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Original article

Synergistic effects of imidacloprid and high temperature on honey bee colonies

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Abstract – With the rising occurrence of sudden heat waves, honey bees are at risk of exposure to unprecedented heat stress. We investigated the synergistic effects of imidacloprid (IMD) and high temperature on honey bees. Mini hives were treated with IMD (20 ppb for 14 days) and high temperature (41 °C for 6 h) either singly or in combination. Heat shock protein 70 and 90 genes were upregulated in bees exposed to the combined treatment compared to those exposed to each single treatment. Transcriptome analysis revealed that metabolic pathways remained intact in the high temperature treatment, whereas several metabolic pathways were altered by either the IMD treatment (downregulation of cellular respiration pathways) or the combined treatment (upregulation of protein synthesis and signaling pathways). These findings suggest that IMD and high temperature have negative synergistic effects on honey bees.

Honey bee / Imidacloprid / Heat wave / Heat shock response / Thermoregulation

1. INTRODUCTION

Honey bees, as individuals, are highly tolerant to heat stress. During flight, the temperature of the honey bee thorax rises up to 49 °C (Stabentheiner et al. 2002), and honey bees can survive for 24 h at 45 °C, far beyond the typical temperature level that other species can endure (Abou-Shaara et al. 2012; Ranga et al. 2017). Although the preferred temperature for individual honey bees is 28 °C (Schmolz et al. 2002; McAfee et al. 2020), the honey bee colony thermoregulates efficiently via active heat production, evaporation, or ventilation and maintains the in-hive temperature at 32–36 °C for proper development of the brood (Jones and Oldroyd 2006). Failure of thermoregulation during the brood and pupal stages of honey bee colonies is known to negatively affect susceptibility to pesticides (Medrzycki et al. 2010), behavioral performance (Tautz et al. 2003), synaptic organization of the brain (Groh et al. 2004), and memory of adult bees (Jones et al. 2005).

A heat wave can be defined as a period of more than five consecutive days with daily maximum temperatures being 5 °C higher than average temperatures (Frich et al. 2002). As a result of climate change, heat waves occur more frequently in general and are predicted to occur up to four times more frequently by the late twenty-first century (Lhotka et al. 2018; Marx et al. 2021). Heat waves are known to increase the mortality of humans and birds (McKechnie

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and Wolf 2010; Mitchell et al. 2016). It was previously reported that honey bees, through recruitment of backup foraging forces, can adapt to a simulated heat wave of 37 °C without cost (Bordier et al. 2017a, b). However, this does not necessarily mean that honey bees maintain normal physiologies under high temperatures. At the individual level, heat stress damages the fertility of queen bees and the digestive tracts of worker bees (McAfee et al. 2020; Bach et al. 2021). Additionally, frequent heat waves in conjunction with other stress factors might result in severe disorders in honey bees.

Imidacloprid (IMD) is a representative neonicotinoid insecticide that has been reported to be potentially responsible for colony collapse disorder (CCD) (Lu et al. 2014). It is known to negatively affect honey bee foraging behavior, development (Tome et al. 2020), motility (Delkash-Roudsari et al. 2020), and learning and memory (Decourtye et al. 2004). In addition, IMD exposure alters the metabolic rate of honey bees (Nicodemo et al. 2014; Cook 2019; Gooley and Gooley 2020) and affects thermoregulation (Meikle et al. 2018).

Since honey bees are usually exposed to multiple stressors concurrently in the natural agroecosystem (Barron 2015; Hristov et al. 2020), it is crucial to understand the interactions between different stressors and their combined effects. It has been reported that the combinations of two different stress factors can induce synergistic negative effects on honey bees: *Nosema* and virus (Zheng et al. 2015), *Nosema* and pesticide (Retschnig et al. 2014), two pesticides (Wang et al. 2020), and pesticide and nutritional stress (Tosi et al. 2017). IMD is known to disrupt respiratory metabolism and thermoregulation of honey bees (Tosi et al. 2016; Cook 2019; Gooley and Gooley 2020; Vergara-Amado et al. 2020). Thus, if honey bees are exposed to IMD and heat stress simultaneously, the resulting negative impacts would not be additive but rather synergistic. With a wide use for seed coating, IMD residue has been detected in nectar and pollen with average concentration of 0.9–41.9 ng/g (Stoner and Eitzer 2012; Jiang et al. 2018; Tong et al. 2018; Tosi et al. 2018; Wen et al. 2021). Considering the increased occurrence of heat waves in recent years, such a double exposure scenario is highly likely. If an IMD-exposed honey bee colony is under a sudden heat wave, the resulting failure of in-hive temperature stabilization due to deteriorated thermoregulation would affect honey bee physiology, thereby inducing a negative feedback loop between metabolic changes and in-hive temperature control.

