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Original Article

Mediating a host cell signaling pathway linked to overwinter mortality offers a promising therapeutic approach for improving bee health

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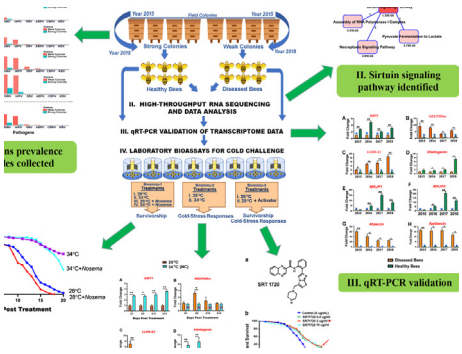
HIGHLIGHTS

- Microsporidia parasite *Nosema ceranae* and Black queen cell virus are significant predictors of overwintering colony losses when the prevalence of Deformed wing virus is low after acaricide treatment.
- The major royal jelly proteins (MRJPs) not only play nutritional roles in honey bees but also are linked to honey bees' immune responses. Therefore, they can serve as molecular markers for measuring overwintering stress in honey bees.
- Sirtuin signaling pathway is the most significantly suppressed pathway in overwintering diseased bees.
- SIRT1, a major sirtuin protein that regulates energy and immune metabolism, was significantly downregulated in bees merely exposed to cold challenges. Activation of SIRT1 expression by SRT1720, a SIRT1 activator, could improve the physiology and extend the lifespan of cold-stressed bees.

GRAPHICAL ABSTRACT



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ABSTRACT

Introduction: Honey bees provides valuable pollination services for world food crops and wild flowering plants which are habitats of many animal species and remove carbon dioxide from the atmosphere, a powerful tool in the fight against climate change. Nevertheless, the honey bee population has been declining and the majority of colony losses occur during the winter.

Objectives: The goal of this study was to understand the mechanisms underlying overwinter colony losses and develop novel therapeutic strategies for improving bee health.

Methods: First, pathogen prevalence in overwintering bees were screened between 2015 and 2018. Second, RNA sequencing (RNA-Seq) for transcriptional profiling of overwintering honey bees was conducted and qRT-PCR was performed to confirm the results of the differential expression of selected genes. Lastly, laboratory bioassays were conducted to measure the effects of cold challenges on bee survivorship and stress responses and to assess the effect of a novel medication for alleviating cold stress in honey bees.

Results: We identified that sirtuin signaling pathway is the most significantly enriched pathway among the down-regulated differentially expressed genes (DEGs) in overwintering diseased bees. Moreover, we showed that the expression of SIRT1 gene, a major sirtuin that regulates energy and immune metabolism, was significantly downregulated in bees merely exposed to cold challenges, linking cold stress with altered gene expression of SIRT1. Furthermore, we demonstrated that activation of SIRT1 gene expression by SRT1720, an activator of SIRT1 expression, could improve the physiology and extend the lifespan of cold-stressed bees.

Conclusion: Our study suggests that increased energy consumption of overwintering bees for maintaining hive temperature reduces the allocation of energy toward immune functions, thus making the overwintering bees more susceptible to disease infections and leading to high winter colony losses. The novel information gained from this study provides a promising avenue for the development of therapeutic strategies for mitigating colony losses, both overwinter and annually.

Introduction

European honey bees (*Apis mellifera*) provide pollination services to over 100 agricultural food crops. Moreover, honey bees provide an invaluable ecosystem service to wild flowering trees and plants, which are habitats for a wide range of animal species and also contribute to removing carbon dioxide from the atmosphere and reducing climate change [1]. However, populations of honey bees have been decreasing since 2006, when Colony Collapse Disorder (CCD), a mysterious disappearance and extensive die-off of honey bees, wiped out bees by the millions in the U.S. during the winter of 2006–2007 [2,3]. While the causes of the CCD still remain elusive, CCD is not the only problem honey bees are facing. In fact, over the past decade, beekeepers, primarily in the U.S. and Europe, have been suffering high levels of annual losses and overwinter colony mortality [4,5]. To further complicate the panorama, the widespread reduction of bee populations has coincided with an increasing demand for honey bees and the work they perform [6]. For example, California's almond production alone, the world's largest pollination event that typically takes place in February when bees are overwintering, requires over 1.5 million bee hives for adequate pollination each spring [7]. As a result of high colony losses and the rising cost of colony management and transport, the costs associated with pollination services have increased dramatically. This, in turn, has led to increased costs for crop production.

A number of factors including parasites and disease, habitat loss, poor nutrition, pesticide exposure, and lack of genetic diversity among others have been suggested to play roles in the alarming decline of honey bee populations [8,9]. Of the issues adversely affecting honey bees, pathogens, especially viruses, often have been linked to colony losses and it has been repeatedly reported that pathogens, especially viruses detrimentally interfere with honey bees' social system, accelerate maturation, impair immune function, and shorten life spans [2,10–12]. In particular, the prevalence of a virulent strain of *Deformed wing virus* (DWV) transmitted and exacerbated by the ectoparasitic mite *Varroa destructor,* which is the most severe biotic threat to honey bees, has caused the death of millions of honey bee colonies worldwide [13].

Most managed bee colony losses occur during the winter with average losses of over 30 % since the CCD outbreak (https://beeinformed.org). Though honey bees do not hibernate during the winter, they have evolved remarkable adaptations to survive winter conditions by replacing summer bees with a completely new population of overwintering (diutinus) bees when a colony is transitioning from summer to winter and developing distinctive physiological and behavioral traits [14]. Winter bees are characterized by enlarged fat bodies and hypopharyngeal glands for producing large amounts of vitellogenin (*Vg*), a storage and antioxidant protein. Once the outside temperature drops to 10 °C or below, honeybee workers inside beehives will form a cluster around the queen/brood and vibrate their wing muscles and bodies to generate heat to keep warm. On the exterior surface of the cluster, the bees pack tightly to form an insulating layer to retain heat. They remain in the cluster until pollen becomes available again at the start of the next foraging season [15–17]. Despite the physiological and behavioral adaptations of winter bees, winter weather conditions have created a survival crisis for honey bees in temperate climates, and honey bees have been dying off at an economically unsustainable rate during the winter over the past decades. Among the potential causes of colony loss, the infestation levels of parasitic *Varroa* mites and levels of the viruses they transmit are the most strongly correlated causes of overwintering declines of bee populations [8,9,18,19]. Because the presence and diversity of pathogens especially viruses negatively influence colony overwinter survivorship, they are considered predictive markers of winter losses and colony collapse [11,16,20–23].

While we are increasingly concerned about the threat posed by the winter colony losses, there is limited information about the mechanism underlying the winter mortality of honey bees and the molecular basis of honey bees' responses to pathogenic infections during their overwintering period. In the present study, we conducted an investigation to monitor pathogen prevalence in overwintering bee colonies between 2015 and 2018. Then, we per-

formed RNA sequencing (RNA-Seq) analysis and qRT-PCR assay and compared RNA transcripts between overwintering bees from both healthy colonies and weaker counterparts. Furthermore, we conducted laboratory bioassays to measure the effects of cold challenges on bee survivorship and to assess the impact of a novel medication which was identified based on our transcriptome sequencing data for alleviating cold stress in honey bees. The long-term implication of this research is to understand the mechanisms underlying overwinter colony losses and develop effective management strategies to improve the overwintering success of honey bee colonies, thereby restoring bee populations to healthy levels and safeguarding their pollination services.

Materials and methods

Ethics statement

No specific permits were required for the described studies. Observations were made in the USDA-ARS Bee Research Laboratory apiaries, Beltsville, Maryland, USA. Studies involved the European honey bee (*Apis mellifera*), which is neither an endangered nor protected species. The experimental procedures are outlined as a flow diagram in Supplementary Fig. 1.

Bee colonies

The experimental bee colonies (*A. mellifera ligustica*) that were maintained at the apiaries of the USDA-ARS Bee Research Laboratory, Beltsville, Maryland, USA were monitored for *Varroa* mite populations regularly using sticky boards and sugar roll methods [24] and varroacide treatments were applied in the summer and fall to keep *Varroa* populations low. For a three-month period associated with the coldest average temperatures in the region (December, January, and February) from 2015 to 2018, four strong and four weak colonies were randomly selected for monthly pathogen screening throughout the study. The strength of the honey bee colonies was determined based on the number of adult bees and brood as well as the amount of food stored in the combs as described previously. Briefly, the bee colonies that had more than ten frames covered with adult workers and more than six frames filled with brood and food stores were defined as strong colonies, while bee colonies that had a small number of foraging bees flying in and out, less than ten frames of adult bees, less than six combs with brood and small patches of food stores were defined as weak colonies. Samples of nurse workers (N = 100) that usually-three to ten days old and perform tasks inside the hive,were collected from the brood frame by gently scraping worker bees into 50-mL conical tubes from each of the four strong and four weak colonies. In addition, foraging workers (N = 50) that specialize on collecting pollen and nectar.were collected at the hive entrance of each colony. All collected bee samples (total N = 150) were stored at −80 °C until subsequent analyses.

Pathogen detection

Each month, twenty adult workers from each of the four strong and four weak colonies were individually homogenized in 1 ml of TRIzol (Invitrogen, Carlsbad, CA, USA) for RNA isolation, following the manufacturer's instructions. Individual RNA samples were examined for the presence of the most common viruses including *Acute bee paralysis virus* (ABPV), *Black queen cell virus* (BQCV), *Chronic bee paralysis virus* (CBPV), DWV, IAPV, *Kashmir bee virus* (KBV), and *Sacbrood bee virus* (SBV) using Reverse Transcription-Polymerase Chain Reaction (RT-PCR) with the primer pairs described previously [25]. The Brilliant II SYBR Green qRT-PCR 1-Step master mix (Agilent Technologies, Santa Clara, CA, USA) was used for the amplification of targeted viruses, according to the manufactureŕs instructions for reaction mixture and thermal profile.

Thirty foraging workers from each colony were used for *Nosema* detection. The abdomens were removed from the foraging workers and ground thoroughly in 30 ml of deionized H_2O. 10 µl of the homogenate was loaded onto a hemocytometer (Neubauer-ruled Bright Line counting chambers; Hausser Scientific, Horsham, PA, USA). The presence of *Nosema* spores and their numbers were counted under light microscopy. The colony was recorded as infected with *Nosem*a if the spore count result was $\geq 10^5$ /per bee.

