Genes associated with hot defensive bee ball in the Japanese honeybee, Apis cerana japonica

Takahiro Kamioka1†, Hiromu C. Suzuki1,2†, Atsushi Ugajin3, Yuta Yamaguchi4, Masakazu Nishimura4, Tetsuhiko Sasaki4,5, Masato Ono4,5 and Masakado Kawata1*

Abstract

Background: The Japanese honeybee, Apis cerana japonica, shows a specific defensive behavior, known as a “hot defensive bee ball,” used against the giant hornet, Vespa mandarinia. Hundreds of honeybee workers surround a hornet and make a “bee ball” during this behavior. They maintain the ball for around 30 min, and its core temperature can reach 46. Although various studies have been conducted on the characteristics of this behavior, its molecular mechanism has yet to be elucidated. Here, we performed a comprehensive transcriptomic analysis to detect candidate genes related to balling behavior.

Results: The expression levels of differentially expressed genes (DEGs) in the brain, flight muscle, and fat body were evaluated during ball formation and incubation at 46 °C. The DEGs detected during ball formation, but not in response to heat, were considered important for ball formation. The expression of genes related to rhodopsin signaling were increased in all tissues during ball formation. DEGs detected in one or two tissues during ball formation were also identified.

Conclusions: Given that rhodopsin is involved in temperature sensing in Drosophila, the rhodopsin-related DEGs in A. cerana japonica may be involved in temperature sensing specifically during ball formation.

Keywords: Apis cerana japonica, Hot defensive bee ball, RNA-seq, Differential gene expression, Thermal sensitivity, Rhodopsin

Background

Temperature is one of the abiotic factors that affects insects. Given that climate change may affect the distribution of organisms [54], it is essential to understand the thermal adaptation mechanisms of insects. Exceptionally for insects, two species of honeybee, Apis mellifera and Apis cerana, inhabit regions with a wide temperature range from the tropical to the temperate zones [10]. Honeybees may have adapted to living in such a wide range of temperatures by acquiring temperature regulation abilities. Therefore, studies on honeybee thermoregulation are important for achieving a better understanding of insect temperature adaptation.

The body temperature of insects is controlled by the heat production via flight muscle [23–25] and by selecting the optimal temperature via temperature sensors [33, 61]. Honeybees maintain their nest temperature at 33–36 °C, the optimal temperature range for larval growth [15, 60, 74]. Nest temperature is maintained by worker bees that regulate the heat production using their flight muscles [32, 64]. Thus, nest temperature control requires heat regulation via both flight muscles and temperature sensing. Although several studies have investigated the molecular mechanism of temperature sensing in insects [7, 21, 47, 58, 61], the molecular mechanism
underlying the heat regulation system in honeybees has yet to be clarified.

Apis cerana, a native species of Asia, has different characteristics from A. mellifera, such as being resistant to certain diseases [55, 76]. A. cerana also shows different thermal characteristics from that of A. mellifera such as a high heat production capacity [15] and a high activity level at low temperature [1, 72]. Thus, A. cerana may have different thermoregulation abilities than A. mellifera.

Additionally, A. cerana exhibits unique anti-predator behavior against hornets, one of the primary predators of honeybees in Asia [49], which involves precise temperature regulation [50, 51, 65]. In Japan, the giant hornet, Vespa mandarinia, attacks the honeybee nests en masse in autumn. Indeed, V. mandarinia frequently destroys entire colonies of A. mellifera [39], which was introduced to Japan only about 150 years ago (http://www.beekeeping.or.jp/beekeeping/history/japan) and does not have effective countermeasures against the hornets. By contrast, A. cerana (Eastern honeybee) displays a collective defensive behavior, first reported in the Japanese honeybee, A. cerana japonica. This behavior is known as a “hot defensive bee ball” because honeybee workers can kill hornets by surrounding them and producing the heat from their flight muscles (Fig. 1). Nest defense of A. cerana japonica using hot defensive bee ball consists of multiple steps [51]. First, when an individual V. mandarinia attaches markings to attract its nestmates toward the nest of honeybees, worker bees warm their flight muscles to prepare for the hot defensive bee ball. When V. mandarinia enters the nest, approximately 500 worker bees surround the hornet. These workers rapidly raise the temperature inside the ball, which can reach 46 °C, higher than the lethal temperature of the hornet, and they maintain the ball for around 30 min [51, 65, 66]. Using this balling behavior, honeybees can effectively kill the hornet while many workers survive [29, 51, 65], although the exposure to high heat during the bee ball affects the viability of A. cerana japonica [77]. Thus, the hot defensive bee ball is a specific thermoregulatory behavior that produces and maintains a dangerous temperature even for this species.

