This study combined behavioral, pharmacological, transcriptomic, and RNAi approaches to compare the defensive responses in both bee species. We found that *V. velutina* preferentially attacks *A. mellifera* and less frequently target *A. cerana* colony, reflecting distinct opportunistic predation strategies. Behaviorally, *A. cerana* initiated defense earlier and displayed a higher level of alertness, whereas *A. mellifera* reacted at closer ranges. Pharmacological interventions demonstrated that octopamine (OA) significantly modulated defensive responses in both species. Critically, transcriptomic analysis identified a key molecular divergence: under hornet threat, *A. mellifera* exhibited upregulated expression of the octopamine receptor *Octβ2R*, while *A. cerana* did not. RNA interference (RNAi) mediated suppression of tyramine beta-hydroxylase (*Tbh*), a key OA synthesis gene, significantly reduced defensive behavior in both species, confirming the important role of this neurotransmitter. Furthermore, we revealed that *A. mellifera* defense involved activation of energy metabolism pathways such as the TCA cycle and oxidative phosphorylation. In contrast, *A. cerana* employed broader metabolic regulation, including unsaturated fatty acid biosynthesis. The findings not only provide new insights into the evolution of honeybee defensive behaviors but also offer important references for exploring the relationship between gene regulation and behavioral evolution in social insects.

Keywords: Honeybee; Hornet; Defensive behavior; Transcriptome; RNAi

INTRODUCTION

In social insects, defensive behavior is strongly influenced by the species of predator and prey (MD Breed et al., 1978). In nature, honeybees face a variety of threats, primarily from various natural enemies (predators) and parasites both from outside and inside the hive. During the long-term natural evolution, honeybees have developed various strategies to defend against microbial infections, parasitic invasions, and predatory threats, ensuring the survival and reproduction of their colonies. Through their "wisdom", honeybees adapt to adverse environmental pressures by reducing the natural mortality of foragers, releasing alarm pheromones, and increasing the oviposition of queens (RD Booton et al., 2017; JD Evans and RS Schwarz, 2011; M Nouvian et al., 2016; FL Ratnieks and NL Carreck, 2010; AJ Vanbergen and TIP Initiative, 2013). In contrast, behavioral defenses are against a specific predator, requiring prior recognition of threats through olfactory, visual, or tactile cues, or learned from previous encounters with danger (MD Breed et al., 2004; K Tan et al., 2012; M Wood and F Ratnieks, 2004). Honeybees exhibit diverse defensive behaviors, including selecting optimal hive or cavities to withstand harsh environments and predators, attacking intruders with powerful mandibles, launching collective assaults, and performing absconding flights (X Jin, 1992).

Hornets are primary predators of honeybees. They invade beehives to plunder honey, pollen, larvae, and adult bees as nutritional resources for themselves and their offspring (D Baracchi et al., 2010). Defensive behavior initiates with the recognition of threats and intruders. Both Western honeybees (*Apis mellifera*) and Eastern honeybees (*Apis cerana*) demonstrate effective discrimination of hornet odors and exhibit similar avoidance responses to these chemical cues, indicating no significant interspecific difference in their olfactory detection capabilities (GJ Hunt, 2007). Guard bees dynamically modulate their defensive responses based on threat intensity and environmental context, by releasing alarm pheromones, they can recruit more cohorts to collectively counter hornet attacks (M Nouvian et al., 2016). Studies have found that *A. cerana* has an extremely strong defensive response to *V. velutina*. The guards will vibrate their abdomens to scare off the hornets near the beehive entrance. When hornets approach the hive entrance, they will be killed by honeybees via a

way of collective mobbing (K Tan et al., 2012). Similarly, *Apis cerana japonica* also exhibits a special defensive behavior (known as a 'hot defensive bee ball') against *Vespa mandarinia* (T Kamioka et al., 2022). Mattila *et al* (2021) found that when hornets appear at the beehive entrance of *A. cerana*, the colony emits an alarm signal – termed 'antipredator pipes', which has the same characteristics as the alarm screams and panic calls in primates and birds. Compared to *A. cerana*, *A. mellifera* exhibits comparatively weaker defensive capabilities when confronted with hornet threats. Nevertheless, *A. mellifera* does not remain passive in the face of danger, the bees will take several effective countermeasures against hornet attacks. Papachristoforou *et al* (2011) have shown that the cyprian honeybees (*Apis mellifera cypria*) exhibit two defensive strategies when attacked by hornet (*Vespa orientalis*): some guard bees attack the hornets, and the remaining guards avoid conflict with the hornets by gradually retreating and forming a defensive line of honeybees at the hive entrance. Another study showed that when honeybees (*Apis mellifera ligustica*) and European hornets (*Vespula germanica*) were fighting, honeybees attracted their cohorts' support and showed a correlation between the degree of support and the duration of attack (M Pusceddu et al., 2017). Currently, the observed differences in defensive strategies between *A. cerana* and *A. mellifera* against hornet predation are primarily explained by coevolution, while the underlying mechanisms remain largely unexplored.

To interpret honeybee behavior, numerous studies have focused on biogenic amines, which serve as primary neuromodulators of aggressive behaviors in invertebrates (RN Abbey-Lee et al., 2018; OV Alekseyenko et al., 2010; VE Dyakonova et al., 1999; C Zhou et al., 2008). Octopamine (OA), a biogenic amine in insects, functions as a neurotransmitter, neurohormone, and neuromodulator, regulating critical physiological processes including growth, behavior, and metabolism (R Mezawa et al., 2013; G Robinson et al., 1999). Previous studies have demonstrated the involvement of OA in modulating aggression in *Drosophila*, ants, and crickets. Specifically, OA regulates aggressive behavior in *Drosophila* through Drosulfakinin (Dsk) signaling (MJ Williams et al., 2014), while OA-deficient mutants don't exhibit confrontational behaviors toward conspecifics (S Kaya-Zeeb et al., 2022). The octopaminergic (OAergic) system in the ant brain has been functionally linked to the

regulation of aggressive motivation (H Aonuma and T Watanabe, 2012), while OA also facilitates post-conflict aggression recovery in crickets (J Rillich and PA Stevenson, 2014). Simultaneously, OA has been demonstrated to modulate olfactory processing in honeybees and enhance nestmate recognition—a critical component of their defensive behavioral repertoire (S Akasaka et al., 2010; J Erber et al., 1993; T Farooqui et al., 2003; G Robinson et al., 1999). Although previous studies have established OA's involvement in regulating various honeybee behaviors—including dance communication, foraging, learning, flying, and information transfer (AB Barron et al., 2007b; A Behrends and R Scheiner, 2012; BL Fussnecker et al., 2006; S Kaya-Zeeb et al., 2022; M Mizunami and Y Matsumoto, 2017; DJ Schulz and GE Robinson, 1999;2001), its potential role in modulating defensive behaviors remains unexplored. Notably, bee behavior is not governed by a single neurotransmitter but is modulated by a synergistic network of biogenic amines, including OA and dopamine (DA). Within this network, DA exhibits distinct and complementary functions compared to OA: it is more prominently involved in evaluating behavioral motivation and responding to adverse stimuli. Recent studies have shown that DA levels in the honeybee brain decrease following exposure to hornets or after receiving vibrational alarm signals indicating hornet attacks (S Dong et al., 2023b). Similarly, a decline in brain DA levels was observed 24 hours after hornet encounters, accompanied by increased defensive aggregation, impaired olfactory sensitivity, and deficits in associative learning (G Gu et al., 2025). These findings suggest that DA may play a unique role in regulating more complex defensive and avoidance behaviors triggered by external threats.

To investigate the defensive strategies adopted by honeybees in response to hornet predation threats, we initially performed behavioral analyses of bee–hornet interactions under natural conditions. Subsequently, laboratory experiments were conducted to quantify defensive responses through pharmacological treatments, specifically examining the roles of OA in modulating these behaviors. Meanwhile, in order to screen the key genes and signaling pathways that are activated or inhibited in honeybees in response to hornet attacks, we performed transcriptome high-throughput sequencing analysis, which may lead to the discovery of unprecedented new molecular targets related to species-specific defense

strategies. Considering that tyramine beta-hydroxylase (Tbh) is a crucial enzyme for OA synthesis, we employed RNA interference (RNAi) technology to investigate the silencing effect of dsRNA on *Tbh* gene and its impact on honeybee defensive behavior. Given the distinct defensive strategies observed among honeybee species, this study further compares the differences in defensive behavior between *A. mellifera* and *A. cerana* against *V. velutina*, and explores potential underlying mechanisms.

MATERIALS AND METHODS

Experimental subjects

A. mellifera and *A. cerana* were obtained from the experimental apiary of Li's lab of Yunnan Agricultural University (Kunming, Yunnan Province, China, GPS coordinates: 102°44'56.12"E, 25°7'41.75"N). The *V. velutina* that intruded the experimental colonies was captured and used as an attacker. It is the most common and dominant hornet species exerting significant predation pressure on our local honeybee colonies (*A. cerana* and *A. mellifera*) throughout the experimental season.

Behavioral response analysis

Field observations were conducted in August of both 2023 and 2024. Experiments were performed on sunny days, starting at 13:00 daily. Three healthy colonies per species were used for the study of defensive behavior at the beehive entrance, with five replicated trials conducted per colony. For quantifying the behavior of hornets preying on bees, we observed 4 healthy colonies per species, with the observation period for each trial lasting 1 hour. Statistics of defensive response indexes of guard bees against hornets: healthy colonies of *A. cerana* and *A. mellifera* were observed under natural conditions to quantify four key defensive parameters.

Defense initiation distance: The spatial interval at which honeybees first exhibit defensive responses upon hornet approach.

Attack distance: The distance at which honeybees initiate physical counterattacks toward approaching hornets.

Latency to defense initiation: The time interval between hornet appearance and the first observable defensive response by honeybees.

Number of defending bees: Number of bees involved in defending hornets.

Statistics of behavioral indicators of hornets preying on guard bees: predatory behaviors were recorded, these include: hornet appearing frequency at beehive entrances of honeybees, number of successful predation, the predation success rate of hornets and the time required for successful predation.

Observation chamber

The defensive behavior observation chambers were custom-designed and consisted of an open—topped cardboard box measuring 12 cm (L)×12 cm (W)×3 cm (H). The top was covered with a colorless transparent glass plate (20 cm (L)×20 cm (W)×3 mm (H)) to facilitate behavioral observation and video recording. A white background paper, cut to match the exact base dimensions (12 cm (L)×12 cm (W)) of the observation chamber, was installed to enhance visual contrast. Schematic diagrams of the observation chamber are provided in Supplementary Figure S1.

Pharmacological treatments

We used 3 healthy colonies per honey bee species. For each unique treatment (including all controls and drug doses), we performed three chamber trial per colony. Guard bees were anesthetized with CO_2 and then injected 1 μL of test compounds via dorsal thoracic microinjection using Microliter Syringe (HAMILTON, 1701RN, volume 10 μL).

Honeyees were injected with physiological saline (Shanghai yuanye Bio-Technology Co., Ltd., Cat: S5886-500G) as a control. OA (Merck, Lot: 68631), DA (Merck, Lot: H8502), mianserin (octopamine receptor antagonist) (Shanghai Yien Chemical Technology Co., Ltd., Lot: R019036), amitraz (octopamine receptor agonist) (Shanghai Yien Chemical Technology Co., Ltd., Lot: R051466) were prepared as solutions with the concentration of 1 mg/mL, 2 mg/mL, respectively (AB Barron et al., 2007a). DA, OA, and Mianserin were dissolved in physiological saline, amitraz was dissolved in physiological saline containing 5% methanol (the solubility of amitraz in water is very low, and it can be dissolved in many organic solvents such as acetone, xylene and methanol). Methanol was purchased from Guangdong Guanghua Technology Co., Ltd., Lot: 20230410.

The injected bees were transferred to observation chambers (10 bees per chamber) and

placed in an artificial climate incubator (32°C, 75% humidity) (Shanghai Yiheng Scientific Instrument Co., Ltd., MGC-H series) for 15-30 min, to ensure full recovery and reagent efficacy. After regaining stability, the chamber was removed from the artificial climate incubator, and a hornet was introduced into each chamber. At the same time, the interactions were recorded with a smartphone camera (OPPO Guangdong Mobile Communication Co., Ltd., PDKM00) and the video time is 5 minutes. Individual hornets were never reused across trials. A total of 180 hornets were used in this study, each for a single chamber assay only.

Behavioral quantification

The identification of defensive behavior of guard bees was from previous studies with brief modification (H Li-Byarlay et al., 2014; M Pusceddu et al., 2017). In this study, we systematically observed and quantified five distinct defensive behaviors:

Evasion—bees retreat or flee when the hornet approaches.

Pursuit—bees actively chase or closely follow the hornet, often initiating attack maneuvers.

Biting—bees open their mandibles to bite any body part of the hornet upon physical contact.