For each colony, the rate of the virus infection, the status of *Nosema* infection, and the strength of individual colonies were recorded monthly. For both strong and weak colonies, the infection rate of each virus every month was calculated based on the percentage of tested bees that were infected (N = 20 bees/per colony X 4 colonies = 80 bees). The average infection rate for each year was then calculated by combining the data from three months of the same year (N = 80 bees × 3 months = 240 bees). The average infection rate over the four-year period was calculated by combining the data from the four years (N = 240 bees × 4 years = 960 bees). For both strong and weak colonies, the *Nosema* infection rate every month was calculated based on the percentage of colonies where spores were detected in bees (N = 4 colonies). The average *Nosema* infection rate each year was calculated by combining the data from three months of the same year (N = 4 colonies X 3 months = 12 colonies). The average *Nosema* infection rate over the four-year period was calculated by combining the data from the four years (N = 12 colonies X 4 = 48 colonies).

High-throughput RNA sequencing and data analysis

Individual bees that were collected from strong colonies and identified as containing less than two pathogens were defined as healthy bees. Meanwhile, individual bees that were collected from weak colonies and found to contain at least three different pathogens were defined as diseased bees. High throughput sequencing was performed with bees from three health colonies (five bees/-colony) and three diseased colonies (five bees/per colony) that were collected in late December 2015 (N = 10 Illumina RNA libraries). A flowchart of high-throughput RNA sequencing and data analysis is shown in Supplementary Fig. 2. RNA samples that were extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) were further processed with a Qiagen RNeasy purification kit (Germantown, MD). The quantity and purity of RNA samples were measured using a NanoDrop 8000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). The integrity of RNA samples was evaluated using the Agilent Bioanalyzer 2100 (Santa Clara, CA). Only RNA samples with an RNA integrity number (RIN) value of over eight were used to generate first and second-strand cDNAs using random hexamer primers and the reverse transcriptase Superscript II (Invitrogen, Carlsbad, CA). The overall level of ribosomal RNA present in total RNA samples was reduced prior to library construction using the ribo-Zero™ Magnetic (Human/Mouse/Rat) rRNA Removal Kit (Epicentre Technologies, Madison, WI).

Illumina RNAseq libraries were prepared with the TruSeq RNA Sample Prep kit (Illumina, San Diego, CA) per the manufacturer's protocol, without the poly-A isolation steps. Adapters containing six nucleotide indexes were ligated to the double-stranded cDNA. The DNA was purified between enzymatic reactions and the size selection of the library was performed with AMPure XT beads (Beckman Coulter Genomics, Danvers, MA). Libraries are assessed for concentration and fragment size using the DNA High Sensitivity Assay on the LabChip GX (Perkin Elmer, Waltham, MA). The

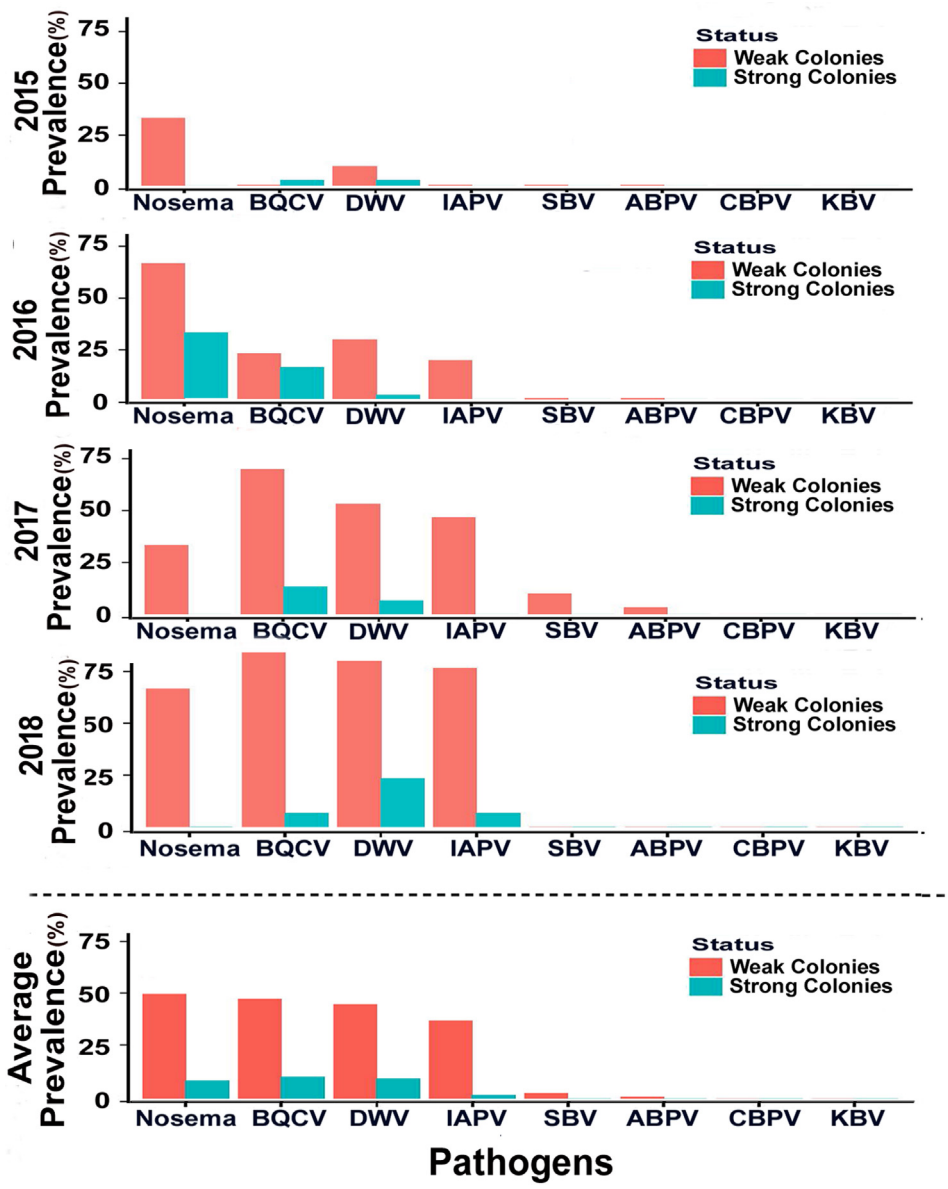

Fig. 1. The prevalence of pathogens in both weak and strong colonies from 2015 to 2018 as well as the average prevalence of pathogens within the four-year period. The virus prevalence in both the weak and strong colonies is shown as a percentage of the total number of bees (N = 240) that tested positive for the viruses via qRT-PCR. The frequency of *Nosema* in both weak and strong colonies (N = 10) is shown as a percentage of the total number of bee colonies that exhibited the presence of *Nosema* spores where the spore count result was $\geq 10^5$ / per bee.

libraries were sequenced on a 100PE Illumina HiSeq 2500 run at the University of Maryland Institute of Genome Sciences.

The clean read data prepared and provided by the service provider was analyzed by the following steps: First, after trimming and quality control of the generated sequences, transcriptomic analysis was performed with TopHat (version 1.4.1 at https://ccb.jhu.edu/software/tophat/index.shtml) by mapping sequencing reads and counting the reads across genes and transcripts based on annotated reference genes from the honey bee reference assembly Amel_4.5. During the alignment, three mismatches with maximum 100 multiple hits for a read were allowed; Second, from the mapped reads, a list of differentially expressed genes was generated by running DEseq (one of R/Bioconductor packages version 3.8) under its default settings [26]. Genes with a log 2 fold-change value > 1 and an FDR-adjusted p-value < 0.01 by the Benjamini-Hochberg method were considered differentially expressed genes (DEGs). The gene expression volcano plot was generated using the web tool, which is freely available at https://paolo.shinyapps.io/ShinyVolcanoPlot/. Using PANTHER (Version 14.0 at http://www.pantherdb.org/)[27,28] and DAVID (Bioinformatics Resources 6.8, https://david.ncifcrf.gov/), gene ontology (GO) enrichment analysis was performed on FlyBase orthologs of bee differentially expressed genes (DEGs). The terms with gene count more than five and *P*-value < 0.05 after the false discovery rate (FDR) correction for multiple testing were considered; Third, the unmapped reads for each sample were processed using Metaphlan2 (version 2.0 at http://huttenhower.sph.harvard.edu/metaphlan2) [29,30]. Finally, in order to better explore exogenous pathogen transcripts, unmapped reads by removing reads which aligned to the Apis rRNA sequence from http://www.rna.icmb.utexas.edu/ [31] using Bowtie2 (version2.2.3 http://bowtie-bio.sourceforge.net/bowtie2/index.shtml) with its default parameters were further filtered [32]. The remaining unmapped reads

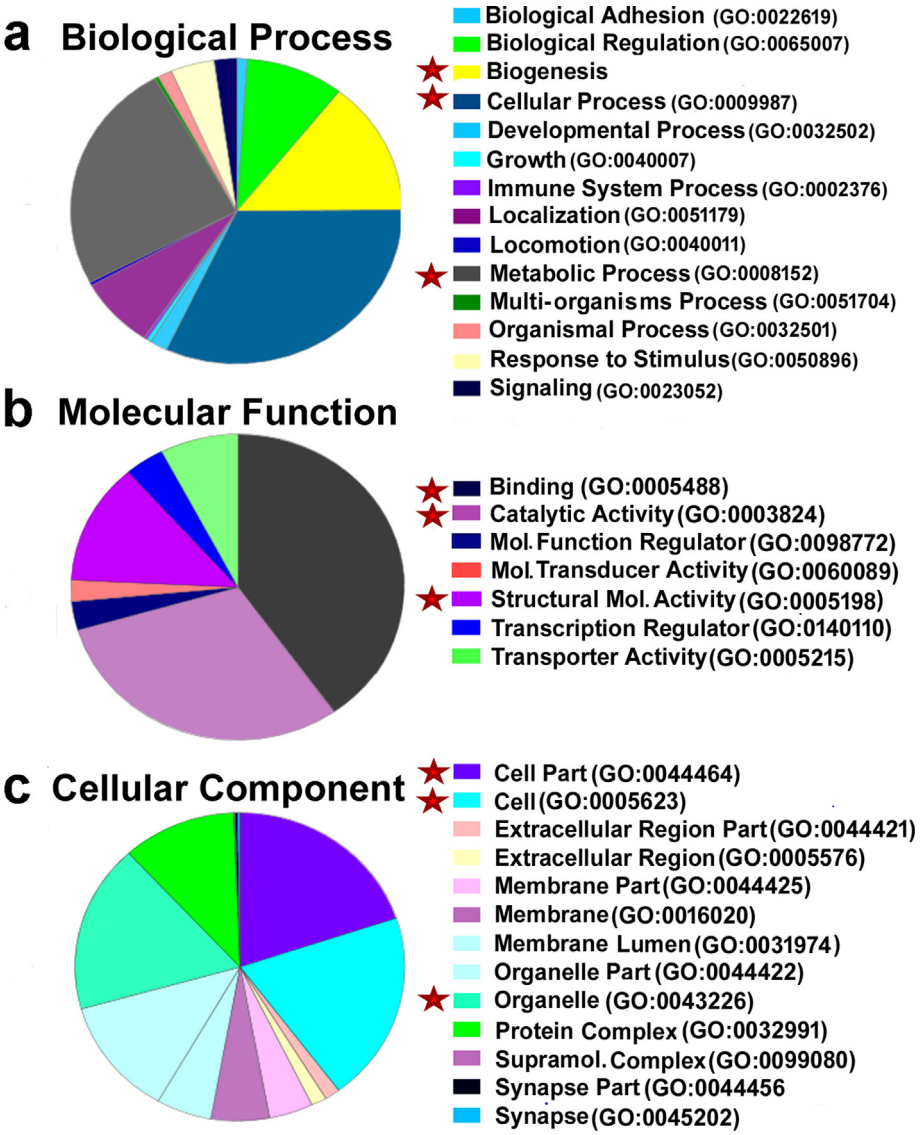

Fig. 2. Distribution of enriched functions of DEGs. (*A*) Biological Process (BP), (*B*) Molecular Functions (MF) and (*C*) Cellular Component (CC). Each pie displays the distribution of DEGs according to BP, MF, or CC. The top three significantly enriched GO terms of the BP, MF, or CC are marked with the stars.