Although A. mellifera also makes bee balls to defend against hornets [5, 26, 41, 48, 50, 53], the hornet-killing efficiency of their behavior is lower than that of A. cerana japonica [2]. This low efficiency may be due to the lower temperature produced within the A. mellifera bee ball [29] and/or the higher mortality of workers during balling [69]. This comparison suggests that high predation pressure from hornets may have led to the refinement of balling behavior in A. cerana japonica.

The formation of the hot defensive bee ball of A. cerana japonica is a multifaceted process involving thermoregulation near the sublethal temperature, which provides a rare opportunity to elucidate the molecular basis of thermoregulation in honeybees. However, despite multiple studies into the characteristics of bee balling, few have attempted to clarify its molecular mechanism, except for one study that investigated neural activity during balling behavior [71].

Therefore, in this study, we performed comprehensive gene expression analysis using RNA sequencing (RNA-seq) to identify candidate genes related to balling behavior in A. cerana japonica. We identified genes for which expression levels changed during the bee balling process in the brain, fat body, and flight muscle, respectively.

Results

Global gene expression patterns

Worker bees after bee ball formation (“balling”, n = 4), short 46 °C incubation (“heated”, n = 4), or normal incubation (“control”, n = 6) were sampled for RNA extraction and the subsequent sequencing (Fig. 2; see Methods for the detailed sampling procedure). RNA-seq produced 1,509,857,212 reads in total from three tissues (i.e., the brain, fat body, flight muscle) of 14 A. cerana japonica individuals (Table 1). The maximum and minimum number of reads was 43,811,508 and 28,303,868, respectively. Total length of reads was 190,309,614 bp. These reads yielded 218,202 contigs after de novo assembly using Trinity (Table 1). The reciprocal BLAST search narrowed these down to 10,712 contigs that represented orthologs of A. mellifera. We used this gene set as a reference for short-read mapping to calculate FPKM using RSEM. The RNA-seq count data are listed in Additional file 1, and FPKM values are listed in Additional file 2. Approximately 90.95% of total reads were aligned onto the reference contigs (Table 1); the maximum and minimum rates were 92.73% and 89.23%, respectively. Principal
Component analysis (PCA) using the FPKM data showed that expression patterns were tightly clustered by tissues, not by experimental treatments (Fig. 3). The PC1 axis separated the brain samples from the fat body and the flight muscle, whereas PC2 separated all organs equally (Fig. 3). The FPKM density distribution and violin plots also showed that gene expression profiles were similar among treatments but differed among tissues (Additional file 5: Fig. S1).

DEGs specific to balling behavior
Differentially expressed genes (DEGs) were detected among the three experimental treatments using the TCC package [67]. The pattern of DEG is shown in the MA plot (Additional file 6: Fig. S2). The numbers of DEGs in Comparison 1 ("balling" vs. "control") were 82, 101, and 28 in the brain, fat body, and flight muscle, respectively (Fig. 4 and Additional file 7: Fig. S3a). The numbers of DEGs in Comparison 2 ("heated" vs. "control") were 106,
127, and 91, respectively (Fig. 4 and Additional file 7: Fig. S3b). We defined DEGs from Comparison 1 after removing the overlapped DEGs with Comparison 2 as genes related to the bee ball formation (“ball-only”). In this group, 47, 75, and 15 DEGs were identified in the brain, fat body, and flight muscle, respectively (Fig. 5). A complete list of DEGs is provided in Additional file 3.

Surprisingly, two genes stood out as “ball-only” DEGs that are common across all tissues: rhodopsin long wavelength and arrestin 2 (Additional file 3). These genes were upregulated in all tissues in the “balling” treatment (Additional file 3).

In the brain, genes associated with rhodopsin signal transduction were upregulated, such as 1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase (PLC), arrestin 1, and carotenoid isomeroxygenase (ninaB). The gene expression of nuclear receptor subfamily 4 group A member 2 (NR4A2) and atrial natriuretic peptide receptor 1 (NPR1), which are related to dopamine metabolism, increased and decreased, respectively.

Furthermore, the expression of 15-hydroxyprostaglandin dehydrogenase (HPGD), related to inflammation, increased, as did the expression of malate dehydrogenase and NADH dehydrogenase subunit 5, which are associated with glucose metabolism.

In the fat body, gene expression of three Tret1 genes related to trehalose transport increased, whereas the expression of ACC associated with fatty acid synthesis
decreased. Several HSP genes, such as HSP90-alpha and Hsc70-4, were upregulated, whereas the expression of some immunity-related genes, such as abaecin-like, hymenoptaecin, and defensin-1, were downregulated.

In the flight muscle, the expression of genes that encode endochitinase and cuticle protein, which are involved in exoskeleton formation, increased and decreased, respectively.

**Pathway analysis and GO analysis**

We also performed pathway analysis to reveal the overall trend of DEGs. The longevity regulating pathway was enriched in most of the categories we tested (Table 2 and Additional file 4: Table S1). Phototransduction, inositol phosphate metabolism, and phosphatidylinositol signaling were enriched in the "ball-only" category (Table 2). Protein processing in the endoplasmic reticulum and spliceosome activity were involved in Comparisons 1 and 2 (Table 2 and Additional file 4: Table S1).