In this study, the synergistic effects of IMD exposure and heat shock on honey bees were investigated at the colony level. To determine a colony-level response to the stressors, whole mini hives containing a queen and brood were used for the experiment. Following treatment with IMD and high temperature, singly or in combination, fluctuations in relative humidity (RH) and temperature inside hives were measured to determine their individual or combined adverse effects on thermoregulation. Transcription profiles of heat shock protein 70 and 90 genes (*Hsp70* and *Hsp90*) and the vitellogenin gene (*Vg*), which are considered general stress markers for honey bees (Even et al. 2012; Kim et al. 2019), were analyzed by real-time quantitative PCR (qPCR), and any changes in metabolic pathways were investigated by whole transcriptome analysis.

2. MATERIAL AND METHODS

2.1. IMD and high-temperature treatment of honey bees

Information on reagents used in this study is listed Supplementary Table 1. Analytical standard grade IMD was purchased from Merck (Darmstadt, Germany), diluted with acetone (≥ 99.5% purity, Merck) to 20 mg/kg and stored at −20 °C until use. The storage period did not exceed 2 months. Italian hybrid honey bee colonies whose queens were all siblings were purchased from a neighboring apiary. They were kept on a rooftop apiary at the Gwanak campus of Seoul National University (Seoul, Korea, 37° 27′ 46.8″ N, 126° 57′ 06.9″ E). The experiments were performed in July to August, 2020,

the summer season in Korea. Three independent experiments were performed, each of which had a single colony per treatment. For each experiment, honey bee samples were collected from four sets of treatment groups: untreated control (CC), high temperature-treated (CH), IMD-treated (IC), and both IMD and high temperature-treated (IH) groups. The procedure for the experiment is illustrated in Figure 1.

First experiment. Four mini hives ($200 \times 265 \times 135$ mm^3) were prepared in the apiary. Five frames (one full of eggs and larvae, one full of pollen, and three empty cell-built frames), approximately 2000 nurse bees, and an egg-laying queen were placed into each hive. IMD-acetone stock solution (20 mg/kg) was 1000-fold diluted with 50% sucrose solution (w/v) to the final concentration of 20 µg/kg IMD with 0.1% acetone. For the control, only acetone was added to the 50% sucrose solution to obtain the final 0.1% acetone concentration. Fifty milliliters of 50% sucrose solution containing 20 µg/kg IMD, which was an average detection concentration for pollen of winter oilseed rape in the field (Wen et al. 2021), and 10 g of commercial pollen bread (composed of 20% rapeseed pollen, 20% yeast, 20% soybean flour, and 40% white sugar, Bullo Apiary, Guri-si, Korea) were provided to each of two hives, and the same amount of sucrose solution without IMD and pollen were provided to each of another two hives every day until the end of experiment. Control and IMD-treated hives were located more than 10 m away from each other with walls between them, and food was supplied just before sunset to prevent robbing from other bees. After 7 days, 1000 newly emerged bees from other regular hives were marked on thoraces with paint (Uni posca, Mitsubishi pencil, Japan) and introduced to each hive; thus, the total number of worker bees/hive became approximately 3000. After 14 days, the entrances of all four hives were blocked by plastic screen slides with holes to prevent bees from escaping from the hives during the following heat stress period. Every thermal treatment started at 9 a.m. A set of one control hive and one IMD-treated hive was placed into a growth chamber, while the other set of hives was placed into another growth chamber. One growth chamber was set for normal temperature conditions at 25 °C for 30 min and then at 30 °C for 7 h under 60% RH and light conditions (1_CC and