were then aligned against nr/nt (NCBI non-redundant nucleotide database) using megaBLAST (with an option of -W 16, i.e. a word size of 16). The matches in megaBLAST results were parsed into a taxonomic representation listing using KronaTool (a set of computer scripts at https://github.com/marbl/Krona/wiki/KronaTools) to represent the LCA (lowest common ancestor). These lists were formatted into an HTML style of files (Krona charts), containing the percentage of reads that match a specific organism. Krona charts allow hierarchical data to be interactively visualized and explored in multi-layered pie charts with zooming (https://github.com/marbl/Krona/wiki) [33] in a web browser like Google Chrome or Internet Explorer.

Additionally, genes that are homologous to their *Drosophila* gene counterparts were further analyzed for canonical pathways, biological functions/diseases, and functional molecular networks via Ingenuity Pathway Analysis (IPA) (Ingenuity Systems, Redwood City, CA). The Fisher's exact test was employed to calculate a ρ-value to determine the probability that the association between the gene in the dataset and the predefined pathways and functional categories in the Ingenuity Pathway Knowledge Base are due to random chance alone.

qRT-PCR validation of differentially expressed genes detected by RNA-Seq

A list of subset genes from the protein synthesis, immune responses, sirtuin signaling pathway, oxidative phosphorylation, mitochondrial dysfunction, and metabolism that were identified by RNA-seq analysis as differentially regulated were subjected to RT-qPCR validation using *β-actin* as a reference gene for normalization. qRT-PCR was performed in fifteen healthy bees and fifteen diseased bees for each year that were collected in 2015, 2016, 2017, and 2018, individually. The primers used in qRT-PCR are included in Table 1. The approximately equal amplification efficiencies of the RT-qPCR assay for each of the target genes and housekeeping gene *β-actin* were confirmed individually (the slope of normalized Ct *vs* log input RNA $\leq$ 0.1). Among multiple evaluated reference genes including β-actin, glyceraldehyde-3-phosphate dehydro- genase (GAPDH) and ribosomal 18S (18S), β-actin showed the highest level of expression stability and therefore was chosen as a normalizer in our study. The Brilliant III SYBR Green qPCR master mix (Agilent Technologies, Santa Clara, CA, USA) was used for the amplification of targeted genes, according

Table 1
qPCR Primers used for validation.

Primer	Sequence (5′-3′)	Code gene	Annealing temperature (°C)	Reference and notes
Mrjp1-F	AGCAGACGAGAAAGGTGAAGG	*mrjp1*	59	Li *et al.*, 2019
Mrjp1-R	TTGGACTCCTTCGTAATGTATGTCA			
Mrjp2-F	CCTGATTGGTCGTTTGCAGAG	*mrjp2*	59	Li *et al.*, 2019
Mrjp2-R	TGGTCTGCCATGTACACTAAAG			
Mrjp3-F	AACAAGCGCAGCTGTGAATC	*mrjp3*	59	Li *et al.*, 2019
Mrjp3-R	TGTCTTATCACGCCATCTGTCC			
Mrjp4-F	TAGAGGTGGCGTTGTTCGAG	*mrjp4*	59	Li *et al.*, 2019
Mrjp4-R	CGAGAAAAGTCTTGTTGTGCCA			
Yellow-e-F	CAACTACCTGCGACGACATG	Yellow-e	58	This paper
Yellow-e-R	CGGAGAATGCTAGGCCAATG			
Sirt1-F	AGAACAAGTCGCAGGCATTG	Sirt1	58	This paper
Sirt1-R	CGAAATGGGAGGCAAAGTGT			
LLDH-X2-F	CAAGTGATCGGAAGTGGCAC	LLDH-X2	58	This paper
LLDH-X2-R	AGCTATGTTCACTCCCGACC			
NDUFIS6m-F	AAAAGTCACTCATACTGGTCAGA	NDUFIS6m	58	This paper
NDUFIS6m-R	GTCCACCACCTCCATCACAT			
Vg-F	TTAAGGAATTGGTCGGGGCT	Vitellogenin	58	This paper
Vg-R	TCAAGCTGAAGAGACCCTGG			
β-actin-F	TTGTATGCCAACACTGTCCTTT	Housekeeping gene	55	Chen et al., 2005
β-actin-R	TGGCGATGATCTTAATTT			

to the manufactureŕs instructions. The comparative Ct method (ΔΔ Ct Method) [34] was chosen for the interpretation of gene expression of overwintering honey bees in response to pathogenic infections. For each gene, the expression level was quantified based on the value of the cycle threshold (Ct) and was expressed as means + SE. The average Ct value of the target gene was normalized with the corresponding *β-actin* value using the formula ΔCt = average Ct (target) – average Ct (*β-actin*). One of the two bee groups (either healthy bees or diseased bees) with a lower level of gene expression was chosen as the calibrator. The value of the calibrator was set to one. The relative concentration of each target gene was expressed as the fold-difference relative to the calibrator which was calculated using the formula $2^{-\Delta\Delta Ct}$.

Laboratory bioassays

To set up laboratory bioassays, frames with sealed brood from healthy colonies identified as pathogen-free by our monthly disease survey were removed from bee colonies and placed individually in a mesh-walled cage and incubated in an insect growth chamber at 34 ± 1 °C, 55 ± 5 % RH overnight. The newly emerged bees roamed on the frame containing of brood, pollen, and honey for 24hrs to acquire the necessary gut microbiota, the newly emerged worker bees were collected and transferred separately to the ''top feeder" rearing cages [35] (35 bees/per cage). The bees were fed a sucrose solution (50 %) that was replaced every-two days.

To evaluate the effect of cold stress on bee survivorship, the caged bees were divided into four groups based on the treatment they received: 1) bees maintained at optimized temperature (34 °C), 2) bees exposed to cold challenge (28 °C), 3) bees inoculated with *Nosema ceranae* spores ($1x10^5$ spores/per bees) then maintained at 34 °C, and 4) bees inoculated with *N. ceranae* spores ($1x10^5$ spores/per bees) then maintained at 28 °C. We followed our previously described method for *N. ceranae* spores purification and inoculation [36]. Each experimental group consisted of three cages (35 bees/cage). Different groups of cup cages were maintained in insect incubators (28 °C or 34 °C) based on the temperature they received. Dead bees were removed and their numbers were recorded daily. The observation of survivorship was completed when one group achieved 100 % mortality.

To assess the effects of cold stress alone on the host metabolic and physiological processes, the expression level of genes encoding SIRT1, LLDH-X2, NDUFIS6m, and Vg that are involved in stress response and energy metabolism was compared between bees exposed to cold challenge (28 °C) and bees maintained at optimized temperature (34 °C) on day-1, day-5, day-10 and day-15 post-treatment by RT-qPCR assay. Each experimental group consisted of three cages (35 bees/cage). At each time point post-treatment, five bees were sampled from each cage (N = 5 X 3 cages = 15 bees) for RNA extraction. RT-qPCR and data analysis were performed following the same methods described above.

To investigate the effect of the sirtuin-activating compound (STAC) on alleviating cold induced stress response in honey bees, SRT1720, a specific SIRT1 activator that was developed by Sirtris Pharmaceuticals (Cambridge, MA), was purchased from Sigma - Aldrich (CAS 925434–55-5; St. Louis, MO) and used in our bioassays. SRT1720 was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) to prepare the stock solution and then diluted in 50 % sucrose water to feed the bees. The cold-challenged honey bees were divided into four groups and fed with 50 % sucrose containing 0, 0.4 μg/ml, 2 μg/ml, and 10 μg/ml of SRT1720, respectively, for 15 days. Each experimental group consisted of six cages (35 bees/cage), three cages for observation on bee survivorship to define optimized SRT1720 concentration and three cages for sample collection. All cup cages were maintained in 28 °C insect incubators. Only bee samples collected from the control group without SRT1720 treatment and the group that had the highest survival rate were used for downstream molecular analysis. RT-qPCR analysis was performed for measuring expression levels of genes encoding SIRT1, LLDH-X2, NDUFIS6m, and *Vg* following the same methods as described above.

Statistical analyses

Differences in pathogen prevalence between bees from strong *vs* weak colonies were analyzed by binomial models. After confirming the presence of a normal distribution and an equal variance of data, comparisons in mRNA levels between healthy bees and their diseased counterparts as well as between bees challenged at 28 °C and bees kept at 34 °C were conducted using a student's *t*-test. In all cases, a P value of ≤ 0.05 was considered to be statistically significant. Survival analysis was performed using the Kaplan-Meier method. LOG-rank and Wilcoxon tests were used to assess the impact of stressors (28 °C or/and *N. ceranae*) on bee survivorship as well as the impact of SRT1720 treatment on the improvement of survivorship of cold challenged bees in comparison to bees in the control group. The Cox proportional-hazards regression model was used to estimate the dependency of survival time on two vari-

ables, cold temperature and *Nosema* infection. All analyses were carried out using SPSS software 18.0 (Cary, NC, USA) and R version 4.0. Figures were generated with Graphpad Prism 8.3 (GraphPad Software, Inc., San Diego, CA, USA) and the R package ggplot2.

Availability of data and materials

RNA-seq data for all six libraries have been deposited at the U.S. National Institutes of Health NCBI under GEO accession ID Series of GSE 196998.