During the gene ontology (GO) analysis, we found that the GO term "Defense response to bacterium" was enriched in the fat body in all categories (Additional file 4: Table S2). We found no enrichment of GO terms in the brain or flight muscle.

**Discussion**

The formation of a bee ball is a defensive strategy widely observed among honeybee species. However, balling by the Japanese honeybee, *A. cerana japonica*, is unique
because of the precise temperature regulation and extreme heat production that are part of the process. To better understand the molecular basis of this behavior, we measured gene expression levels during bee ball formation in the brain, fat body, and flight muscle of worker honeybees using RNA-seq.

Role of the rhodopsin signaling pathway in bee ball formation

A surprising result of this study was that several genes related to the rhodopsin signaling pathway were included in the DEGs of the “ball-only” group. The rhodopsin long wavelength gene and arrestin 2 genes were upregulated during the balling in all three tissues. Three other genes related to rhodopsin signaling were also upregulated in the brain, namely PLC, arrestin 1, and carotenoid isomeroxygenase. Pathway analysis revealed that DEGs related to phototransduction were enriched in the brain and flight muscle, and those related to inositol phosphate metabolism and the phosphatidylinositol signaling, which often involve G-protein-coupled receptor (GPCR) signaling, were enriched in the brain and fat body.

Rhodopsin is primarily known as a photosensory protein involved in the downstream GPCR cascade with the transient receptor potential (TRP) channel family [42]. However, recent studies have found that rhodopsin also plays a role in thermal preference in Drosophila [7, 40, 61]. In animals, some of the TRP channel subfamilies (e.g., TRPV, TRPM, and TRPA) function as thermosensors [12]. In Drosophila, several channels of the TRPA group, such as TRPA1 and painless, act as heat sensors at the periphery, driving thermotaxis or avoidance of noxious heat [21, 36, 58, 61, 63]. For example, the preference of adult D. melanogaster toward temperatures of 24 °C is controlled by TRPA1, and its knockout results in defects in optimal temperature selection [21, 59]. In contrast, Drosophila larvae have a different temperature preference [36], which is under the control of several rhodopsin genes [61, 63]. Shen et al. [61] demonstrated that wild-type larvae preferred temperatures of 18 °C, whereas Rh1 mutant larvae lost this preference. Drosophila individuals carrying mutations in a PLC gene (norpA), which is involved in the amplification of rhodopsin signals during phototransduction, also showed similar defects in thermotaxis [36, 61]. Sokabe et al. [63] showed that Rh5 and Rh6, rather than Rh1, are required for the temperature preference toward 18 °C in late third-instar larvae. They also showed that Rh5 and Rh6 are co-expressed in TRPA1-neurons and function together with some molecules involved in Gq/PLC/TRP signaling cascade [63].

These studies consistently suggest that, in Drosophila, the thermal sensing function of rhodopsin is dependent on the G-protein signaling cascade and the TRPA1 channel being expressed in the same neurons. Although hymenopteran insects have lost TRPA1 homologs, they have acquired another gene in the TRP family called HsTRPA, which has a similar thermal and chemical sensing property as those of Drosophila TRPA1 [33]. The HsTRPA channel of A. mellifera (AmHsTRPA) is activated at around 34 °C [33], and might contribute to the maintenance of nest temperature. Therefore, it is possible that rhodopsin and its downstream G-protein pathway coordinate with HsTRPA to detect thermal stimuli in honeybees. Our RNA-seq data also confirmed that AmHsTRPA mRNA was present in all three tissues (Fig. 5).

Arrestin 2, another universally upregulated gene in the “ball-only” group, is known to interact with the GPCRs, such as the rhodopsin in animals [37, 73]. In the visual system of Drosophila, an excess of activated rhodopsin causes prolonged depolarized afterpotential (PDE), during which rhodopsin does not respond to new light stimuli. Arr1 and Arr2 of Drosophila desensitize rhodopsin

| Organ         | KEGG pathway                                      | ID          | Number of Genes | Corrected P-value |
|---------------|---------------------------------------------------|-------------|-----------------|-------------------|
| Brain         | AGE-RAGE signaling pathway in diabetic complications | ame00270    | 2               | 0.0346            |
|               | Phototransduction—fly                             | ame04745    | 2               | 0.0346            |
|               | Longevity regulating pathway—multiple species     | ame04213    | 2               | 0.0359            |
|               | Inositol phosphate metabolism                     | ame00562    | 2               | 0.0359            |
|               | Phosphatidylinositol signaling system              | ame04070    | 2               | 0.0359            |
|               | Wnt signaling pathway                             | ame04310    | 2               | 0.0441            |
| Fat body      | Inositol phosphate metabolism                     | ame00562    | 3               | 0.0364            |
|               | Phosphatidylinositol signaling system              | ame04070    | 3               | 0.0364            |
|               | Pentose and glucoronate interconversions          | ame00040    | 2               | 0.0407            |
| Flight muscle | Terpenoid backbone biosynthesis                   | ame00900    | 1               | 0.0299            |
|               | Phototransduction—fly                             | ame04745    | 1               | 0.0299            |
and terminate the PDE [13]. If rhodopsin functions as a temperature sensor during bee balling, the role of arrestin 2 might be to desensitize rhodopsin to ensure that it maintains its sensitivity to temperature changes.