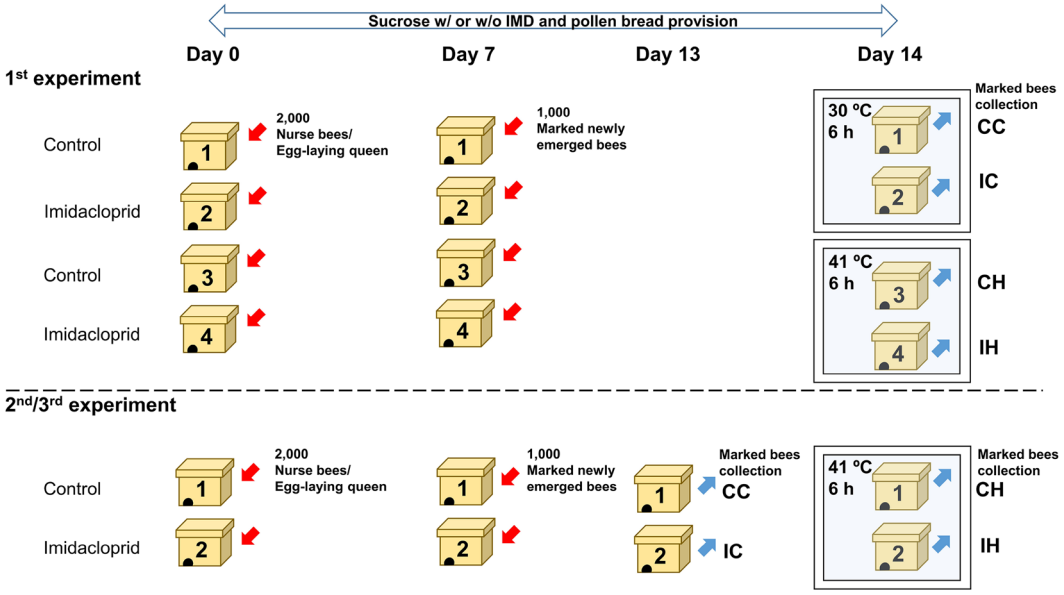

Figure 1. Illustration of the imidacloprid/high temperature exposure experiment

1_IC). The temperature 30 °C was considered a typical sunny day temperature in the summer of Seoul, Korea. For the high temperature treatment, the other growth chamber was set at 25 °C for 30 min, 30 °C for 30 min, 35 °C for 30 min, and then 41 °C for 6 h under 60% RH and light conditions (1_CH and 1_IH). During incubation, temperature and humidity were constantly recorded using in-hive sensors (BroodMinder, Stoughton, Wisconsin, USA) attached to the brood frames in each hive. After incubation, bees were immediately flash-frozen with liquid nitrogen, and marked bees were sorted.

Second experiment. A set of one control mini hive and one IMD-treated mini hive was prepared outside the apiary following the 1st experimental procedure. After 13 days, 100 marked bees were collected from each hive and flash-frozen (2_CC and 2_IC). After 14 days, the two mini hives were heat-treated following the protocol from the 1st experiment, and honey bees from the respective hives were catalogued as 2_CH and 2_IH.

Third experiment. This was a replicate of the 2nd experiment.

2.2. Quantitative real-time PCR

Total RNA was extracted from pooling of eight honey bee abdomens (three replicates) from every experimental condition of each colony (total $n = 36$, pooling of eight bees × 3 replicates from a colony × 4 experimental conditions × 3 independent experiments) with TRI reagent (MRC, Cincinnati, OH, USA) following the instructions of the manufacturer. After DNase I treatment (TAKARA Korea Biomedical Inc., Seoul, Korea), cDNA was synthesized using Superscript IV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and oligo$(dT)_{20}$ primer (Macrogen, Seoul, Korea) at 55 °C following the instruction manual. The synthesized cDNAs were used as templates for qPCR.

qPCR was conducted using SYBR Premix Ex Taq (TAKARA Korea Biomedical Inc) in 40 cycles of the following thermal program: 30 s at 95 °C, 15 s at 56 °C and 30 s at 72 °C. cDNA (12.5 ng) was added to a 10 µl total reaction volume. The ADP-ribosylation factor 1 (*arf1*) and Ras-related protein Rab-1A (*rab1a*) genes were used as reference genes for normalization of target gene expression in pesticide-treated honey bees (Kim et al. 2022a). Transcription levels of *Hsp70*, *Hsp90*, and the *Vg* were measured to evaluate stress levels of each experimental group following treatment (Kim et al. 2019). Information on the primer sets is listed in Supplementary Table 2.