Stinging—bees curve their abdomens to attack the hornet with their stingers.

Collective assault—two or more bees jointly attack the hornet through coordinated biting or stinging.

The behaviors were counted as the total number of occurrences per chamber trial over the entire fixed 5 min observation period following the introduction of the hornet. All behavioral scoring was conducted manually by a single, trained observer to maintain consistency throughout the experiment.

Transcriptomic sequencing

To mimic a natural attack, a live hornet was tethered 5 cm from the hive entrance. Guard bees that exhibited defensive behavior were then collected and snap-frozen in liquid nitrogen. We define guard bees as individuals that attack hornets directly, or move and stay themselves at the entrance of the hive, usually accompanied by mandibular spread, especially in response to hornets. We used pre-cooled forceps to carefully separate the head from the thorax for each

bee. The heads from bees belonging to the same experimental group and colony were then pooled together to form one biological replicate. Then heads were collected in 1.5 mL microcentrifuge tubes (Nanning Life Sciences (Wujiang) Co., Ltd., batch no. 30523392) and stored at -80°C refrigerator (Qingdao Haier Biomedical Co., Ltd., Model: DW-86L388J) for transcriptomic analysis. Bees from control groups (bees in the absence of hornet threats) underwent identical procedures. We used 3 biological replicates per condition. Each replicate consisted of bee heads pooled from a single, independent colony (i.e., 1 colony=1 replicate). Each RNA-seq sample comprised approximately 0.5 grams of honey bee heads.

Total RNA was extracted from head tissues using the TRIzol method, and RNA integrity was assessed using the RNA Nano 6000 Assay Kit on a Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

Library preparation and quality control for transcriptome sequencing: total RNA was used as input material for the RNA sample preparations. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in first strand synthesis reaction buffer(5X). First-strand cDNA was synthesized using fragmented mRNA as template with random hexamer primers in an M-MuLV reverse transcriptase system, followed by RNA strand degradation with RNase H. Second-strand cDNA was then generated using DNA polymerase I and dNTPs. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of 3' ends of DNA fragments, Adaptor with hairpin loop structure were ligated to prepare for hybridization. In order to select cDNA fragments of preferentially 370 ~ 420 bp in length, the library fragments were purified with AMPure XP system. Then PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and index (X) primer. Finally，PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system.

Clustering and sequencing (Novogene Experimental Department): the clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumina) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina Novaseq platform and

150 bp paired-end reads were generated.

Quality control: raw data (raw reads) of fastq format were firstly processed through fastp software. To ensure data quality and reliability, raw sequencing reads were subjected to rigorous filtering: clean data (clean reads) were obtained by removing reads containing adapter, reads containing poly-N and low quality reads (Reads with Qphred≤5 bases constituting≥50% of their length) from raw data. At the same time, Q20, Q30 and GC content in clean data were calculated. All subsequent analyses were performed using this high-quality filtered dataset.

Reads mapping to the reference genome: the reference genome and gene model annotation files were directly downloaded from the genome website (*A. mellifera*: NCBI Assembly GCF_003254395.2 (Amel_HAv3.1), *A. cerana*: NCBI Assembly GCF_029169275.1 (AcerK_1.0)). Using HISAT2 v2.0.5, we first built the genome index and then aligned the paired-end clean reads to the reference genome. We selected Hisat2 as the mapping tool for that Hisat2 can generate a database of splice junctions based on the gene model annotation file and thus a better mapping result than other non-splice mapping tools. Subsequently, featureCounts v1.5.0-p3 was used to count the reads numbers mapped to each gene. And then FPKM of each gene was calculated based on the length of the gene and reads count mapped to this gene. FPKM, expected number of Fragments Per Kilobase of transcript sequence per Millions base pairs sequenced, considers the effect of sequencing depth and gene length for the reads count at the same time, and is currently the most commonly used method for estimating gene expression levels.

Analysis of differential expressed genes: differential expression analysis of two conditions (three biological replicates per condition) was performed using the DESeq2 R package (1.20.0). DESeq2 provide statistical routines for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. The resulting P-values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate. Genes with a FDR<0.05 & |log2FC|≥1 found by DESeq2 were assigned as differentially expressed.

GO and KEGG enrichment analysis of differentially expressed genes: gene Ontology

(GO) enrichment analysis of differentially expressed genes (DEGs) was implemented by the clusterProfiler R package, in which gene length deviation was corrected. The Benjamini-Hochberg (BH) method is used to correct the p value, which is achieved by the p.adjust function : Gene length bias correction is generally performed when the count value has been corrected during the difference. GO terms with Pvalue less than 0.05 were considered significantly enriched by DEGs. The open source code involving the clusterProfiler software can be referred to the following links :

http://bioconductor.org/packages/release/bioc/html/clusterProfiler.html

https://zhuanlan.zhihu.com/p/35510434

KEGG is a database resource for understanding high-level functions and utilities of the biological system, such as the cell, the organism and the ecosystem, from molecular-level information, especially large-scale molecular datasets generated by genome sequencing and other high-through put experimental technologies (http://www.genome.jp/kegg/).

RNA interference

dsRNA interferes with the *Tbh* gene in honeybees: total RNA was isolated using TRIzol® reagent (TIANGEN BIOTECH (Beijing) Co., Ltd.,Cat: DP424), with integrity and purity verified by agarose gel electrophoresis and A360 UV spectrophotometer (Aoyi Instruments (Shanghai) Co., Ltd.). cDNA synthesis was performed using a reverse transcription kit (Yeasen Biotechnology (Shanghai) Co., Ltd., Cat:11141ES60) following the manufacturer's protocol. Coding sequences of *AmTbh* and *AcTbh* were identified via NCBI, and gene-specific primers were designed for amplification. The Enhanced Green Fluorescent Protein (*EGFP*), which has no homologous sequence in honeybees was served as the control (primer sequences were synthesised by Beijing Tsingke Biotech Co., Ltd. and were shown in Table 1). PCR amplification of cDNA yielded products of 585 bp (*AmTbh*), 594 bp (*AcTbh*), and 680 bp (*EGFP*), separated by 1% agarose gels. The cycling conditions were as follows: 98℃ for 1 min; followed by 38 cycles at 98℃ for 10 s, specific annealing temperature for 20 s, and 72℃ for 5 s; 72℃ extension for 5 min. Amplified fragments were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega). The amplified *Tbh* gene fragment was cloned into pGM-T vector, and the recombinant plasmid was transformed into DH5α

(TIANGEN, Cat: CB101) competent cells according to the instructions of pGM-T blunt cloning kit (TIANGEN, Cat: VT402). The positive clones were identified by PCR and the size of PCR product was detected by 1% agarose gel electrophoresis, so as to screen out the positive clones of recombinant plasmid carrying *Tbh* gene. Plasmid extraction was carried out according to the instruction manual (TIANprep Mini Plasmid Kit, TIANGEN, Cat: DP103). The quality and concentration of plasmid DNA were determined by A360 UV spectrophotometer, and then the plasmid DNA was identified by enzyme digestion (Promega). The qualified plasmid DNA was entrusted to Beijing Tsingke Biotech Co., Ltd. for sequencing verification. Using the plasmid DNA of the positive clone as a template, the recombinant plasmid containing *Tbh* and the *Tbh* primer labeled with T7 promoter were used for *Tbh* dsRNA synthesis according to the instructions from the T7 RiboMAX™ Express RNAi System Kit (Promega, Lot:0000600005). *EGFP* dsRNA was synthesized using the same procedure.

dsRNA purification protocol is as follows: precipitation: 4 μL of 3 mol/L NaAC (pH 5.2) was added to the centrifuge tube at a 10:1 (v/v) ratio (dsRNA : NaAC), and 40 μL of isopropanol (Xilong Chemical Co., Ltd., Cat:1211011) was added at a 1:1 (v/v) ratio (dsRNA : isopropanol). After mixing evenly, the tube was incubated on ice for 5 min, and turbidity occurred under the action of NaAc and isopropanol precipitation. The sample was spun at 13000×g for 10 min (Eppendorf, 5424R); washing: precipitates pelleted by centrifugation (13,000×g, 10 min) were washed twice with 0.5 mL of ice-cold 75% ethanol (Guangdong Guanghua Technology Co., Ltd., Lot: 20240920); drying: the sample was left to dry for 15 min at room temperature in a fume hood to remove residual ethanol. The RNA precipitate was re-suspended in Nuclease-Free Water; quality control: the integrity of dsRNA was examined by 1% agarose gel electrophoresis, the absorbance of the sample at 260 nm and 280 nm wavelengths was measured by A580 ultraviolet spectrophotometer (Aoyi Instruments (Shanghai) Co., Ltd.,) to determine the purity of dsRNA, and quality qualified dsRNA stored in a refrigerator at -80°C.

RNAi efficiency was tested. We tested dsRNA at concentrations of 1, 3, and 5 μg/μL (in a 1 μL injection volume) in small groups of bees prior to the main experiment. We found that

5 μg/μL dose frequently induced pronounced adverse effects, including abnormal behaviors such as convulsions and marked locomotor impairment. Therefore, we chose the injection concentration of 1 ug/uL, 3 ug/uL. From each of these colonies, bees were allocated to both the treatment groups and control groups. guard bees were anesthetized with CO_2 and injected dorsally with 1 μL of 1 μg/μL and 3 μg/μL dsRNA *AmTbh* or *AcTbh* (treatment groups), ds*EGFP*, or physiological saline using a microsyringe. Following 20 ~ 30 min recovery, bees were plunged into liquid nitrogen. RNA extraction and RT-qPCR were performed to quantify *AmTbh*/*AcTbh* expression relative to controls, by which RNAi efficiency was confirmed.

Guard bees were anesthetized with CO_2 and injected with reagents through the dorsal thoracic plate with a microsyringe. The needle was inserted to a depth of approximately 0.5 - 1.0 mm. Each bee in the treatment group was injected with 1 μL of dsRNA at a concentration of 3 μg/μL, the control group bee was injected with ds*EGFP*, and the blank control group bee was injected with physiological saline. Any injected bee showing hemolymph leakage was immediately discarded (10 guard bees were injected in each group, and the experiment was repeated 4 times). Injected bees were allowed to recover for 20 ~ 30 min until normal behavior resumed, then hornet defense experiments were conducted following identical procedures. After the experiment, the guard bees were directly frozen in liquid nitrogen and transferred to -80°C refrigerator for subsequent RT-qPCR experiments.

RT-qPCR

Total RNA was extracted using TRIzol® reagent (Tiangen Biochemical Technology (Beijing) Co., Ltd.,Cat: DP424), and cDNA synthesis was performed according to the instructions of the Reverse Transcription Kit (Yeasen Biotechnology (Shanghai) Co., Ltd., Cat:11141ES60). PCR was performed using the Hieff UNICON® Universal Blue qPCR SYBR Green Master Mix (Yeasen Biotechnology (Shanghai) Co., Ltd., Cat:11184ES08) on the CFX Connect Real-Time System (Bio-Rad Laboratories), and *β-actin* was used as an internal reference gene. The PCR program cycling parameters were as follows: 95℃ for 2 min, followed by 40 cycles at 95℃ for 10 s, specific annealing temperature for 30 s, and 72℃ for 30 s. Results were calculated and analysed using the $2^{-\Delta\Delta CT}$ method. The experiment was performed three times utilizing three independent biological samples. Primers (Supplementary

Tables S1) were designed using Primer 5.0 software and synthesised by Beijing Tsingke Biotech Co., Ltd.

Data processing

The data were processed using SPSS 26.0 and GraphPad Prism 10.0 software. The results were expressed as mean. For the defense index, we first performed the normality test (Shapiro-Wilke test) and the homogeneity of variance test (Levene's test) for the selection of parameters, excluding the availability of linear mixed model analysis (Supplementary Tables S2 Liner-Mixed Model). For all chamber assays, the observation chamber was defined as the experimental unit. The behavioral outcome (e.g., defensive index) for the 10 bees in a chamber was treated as a single data point.

A. mellifera defense index calculation: evade=-1, pursue=1, bite=2, sting=3, small mass attack (SMA) (2≤number of bees<5)=4, big mass attack (BMA) (5≤number of bees)=5.

Defense index: $=$

$$\frac{-1\times evade\ times+1\times pursue\ times+2\times bite\ times+3\times sting\ times+4\times SMA\ times+5\times BMA\ times}{10} \quad (1)$$

We analyzed the defensive behavior of the control groups of *A. cerana* and *A. mellifera*. The duration of SMA by *A. cerana* was 2 times that of *A. mellifera*, and the duration of BMA was 3 times that of *A. mellifera* (Supplementary Tables S3).

A. cerana defense index calculation: evade=-1, pursue=1, bite=2, sting=3, SMA(2≤number of bees<5)=8, BMA (5≤number of bees)=15.