Results

Prevalence of pathogenic infections in overwintering bee colonies

Field bee colonies were classified as strong and weak colonies based on the size of the adult populations, the amount of sealed brood, and the presence of food stores at the end of summer, as described previously [37]. The prevalence of pathogens in both weak and strong colonies in 2015, 2016, 2017, and 2018 as well as the average occurrence of pathogens across all four years combined are presented in Fig. 1A, 1B, 1C, and 1D). The most prevalent pathogens overall were *N. ceranae* (29 % prevalence), BQCV (28 %), and DWV (26 %), followed by IAPV (19 %). While co-occurrence of multiple pathogens was found in both strong and weak colonies as a few bee viruses such as DWV are ubiquitous in honey bees [38], the prevalence of each of the four most common pathogens was significantly higher in weak colonies than their strong counterparts. This included sixfold higher prevalence of *N. ceranae,* 4.6-fold higher prevalence of BQCV, 4.7-fold higher prevalence of DWV, and 21.5-fold higher prevalence of IAPV (Binomial model, weak colonies *vs* strong colonies, *N. ceranae, P* = 1.78 E^{-11}; BQCV, *P* = 1.81 E^{-11}; DWV, *P* = 8.04 E^{-11}; IAPV, *P* = 5.92 E^{-14}). The year with the fewest pathogens was 2015, while 2018 had the highest rates of pathogens across the duration of this study. All weak colonies that had multiple virus infections began collapsing in December and collapsed completely after February of the following year. Meanwhile, most strong colonies survived the winters from 2015 to 2018.

The combination of co-circulating pathogens in honey bees was similar across the years. While bees from strong colonies were identified as positive for a few pathogens during the winter season, the prevalence was fairly low. At the same time, bees from weak colonies carried three or more pathogens simultaneously and the infection rate of each pathogen was high in these groups. Among the pathogens that were examined, *N. ceranae* occurred each year in weak colonies but only in 2016 for strong colonies (Fig. 1A, 1B, 1C, and 1D).). The average infection rate of *N. ceranae* over the four-year period was 50 % in the weak colonies and 8 % in the strong colonies (Fig. 1E). Among the viruses that were tested for, the most common ones identified in weak colonies in order from the highest to the lowest average infection rates were BQCV, DWV and IAPV. Other viruses such as SBV and ABPV were also detected in weak colonies. The average infection rate of BQCV, DWV, and IAPV in the weak colonies over the four-year duration was 46 %, 43 %, and 36 %, respectively. Meanwhile, the average infection rate of BQCV, DWV, and IAPV in the strong colonies over the four-year period was 10 %, 9 %, and 2 %, respectively.

Differential gene expression profiles between healthy and diseased bees revealed pathways and genes associated with diseased overwintering honey bees

The high-quality paired-end reads from the cDNA libraries of healthy bees (from strong colonies) and diseased bees (from weak collapsing colonies) were obtained and were found to have an average length of 100 bp at each end; on average 60 % of them were uniquely aligned and mapped on annotated reference genes from the honey bee reference assembly Amel_4.5. The number of raw sequence reads, sequencing quality assessments, and alignment summaries for each sample are provided in Table S1.

To ascertain the genes associated with the diseased bees, the differential gene expression analysis was performed using an R package, DEseq, at the threshold of false discovery rate (FDR), P < 0.01 and log2 [(fold change FC)] > 1 [26]. The results showed that 593 genes (459 upregulated and 134 downregulated) were differentially expressed (DEGs) when comparing healthy to diseased bee samples (Table S2). It is noted that more DEGs are upregulated in diseased bees and the ratio between up- and downregulated DEGs is 3.4: 1.

Since the honey bee genome assembly (Amel_4.5) has not been fully annotated [39], the FlyBase gene orthologs were used to annotate our bees' DEGs in PANTHER and David as *Drosophila melanogaster* (hosted in the FlyBase at https://flybase.org/) is a model organism and shows similarities to honey bees (See Table S3 and Table S4 for mapped and unmapped genes, respectively). The gene ontology (GO) enrichment analysis was performed using the fruit fly (*D. melanogaster*) reference genes as the background data. GO is an important bioinformatics tool for annotating genes and is divided into three non-overlapping ontologies: Biological Process (BP), Molecular Function (MF), and Cellular Component (CC). The top three significantly enriched BP terms were the cellular process (GO:0009987), metabolic process (GO:0008152), and cellular component organization or biogenesis (GO:00071840). The top three significantly enriched MF terms were binding (GO:0005488), catalytic activity (GO:0003824), and structural molecule activity (GO:0005198). The top three significantly enriched CC terms were cell part (GO:0044464), cell (GO:0005623) and organelle (GO:0043226) (Fig. 2, Table 2).

To further define the biological functions of the identified DEGs, IPA, KEGG pathways and enrichment analyses were performed. The results showed that DEGs are involved in oxidative stress, immune responses, metabolism, and transcription-related pathways, including sirtuin signaling pathway, oxidative phosphorylation, mitochondrial dysfunction, Toll pathway, JAK/Stat pathway, insulin/insulin-like signaling (IIS), Wnt signaling pathway, apoptosis, Notch pathway, etc. In addition, the DEG were also mapped on xenobiotic metabolism signaling and neuronal synapse pathways that are catalyzed by the cytochrome P450s and are responsible for removing toxic agents. The top three canonical pathways were sirtuin signaling pathway (inhibited, 12 molecules, z-score = -2.646, p-value = 1.3 E-08,), oxidative phosphorylation (activated, 8 molecules, z-score = 2.828, p-value = 4.75 E-08,) and mitochondrial dysfunction (altered, 8 molecules, z-score = N/A, p-value = 1.51E-06,) (Fig. 3, Table S3).

A network was produced by the highest scoring network with a total of 32 DEGs and a highly significant score of 77 (ATP 5F1D, ATP5 MG, ECSIT, GRPEL1, HPF1, Mitochondrial complex 1, MRPL11, MRPL40, NRPL44, MRPS18C, MRPS23, MRPS24, MRPS25, MRPS28, MRPS7, MRPF, NADH-dehydrogenase, NDUFA6, NDUFA68, NDUFA6, NDUFA10, NDUFB5, NDUFB7, NDUFS6, NFkB (complex), RTF1, TIMM21, TIMMDC1, TMEM223, TEME70, TRMT10C, UBXN1) (Fig. 4). The top three functions of this network involve cell signaling, post-translational modification, and protein synthesis.

Validation of transcript expression of selected genes confirmed RNA-seq results and identified diagnostic biomarkers and therapeutic targets

A subset of the genes from sirtuin signaling pathway, oxidative phosphorylation, mitochondrial dysfunction, protein synthesis,

Table 2
GO term enrichment of DEGs. Enriched GO terms of differentially expressed genes between healthy and diseased honey bees.

GO ID	Term	*D. melanogaster* (REF) Count	Count	Expected	Fold Enrichment	+/-	FDR
GO:0001677	formation of translation initiation ternary complex	175	7	0.95	7.34	+	8.50E-02
GO:0022618	ribonucleoprotein complex assembly	254	8	1.38	5.78	+	2.51E-02
GO:0071826	ribonucleoprotein complex subunit organization	257	8	1.4	5.71	+	1.94E-02
GO:0043933	protein-containing complex subunit organization	309	8	1.68	4.75	+	4.63E-02
GO:0016043	cellular component organization	1007	16	5.49	2.92	+	1.78E-02
GO:0071840	cellular component organization or biogenesis	1028	16	5.6	2.86	+	2.01E-02
GO:0006415	translational termination	175	7	0.95	7.34	+	4.25E-02
GO:0043624	cellular protein complex disassembly	181	7	0.99	7.1	+	2.61E-02
GO:0032984	protein-containing complex disassembly	189	7	1.03	6.8	+	2.26E-02
GO:0006414	translational elongation	175	7	0.95	7.34	+	2.83E-02

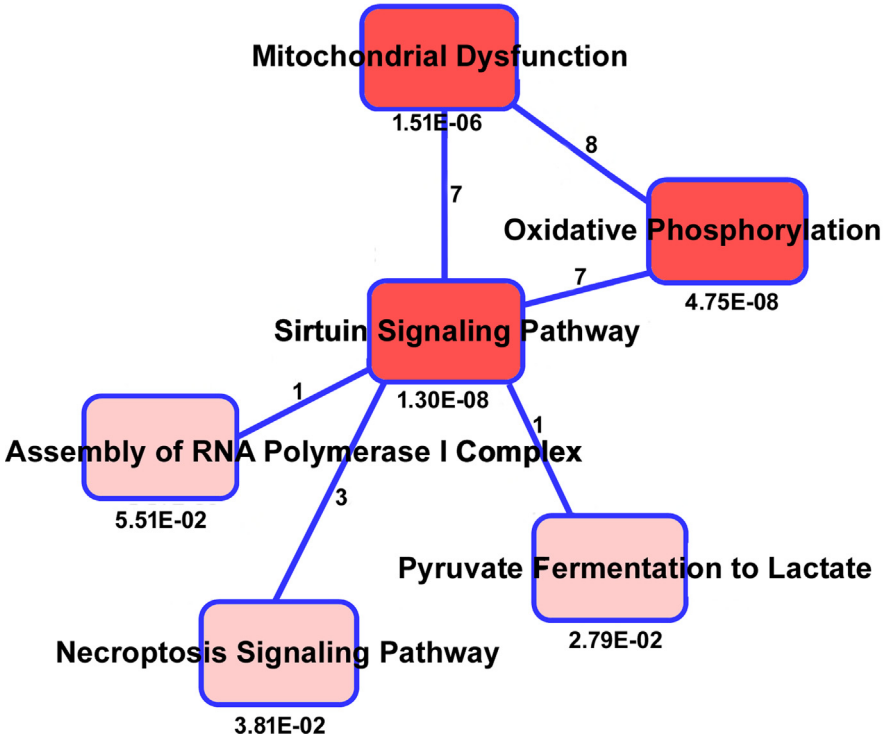

Fig. 3. Overlapping canonical pathways. Sirtuin signaling pathway is the top enriched signaling pathway and shares the large number of molecules with oxidative phosphorylation (seven) and mitochondrial dysfunction (seven), indicating shared biology across the three pathways. Sirtuin signaling pathway also share molecules with assembly of RNA polymerase I complex, necroptosis signaling pathway, and pyruvate fermentation to lactate. The network of overlapping canonical pathways shows each pathway as a single "node" colored proportionally to the right-tailed Fisher's Exact Test p-value, where brighter red = more significant. The p-value of each pathway is shown below the node. The number near the solid line connecting two pathways indicates the number of molecules shared by both pathways. The overlapping canonical pathways were generated through IPA (QIAGEN Inc., https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

immune response, and metabolism that were identified by RNA-seq analysis as differentially regulated was subjected to RT-qPCR validation using *β-actin* as a reference gene for normalization. High consistency between RNA-seq and qRT-PCR results was observed in genes tested.