Overall, our results raise the possibility that rhodopsin and its associated molecules function with HsTRPA during the detection of the thermal stimuli by *A. cerana japonica*. The putative molecular mechanism of temperature sensing via rhodopsin signaling during balling behavior is shown in Fig. 6. We confirmed the expression of multiple genes related to rhodopsin signaling in all tissues, although many of these genes were neither up- nor downregulated during bee ball formation (Additional file 4: Table S3). We suggest that the upregulation of rhodopsin and arrestin 2 alter the heat preference during the balling behavior to ensure that honeybees can continue monitoring the temperature that can sufficiently kill the attacker. In addition, *A. cerana japonica* workers are expected to make bee ball multiple times during a mass attack of *V. mandarinia*; therefore, changes in heat preference may be important for the formation and maintenance of multiple bee balls. Upregulation of rhodopsin and arrestin 2 in all tissues is also consistent with a previous report that adult honeybees were capable of sensing temperatures throughout their body [28]. However, our results are not entirely consistent; we observed the upregulation of other genes in rhodopsin signaling (e.g., PLC and arrestin 1) only in the brain and not in the fat body and flight muscle. This result may suggest that different sets of molecules are involved in the thermosensory rhodopsin pathway in other tissues. At present, we cannot entirely rule out the possibility that these molecules function in processes other than temperature sensing. Further study is warranted to elucidate the role of rhodopsin signaling during bee ball formation.

**DEGs in the brain**

Our results showed that the expression of genes related to behavior, inflammation, metabolism, and visual signals changed in the brain during balling behavior. Dopamine influences locomotor activity in invertebrates [9, 14], and it affects the behavioral activity in honeybees [22, 44]. In the present study, expression of NR4A2 increased and NRP1 decreased in the “ball-only” condition; in previous studies, NR4A2 and NRP1 affected dopamine levels in the brains of mice and rats, respectively [16, 45]. Therefore, these genes could be involved in some actions during balling behavior via the regulation of dopamine levels. Moreover, Hunt et al. [27] found that arrestin 1 was included in the QTL related to stinging behavior, and that its expression level was higher in stinging *A. mellifera* relative to non-stinging young *A. mellifera*. Honeybees often sting the hornet during the bee ball formation [20, 50, 77]. *A. cerana japonica* was previously reported not to sting during the balling [50], but a recent study found frequent stinging by *A. cerana cerana* during

![Fig. 6](image-url)
the ball formation [20]. Therefore, the upregulation of arrestin 1 may induce the stinging behavior or enhance an alternative signal that acts specifically on attacking behaviors in A. cerana japonica. Amino acid sequence comparison of Arrestin 1 between A. cerana japonica and A. mellifera revealed that some of the amino acid mutations between the two species were deleterious (Additional file 6: Table S4). The difference in the amino acid sequence of arrestin 1 between these species could be related to their behavioral difference during balling.

Inflammation is a reaction caused by injury or infection with several symptoms, including rashes, fever, swelling, and pain [57]. Prostaglandins are bioactive substances involved in inflammation [18, 57]. We observed the upregulation of prostaglandins in the “ball-only” condition [78], indicating that HPGD is involved in the suppression of inflammation caused by the high-temperature experienced during balling.

Carbohydrates are required for brain activity in many animals [38]. In the current study, expression of the genes encoding malate dehydrogenase (MDH) and NADH dehydrogenase subunit 5, which are involved in sugar metabolism, increased in the “ball-only” condition. MDH is involved in the Krebs cycle [46], while NADH dehydrogenase is involved in mitochondrial electron transport [43]. Glucose metabolism reportedly increases during long-term memory formation in mushroom bodies in Drosophila [56], suggesting that carbohydrates are used for higher-order functions in the insect brain. In A. cerana japonica, higher information processing in mushroom body is reportedly activated during bee balling [71]; therefore, the upregulation of MDH- and NADH-related genes could promote sugar metabolism in the brain of workers during balling behavior.

**DEGs in fat body**

Fat bodies are insect organs that store and transport energy substrates such as carbohydrates [6]. Since honeybees mainly use carbohydrates during high-energy metabolic processes, such as flight and heat production [52], the fat body likely plays a vital role in the formation of hot defensive bee ball. Our results showed that the expression levels of genes related to energy metabolism, stress tolerance, and immunity were altered in the fat body during balling.