Defense index: $=$

$$\frac{-1\times evade\ times+1\times pursue\ times+2\times bite\ times+3\times sting\ times+8\times SMA\ times+15\times BMA\ times}{10} \quad (2)$$

RESULTS

Distinct anti-predatory strategies of *A. cerana* and *A. mellifera*

We tested behavioral parameters of honeybee-hornet interactions under natural conditions. The results demonstrated that *A. cerana* start defense and initiate counterattacks at significantly greater distances from hornets than *A. mellifera* (P≤0.05) (Figure 1A, C). However, the time between a hornet's appearance at the beehive entrance and the bees' first defensive response showed no significant difference between the two bee species (Figure 1B).

The number of *A. mellifera* attacking hornet was significantly lower than that of *A. cerana* (*P*≤0.05) (Figure 1D). We also found that there was a significant difference in the number of hornets appearing at the hive entrance of both bee species, with more invaders for *A. mellifera* than that of *A. cerana* (*P*≤0.05) (Figure 1E). There was no significant difference in the number of bees that successfully preyed on *A. mellifera* and *A. cerana*, and there was no difference in the successful predation rate of hornets (Figure 1F, G). The time spent by hornets to capture *A. cerana* was significantly greater than that to capture *A. mellifera* (*P*≤0.01) (Figure 1H).

Pharmacological evidence for the roles of OA and DA in modulating defensive behavior

We quantified the five observed defensive behaviors of bees and excluded the effect of 5% methanol itself (Supplementary Tables S4). The results are shown in Figure 2: injection of OA and 1 mg/mL DA significantly increased the defensive index in *A. mellifera* (*P*≤0.05), while *A. cerana* exhibited a highly significant increase in defensive index only after injection of 2 mg/mL OA (*P*≤0.01). Injection of the OA receptor antagonist mianserin extremely significantly reduced defensive index in both *A. mellifera* and *A. cerana* (*P*≤0.01), whereas injection of the OA receptor agonist amitraz significantly increased the defensive index in both bee species (*P*≤0.05). Subsequently, we compared the effects of DA on honeybee defensive capacity and found that only injection at a concentration of 1 mg/mL DA significantly enhanced bees' defensive index, whereas increasing the DA concentration to 2 mg/mL did not yield any significant effect.

OA and DA affects the choice of bee defense behavior

When facing attacks from yellow legged hornets, honeybees defensive themselves by employing various behaviors including evading, pursuing, biting, and stinging. The proportion of each type of behavior relative to the total defensive responses is shown in Figure 3. Compared to the control group, injections of OA and DA in both *A. mellifera* and *A. cerana* significantly reduced the proportion of evasion behavior and significantly increased the proportions of biting and stinging behaviors.

Without pharmacological treatment, *A. mellifera* exhibited a significantly lower defensive index than *A. cerana* (Figure 3E), with *A. mellifera* bees showing a markedly

higher proportion of evasion behavior compared to their Eastern counterparts ($P \leq 0.01$) (Figure 3F). The proportion of *A. cerana* responding to hornets attacks via BMA is approximately 2.5 times that of *A. mellifera* (Figure 3G), indicating that without drug treatment, *A. mellifera* have significantly inferior defensive capabilities against hornets compared to *A. cerana*.

The defensive behaviors of *A. mellifera* and *A. cerana* under drug treatment were compared as shown in Figure 4. We further analyzed the proportion of the type of mass attack adopted by Eastern and Western bees. On the whole, the *A. cerana* took BMA to defend themselves against the hornet attack, while the *A. mellifera* took SMA (Figure 5).

Quality analysis of sequencing data

The transcriptome sequencing yielded a total of 552 157 210 clean reads, with Q20 and Q30 scores exceeding 98.27% and 94.96%, respectively. The GC content in clean reads ranged from 37.90% to 41.21% of the total nucleotides. The clean reads from each sample were aligned to the reference genome, with a mapping rate of over 88.23%, indicating balanced base composition and distribution after filtering. The data quality control was high, ensuring reliability for subsequent accurate analysis (Supplementary Tables S5–S6).

Transcriptomic analysis

Differentially expressed gene analysis: in the Amh-vs-Am (Amh: *A. mellifera* guard bees under hornet threat; Am: *A. mellifera* guard bees without hornet threat.) comparison, we identified 180 up-regulated (UR) and 186 down-regulated (DR) genes (Figure 6A), while the Ach-vs-Ac (Ach: *A. cerana* guard bees under hornet threat; Ac: *A. cerana* guard bees without hornet threat) comparison showed 74 UR and 71 DR genes (Figure 6B). These results demonstrated significant differences in gene expression between guard bees exposed to hornet threats versus untreated controls. In addition, *A. mellifera* exhibited heightened sensitivity in gene expression changes when confronted with hornet threats. exhibited more sensitive changes of gene expression.

DEGs enrichment analysis: Gene Ontology (GO) enrichment analysis was performed on differentially expressed genes (DEGs) from the Amh-vs-Am and Ach-vs-Ac comparisons. The top 20 enriched GO terms revealed that Amh-vs-Am DEGs (Figure 6C) were

predominantly associated with oxidation-reduction process, proteolysis, sensory perception of smell, cofactor binding, and transmembrane signaling receptor activity. Ach-vs-Ac DEGs (Figure 6D) showed significant enrichment in oxidation-reduction processes, oxidoreductase activity, cofactor binding, ion transport, and transmembrane transporter activity.

We performed KEGG pathway enrichment analysis on the identified differentially expressed genes (DEGs) (Supplementary Tables S7 differentially expressed genes (DEGs)) and selected the top 30 most significantly enriched pathways (P<0.05) for further characterization. In the Amh-vs-Am comparison, DEGs were primarily enriched in metabolic pathways including the citrate cycle (TCA cycle), carbon metabolism, 2-oxocarboxylic acid metabolism, pyruvate metabolism, glyoxylate and dicarboxylate metabolism, and oxidative phosphorylation (Figure. 6E). In the Ach-vs-Ac comparison, DEGs were predominantly enriched in fatty acid elongation, biosynthesis of unsaturated fatty acids, pyruvate metabolism, cysteine and methionine metabolism, and fatty acid metabolism (Figure 6F).

Screening of defense-related DEGs

Based on the results of transcriptome sequencing, we performed quantitative analysis of key genes and randomly selected eight genes for RT-qPCR validation (*A. mellifera*: *LOC412896*, *LOC725215*, *LOC411672*, *LOC412382*; *A. cerana*: *LOC107993664*, *LOC107999936*, *LOC108002222*, *LOC108002738*). The results are shown in Figure 7. The expression patterns of the 8 key genes selected in guard bees were consistent with the transcriptomic sequencing results, demonstrating the high reliability and strong reference value of our sequencing data.

The influence of RNAi on defensive ability of honeybees

The effect of *Tbh* silencing on honeybee defensive ability is shown in Fig 8. The gene silencing efficiency was detected by RT-qPCR. The results showed that the RNAi effect of ds*AmTbh*/ds*AcTbh* was not significant at a concentration of 1 μg/μL, whereas ds*AmTbh*/ds*AcTbh* effectively functioned in honeybees at 3 μg/μL (Figure 8A). At 3 μg/μL dsRNA, *A. mellifera* injected with ds*AmTbh* exhibited significantly lower defensive index compared to ds*EGFP* and saline controls (P≤0.05). Similarly, *A. cerana* injected with ds*AcTbh* showed significantly reduced defensive index relative to both control groups

($P \leq 0.05$) (Figure 8B). ds*AmTbh*/ds*AcTbh*-injected bees displayed elevated evasion behaviors compared to ds*EGFP* and saline controls. Silencing *Tbh* significantly reduced stinging behavior in *A. mellifera* but had no significant effect on *A. cerana* (Figure 8C). After the defensive experiments, *A. mellifera* and *A. cerana* were frozen in liquid nitrogen. Subsequent relative quantitative analysis of *AmTbh/AcTbh* gene expression in their brains revealed that the *AmTbh/AcTbh* mRNA levels in the ds*EGFP*-injected and saline-injected control groups were significantly higher than those in the ds*AmTbh*/ds*AcTbh*-injected groups ($P \leq 0.01$), confirming successful RNAi-mediated suppression of *Tbh* transcripts and its correlation with impaired defensive capacity (Figure 8D).

DISCUSSION

Insects face persistent threats from predators, parasites and competitors, making defense an organizational feature in eusocial insects (CK Starr, 1991; EO Wilson, 1971). Social insects have evolved diverse, sophisticated, and intelligent defensive strategies (B Hölldobler and EO Wilson, 1990).

In this study，we first evaluated the attack-defense behavior between hornet (*V. velutina*) and honeybees under natural conditions. We found that *A. cerana* began to show defensive alertness when hornets approached the hive entrance about 25 cm, whereas *A. mellifera* responded only at closer distances (within about 15 cm). Additionally, different distances at which they initiate attacking hornets were also observed: approximately 6 cm for *A. cerana* and about 4 cm for *A. mellifera* (Supplementary Tables S8). These results demonstrate that when facing the threat of hornet predation, *A. mellifera* does not perform as effectively as *A. cerana*. A previous study has also found that Eastern honeybees exhibit stronger defensive responses against intruders compared to their Western counterparts (HR Mattila et al., 2020). It also explains the lack of co-evolution between *A. mellifera* and hornet (S Dong et al., 2023a). We also found that the number of *A. mellifera* attacking hornet was significantly lower than that of *A. cerana* and the successful capture of *A. mellifera* by hornets was significantly higher than that of *A. cerana.* This behavioral asymmetry reflects an evolutionary trade-off between resource acquisition and predation risk (M Pusceddu et al., 2017), where hornets perceive less risk in preying on *A. mellifera* than on *A. cerana*. Similar

observation has been reported in *Apis dorsata* (G Kastberger et al., 2008).

Through pharmacological experiments, we found that after OA treatment, the defense index of both *A. mellifera* and *A. cerana* increased significantly. This indicates that OA can enhance the ability of bees to defend against *V. velutina*, and the higher the concentration, the more pronounced the enhancement of defense. This also shows that OA's regulation in bee defensive behavior is evolutionarily conserved. Injection of OA receptor antagonist (i.e. mianserin) will reduce the defense ability of bees, while OA receptor agonist (i.e. amitraz) can improve the defense ability of bees. Zhou *et al*. (2008) found that reducing OA also reduced the aggression of male and female *Drosophila*, and enhancing octopaminergic signaling promoted the aggression of *Drosophila* in social groups. Injection of biogenic amines such as OA, DA, or serotonin into the hemolymph of *Trichoplusiani* has been shown to significantly increase the levels of injected compounds in the hemolymph, brain, and thoracic ganglia within 30 min (CEL Jr. et al., 1994). Behavioral studies found that OA treatment improved the ability of guard bees to distinguish between nestmates and intruders (G Robinson et al., 1999). Fussnecker *et al*. (2006) have proved that OA can affect the movement behavior of adult bees by injecting OA and mianserin into bees, which can influence their walking, grooming, fanning and flying. These results suggest that OA acts as a stress hormone ('flight-or-fight' hormone) that adjusts the body to meet the animal's energy needs when required (JC David and JF Coulon, 1985; PD Evans and S Robb, 1993; T Mentel et al., 2003; HJ Pfluger et al., 2004; T Roeder, 1999).

Furthermore, this study investigated the relationship between DA—a neuroactive substance linked to insect neural processes—and honeybee defense mechanisms. The results demonstrate that DA enhances defensive responses in honeybees only at a concentration of 1 mg/mL. Previous studies on the fighting behavior of virgin queens have found that dopamine receptor antagonists can inhibit the aggressive behavior of a virgin queen against another counterpart (S Farkhary et al., 2017) and inhibit the autonomous activity of the former (K Harano et al., 2008). The level of DA in the winner' brain is significantly higher than that of the loser (K Sasaki and M Harada, 2020). DA may increase the aggression of bees by enhancing their movement and flight abilities (S Akasaka et al., 2010; R Mezawa et al., 2013).

Our results suggest that the defensive behavior may reach a plateau beyond a certain DA level, as a further twofold increase in concentration did not lead to a significant additional effect. Additionally, a study has shown that increasing DA levels in bees can also reduce the fear effect on hornets (S Dong et al., 2023b). The defensive response of bees after DA injection in this study also seems to prove this. The high concentration of DA will reduce unnecessary attacks and generate more evasive behavior. However, this study is to study foraging-related behaviors. Artificially increasing DA levels in their brains can offset the negative effects of danger. In the foraging scenario, high concentrations of DA indicate 'high resource value', prompting bees to ignore risks. Our result is a direct counterattack to the threat, high concentrations may play a 'stable mood' or 'transfer behavior priority' role, reducing unnecessary aggression.