The expression level of gene encoding SIRT1 protein, a key member of the sirtuin family and a regulator of proteins and genes involved in immune response and energy metabolism, was down-regulated in diseased honey bees collected in 2015, 2016, 2017, and 2018, compared to the healthy bees (2015, $P = 0.001$; 2016, $P = 0.0004$; 2017, $P < 0.0051$; 2015, $P = 0.0029$). Meanwhile, the expression of genes encoding LLDH-X2 and NDUFIS6m which are protein substrates of the sirtuin signal pathway and are involved in energy metabolism and mitochondrial function, was found to be significantly upregulated in diseased honey bees collected in 2015, 2016, 2017, and 2018, in comparison with the healthy bees examined in the same years (Unpaired student's *t*-test, LLDH-X2: 2015, P = 0.002; 2016, P < 0.001; 2017, P = 0.011; 2018, P = 0.002; NDUFIS6m: 2015, P = 0.0037; 2016, P = 0.006; 2017, P = 0.046; 2018, P = 0.006) (Fig. 5A, 5B, 5C).

The expression of genes encoding *Vitellogenin* (*Vg*) and major royal jelly proteins (*MRJP1, MRJP2*) which are produced in the hypopharyngeal gland and are an important protein resource for overwintering bees, was significantly downregulated in the diseased honey bees collected in 2015, 2016, 2017, and 2018, in comparison with the healthy bees gathered during the same yearly intervals (*Vg*: 2017, P = 0.019; 2018, P = 0.001, Fig. 5D) (*MRJP1:* 2016, P = 0.05; 2017, P = 0.002; and 2018, P = 0.005, Fig. 5E) and (*MRJP2:* 2016, P = 0.003; 2017, P < 0.001;2018, P = 0.005, Fig. 5F).

The significant upregulation of expression was also seen in genes encoding antimicrobial peptides *Abaecin* (Fig. 5G: 2015, P < 0.001; 2016, P < 0.001;2017, P = 0.008; 2018, P = 0.001) and *Apidaecin* (Fig. 5H: 2015, P = 0.002; 2016, P = 0.004; 2017, P = 0.004; 2018, P < 0.001) in diseased honey bees collected in 2015, 2016, 2017, and 2018, in comparison with the healthy bees examined in the same years. The expression pattern between diseased bees

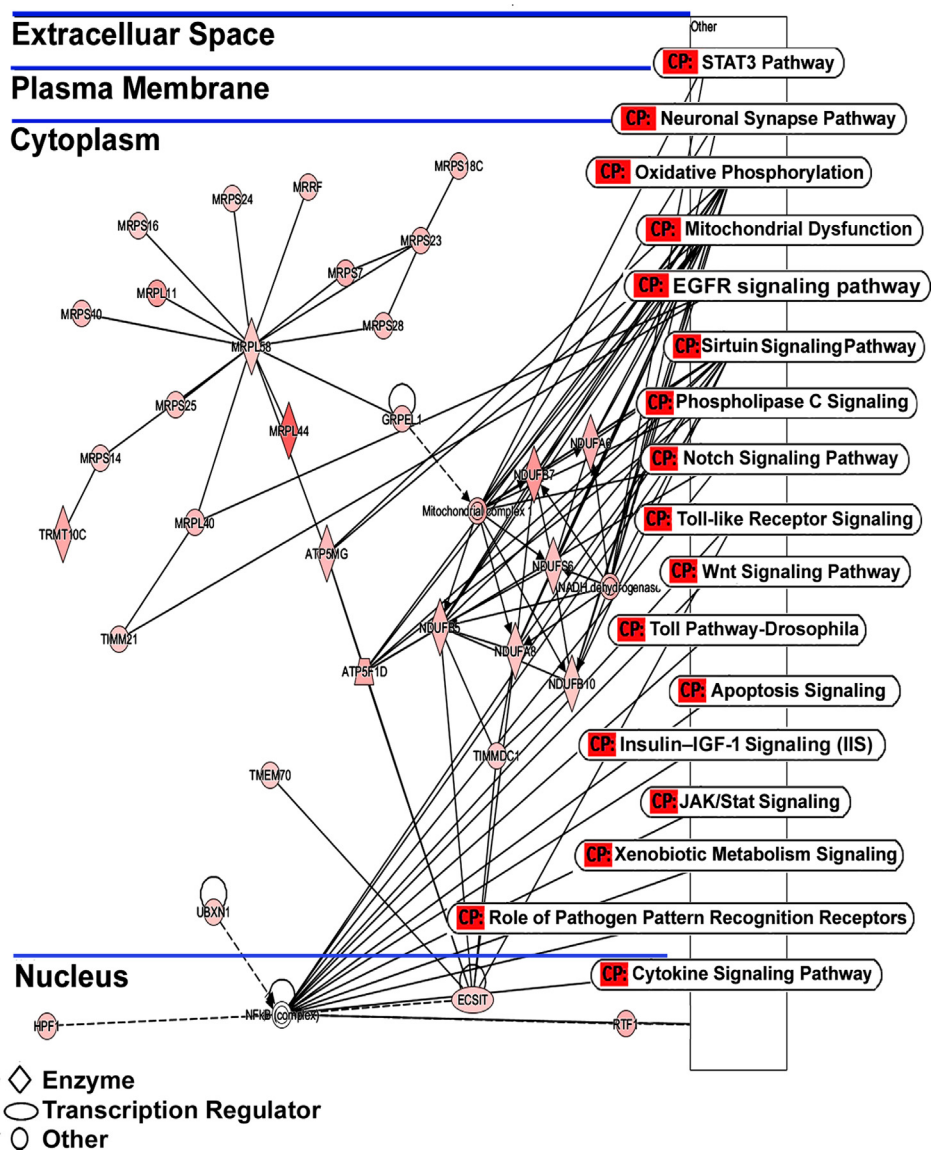

Fig. 4. The network depicting the connectivity of DEGs in the significant signaling pathways in diseased bees, as relative to healthy bees. This network was produced by the highest scoring network which had a total of 32 DEGs and a highly significant score of 77. Canonical pathways are shown in each of the boxes. Nodes characterize genes and solid and dashed lines represent direct and indirect relations between genes. The intensity of the node color indicates the degree of upregulation (red) of significant genes. Different shapes are indicated in the legend. Horizontal lines specify the most likely subcellular location for the protein encoded by each node. The networks were generated through IPA (QIAGEN Inc., https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and their fitter counterparts was very similar across four consecutive years.

Cold challenge triggered stress responses mediated by the sirtuin pathway

While our RNA-Seq differential expression analysis showed that sirtuin is the most significantly enriched pathway among the down-regulated differentially expressed genes (DEGs) in overwintering diseased bees that carried higher pathogen loads, it was unclear if perturbed sirtuin signaling was triggered by the combination of cold stress and pathogens or by cold temperature alone. Our pilot study showed that 28 °C cold challenge did not result in an immediately lethal effect but had a significantly negative impact on bees' survival compared to bees maintained in 34–35 °C in-hive optimum temperature. Our further laboratory bioassays for evaluating responses of honey bees to the cold challenge showed that transcript level of of SIRT1 in bees exposed to 28 °C was decreased on day-1 post-treatment, and continued to decrease gradually, reaching its lowest expression on day-15 post-treatment. Expression levels differed significantly from control bees that were maintained at 34 °C (Unpaired student's *t*-test, Day-1, $P < 0.0001$; Day-5, $P = 0.0011$; Day-10, $P = 0.0008$; Day-15, $P = 0.0007$) (Fig. 6A). Meanwhile, the transcript level of of NDUFIS6m and LLDH-X2 which encodes proteins involved in energy metabolism and are regulated by the sirtuin signal pathway in cold challenged bees was upregulated relative to control bees maintained at 34 °C. The greatest fold change in NDUFIS6m transcript level was seen on day-5 post-treatment where the gene expression in the cold stressed bees was 2.8- fold higher than in control bees ($P = 0.0062$). The difference in NDUFIS6m transcript level was not significant at other time points post-treatment (Fig. 6B). The expression of gene encoding LLDH-X2 was sevenfold higher in cold challenged bees than in bees kept at 34 °C on day-1