Of the “ball-only” DEGs involved in energy metabolism, the expression of three Tret1 genes increased, whereas that of acetyl-CoA carboxylase decreased during balling. Insects use trehalose, generated from glycogen in the fat body, as a major hemolymph sugar [75]. In Polypedilum vanderplanki, Tret1 transports trehalose, synthesized in the fat body, into the hemolymph [30]. Therefore, these genes are expected to be used to transport carbohydrates for energy metabolism in the flight muscle for heat production during balling. Acetyl-CoA carboxylase is a gene involved in fatty acid synthesis [43]. During balling behavior, fatty acid synthesis may be suppressed because carbohydrates are instead required for heat production.

Immunity is an energetically costly function in insects [4, 17]. In A. mellifera, for example, the number of foraging flights was reduced in immune-activated workers [3]. Our GO analysis revealed that “defense response to bacterium” was enriched in the fat body. This GO term is associated with abaecin-like hymenoptaecin and defensin-1, which were all downregulated in the “ball-only” condition, indicating that immunity may be suppressed during bee ball formation because energy needs to be allocated for metabolism.

High temperatures are a major stressor for insects. In A. cerana japonica, the survival rate of workers that participate in balling is reduced [77], suggesting that the high temperature in the bee ball affects the honeybee workers as well as the hornet. We found that gene expression of three heat shock proteins, HSP90, HSc70, and HSP97, increased in the “ball-only” condition. Such heat shock proteins are involved in response to temperature stresses in insects [11, 31, 34, 62, 68, 70]; thus, the aforementioned HSP genes may function in heat tolerance during balling behavior.

**DEGs in flight muscle**

Flight muscles are the primary heat-generating organs in insects [25]. Although we did not identify strong candidate genes that enabled extraordinary heat production in the flight muscle of A. cerana japonica, we were able to detect several “ball-only” DEGs related to the exoskeleton. Insect flight muscles adhere to the thorax exoskeleton, which is composed of chitin, cuticle protein, phenols, and lipids [46]. We found that the expression of genes encoding endochitinase A1 increased, while those of cuticle protein genes decreased. Chitinase is used to degrade chitin in insects [35, 46]; chitin is a polymer of N-acetylglucosamine, which is the raw material of trehalose. Thus, our results may imply that honeybees produce additional sugar for energy metabolism by digesting their exoskeleton during bee ball formation. Additionally, these genes may influence the contraction of flight muscles by regulating the strength of the exoskeleton.

**Conclusion**

To detect the candidate genes underlying the defensive balling behavior of A. cerana japonica, we conducted extensive gene expression analysis. Intriguingly, we found that the expression level of several genes involved in rhodopsin signaling increased in the brain, fat body, and
flight muscle during the balling behavior. Our results also revealed expression changes in genes related to energy metabolism and heat-stress response. These results will provide a new perspective on the specific defense behavior of Japanese honeybees.

**Methods**

**Bee sampling**

We conducted a series of experiments to induce bee ball formation in *A. cerana japonica* and collected samples for RNA extraction. We used a colony of *A. cerana japonica* from Nagano Prefecture that was reared at the apiary of Tamagawa University in Tokyo. Experiments were performed in the autumn of 2015. A *Vespa mandarina* worker used in the experiment was captured on the campus of Tamagawa University in Tokyo.

Bees were sampled for RNA extraction at several different stages of the experiment; these samples were later used for comparison of gene expression. The experimental outline is shown in Fig. 2.

Worker honeybees show age-dependent division of labor. Therefore, the following process was undertaken to collect 15-day-old bees (known to participate in bee balling [77]; from the nest and the bee ball, the following operation was performed. A brood comb containing pupae was collected from a hive and reared in an incubator at 34 °C. Newly emerged bees were marked on the thorax using colored paint markers (PX-21; Mitsubishi Pencil, Japan) and returned to the hive. The balling experiment was conducted when the marked worker bees were 15 days old. Before the experiment, the marked workers were collected from the colony. Some of the workers were immediately anesthetized on ice water for tissue dissection (denoted "before," n = 3), and the remaining bees were placed in plastic cups with 1 mol/L sucrose solution and incubated overnight at 31 °C in the dark. The incubated bees underwent further incubation, either for an additional 30 min at 31 °C in the dark (denoted "normal"; n = 3), or for 30 min at 46 °C in the dark (denoted "heated," n = 4). After the additional 30-min incubation period, workers were collected and anesthetized on ice water. In the following analysis, “before” and “normal” were treated as “control” group to improve the statistical power; this was appropriate because two groups were placed under the essentially same environmental conditions.