Data analysis revealed that untreated *A. mellifera* exhibit a significantly lower defense index than *A. cerana*. In China, *A. mellifera* is an introduced species. Due to a lack of co-evolution with the "novel predator (*V. velutina*)" (S Dong et al., 2023a), they do not launch a mass attack when threatened by these hornets. Instead, they employ repeated, brief defensive maneuvers. By comparing the types of defensive behaviors between the two bee species, we found that Western honeybees typically resort to evasive tactics initially, avoiding direct confrontations with hornets, whereas Eastern honeybees actively confront the adversaries. This aligns with prior studies documenting non-contact defense strategies in honeybee colonies, such as intimidation displays or physical barriers, to deter predators without direct combat. For example, the swarm gathers on the hive platform in a behavior known as "bee carpets" to reduce colony activity. Simultaneously, they perform abdominal shaking (referred to as shimmering) (G Kastberger et al., 2008), emit hissing alarm signals (A Papachristoforou et al., 2008) and construct propolis walls to prevent hornets from entering the hive (A Papachristoforou et al., 2011). Our study further demonstrates that *A. cerana* tend to engage in intense collective defense, while *A. mellifera* tend to exhibit more gradual and mild defensive response. *A. cerana* recruit robust cohorts' support during hornets' threats (K Tan et al., 2016) and employ an evolved extraordinary strategy: forming a heating bee ball to thermally kill predators (M Ono et al., 1995). This also prove that *A. mellifera* consider the

553 trade-off between collective defense and the danger from predators, and adjust their defensive
554 behavior based on the strength of the threat to prevent unnecessary sacrifice (G Helfman,
555 1989; K Tan et al., 2012).

556 The levels of neurotransmitters such as OA are dynamically regulated by the rates of
557 presynaptic synthesis, release, synaptic reuptake, and degradation, which are governed by
558 interconnected elements. The biosynthesis of OA in insects depends on the activity of two
559 enzymes: tyrosine decarboxylase (Tdc) and Tbh (MS Livingstone and BL Tempel, 1983). Tdc
560 and Tbh mutants were isolated from *Caenorhabditis elegans*, providing evidence that OA
561 plays different roles in motor behavior (MJ Alkema et al., 2005). Previous studies have
562 shown that Tbh activity is not related to bee behavior, however, Tbh mRNA levels are closely
563 positively correlated with OA levels during behavioral development, and Tbh mRNA is
564 localized to previously identified octopaminergic neurons in the bee brain (HK Lehman et al.,
565 2006). This study significantly inhibited the defense ability of bees by suppressing the
566 expression of *Tbh* gene through RNAi, indicating that *Tbh* may be one of the important genes
567 regulating bee defense, and also proving that OA plays a crucial role in bee defense function.
568 These findings are consistent with Ishii *et al.*'s (2022) work in *Drosophila*, where knockdown
569 of *Tbh* and *Tdc2* by RNAi reduced the defensive of fruit flies raised alone, further validating
570 OA's conserved neuromodulatory function across species. Simultaneously, in *Drosophila*
571 *melanogaster*, *Tribolium castaneum*, *Nilaparvata lugens*, and *Mythmina separata*, OA levels
572 also decrease when *Tbh* expression is downregulated or mutated (S Ahmad et al., 2021; SJ
573 Certel et al., 2007; H Kong et al., 2019; T Roeder, 2005; L Xu et al., 2018).

574 Interestingly, we found that OA receptor and OA receptor β-2R (*Octbeta2R*), which are
575 involved in biogenic amine signal transduction, were significantly up-regulated only in *A.*
576 *mellifera*, and there was no difference in the expression of OA and its receptors in *A. cerana*.
577 *Octbeta2R* is the only molecular target of amitraz in insects. This finding explains why
578 Western bees are more sensitive to the behavioral regulation of OA and its agonist amitraz at
579 the molecular level: the increased expression of *Octbeta2R* may enhance the efficiency of OA
580 energy signal transmission, thereby improving the strength and flexibility of defense response.
581 In contrast, the defensive behavior of *A. cerana* may be more dependent on other neural

582 regulation mechanisms or solidified behavioral programs, and its immediate dependence on
583 OA signals is lower. This discrepancy may explain why RNAi-mediated suppression of *Tbh*
584 more effectively impaired defensive ability in *A. mellifera* than in *A. cerana*. This finding is
585 consistent with the up-regulation of *Octbeta2R* in *A. mellifera*, suggesting that its defensive
586 behavior is more dependent on a fully functional OA synthesis and signaling pathway. The
587 difference in gene expression patterns may also explain the differences in the strategies
588 adopted by the two bee species in response to the predation threat of the hornet. The guarding
589 behavior of *A. mellifera* stimulates the release of neurohormones (neuropeptides or biogenic
590 amines) to enhance their own defensive function (GJ Hunt, 2007), while *A. cerana* has
591 formed a mechanism of mutual adaptation during long-term evolution.

592 In addition to the *Octbeta2R*, transcriptome analysis showed that the defense function of
593 honeybees involves other genes and pathways. Our study also showed that in the face of
594 hornet threat, genes involved in sensory perception of odors in bees are significantly enriched,
595 and the expression of multiple odor receptor genes also shows significant differences (*Or20*,
596 *Obp14*, *Or14*, *Obp11*, *Or105*, *Obp9*, etc.). The gene expression of venom acid phosphatase
597 Acph-1 in *A. mellifera* and venom acid phosphatase Acph-1-like in *A. cerana* were
598 up-regulated. The venom acid phosphatase Acph-1 is an acid phosphatase identified in the
599 venom of Western bees (T Grunwald et al., 2006). The venom acid phosphatase Acph-1-like
600 protein is also a venom component expressed in the venom glands of Eastern bees (B Kim
601 and B Jin, 2014). Interestingly, the expression of neuropeptides CAPA receptor-like was also
602 up-regulated in honeybees under hornet stress, suggesting that neuropeptide CAPA receptor is
603 a key gene for honeybees to defend against hornet's attack. Stabentheiner *et al*. (2007) found
604 that highly aggressive guard bees have strong muscle activities, while CAPA peptides have
605 key muscle-regulating functions (R Predel and C Wegener, 2006) and may also play a key
606 role in odor perception (X Kong et al., 2021).

607 In this study, we do see the characteristics of carbohydrate metabolism in DGEs, which
608 is consistent with the studies linking glycolysis and oxidative phosphorylation with defensive
609 and aggressive behavior (S Chandrasekaran et al., 2015; H Li-Byarlay et al., 2014; CC
610 Rittschof et al., 2019b), and is also similar to the results of honeybee aggressive

transcriptomics published by Rittschof (2019a). Meanwhile, KEGG enrichment analysis suggests that energy metabolism is likely a key pathway involved in regulating the defense function of honeybees, and multiple pathways related to energy metabolism were significantly enriched, such as TCA cycle, pyruvate metabolism, oxidative phosphorylation, etc. In addition, transcriptome analysis of *A. cerana* suggests that unsaturated fatty acid synthesis and fatty acid metabolism may also be important pathways involved in its defense behavior. There is evidence to suggest that honeybee aggression is regulated by neuropeptides in the brain, and neuropeptide regulation is associated with lipid metabolism (GM Paula et al., 2024). The activity of lipoprotein lipase has been measured in the flying muscles of bees, suggesting that fat is used as a fuel for flight (B Crabtree and EA Newsholme, 1972). There is also evidence that the supply of unsaturated fatty acids in the pectoral muscles is important in the flight of the orchid bee (*Euglossini*) (E Rodriguez et al., 2015). The metabolic regulation pathways of *A. mellifera* and *A. cerana* are significantly different under the stress of hornets. The reasons may be as follows: the defensive behavior of *A. mellifera* mainly relies on the activation of energy metabolism-related pathways. It may be because *A. mellifera* tends to have fast, relatively simple and high-intensity defensive behavior when facing *V. velutina*, which requires a large amount of energy support. The efficient activation of pathways such as TCA cycle, pyruvate metabolism and oxidative phosphorylation can rapidly provide ATP to meet its high-intensity defense needs and adapt to predation pressure. The defensive behavior of *A. cerana* involves a broader metabolic regulation, probably because *A. cerana* prefers to adopt persistent, cooperative and diverse defense strategies (such as gate guarding, group collaboration, and multiple defense behaviors).

This study systematically revealed the core role of OA signaling pathway, especially its key enzyme gene *Tbh*, in regulating bee defense strategies, and pointed out that other key molecular targets such as *Octbeta2R*, neuropeptide CAPA receptor and bee venom acid phosphatase Acph-1 are involved in the regulation of defense responses. The differential expression of *Octbeta2R* under the threat of hornets is the basis of species-specific defense strategies between *A. mellifera* and *A. cerana*. However, the study failed to directly determine

the endogenous concentration of OA in the brain of honeybees, and its behavioral effects were mainly inferred based on functional activation at the receptor level. Future work needs to further explore the synergistic effect of key targets in the defense neural circuit.

DATA AVAILABILITY

The authors confirm that the data supporting the findings of the present study are available from the corresponding author upon reasonable request. The raw reads of transcriptome data have been deposited into the NCBI Sequence Read Archive under the accession number PRJNA1376211.

SUPPLEMENTARY MATERIAL

Supplementary Tables S1–S9, Figure S1.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

T. X. (Data curation , Formal analysis, Methodology, Visualization, Writing – original draft, Writing – review & editing), Z.Y.Y. (Conceptualization, Data curation, Methodology, Writing – review), D.H.X. (Investigation, Methodology), H.O.K. (Investigation, data acquisition), Z.T.L. (Investigation, Methodology), M.H.Y. (Conceptualization, Supervision, Project administration, Writing – review & editing), and Y.H.L. (Conceptualization, Data curation, Funding acquisition, Investigation, Project administration, Supervision, Writing – original draft, Writing – review & editing). All authors read and approved the final version of the manuscript.

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850

851 **Table 1** RNAi primer information

Gene name	Primer sequence（5’-3’）	Product length	Tm
AmTbh	F：GGCATTGGTCTTCCACAACA R：TGGCGGAGGTAAGGGTAGTT	585bp	56.8℃
*AmTbh*T7	F：TAATACGACTCACTATAGGGGCATTGGTCTTCCACAACA R：TAATACGACTCACTATAGGTGGCGGAGGTAAGGGTAGTT	585bp	67℃
AcTbh	F：CAGGCATTGGTCTTCCACAAC R：TTCGAGTTGGCGGAGGTAAG	594bp	58℃
*AcTbh*T7	F：TAATACGACTCACTATAGGCAGGCATTGGTCTTCCACAAC R：TAATACGACTCACTATAGGTTCGAGTTGGCGGAGGTAAG	594bp	68℃
EGFP	F：TGCTTCAGCCGCTACCC R：TCCAGCAGGACCATGTGAT	680bp	56.2℃
EGFPT7	F：TAATACGACTCACTATAGGTGCTTCAGCCGCTACCC R：TAATACGACTCACTATAGGTCCAGCAGGACCATGTGAT	680bp	65.5℃
AmTdc	F：GTTTCAAGTACGCGGCAAGG R：TCTTCGGTGTTCGGGTCTTG	516	58℃
AcTdc	F：GGAAGGAAAGAGGGTGACGG R：GCGGCCCAAGAGAAACCTAT	249bp	58.1℃
Amβ-actin	F：AGGCTCTTTTCCAACCATCC R：GGTGCTAGGGCAGTGATTTC	190bp	56℃
Acβ-actin	F：ATCACCATCGGCAACGAGAG R：ACGTTGTTGGCGTACAGGT	152bp	57℃

852 Notes: Am: *Apis mellifera*; Ac: *Apis cerana.*

Figure legends

Figure 1 Behavioral response between honeybee and yellow-legged hornet. Each data point for behavioral metrics (A-D, E-G) represents the value from one independent colony (colony-level). Each data point for predation time (H) represents an independent predation event. A, defense initiation distance (n=15): data normal (P>0.05) but variances unequal (P=0.031). B, start defense time (n=15): data for *A. cerana* non-normal (P=0.004). C, attack distance (n=15): data for *A. mellifera* non-normal (P=0.002). D, number of defending bees (n=15): data for both species non-normal (P<0.05). E, hornet appearing frequency (n=4): data normal (P>0.05) but variances unequal (P=0.03). F, number of successful predation (n=4): data normal (P>0.05) but variances unequal (P=0.032). G, successful predation rate (n=4): data normal (P>0.05) with equal variances (P=0.183). H, time required for successful predation (*A. mellifera*: n=174; *A. cerana*: n=66): data for both species severely non-normal (P<0.001). Statistical analyses: Welch's t-test was applied to panels A, E and F; the Mann-Whitney U test was used for panels B, C, D and H; Student's t-test was used for panel G. ns, P>0.05, *, P≤0.05, **, P≤0.01, ***, P≤0.001, ****, P≤0.0001. Error bars: mean ± SD.