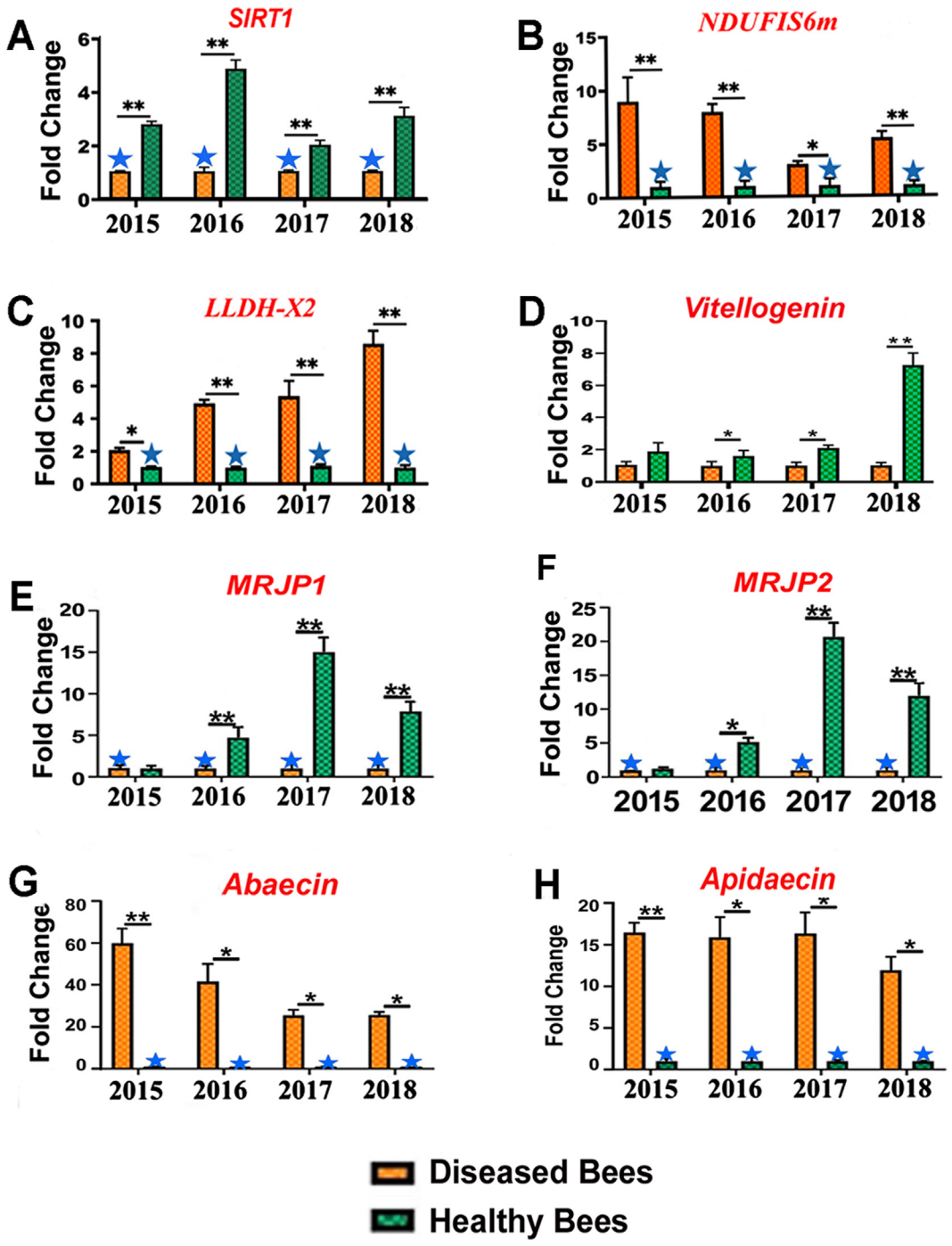

Fig. 5. Validation of RNAseq results by qRT-PCR. The fold change in the expression level of genes involving in sirtuin signaling pathway, metabolism, mitochondrial function, protein synthesis, immune response and metabolism between the diseased bees and the healthy bees collected in 2015, 2016, 2017, and 2018. (A) Sirt1, (B) NDUFIS6m, (C) LLDH-X2, (D) Vitellogenin, (E) MRJP1, (F) MRJP2, (G) *Abaecin*, and (H) *Apidaecin*. The Y-axis shows the relative expression which was expressed as an *n*-fold difference relative to the calibrator (marked by a star) by the $2^{-\Delta\Delta Ct}$ method. Meanwhile, the X-axis shows the diseased bees and healthy bees collected during the winter of 2015, 2016, 2017 and 2018. The asterisk (*) denotes a statistically significant difference between the groups (* $P \leq 0.05$, ** $P < 0.01$, unpaired student's *t*-test).

post-treatment (P = 0.0027). The difference in LLDH-X2 transcript level revealed a more than twofold reduction after day-1 post-treatment. However, the difference in the gene expression between the two groups was significant until day-10 post-treatment (Day 5, P = 0.0292; Day 10, P = 0.0087) (Fig. 6C). The expression level of gene encoding *Vg* in cold-stressed bees was also significantly downregulated on day-1 post-treatment (P = 0.0047) and reached its lowest expression by having a>10-fold decrease relative to the uninfected controls on day-5 post-treatment (P = 0.0093). While the fold-change of expression levels of the gene encoding *Vg* decreased substantially after day-5 post-treatment, cold-stressed bees had a significantly lower transcript level of *Vg* than bees kept at 34 °C (Day-10, P = 0.0213; and Day-15, P = 0.020) (Fig. 6D).

Cold challenge resulted in significantly shortened lifespans of adult bees, particularly those infected with Nosema

As shown in Fig. 7, survivorship analysis showed that cold stress not only exerted a direct negative impact on bee survivorship but also triggered accelerated mortality in pathogen-infected bees.

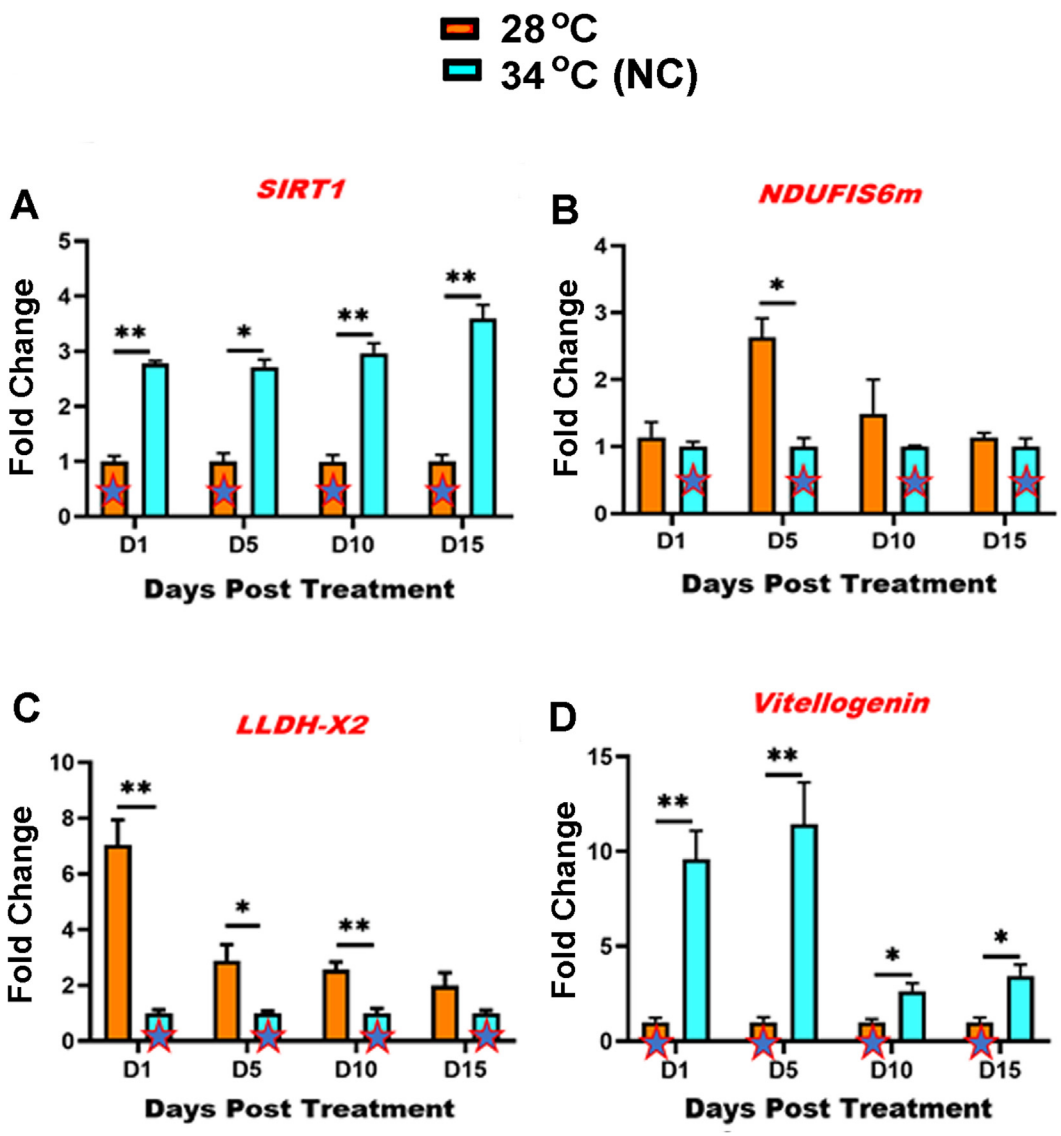

Fig. 6. The fold change in the expression level of genes that are involved in stress response and energy metabolism between cold-challenged bees (28 °C) and bees kept at an optimized temperature (34 °C). (A) SIRT1, (B) NDUFIS6m, (C) LLDH-X2, and (D) *Vitellogenin* (*Vg*). The Y-axis shows the relative expression, which was expressed as an *n*-fold difference relative to the calibrator (marked by a star) by the $2^{-\Delta\Delta Ct}$ method. Meanwhile, the X-axis shows different time points post-treatment. The asterisk (*) denotes a statistically significant difference between the groups (* $P \leq 0.05$, ** $P < 0.01$, unpaired student's *t*-test).

While the group of adult workers that were exposed to 28 °C reached 100 % mortality at day 20 post-treatment, the survival rate of the control group of bees that were maintained at an optimized temperature (34 °C) was 68.29 %, which was significantly greater than that of cold-challenged bees (Log-rank test: Chi-square = 104.5, P < 0.0001; Gehan-Breslow-Wilcoxon test: Chi-Square = 85.63, P < 0.0001).

Following experimental inoculation with intracellular microsporidia parasite *N. ceranae*, the survival rate of the group of *N. ceranae* infected bees maintained at 34 °C was 41.18 % by day 18. Meanwhile, the **mortality** rate of bees exposed to 28 °C cold challenge was 100 %, and the survival rates differed significantly between the two Nosema-infected groups that were exposed to different temperatures (28 °C *vs* 34 °C) (Log-rank test: Chi square = 159.1, P < 0.0001; Gehan-Breshlow-Wilcoxon test: Chi square = 137.2, P < 0.0001). Notably, *Nosema* infected bees exposed to the cold challenge (28 °C) displayed higher levels of mortality than bees kept at the same temperature without *N. ceranae* infection, demonstrating a collective impact of cold stress and pathogenic infection in colony survival and confirming that overwinter cold stress could profoundly alter the course of pathogen-host interactions. The Cox proportional hazards regression analysis to estimate the dependency of survival time on two variables, cold temperature and *Nosema* infection, indicated that bees under cold challenge had a higher risk than bees infected by *Nosema* (COX proportional hazards analysis, omnibus tests of model coefficients, Chi square = 243.114, *P* = 0.000. Temperature, *P* = 0.000, HR = 8.626 (95 % CI: 6.374–11.673), *N. ceranae*, *P* = 0.000, HR = 1.694, 95 % CI: 1.313–2.184).

SRT1720, an activator of SIRT1, could significantly extend the lifespan of honey bees exposed to cold challenges

To confirm whether the sirtuin signaling pathway is implicated in regulating survivorship of overwintering bees, we examined the effects of SRT1720, an activator of SIRT1, on the lifespan of cold stressed bees by comparing cumulative mortality in the negative control group (without treatment) and groups treated with differ-

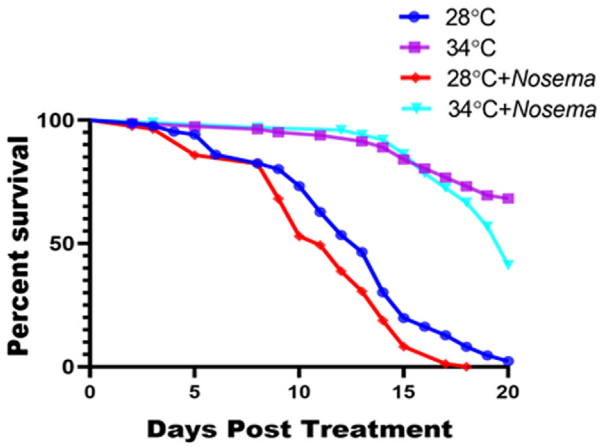

Fig. 7. Survivorship of honey bees in laboratory bioassays. Comparison of the survivorship curves among honey bees exposed to 1) cold challenge (28 °C), 2) maintained at a standard temperature (34 °C), 3) *Nosema ceranae* infected honey bees exposed to cold challenge (28 °C) and 4) *Nosema ceranae* bees maintained at a standard temperature (34 °C). The X-axis indicates the days post-treatment, while the Y-axis represents the survival rate (%) based on the daily accumulated mortality. Significant differences between the two different groups were analyzed using the Kaplan-Meier Gehan-Breslow-Wilcoxon method. Also, log-rank was employed to assess the overall homogeneity between the treatments. In all cases, $P \leq 0.05$ was considered to be significant.

ent concentrations of SRT1720 over a twenty-day observation. The results showed that, while the negative control group reached 100 % mortality at day 20 post-treatment, SRT1720 at concentrations of 10 μg/mL, 2 μg/mL, or 0.4 μg/mL increased the mean lifespan of cold-stressed honey bees at the same time point by 21.59 %, 34.83 %, and 31.87 %, respectively. The percent survival among the different groups was significantly different on day 20 after treatment (Log-rank test, Chi-Square = 46.35, P < 0.0001; Gehan–Breslow–Wilcoxon test, Chi-Square = 26.22, P < 0.0001) (Fig. 8). The maximal effect was observed at 2 μg/ml SRT1720 and therefore, this concentration was used for the subsequent SRT1720-mediated gene expression analysis.