2.3. Transcriptome analysis

For transcriptome analysis, the total RNA from honey bee abdomen samples from the 1st experiment was used. Three biological replicates (pooling of eight bees per replicate) from each of the 1_CC, 1_CH, 1_IC, and 1_IH experimental groups (total 12 samples) were sequenced. The RNA quality and quantity of each sample were analyzed using a Bioanalyzer (Agilent Technologies, USA). TruSeq mRNA library kits (Illumina, Inc, San Diego, CA, USA) were used to construct RNA-Seq libraries with insert sizes of 300 bp from 2 µg of total RNA for each experimental group. Pooled libraries were sequenced using the Illumina HiSeq X platform with paired-end (PE) reads of 151 bp. Low-quality and duplicated reads and adapter sequences were removed using Trimmomatic ver. 0.39 (Bolger et al. 2014) with default parameters. Sequences contaminated by bacteria, viruses and humans were removed using BBDuk (ver. 38.87) with default parameters. For the expression profiling of genes, trimmed RNA-Seq reads were mapped to the *A. mellifera* (DH4) genome sequences (v3.1, enBank acc. GCF_003254395.2) using HISAT2 ver. 2.1.0 (Kim et al. 2015), and then RNA reads mapped to protein coding sequences were counted using HTSeq-count ver. 0.11.2 (Anders et al. 2015). The bioconductor package DESeq2 (Love et al. 2014) was used to identify differentially expressed genes (DEGs). Genes with p values of < 0.05 and $|\text{Log2(fold change)}| \geq 1$ were considered to be DEGs.

Up- or downregulated sets of DEGs were uploaded as a query into g:GOSt in g:Profiler (https://biit.cs.ut.ee/gprofiler/gost) for functional

annotation (Raudvere et al. 2019). Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways with a false discovery rate (FDR) < 0.05 were considered to be significant.

2.4. Statistical analyses

To compare multiple sample data, one-way ANOVA was performed with Tukey's multiple comparison test as a post hoc analysis. All statistical analyses and graph designs were performed using Prism 6.0 (GraphPad, San Diego, USA).

3. RESULTS

3.1. Transcription profiles of Hsps and Vg

As representative markers for the stress response of honey bees, the transcription levels of *Hsp70*, *Hsp90*, and *Vg* were determined. The overall transcription profiles of *Hsp70* and *Hsp90* were almost identical in all experiments (Figure 2). Transcription levels significantly increased in the CH group ($p = 0.012$, 0.0001, and 0.008 for *Hsp70* in the 1st, 2nd, and 3rd experiments, respectively, and $p = 0.0001$ and 0.006 for *Hsp90* in the 2nd and 3rd experiments), whereas no difference was observed in the IC group compared to the CC group. The IH group showed significantly higher transcription levels of *Hsp70* and *Hsp90* than the CC group in all experiments. The transcription levels of *Hsp70* and *Hsp90* in the IH group were significantly higher than those in the CH group ($p = 0.010$, 0.036, and 0.0001 for *Hsp70* and $p = 0.019$, 0.044, and 0.004 for *Hsp90* in the 1st, 2nd, and 3rd experiments, respectively) and the IC group ($p = 0.0003$, < 0.0001, and < 0.0001 for *Hsp70* and $p = 0.0002$, < 0.0001, and < 0.0001 for *Hsp90* in the 1st, 2nd, and 3rd experiments,