Figure 2 Effects of reagent injection on the defensive index of honeybees. Each data represents an observation chamber data. After excluding the extreme values (the maximum and minimum) from each group, 7 experimental data were obtained in each group (n=7). A, defensive index of *A. mellifera* at the concentration of 1 mg/mL; B, defensive index of *A. mellifera* at the concentration of 2 mg/mL; C, defensive index of *A. cerana* at the concentration of 1 mg/mL; D, defensive index of *A. cerana* at the concentration of 2 mg/mL; E, defensive index of *A. mellifera* under different concentration of reagents; F, defensive index of *A. cerana* under different concentration of reagents. A, B, C and D were analyzed by one-way ANOVA, Tukey method was used for multiple comparisons. E and F were analyzed by two-way ANOVA. ns, P>0.05, *, P≤0.05, **, P≤0.01, ***, P≤0.001, ****, P≤0.0001.

Figure 3 The distribution of defensive behavior of honeybees and the comparison of the control group. Each data represents an observation chamber data. The figure shows the percentage of the four defensive behaviors of evading, pursuing, biting and stinging. A, c=1 mg/mL, *A. mellifera*; B, c=2 mg/mL, *A. mellifera*; C, c=1 mg/mL, *A. cerana*; D, c=2 mg/mL,

A. cerana; n=9; After excluding the extreme values (the maximum and minimum) from each group, 7 experimental data were obtained in E and F. E, the comparison of the defensive index between *A. mellifera* and *A. cerana* in the control group (n=7), Independent sample *T* test was used for analysis; F, the comparison of the proportion of behavior between *A. mellifera* and *A. cerana* in the control group (n=7), two-way ANOVA was used for analysis. ns, $P>0.05$, *, $P\leq0.05$, **, $P\leq0.01$. G, the proportion of bees in the control adopting mass attack (*A. mellifera*: 100 mass attacks; *A. cerana*, 109 mass attacks).

Figure 4 Comparison of the defensive behavior of *A. mellifera* and *A. cerana*. Each data represents an observation chamber data. After excluding the extreme values (the maximum and minimum) from each group, 7 experimental data were obtained in each group (n=7).. A, the comparison of the defensive index of *A. mellifera* and *A. cerana*; B, c=1 mg/mL, the comparison of the proportion of behavior between the *A. mellifera* and *A. cerana*; C, c=2 mg/mL, the comparison of the proportion of behavior between the *A. mellifera* and *A. cerana*. Two-way ANOVA was used for analysis. ns, $P>0.05$, *, $P\leq0.05$, **, $P\leq0.01$, ***, $P\leq0.001$.

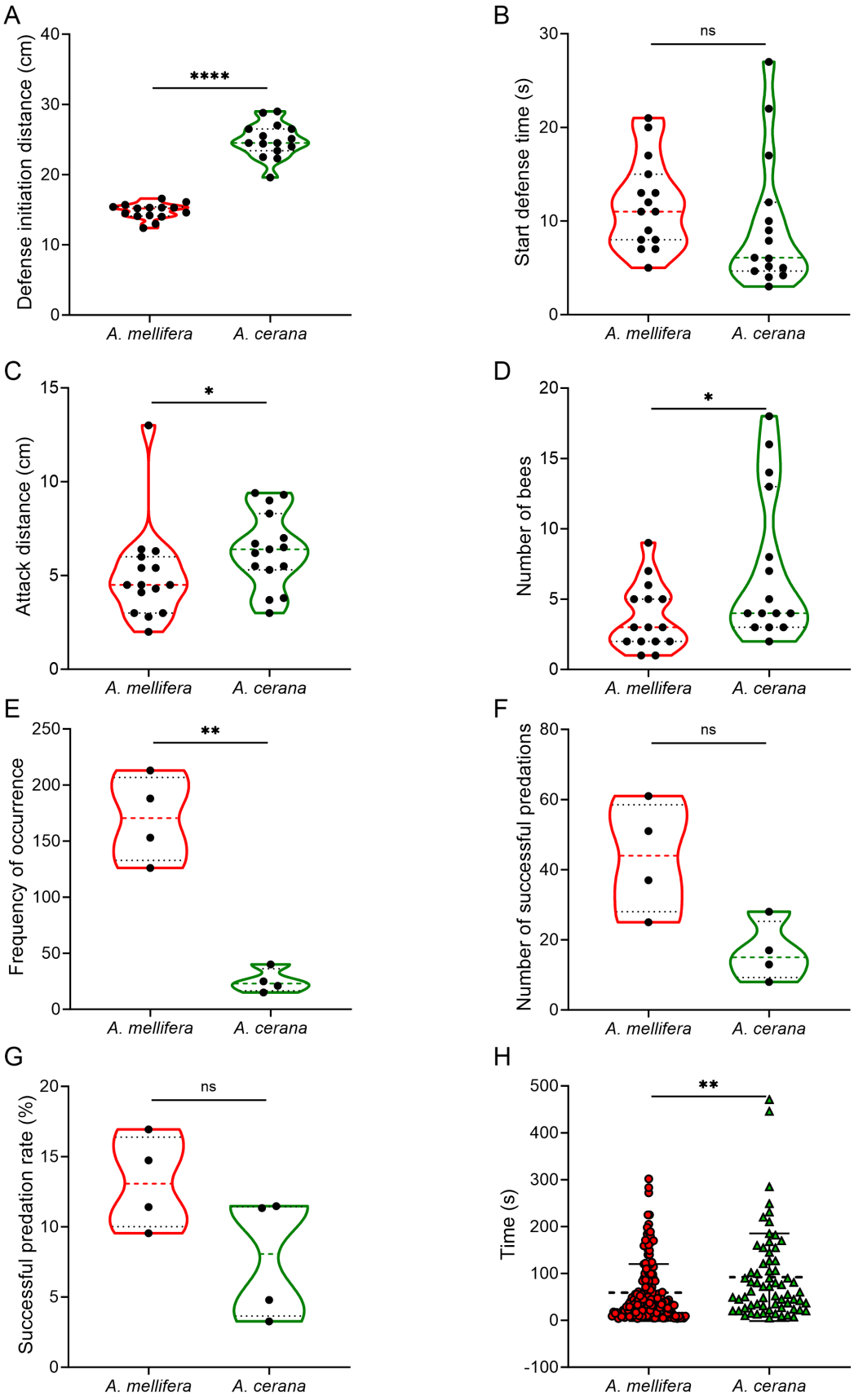
Figure 5 Comparison of the proportion of mass attack in Eastern and Western bees. A, OA — 139 mass attacks, mianserin — 90 mass attacks, amitraz — 140 mass attacks, DA — 151 mass attacks; B, OA — 150 mass attacks, mianserin — 34 mass attacks, amitraz — 286 mass attacks, DA — 172 mass attacks; C, OA — 110 mass attacks, mianserin — 71 mass attacks, amitraz — 146 mass attacks, DA — 96 mass attacks; D, OA — 146 mass attacks, mianserin — 64 mass attacks, amitraz — 142 mass attacks, DA — 72 mass attacks.

Figure 6 Transcriptome data analysis diagram. A, volcano map of DEGs in the transcriptome of guard bees in *A. mellifera*; B, volcano map of DEGs in the transcriptome of guard bees in *A. cerana*; C, Amh-vs-Am: GO enrichment analysis of DEGs; D, Ach-vs-Ac: GO enrichment analysis of DEGs; E, KEGG enrichment analysis of DEGs in Amh-vs-Am; F, KEGG enrichment analysis of DEGs in Ach-vs-Ac. Amh: *A. mellifera* guard bees under hornet threat; Am: *A. mellifera* guard bees without hornet threat; Ach: *A. cerana* guard bees under hornet threat; Ac: *A. cerana* guard bees without hornet threat.

Figure 7 The expression patterns of 8 DEGs in guard bees were detected by RT-qPCR. n=3, each data represents a colony. A, *LOC412896* (*Octbeta2R*, octopamine receptor beta-2R); B,

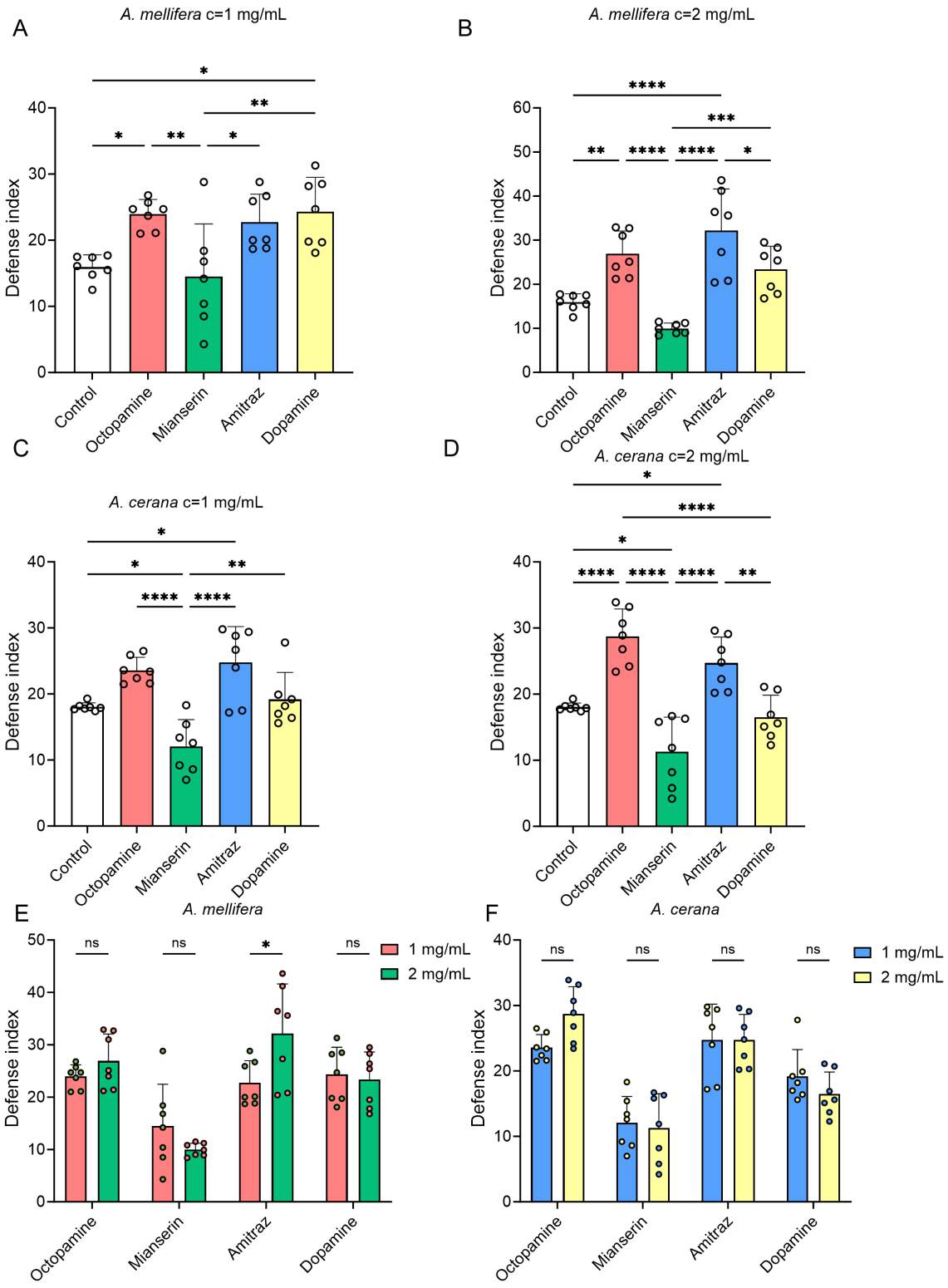
LOC107993664 (CAPA peptides-like); C, *LOC725215* (venom acid phosphatase Acph-1); D, *LOC107999936* (venom acid phosphatase Acph-1-like); E, *LOC411672* (neuropeptides capa receptor-like protein); F, *LOC108002222* (*CytC*, mitochondrial cytochrome C); G, *LOC412382* (*Eaat1*, Excitatory amino acid transporter 1); H, *LOC108002738* (neuropeptides capa receptor-like). Independent sample *T* test was used for analysis. ns, $P>0.05$, *, $P\leq0.05$, **, $P\leq0.01$. Amh: *A. mellifera* guard bees under hornet threat; Am: *A. mellifera* guard bees without hornet threat; Ach: *A. cerana* guard bees under hornet threat; Ac: *A. cerana* guard bees without hornet threat.

Figure 8 The effect of silencing *AmTbh*/*AcTbh* by RNAi on the defensive ability of *A. mellifera*/*A. cerana*. A, the silencing effect was detected by RT-qPCR, n=3, each data represents a colony; B, honeybee defensive index, n=4, each data represents an observation chamber data, the data from one observation chamber corresponded to a single colony; C, the proportion of bee defensive behavior; D, the relative expression of *AmTbh*/*AcTbh*, n=3, each data represents a observation chamber. One-way ANOVA was used for data analysis, and Tukey method was used for multiple comparisons. ns, $P>0.05$, *, $P\leq0.05$, **, $P\leq0.01$, ***, $P\leq0.001$, ****, $P\leq0.0001$.