SRT1720 could significantly elevate the transcript level of SIRT1 and Vg and downregulate transcript level of two downstream targets of SIRT1, LLDH-X2, and NDUFIS6m

Our evaluation of the health benefits of SRT1720 showed that SRT1720 treatment could significantly alleviate the reduction of transcript level of SIRT1 in bees exposed to the cold challenge. The SIRT1 transcript level increased approximately threefold one-day post-treatment in bees exposed to cold challenge, approximately 2.5-fold at five days, but decreased to the same levels as the untreated bees at later time points (unpaired *t*-test, negative control *vs* SRT 1720. Day 1: P = 0.002, t = 6.772, df = 4, F = 2.978; Day 5: P = 0.0295, t = 3.315, df = 4, F = 61.45) (Fig. 9A). Concurrently, the transcript levels of two downstream targets of SIRT1, NDUFIS6m, and LLDH-X2 were found to be downregulated progressively after SIRT1 activation and the expression reached their lowest levels on day 15 post-treatment. At every time point post-treatment, both NDUFIS6m and LLDH-X2 transcript levels were significantly lower in the group of cold-challenged bees treated with SRT1720 compared to the negative control group without treatment (unpaired *t*-test, *NDUFIS6m*. Day-1: P = 0.014; Day-5: P = 0.013, Day-10: P = 0.017; Day-15: P = 0.014) (*LLDH-X2*. Day-1: P = 0.020, Day-5: P = 0.003; Day-10: P = 0.046; Day 15: P = 0.015) (Fig. 9B, Fig. 9C). Meanwhile, the transcript level of *Vg* was upregulated progressively after SIRT1 activation as a result of SRT1720 treatment and reached its maximal level (more than

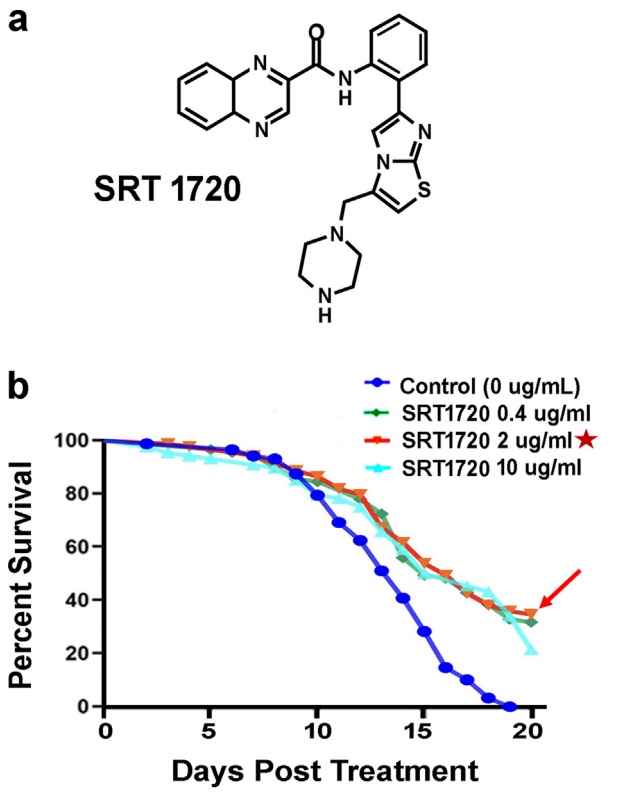

Fig. 8. The effect of SIRT1 activator, SRT1720, on the extension of the lifespan of adult honey bees exposed to cold challenge. (A) The chemical structure of SRT1720. (B). Survivorship curve of honey bees. The survival curves of the negative control challenged by cold and without treatment (0 μg/mL) and the groups challenged by cold and treated with 0.4 μg/mL, 2 μg/mL, or 10 μg/mL SRT1720 are shown. The X-axis indicates the days post SRT1720 treatment, while the Y-axis represents the survival rate (%) based on the daily accumulated mortality. Significant differences between the different groups were analyzed using the Kaplan–Meier Gehan–Breslow–Wilcoxon method. In addition, log-rank was employed to assess the overall homogeneity between the treatments. In all cases, P-value ≤ 0.05 was considered to be statistically significant.

eightfold increase) on day 15 post-treatment. At every time point post-treatment, the transcript level of *Vg* was significantly higher in cold-challenged bees treated with SRT1720 compared to the negative control group without treatment (Day-1: P = 0.144; Day-5: P < 0.001; Day-10: P < 0.001; Day 15: P < 0.001) (Fig. 9D).

Discussion

The identification of underlying mechanisms and pathways that are behind the increased overwinter bee mortality may offer the possibilities for the development of preventive measures and mitigation strategies for colony losses. In this paper, we present a long-term study of the biological and molecular characteristics of overwintering bees under natural field conditions and cold-stressed bees under laboratory conditions.

The ability of overwintering honey bee colonies to combat cold weather depends largely on the population size of summer bees prior to winter as well as the health status of the bee colonies [40–42 11,23,43,44]. Weak colonies with a small cluster of bees are unable to generate enough heat to maintain a stable temperature [45]. Temperature instability has a strong negative impact on many important biological processes including metabolism and innate immune function, thereby leading to increased vulnerability to disease infections and in turn overwintering mortality. In the present study, we revealed that weak colonies [11] with smaller winter bee populations had greater pathogen prevalence than strong colo-

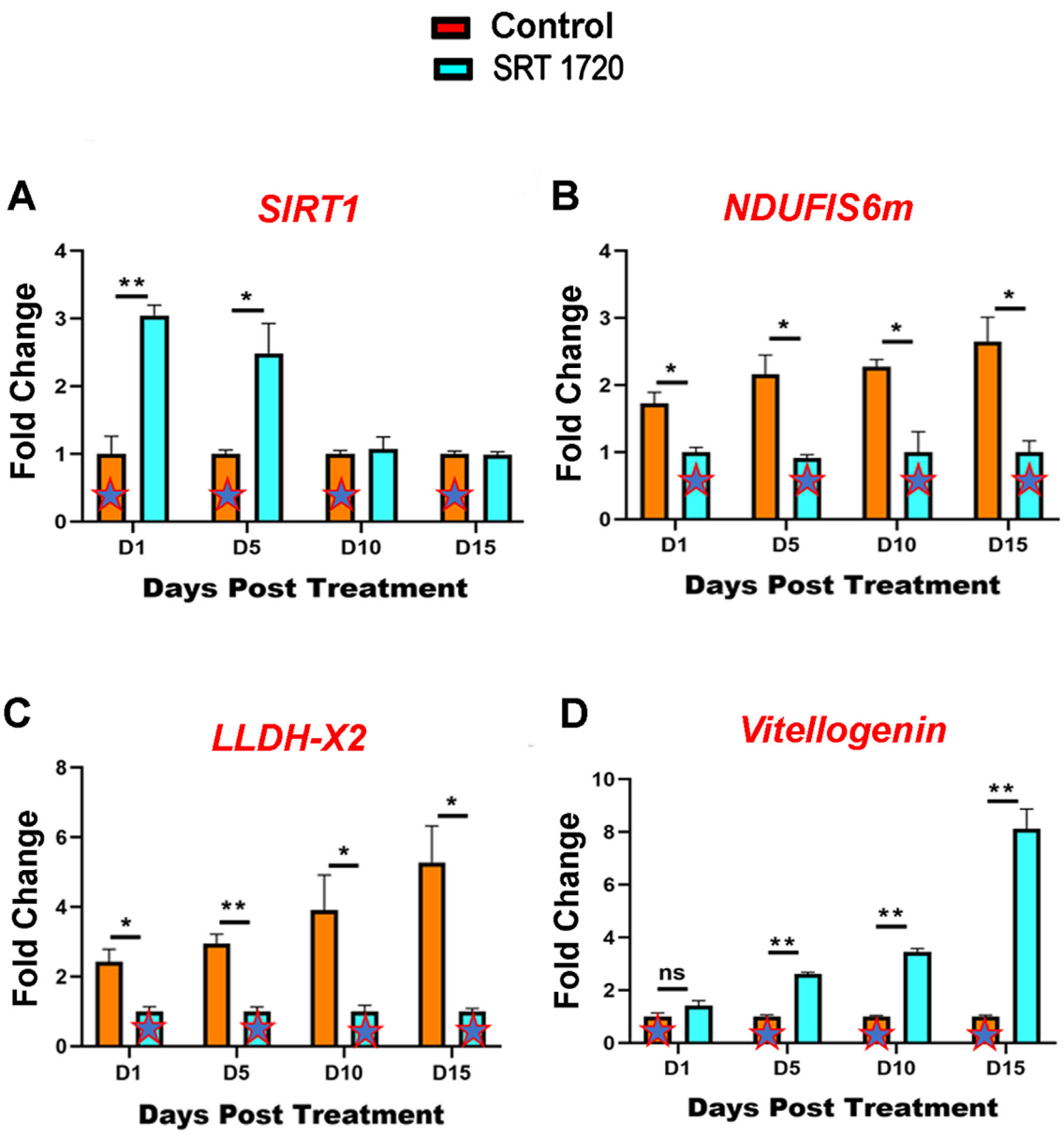

Fig. 9. The effects of SIRT1 activator, SRT1720, treatment on gene expression in honey bees exposed to cold challenge (28 °C). The fold change in the expression level of genes encoding (A) SIRT1, (*B*) NDUFIS6m, (C) LLDH-X2, and (*D*) Vitellogenin (*Vg*) (between cold-challenged bees treated with SRT1720 and the negative control of cold-challenged bees without treatment are shown. The Y-axis shows the relative expression, which was expressed as an *n*-fold difference relative to the calibrator (marked by a star) by the $2^{-\Delta\Delta Ct}$ method. Meanwhile, the X-axis shows the different time points post-treatment. The asterisk (*) denotes a statistically significant difference between the groups (* $P \leq 0.05$, ** $P < 0.01$, unpaired student's *t*-test).

nies and that weak colonies were rarely able to survive through the winter. This result displayed a notable correlation between colony strengths and pathogen prevalence in the winter and demonstrated that cold temperature could alter disease dynamics in infected bees and bring disease-infected bees to the brink of death. Our subsequent laboratory bioassays showed that cold stress not only exerted a direct negative impact on bee survivorship but also triggered accelerated mortality in pathogen-infected bees as shown that bees exposed to the cold challenge (28 °C) displayed higher levels of mortality than bees kept at the same temperature without *N. ceranae* infection. This result demonstrated a collective impact of multiple stressors and confirmed that overwinter cold stress could profoundly alter the course of pathogen-host interactions and affect colony survival[11,23,37,46,47].