For the balling experiment, we used a single *V. mandarina* worker with its stinger removed to induce bee ball formation in *A. cerana japonica*. The hornet was hung from a copper wire and presented at the entrance of the hive. Soon after the ball formation, it was pulled away from the nest. The bee ball was maintained for 30 min. Once the ball had dissipated, worker honeybees that had participated in the balling were collected using a long tweezer and anesthetized on ice water for tissue dissection (denoted “balling,” n = 4).

**RNA extraction**

Brain, flight muscle, and fat body were dissected from sampled bees (n = 4, 4, and 6 in balling, heated, and control groups, respectively). After tissue homogenization, the total RNA of the brain and fat body was extracted using RNeasy Micro Kit (Qiagen) and those of flight muscle were extracted using RNeasy Fibrous Tissue Mini Kit (Qiagen) following the manufacturer’s protocol. Total RNA samples were stored at −80 °C until library construction.

**RNA-seq data analysis**

Library constructions from RNA samples and sequences were conducted at Beijing Genomics Institute (BGI, Shenzhen, China). All libraries were constructed using an Illumina HiSeq 4000 paired-end sequencer. The low-quality raw RNA-seq reads were filtered using the fastx_clipper of FASTX Toolkit version 0.0.13. To reconstruct transcripts, reads derived from 42 samples (3 organs of 14 individuals) were assembled using Trinity version 2.1.1 [19]. Adapter sequences were removed during this process using the Trimomatic tool in Trinity.

We performed reciprocal BLAST searches to annotate orthologous pairs between *A. cerana japonica* and *A. mellifera*. We first downloaded the amino acid sequences of the whole genome of *A. mellifera* from Ensembl Metazoa release 37. We then performed TBLASTN searches (e-value = 0.00001) using the amino acid sequences of *A. mellifera* as input queries and *A. cerana japonica* contigs as databases. Subsequently, we extracted *A. cerana japonica* contigs with the highest match to each *A. mellifera* gene. BLASTX (e-value = 0.00001) was conducted using the selected set of *A. cerana japonica* contigs as input queries and *A. mellifera* protein sequences as databases. These processes allowed us to obtain a set of 10,172 one-to-one orthologous pairs between *A. cerana japonica* contigs and *A. mellifera* proteins.

**Calculating DEGs**

We mapped the reads from each RNA-seq sample onto the de novo transcripts, and calculated the read count and FPKM value for each transcript using RSEM version 1.2.30. PCA analysis was performed with FPKM values using "pcomp" function in R version 3.3.3. Differentially expressed genes (DEGs) were estimated using the TCC package (FDR < 0.05) in edgeR [67]. To detect the genes that were putatively associated with balling behavior, DEGs were identified in the following comparisons: (1) “balling” vs. “control” (Comparison 1) and (2) “control”
vs. “heated” (Comparison 2). The DEGs in Comparison 1 were expected to include not only the genes regulating the bee ball formation but also those functioning in thermal tolerance, whereas the DEGs in Comparison 2 were expected to include genes for thermal tolerance during high-temperature exposure. By removing the DEGs that overlapped between Comparisons 1 and 2 from those of Comparison 1, we narrowed down the DEGs for which expression was altered specifically during the bee ball formation, we denoted these genes as “ball-only” DEGs. The identities of DEGs were estimated by the BLAST search using the NCBI non-redundant database.

Pathway analysis and GO analysis
To detect the functional biases of DEGs in Comparison 1, Comparison 2, and “ball-only”, we performed the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis using KOBAS version 3.0. P values were corrected using the Benjamini-Hochberg correction [8], and terms with a corrected P-value of less than 0.05 were considered significantly enriched terms.

To detect the DEGs’ functional biases in Comparison 1, Comparison 2, and “ball-only”, we performed gene ontology GO analysis using the goseq package in R version 3.3.3. P values were corrected using the Benjamini-Hochberg correction, and terms with a corrected P-value of less than 0.05 were considered significantly enriched terms.

Abbreviations
DEGs: Differentially expressed genes; GO: Gene ontology.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s12862-022-01989-9.

Acknowledgements
We thank T. Makino, S. Maruyama, Y. Ohno, C. Fukazawa, T. Nakayama, T. Inada, M. Takahashi, T. Wakamiya, and S. Aoki for comments on the manuscripts. MK was supported by Research Grants in the Natural Sciences from Mitsubishi foundation.

Authors’ contributions
MK conceived and MK, TK, HCS, AU and MO designed the research. TK and HCS conducted RNA-seq analysis. TK, AU, YY, MN, TS and MO conducted the experiments. TK, HCS and MK analyzed data, and wrote the manuscript. All the authors reviewed the manuscript. All authors read and approved the final manuscript.

Funding
MK was supported by Research Grants in the Natural Sciences from Mitsubishi foundation.