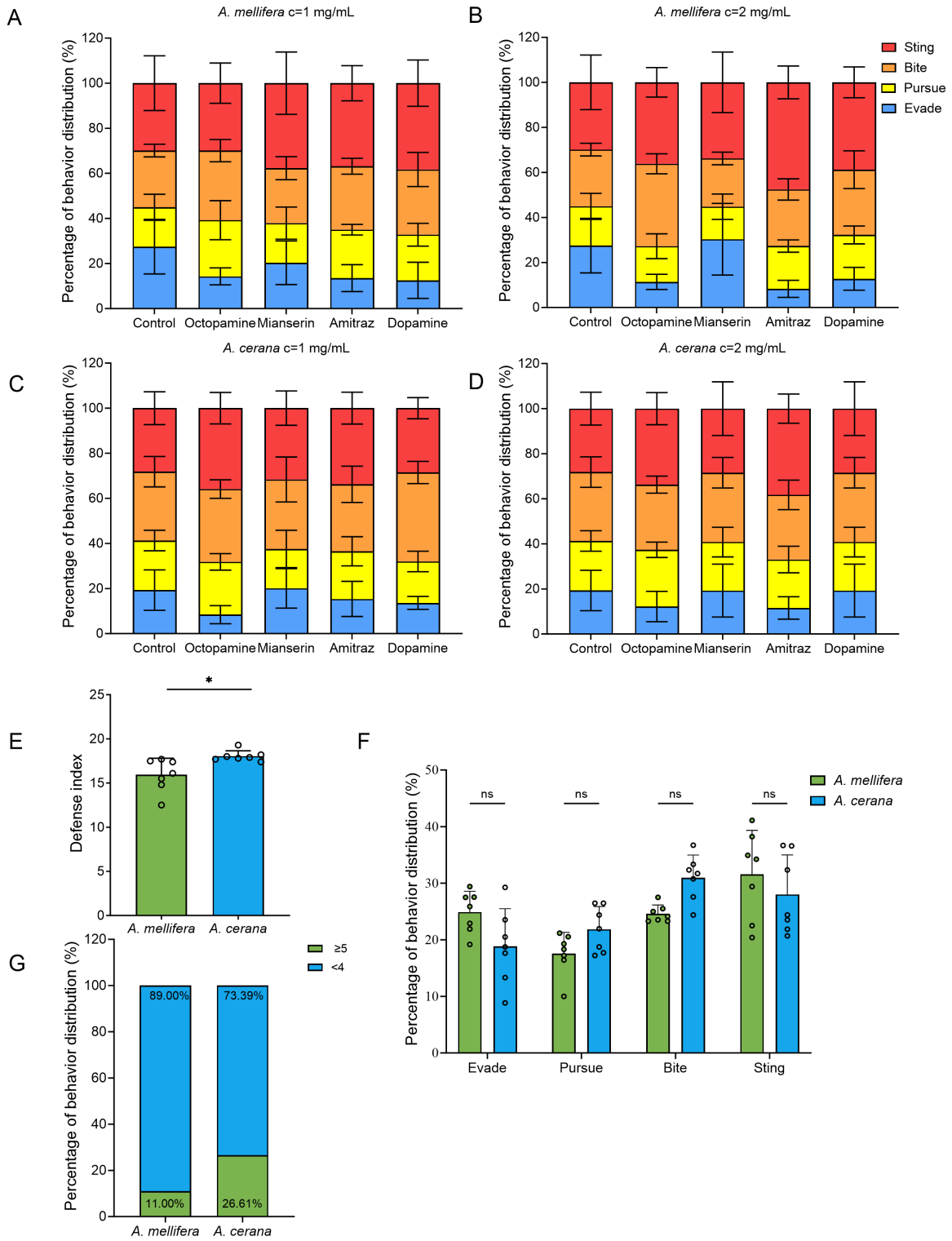
927

928 **Figure 1** Behavioral response between honeybee and yellow-legged hornet.



929

930 **Figure 2** Effects of reagent injection on the defensive index of honeybees.



931

932 **Figure 3** The distribution of defensive behavior of honeybees and the comparison of the

933 control group.

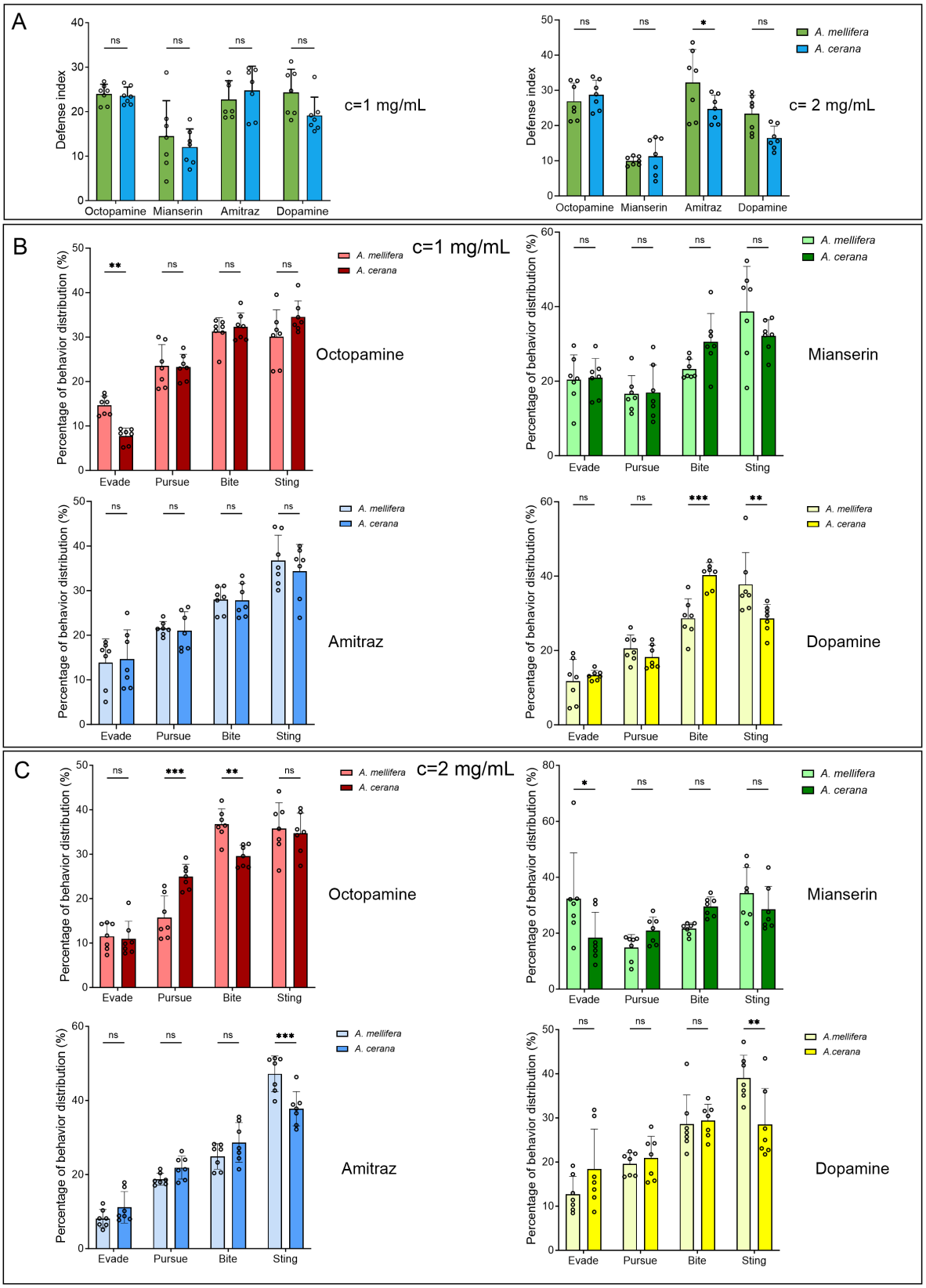
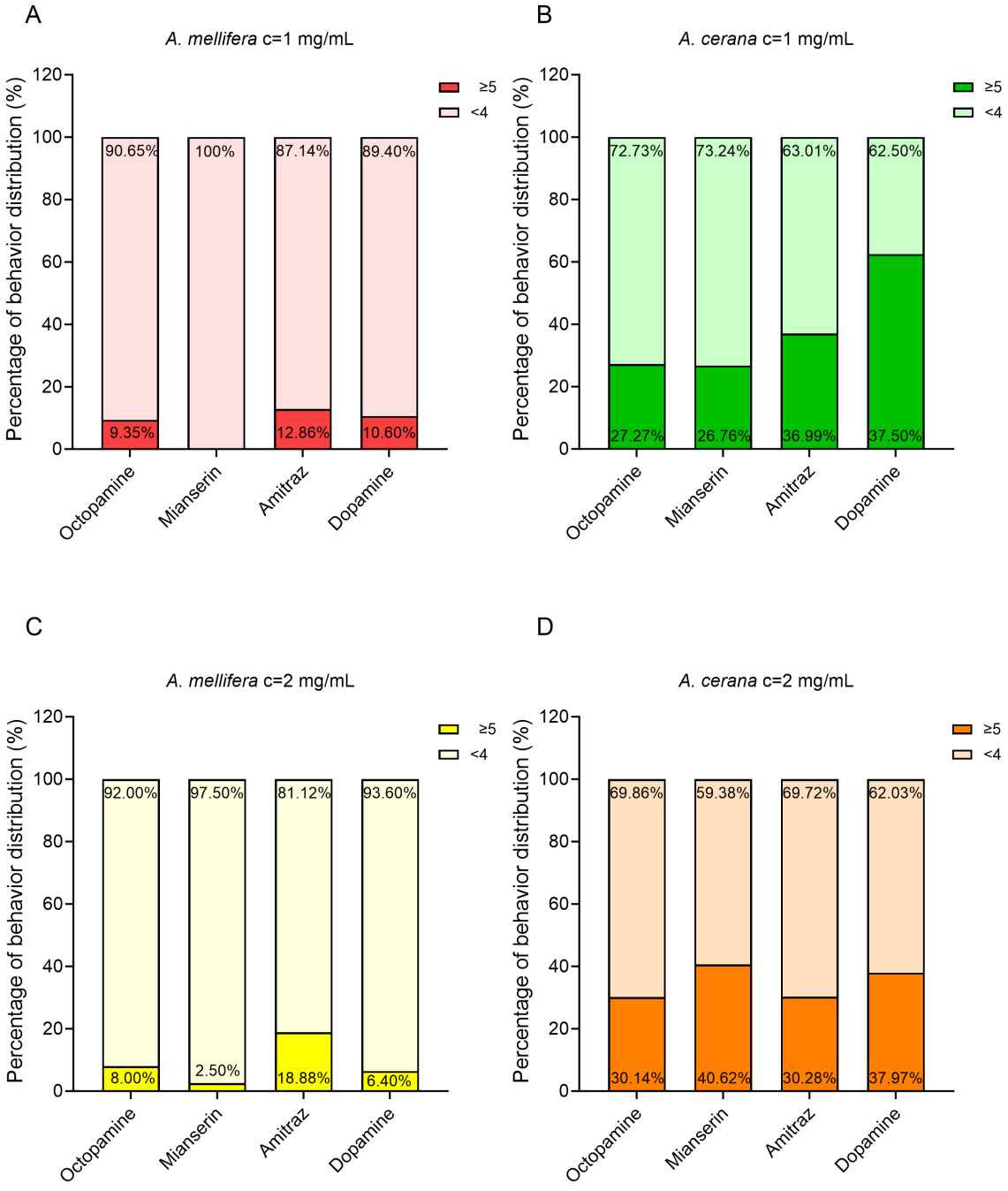


Figure 4 Comparison of the defensive behavior of *A. mellifera* and *A. cerana*.



936

937 **Figure 5** Comparison of the proportion of mass attack in Eastern and Western bees.