While *V. destructor* is considered one of the most important causes of overwintering mortality, the present research indicates that a combination of low-temperature and pathogens, predominantly viruses that are circulated in overwintering bee populations, independent of *V. destructor*; could adversely affect bee health and lead to the collapse of overwintering honey bee colonies. Of the viruses attacking honey bees, DWV is the most widespread and was reported to have infected a minimum average of 55 % of colonies/apiaries across 32 countries [48]. Nevertheless, during our investigation for four consecutive years, DWV was not the most prevalent virus when our bee colonies were properly treated for *Varroa* mites. Instead, infection rates of *N. ceranae* and BQCV were detected at frequencies that exceeded DWV infection. The high level of *N. ceranae* and BQCV is likely due to prolonged winter confinement in the hive without defecating, which provides a favorable condition for the growth and spread of *Nosema* as well as BQCV whose transmission occurs mainly through the fecal-oral route[49]. A previous study also showed that the infection level

of *N. ceranae* and BQCV significantly increased in outdoor colonies during the winter [50]. These findings indicate that *N. ceranae* and BQCV are significant predictors of overwintering colony losses when the prevalence of DWV is low after acaricide treatment.

Sirtuins are stress-responsive proteins and possess the activities of either nicotinamide adenine dinucleotide (NAD +)-dependent deacetylases or mono-ADP-ribosyltransferases [51]. The chemical properties of sirtuins enable them to deacetylate a variety of substrates, thereby regulating diverse biological processes, including energy metabolism, oxidative stress, and survival pathways [52,53]. There are seven types of sirtuins in mammals (SIRT1-7) and different sirtuins occupy different subcellular compartments and are associated with different biological processes [54,55]. SIRT1, the nuclear form of sirtuins, is the best-studied member of the sirtuin family and plays a prominent role in regulating mitochondrial energy metabolism and lifespan extension under conditions of oxidative stress and nutrient availability[56,57,58–60]. The defects in the signaling pathways that are controlled by SIRT1 resulted in various oxidative stress-mediated pathological conditions[56,61]. The biological functions of sirtuins remain relatively unstudied in insects and there is no information available on the involvement of the sirtuin signaling pathway in the metabolic regulation of honey bees. Our RNA-Seq differential expression analysis identified that sirtuin signaling pathway (inhibited), oxidative phosphorylation (activated) and mitochondrial dysfunction (altered) are the top three most significantly enriched pathway among the differentially expressed genes (DEGs) in overwintering diseased bees from collapsing colonies. RT-PCR analyzes showed that SIRT1 was significantly downregulated in bees merely exposed to cold challenges under controlled laboratory conditions. Further, the downregulation of SIRT1 was found to be accompanied by an upregulation of two downstream targets of SIRT1, LLDH-X2 and NDUFIS6m, that are involved in oxidative-energy metabolism and mitochondrial dysfunction, respectively [62]. Mitochondria are not only the powerhouse for major cellular metabolic pathways, but they also possess a highly responsive stimuli-sensing apparatus to elicit an innate immune signaling cascade under diseased conditions. Given the multifunctional nature of mitochondria, it is perceivable that mitochondrial dysfunction could result in a vast array of health problems in overwintering bees, such as metabolic disorder, weakness, and subsequent pathology development. Our results clearly link the cold stress with suppressed transcript level of sirtuin and upregulated mitochondrial dysfunction. This study suggests that increased energy consumption of overwintering bees for maintaining hive temperature could reduce the allocation of energy toward immune functions, thus making the overwintering bees more susceptible to disease infections and leading to high winter colony losses. The identification of significant numbers of specific molecules that the sirtuin signaling pathway shares with mitochondrial dysfunction, oxidative phosphorylation, and other pathways suggests that the sirtuin signaling pathway is a convergent signaling hub that regulates metabolic and immunological processes of cold-stressed honey bees. Consequently, the downregulation of the sirtuin signaling pathway could lead to aberrant energy-generating pathways and immune responses that ultimately result in colony loss in the winter.

Over the past decade, there has been a growing interest in identifying and characterizing compounds that modulate sirtuin functions [63]. Resveratrol, a natural substance found in grapes and red wines, is the first sirtuin-activating compound (STAC) described and was found to activate SIRT1 activities by more than tenfold through binding to an allosteric site of SIRT1 to increase the enzyme's affinity for target substrates[58]. The administration of resveratrol and resveratrol derivatives was shown to activate SIRT1and improve SIRT1-dependent cellular processes, including metabolism and aging in both *in vitro* and *in vivo* studies[64–68]. SRT1720 is a specific synthetic activator of SIRT1 and its affinity with SIRT1 is approximately 1000 times as strong as that with resveratrol[69]. It has been reported that SRT1720 plays various beneficial roles in adult mice including extending life span, preserving bone and muscle mass, and improving the overall health through SIRT1 activation[70,71]. The novel regulatory role of the sirtuin signaling pathway underlying overwintering bee mortality identified in our study suggests a metabolism-based therapeutic strategy for mitigating colony losses. To that end, we tested potential modulators that could boost the activity of SIRT1 and improve honey bees' metabolism and life expectancy and showed that the transcript level of SIRT1 could be elevated upon treatment of cold stressed bees with SRT1720. Moreover, the activation of the expression of gene encoding SIRT1 was accompanied by downregulation of the expression of genes encodingLLDH-X2 and NDUFIS6m as well as a significant elevation of the expression of gene encoding *Vg*. Furthermore, the improvement of the aberrant expression of the gene encoding SIRT1 and its protein substrates resulted in increased robustness and lifespan extension of bees exposed to cold challenges, clearly indicating that SIRT1 is a potential novel target for alleviation of cold stress in honey bees.

The significant transcriptional hallmarks also found in our overwinter diseased bees were the downregulation of genes encoding the major royal jelly proteins (MRJPs), which are synthesized in and secreted by honey bee hypopharyngeal glands and are tightly linked to the overall fitness of honey bee colonies [72,73]. The family of MRJP genes share a common progenitor gene with yellow protein in *D. melanogaster,* which functions in cuticle melanization, and is therefore designated as a member of the yellow family of genes in *D. melanogaster* [74]. Previous studies reported that genes encoding yellow-like proteins were also discovered in *A. mellifera* and that yellow-MRJPs have also been isolated from the honey bee's brain and venom glands [74–76], underlining the potential multifunctionality of the proteins in honey bees. Earlier reports suggested that the transcript level of MRJP1-5 manifested primarily in the nurse bees but not among foragers [74,77–79]. In relation to this, the overwintering honey bees are characterized by halted foraging, cessation of brood rearing and increased transcript level of *Vg* which is the predominant protein supplement when pollen and nectar become scarce in the winter. *Vg* not only acts as a storage protein but also as a direct precursor for the synthesis of food jelly proteins [80] that are produced by the hive bees and used for feeding larvae and the queen. The decreased transcript level of MRJPs as well as *Vg* in diseased overwinter bees and cold-stressed bees indicate that honey bees' physiological functions were perturbed by the external stimuli and suggests that there are additional biological functions associated with MRJPs beyond their nutritional role as a component of royal jelly. To our knowledge, this is the first report linking the MRJPs to the honey bee host's responses to stimuli stemming from cold stress and pathogenic infection and it highlights their possible uses as molecular markers for measuring overwintering stress. Further studies are necessary to determine how cold challenges influence the transcript level of MRJPs and *Vg* and their involvement in bee disease pathogenesis among the aforementioned bees.

The results of this study provide the first evidence linking overwinter bee losses with altered sirtuin gene expression and mitochondrial dysfunction. The modulation of sirtuin activities that would subsequently regulate mitochondrial energy metabolism may be an effective strategy to forestall the occurrence of disease infections, thereby improving colony health and reducing bee mortality, both over winter and annually. The expression of the MRJPs gene family offers reliable new diagnostic biomarkers for monitoring the health status of overwintering honey bees.

Conclusion

Overwinter honey bee colony losses poses a significant threat to agriculture. We identified that sirtuin pathway that plays an important role in animals' physiology including mitochondrial metabolism and immune response is the most significantly enriched pathway in overwintering diseased bees. Further, we showed that the transcript level of SIRT1, a major sirtuin protein that regulates energy and immune metabolism, was significantly downregulated in bees merely exposed to cold challenges, linking cold stress with altered transcript level of sirtuin. Our study led to the identification of a novel therapeutic target for alleviation of cold stress in honey bees and useful biomarkers for measuring the stress levels of overwintering bees. The valuable information obtained from this study might open up a new frontier in relation to developing strategies for monitoring the health status and for mitigating colony losses, both over winter and annually.

Conflict of interests

The authors have declared no conflict of interest.

Ethics statement. No specific permits were required for the described studies. Observations were made in the USDA-ARS Bee Research Laboratory apiaries, Beltsville, Maryland, USA. Studies involved the European honey bee (*Apis mellifera*), which is neither an endangered nor protected species.

CRediT authorship contribution statement

Yi Zhang: Conceptualization, Methodology, Formal analysis, Writing – original draft, Funding acquisition. **Andrew Liu:** Methodology, Formal analysis, Writing – original draft. **Shao Kang Huang:** Methodology. **Jay D. Evans:** Resources, Writing – review & editing. **Steve C. Cook:** Resources. **Evan Palmer-Young:** Formal analysis. **Miguel Corona:** Resources, Writing – review & editing. **Mohamed Alburaki:** Resources. **Ge Liu:** Resources. **Ri Chou Han:** Resources. **Wen Feng Li:** Methodology. **Yue Hao:** Methodology. **Ji Lian Li:** Methodology. **Todd M. Gilligan:** Resources. **Allan H. Smith-Pardo:** Resources. **Olubukola Banmeke:** Resources. **Francisco J. Posada-Florez:** Resources. **Ya Hui Gao:** Formal analysis. **Gloria DeGrandi-Hoffman:** Methodology. **Hui Chun Xie:** Methodology. **Alex M. Sadzewicz:** Methodology. **Michele Hamilton:** Methodology. **Yan Ping Chen:** Supervision, Conceptualization, Formal analysis, Writing – original draft, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jare.2022.12.011.

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