Availability of data and materials
RNA-seq raw sequence reads are available through the DDBJ Sequence Read Archive (https://drrj.nig.ac.jp) under accession no. PRJDB10552. The RNA-seq count data used in the differential gene expression analysis are listed in Additional file 1 (Supporting information). FPKM values used in the principal components analysis are listed in Additional file 2 (Supporting information). A complete list of DEGs is provided in Additional file 3. R scripts used herein are available upon request.

Declarations
Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Author details
1 Graduate School of Life Sciences, Tohoku University, 6-3, Aramaki Aza Aoba, Aoba-ku, Sendai 980-8578, Japan. 2 Department of Integrative Biology, University of California-Berkeley, Berkeley, CA 94720, USA. 3 JT Biohistory Research Hall, Takatsuki, Japan. 4 Graduate School of Agriculture, Tamagawa University, Machida, Japan. 5 Research Institute, Honeybee Science Research Center, Tamagawa University, Machida, Japan.

Received: 5 September 2021 Accepted: 10 March 2022
Published online: 16 March 2022

References
1. Abrol DP. Diversity of pollinating insects visiting litchi flowers (Litchi chinensis Sonn.) and path analysis of environmental flowers influencing foraging behavior of four honeybee species. J Apicultural Res. 2006;45:180–7.
2. Abrol DP. Defensive behavior of Aps cerana F. against predatory wasp. J Apicultural Sci. 2006;50:39–46.
3. Alaux C, Crauser D, Pioz M, Saulnier C, Le Conte Y. Parasitic and immune modulation of flight activity in honey bees tracked with optical counters. J Exp Biol. 2014;217:3416–24.
4. Ardia DR, Gantz JE, Schneider BC, Strebel S. Costs of immunity in insects: an induced immune response increases metabolic rate and decreases antimicrobial activity. Funct Ecol. 2012;26:732–9.

5. Arca M, Papachristoforou A, Mougel D, Portas T, Monceau K, Bonnard O, et al. Defensive behaviour of Apis mellifera against Vespa velutina in France: testing whether European honeybees can develop an effective collective defence against a new predator. Behav Processes. 2014;106:122–9.

6. Arrese EL, Soulages JL. Insect fat body: energy, metabolism, and regulation. Annu Rev Entomol. 2010;55:207–25.

7. BarbagaIlo G, Garrity PA. Temperature sensation in Drosophila. Curr Opin Neurobiol. 2015;34:8–13.

8. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J Royal Stat Soc B. 1995;57:289–300.

9. Chase DL, Pepper JS, Koelle MR. Mechanism of extrasynaptic dopamine signaling in Caenorhabditis elegans. Nat Neurosci. 2004;7:1096–103.

10. Le Conte Y, Navajas M. Climate change: impact on honey bee populations and diseases. Revue Scientifique et Technique (International Office of Epizootics). 2008;27:499–510.

11. Dahlgard J, Loeschke V, Justesen J. Induced thermotolerance and associated expression of the heat-shock protein Hsp70 in adult Drosophila melanogaster. Funct Ecol. 1998;12:786–93.

12. Dhaka A, Vingilis V, Parapatun V, Trp ion channels and temperature sensation. Annu Rev Neurosci. 2006;29:135–61.

13. Dolph PJ, Ranganathan R, Colley NJ, Hardy RW, Socolich M, Zuker CS. Arrestin function in inactivation of G-protein coupled receptor rhodopsin invivo. Science. 1993;260:1910–6.

14. Draper I, Kurshan PT, McBride E, Jackson FR, Kopin AS. Locomotor activity in Drosophila is anatomic and functional analysis. Dev Neurobiol. 2007;67:378–93.

15. Dyer FC, Seeley TD. Interspecific comparisons of endothermy in honeybees (Hymenoptera: Apoidea): deviations from the expected size-related patterns. J Exp Biol. 1987;127:1–26.

16. Eells JB, Lipska BK, Yeung SK, Misler JA, Nikodem VM. Nurr1-null heterozygous mice have reduced mesolimbic and mesocortical dopamine levels and increased stress-induced locomotor activity. Behav Brain Res. 2002;136:267–75.

17. Freidt D, Otis I, Vanatao A, Horák P. Immune response is energetically costly in white cabbage butterfly pupae. Proc R Soc London Ser B. 2003;270:220–2.

18. Golani DE, Armstrong EJ, Armstrong AW. Principles of pharmacology. 4th ed. Wolters Kluver Press; 2017.

19. Grabberey MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, et al. Upregulated energy metabolism in the Drosophila mushroom body, Apis cerana japonica as a defensive behavior against the Asian hornet, Vespa velutina. I. Seasonal changes in the frequency of visits to apiaries by vespie wasps and damage inflicted, especially in the absence of artificial protection. Appl Entomol Zool. 1988;23:418–60.

20. Inada N, Nakao K, Katsura S, Yamao A, et al. Inhibitory effect of centrally administered atrial natriuretic polypeptide on the brain dopaminergic system in rats. Eur J Pharmacol. 1986;131:171–7.