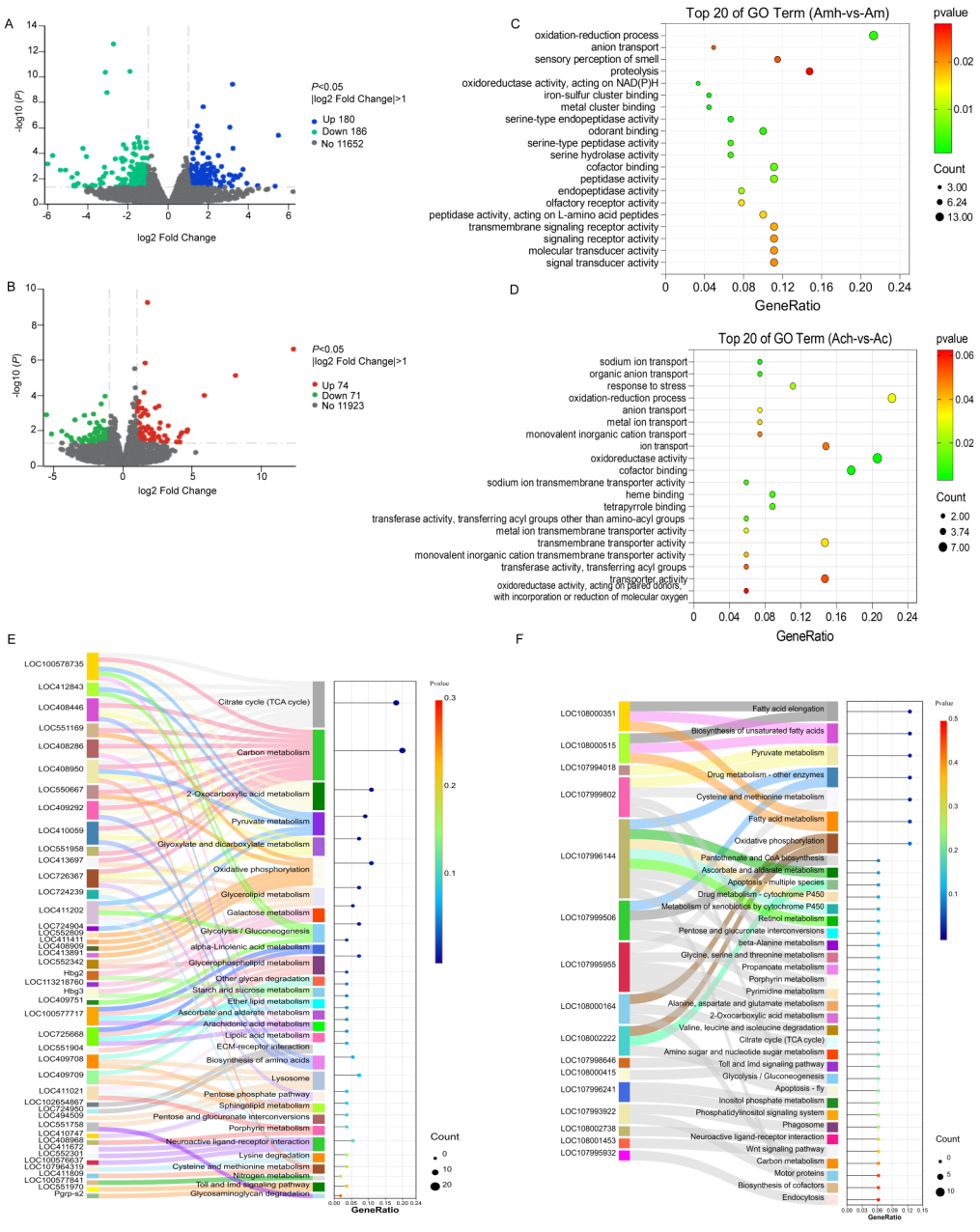
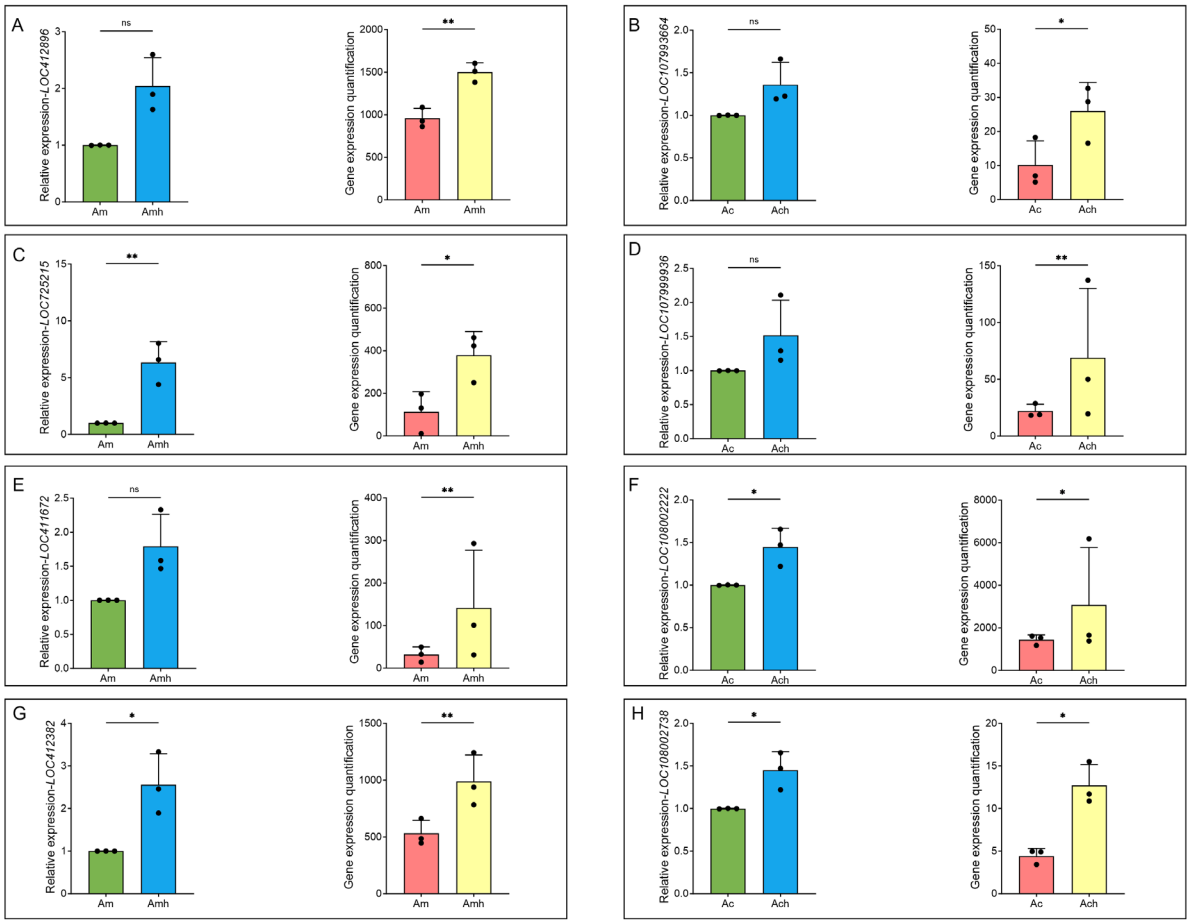


Figure 6 Transcriptome data analysis diagram.



940

941 **Figure 7** The expression patterns of 8 DEGs in guard bees were detected by RT-qPCR.

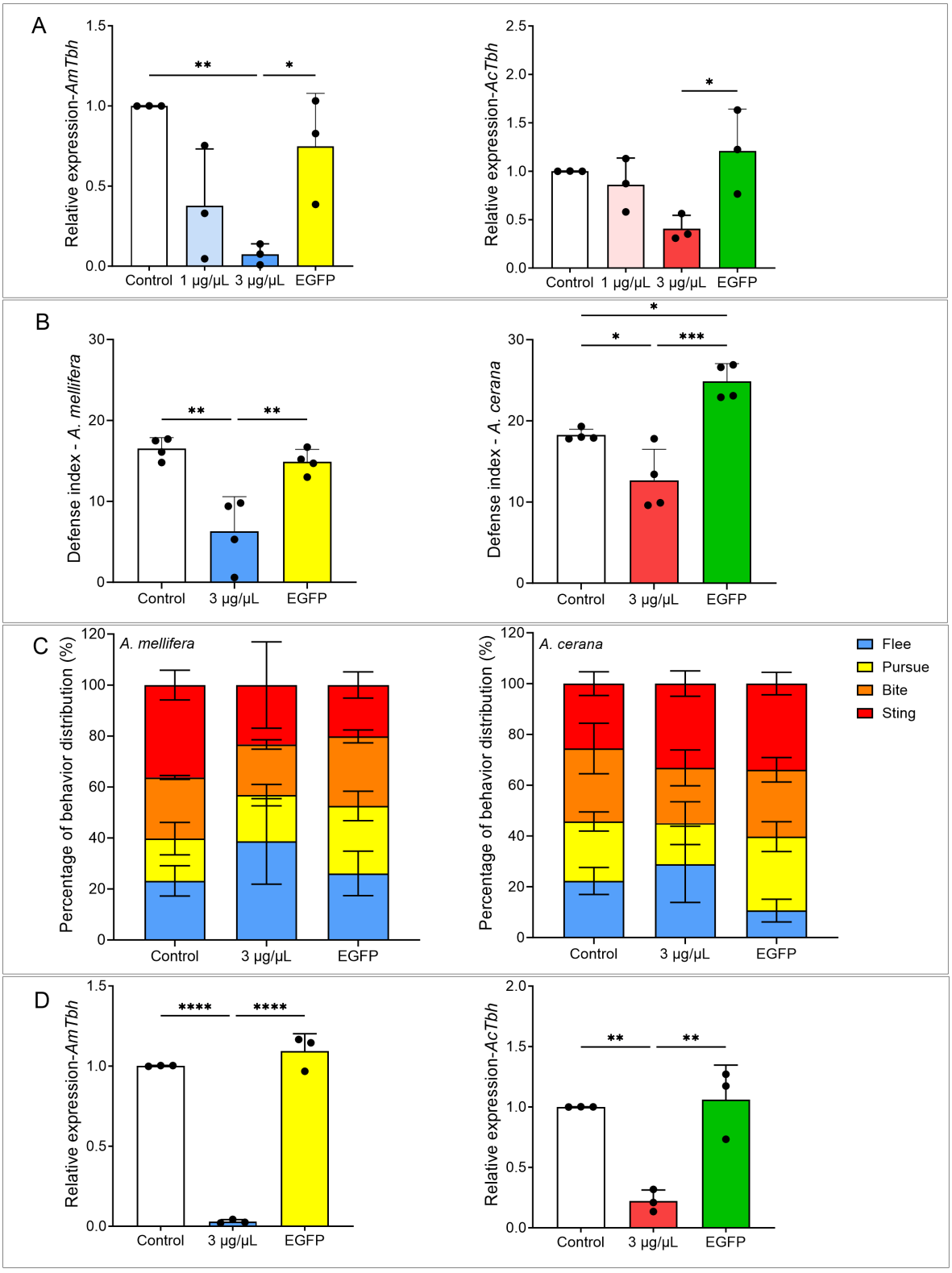


Figure 8 The effect of silencing *AmTbh*/*AcTbh* by RNAi on the defensive ability of *A. mellifera*/*A. cerana*.

945 **Table S1** primer sequence

Gene name	Primer sequence（5’-3’）	Tm
LOC412896	F：TGGTGATGGTGTCGGTGATG R：GATGGAGCTGGTGCTGAAGT	57.9℃
LOC107993664	F：TGGCATGGAATCGGACACAG R：TTGGAGCTGGGGAATCTTCG	57.9℃
LOC725215	F：TGGTGGAAGAAACGGACAAAG R：TGATATTTGCTTCGATTCCAGGGT	58.0℃
LOC107999936	F：TTCAGCTTGGTGCGTTGTTG R：TTGGCACATAAGAAGCGGGT	57.5℃
LOC411672	F：GAGGGGAAACACAGTCAAACTAT R：GTTGTTGTCCTTTTCCGTTGTC	55.5℃
LOC108002222	F：TGGGTATTCCTGCTGGTGAT R：TAACCAGGTGCTTGACCAGT	55.5℃
LOC412382	F：GTCGCCGTTGTAATAGGTGCT R：GCACTGAACTCTCCAGGTCT	58.4℃
LOC108002738	F：CAGAACCGGCCTAAGTAGCG R：TGCAGCTCGATGCGTATATGT	60.0℃
Amβ-actin	F：AGGCTCTTTTCCAACCATCC R：GGTGCTAGGGCAGTGATTTC	56.0℃
Acβ-actin	F：ATCACCATCGGCAACGAGAG R：ACGTTGTTGGCGTACAGGT	57.0℃

946

947 **Table S3** The duration of mass attack in the control group bees

Species			SMA		BMA	
	Colony	Number	Times of occurrence	Average duration (s)	Times of occurrence	Average duration (s)
A	*A. mellifera*	1	4	1.0	N/A	N/A
		2	7	1.0	N/A	N/A
		3	N/A	N/A	N/A	N/A
B		4	17	1.8	5	2.7
		5	16	3.7	N/A	N/A
		6	15	1.9	4	10
		7	12	1.9	1	2
C		8	11	4.3	N/A	N/A
		9	7	2.3	1	15
A	*A. cerana*	1	5	3.7	N/A	N/A
		2	7	2.4	5	7.3
		3	18	2.8	N/A	N/A
B		4	11	6.5	3	12.3
		5	7	3.3	5	10.5
		6	9	6.1	5	12.2
		7	5	4.2	6	11.4
C		8	12	4.9	N/A	N/A
		9	6	3.4	5	12.2

948 Notes: *A. mellifera*: *Apis mellifera*; *A. cerana*: *Apis cerana*. "N/A" denotes Not Applicable.

949

Table S4 The defensive index of the control (5% methanol in saline) group

Species		Behavior times							Defensive index
	Colony	Number	Evasion	Pursuit	Biting	Stinging	SMA	BMA	
		1	21	18	30	25	9	N/A	16.8
A		2	28	26	25	22	12	N/A	16.2
		3	31	17	21	18	9	N/A	11.8
		4	11	20	23	22	11	N/A	16.5
B	*A. mellifera*	5	18	20	20	27	19	3	21.4
		6	28	19	16	30	20	5	21.8
		7	23	12	18	23	16	2	16.8
C		8	9	6	15	17	10	3	13.3
		9	22	20	23	26	18	2	20.4
		1	15	18	23	20	10	2	21.9
A		2	11	9	12	9	5	N/A	8.9
		3	0	4	13	12	7	1	13.7
		4	7	10	27	25	20	6	38.2
B	*A. cerana*	5	6	7	17	13	12	7	27.5
		6	9	15	15	12	8	3	18.1
		7	11	14	13	16	10	N/A	15.7
C		8	12	7	15	18	9	N/A	15.1
		9	2	14	17	10	4	8	22.8

Notes: *A. mellifera*: *Apis mellifera*; *A. cerana*: *Apis cerana*. "N/A" denotes Not Applicable. Statistical analysis indicated that bees injected with the 5% methanol vehicle control showed no significant difference in defensive index compared to those injected with the control (mean difference=-2.34, 95% CI: -7.94 to 3.25, P=0.403) (*A. mellifera*). Statistical analysis indicated that bees injected with the 5% methanol vehicle control showed no significant difference in defensive index compared to those injected with the control (mean difference =-2.122, 95% CI: -8.537 to 4.292, P=0.509) (*A. cerana*). Multiple comparison adjustment : LSD method.

959 **Table S5** Data quality summary

sample	raw reads	raw bases	clean reads	clean bases	error rate	Q20 (%)	Q30 (%)	GC pct (%)
Amh1	49797654	7.47G	48594424	7.29G	0.01	98.54	95.80	39.12
Amh2	42348398	6.35G	41270384	6.19G	0.01	98.90	96.80	40.52
Amh3	50030360	7.5G	48944318	7.34G	0.01	98.27	94.96	39.41
Am1	49334976	7.4G	48196210	7.23G	0.01	98.50	95.70	38.50
Am2	46306192	6.95G	45098412	6.76G	0.01	98.67	96.16	39.26
Am3	47493532	7.12G	46292284	6.94G	0.01	98.41	95.62	37.90
Ach1	49103542	7.37G	47758050	7.16G	0.01	98.64	96.01	39.22
Ach2	46716490	7.01G	45074910	6.76G	0.01	98.61	95.93	40.05
Ach3	48675840	7.3G	47101550	7.07G	0.01	98.72	96.23	39.17
Ac1	42271692	6.34G	41186382	6.18G	0.01	98.44	95.45	41.21
Ac2	46525616	6.98G	45554652	6.83G	0.01	98.74	96.17	40.06
Ac3	48154228	7.22G	47085634	7.06G	0.01	98.63	96.04	39.06

960 Notes: *A. mellifera*: *Apis mellifera*; *A. cerana*: *Apis cerana*. Amh: *A. mellifera* guard bees under hornet
961 threat; Am: *A. mellifera* guard bees without hornet threat; Ach: *A. cerana* guard bees under hornet
962 threat; Ac: *A. cerana* guard bees without hornet threat.

963 **Table S6** Comparison of statistics

sample	total reads	total map	unique map	multi map
Amh1	48594424	44716041(92.02%)	43857673(90.25%)	858368(1.77%)
Amh2	41270384	38412478(93.08%)	37571035(91.04%)	841443(2.04%)
Amh3	48944318	43185276(88.23%)	42503860(86.84%)	681416(1.39%)
Am1	48196210	44177609(91.66%)	43623576(90.51%)	554033(1.15%)
Am2	45098412	41194494(91.34%)	40350468(89.47%)	844026(1.87%)
Am3	46292284	42162342(91.08%)	41417040(89.47%)	745302(1.61%)
Ach1	47758050	44515935(93.21%)	43305933(90.68%)	1210002(2.53%)
Ach2	45074910	42138498(93.49%)	40271146(89.34%)	1867352(4.14%)
Ach3	47101550	44193887(93.83%)	42223820(89.64%)	1970067(4.18%)
Ac1	41186382	38584946(93.68%)	37493609(91.03%)	1091337(2.65%)
Ac2	45554652	42703894(93.74%)	40340229(88.55%)	2363665(5.19%)
Ac3	47085634	43888988(93.21%)	42727856(90.74%)	1161132(2.47%)

964 Notes: *A. mellifera*: *Apis mellifera*; *A. cerana*: *Apis cerana*. Amh: *A. mellifera* guard bees under hornet
965 threat; Am: *A. mellifera* guard bees without hornet threat; Ach: *A. cerana* guard bees under hornet
966 threat; Ac: *A. cerana* guard bees without hornet threat.
967

968 **Table S8** Offensive and defensive distance between hornets and bees

Species	Colony	Bees start reaction distance (cm)	average value (cm)	Bees begin to attack distance (cm)	average value (cm)
A. mellifera	A	15.3	14.8 ± 1.1	6.4	5.0 ± 2.5
		14.1		3.0	
		12.4		4.5	
		14.0		4.1	
	B	14.5		5.4	
		14.6		4.3	
		16.6		3.0	
		15.3		13.0	
		16.1		6.3	
	C	15.3		2.0	
		14.2		6.0	
		15.7		5.4	
		13.0		2.8	
		15.4		4.5	
		15.2		4.5	
A. cerana	A	29.0	24.9 ± 2.4	9.0	6.4 ± 1.9
		25.5		5.3	
		22.3		8.3	
		23.4		6.5	
	B	22.5		5.5	
		27.0		9.4	
		24.5		3.7	
		19.6		7.0	
		28.8		9.3	
	C	24.0		3.8	
		25.1		6.2	
		26.5		5.5	
		24.4		3.0	
		24.5		6.4	
		26.5		6.7	

969 Notes: *A. mellifera*: *Apis mellifera*; *A. cerana*: *Apis cerana*. "N/A" denotes Not Applicable.

970

971 **Table S9 Summary of statistical analysis information for all figures**

Figure	Panel(s)	Unit of Analysis	n (per species/group unless specified)	Number of Colonies	Notes
Figure 1	A, B, C, D	Single trial observation	n=15 (per species)	3	5 trials per colony; each data point represents an observation from one independent trial.
	E, F, G	Colony-level mean	n=4 (per species)	4	Data are the mean value for each independent colony.
	H	Independent predation event	*A. mellifera*: 174, *A. cerana*: 66	4	n values represent counts of independent predation events.
Figure 2	A, B, C, D, E, F	Observation chamber	n=7 (total)	3	Initially 9 chambers were set up; data from 7 chambers were included in the final analysis.
Figure 3	A, B, C, D	Observation chamber	n=9 (total)	3	Initial design included 9 chambers per treatment group.
	E, F	Observation chamber	n=7 (total)	3	Initially 9 chambers were set up; data from 7 chambers were included in the final analysis.
	G	Observation chamber	N/A	3	Derived metric; n is not applicable. Based on data from 3 colonies.
Figure 4	A, B, C	Observation chamber	n=7 (total)	3	Initially 9 chambers were set up; data from 7 chambers were included in the final analysis.
Figure 5	ABCD	Observation chamber	N/A	3	Derived metric; n is not applicable. Based on data from 3 colonies.

Table S9 Summary of statistical analysis information for all figures (Continued)

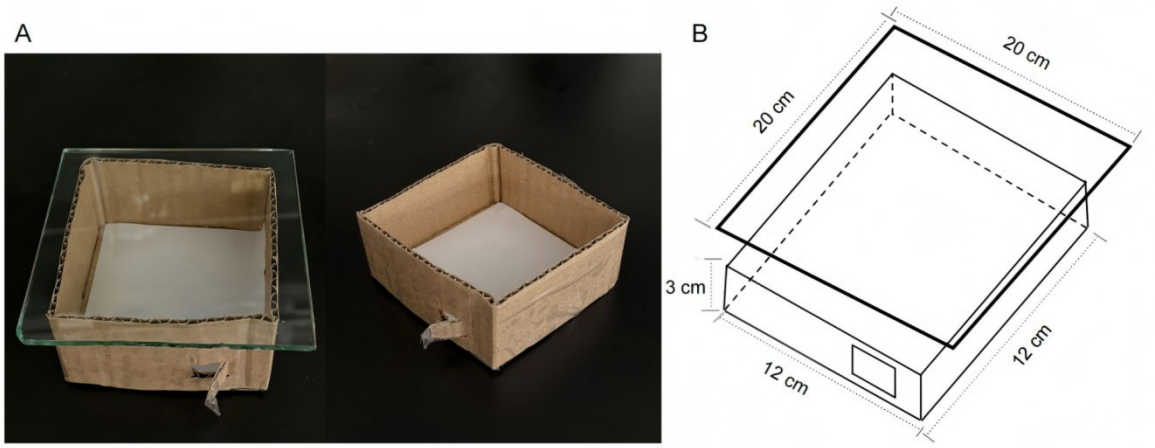
Figure	Panel(s)	Unit of Analysis	n (per species/group unless specified)	Number of Colonies	Notes
Figure 6	A, B, C, D, E, F	Colony (biological replicate)	n=3 (per condition)	3	Each data point represents a pooled sample from one independent colony.
Figure 7	A, B, C, D, E, F, G, H	Colony (biological replicate)	n=3 (per condition)	3	Each data point represents a sample from one independent colony.
Figure 8	A, D	Group of bees (10 bees/group)	n=3 (per treatment)	3	Each data point represents a group of bees from one independent colony.
	B, C	Observation chamber	n=4 (per species)	4	Each data point represents the result from one observation chamber.

Notes: The "n" column generally indicates the number of independent data points per experimental group or per species, unless specified otherwise (e.g., Fig 1H). For derived metrics (Fig 3G, Fig 5), n is not applicable.

"Number of Colonies" refers to the total number of independent colonies that contributed the underlying data for that figure panel.

"Total" indicates that the n value is the sum of data points across all groups in the figure.

"N/A" denotes Not Applicable.



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981 **Figure S1 The defensive behavior observation chambers.** A shows the physical picture of the

982 behavior observation chamber, and B shows the size of the behavior observation chamber.

转录组学结合 RNAi 揭示西方蜜蜂与东方蜜蜂应对黄脚胡蜂攻击的防御策略及分子机制

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摘要

攻击与防御是动物生存的核心问题，捕食者与猎物之间的相互作用驱动了防御行为的进化。然而西方蜜蜂（*Apis mellifera*）与东方蜜蜂（*Apis cerana*）针对黄脚胡蜂（*Vespa velutina*）的防御策略及其差异的分子基础，至今仍未得到充分阐释。本研究综合运用行为学、药理学、转录组学及 RNA 干扰（RNA interference，RNAi）技术，系统分析了两种蜜蜂的防御反应及分子机制。结果显示，黄脚胡蜂优先攻击西方蜜蜂，较少侵扰东方蜜蜂蜂群，这反映了其不同的机会性捕食策略；并且东方蜜蜂启动防御的时间更早，表现出更高的警觉水平，而西方蜜蜂则在更近距离时作出反应。药理学干预实验表明，章鱼胺（OA）在两种蜜蜂的防御反应中均发挥重要的调节作用。转录组分析揭示了一个关键的分子差异：在胡蜂威胁下，西方蜜蜂的章鱼胺受体基因 *Octβ2R* 表达上调，而东方蜜蜂则无此现象。随后，我们通过 RNAi 抑制酪胺 β-羟化酶（*Tbh*）——OA 合成的关键基因，显著降低了两种蜜蜂的防御行为，进一步证实了章鱼胺是调节蜜蜂防御行为的关键神经递质。此外，研究揭示西方蜜蜂的防御涉及三羧酸循环和氧化磷酸化等能量代谢通路的激活；相比之下，东方蜜蜂则采用了更广泛的代谢调节机制，包括不饱和脂肪酸的生物合成。这些发现不仅为蜜蜂防御行为的演化提供了新见解，也为探究社会性昆虫基因调控与行为演化的关系提供了重要参考。

关键词：蜜蜂；胡蜂；防御行为；转录组；RNAi