21. Nation JL. Insect physiology. CRC Press; 2015.

22. Neely GG, Keene AC, Duchek P, Chang EC, Wang QP, Aksoy YA, et al. TrpA1 Arrestin function in inactivation of G-protein coupled receptor rhodopsin. Annu Rev Neurosci. 2006;29:135–61.

23. Neely GG, Keene AC, Duchek P, Chang EC, Wang QP, Aksoy YA, et al. TrpA1 Arrestin function in inactivation of G-protein coupled receptor rhodopsin. Annu Rev Neurosci. 2006;29:135–61.
58. Rosenzweig M, Brennan KM, Taylor TD, Phelps PO, Patapoutian A, Garrity PA. The Drosophila ortholog of vertebrate TRPA1 regulates thermotaxis. Genes Dev. 2005;19:419–24.

59. Sayeed O, Benzer S. Behavioral genetics of thermosensation and hygroosensation in Drosophila. Proc Natl Acad Sci. 1996;93:6079–84.

60. Seeley T. Honeybee ecology. Princeton University Press; 1985.

61. Shen W, Kwon Y, Adgbola AA, Luo J, Chess A, Montell C. Function of rhodopsin in temperature discrimination in Drosophila. Science. 2011;331:1333–6.

62. Shen Y, Gong YJ, Gu J, Huang LH, Feng QL. Physiological effect of mild thermal stress and its induction of gene expression in the common cutworm, Spodoptera litura. J Insect Physiol. 2014;61:34–41.

63. Sokabe T, Chen HC, Luo J, Montel C. A switch in thermal preference in Drosophila larvae depends on multiple rhodopsins. Cell Rep. 2016;17:336–44.

64. Stabenheiner A, Kovac H, Brodschneider R. Honeybee colony thermoregulation—regulatory mechanisms and contribution of individuals in dependence on age, location and thermal stress. PLoS ONE. 2010;5:e8967.

65. Sugahara M, Sakamoto F. Heat and carbon dioxide generated by honeybees jointly act to kill hornets. Naturwissenschaften. 2009;96:1133–6.

66. Sugahara M, Nishimura Y, Sakamoto F. Differences in heat sensitivity between Japanese honeybees and hornets under high carbon dioxide and humidity conditions inside bee balls. J Ethol. 2012;30:2930–6.

67. Sun J, Nishiyama T, Shimiizu K, Kadota K. TCC: an R package for comparing tag count data with robust normalization strategies. BMC Bioinformatics. 2013;14:219.

68. Sun Y, Zhao J, Sheng Y, Xiao YF, Zhang YJ, Bai LX, et al. Identification of heat shock cognate protein 70 gene (Ahsc70) of Apolgyus lucorum and its expression in response to different temperature and pesticide stresses. Insect Sci. 2016;23:37–49.

69. Tan K, Yang MX, Wang ZW, Li H, Zhang ZY, Radloff SE, Hepburn R. Cooperative wasp-killing by mixed-species colonies of honeybees Apis cerana and Apis mellifera. Apidologie. 2012;43:195–200.

70. Torson AS, Yocum GD, Rinehart JR, Nash SA, Kivdera KM, Bowers KE. Physiological responses to fluctuating temperatures are characterized by distinct transcriptional profiles in a solitary bee. J Exp Biol. 2017;220:3722–80.

71. Ugajin A, Inoue T, Kunieda T, Ono M, Yoshida T, Kubo T. Detection of neural activity in the brains of Japanese honeybee workers during the formation of a “Hot defensive bee ball.” PLoS ONE. 2012;7:e32902.

72. Verma LR, Dulta PC. Foraging behavior of Apis cerana indica and Apis mellifera in pollinating apple flowers. J Apic Res. 1986;25:197–201.

73. Whalen EJ, Rajagopal S, Lefkowitz RJ. Therapeutic potential of β-arrestin- and G protein-biased agonists. Trends Mol Med. 2011;17:126–39.

74. Winston ML. The biology of the honey bee. Harvard University Press; 1987.

75. Wyatt GR. The biochemistry of sugars and polysaccharides in insects. Adv Insect Physiol. 1966;4:287–360.

76. Xu P, Shi M, Chen XX. Antimicrobial peptide evolution in the Asiatic honey bee Apis cerana. PLoS ONE. 2009;4:e4239.

77. Yamaguchi Y, Ugajin A, Utagawa S, Nishimura M, Hattori M, Ono M. Double-edged heat: honeybee participation in a hot defensive bee ball reduces life expectancy with an increased likelihood of engaging in future defense. Behav Ecol Sociobiol. 2018;72:123.

78. Zhou H, Tai HH. Threonine 188 is critical for interaction with NAD+ in human NAD+-dependent 15-hydroxyprostaglandin dehydrogenase. Biochem Biophys Res Commun. 1999;257:414–7.

Publisher’s Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.