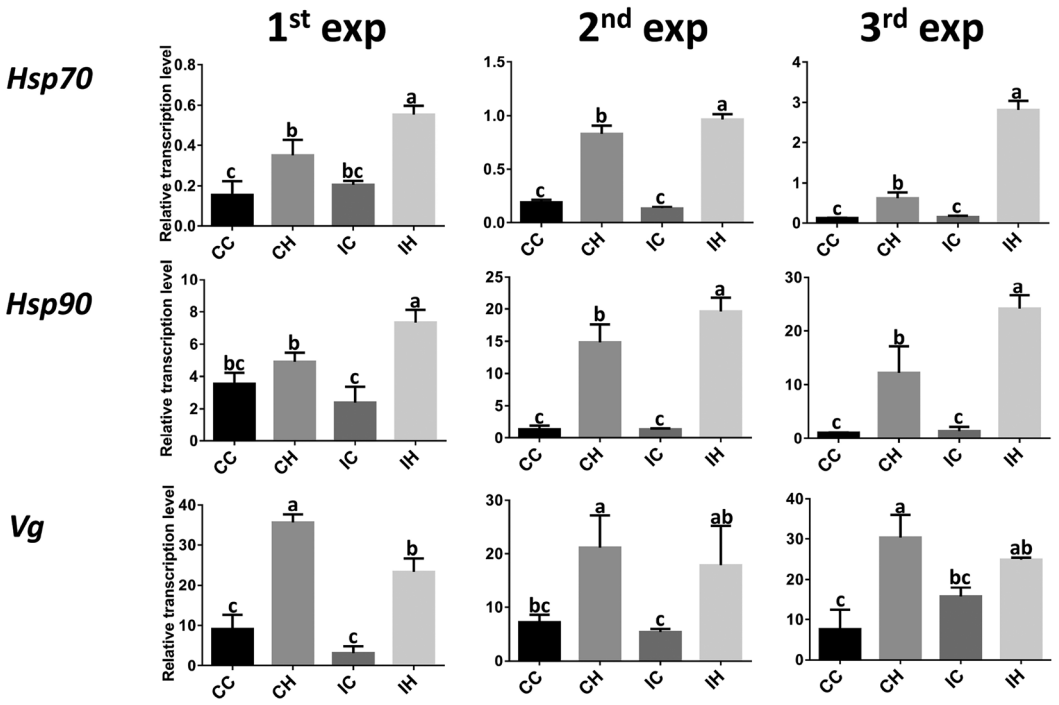

Figure 2. The relative expression levels of high-temperature protein and vitellogenin genes in honey bee abdomen. Three biological replications were used for qPCR analysis for every independent experiment

respectively) in all three experiments, suggesting synergistic effects of IMD and high temperature. The transcription levels of *Vg* significantly increased following heat treatment ($p = 0.0001$, 0.030, and 0.0004 in the 1st, 2nd, and 3rd experiments, respectively) but were not significantly altered by IMD treatment. The simultaneous treatments of IMD and high temperature upregulated *Vg* transcription levels compared to those of the CC group in the 1st and 3rd experiments ($p = 0.001$ and 0.003) and those of the IC group in the 1st and 2nd experiments ($p < 0.0001$ and $= 0.049$), but the levels were statistically indistinguishable from those of the CH group except in the 1st experiment ($p = 0.003$).

3.2. DEGs following IMD and high-temperature treatment

Venn diagrams were drawn with DEGs between the CC group and the CH/IC/IH groups (Figure 3A and B). High-temperature treatment resulted in the least upregulation of genes (141 genes in CH), followed by IMD treatment (872 genes in IC) and the combined treatment of high temperature and IMD (1609 genes in IH, Figure 3A). The IH group shared large fractions [84% (119 in 141 genes) and 87% (761 in 872 genes)] of upregulated DEGs with the CH and IC groups, respectively (Figure 3A). Following the IMD/high temperature combined treatment, 783 genes were uniquely upregulated, accounting for 45% of the total upregulated DEGs. The pattern of downregulated genes was almost identical to that of the upregulated genes (Figure 3B). A total of 163, 825, and 1502 genes were downregulated in CH, IC, and IH, respectively. The IH group shared a total of 75% (123 in 163 genes) and 73% (606 in 825 genes) of downregulated DEGs in the CH and IC groups, respectively. Double treatment with IMD and high temperature led to the downregulation of 814 genes that were not identified in either the IMD- or high temperature-treated group.

The ten most up- or downregulated genes following the treatments in each group are listed in Table I. Upregulation of several *Hsp*s was observed in the high temperature-treated groups: 10 kDa heat shock protein (*Hsp10*), mitochondrial, protein lethal (2) essential for life (*Hsp20*), heat shock protein 60A (*Hsp60*), and endoplasmin (*Hsp90B1*) in the CH group and *Hsp60* and heat shock 70 kDa protein 4 in the IH group. *Vg* was upregulated in the CH group (3.0-fold), whereas it was downregulated in the IC group (7.1-fold). Both the IC and IH groups shared four commonly upregulated genes: cytoplasmic probable aspartate aminotransferase, mitochondrial 2-oxoglutarate dehydrogenase, membrane-associated progesterone receptor component 1, and mitochondrial pyruvate carboxylase. Four genes related to cellular respiration, NADH dehydrogenase 1, 4, and 5 and cytochrome c oxidase subunit III, were commonly downregulated in both the IC and IH groups.

3.3. Functional annotation of DEGs

Enrichment analysis of GO and KEGG pathways was performed with DEGs obtained from the CC groups vs. the CH, IC, and IH groups (Table II). Only two upregulated pathways were identified following high-temperature treatment, whereas 10 and 99 metabolic pathways were upregulated following IMD treatment and the combined treatment of IMD and high temperature, respectively (Figure 3C). Among the two upregulated pathways in the CH group, the "protein processing in endoplasmic reticulum" pathway was commonly upregulated in IH (Table II), suggesting that its upregulation is likely attributed to high temperature. Eight upregulated pathways (e.g., "ribosome" and "thermogenesis") were commonly annotated in the IC and IH groups (Table II), which suggests that those pathways may be induced by IMD treatment. Contrary to the increased number of upregulated pathways in the IH group, the number of downregulated pathways in the IH group was much lower than that observed in the IC group (4 vs. 33, Figure 3D). There was no commonly downregulated pathway between any of the groups.

All the downregulated GO terms or pathways in the IC group were related to cellular respiration. The upregulated GO terms or pathways

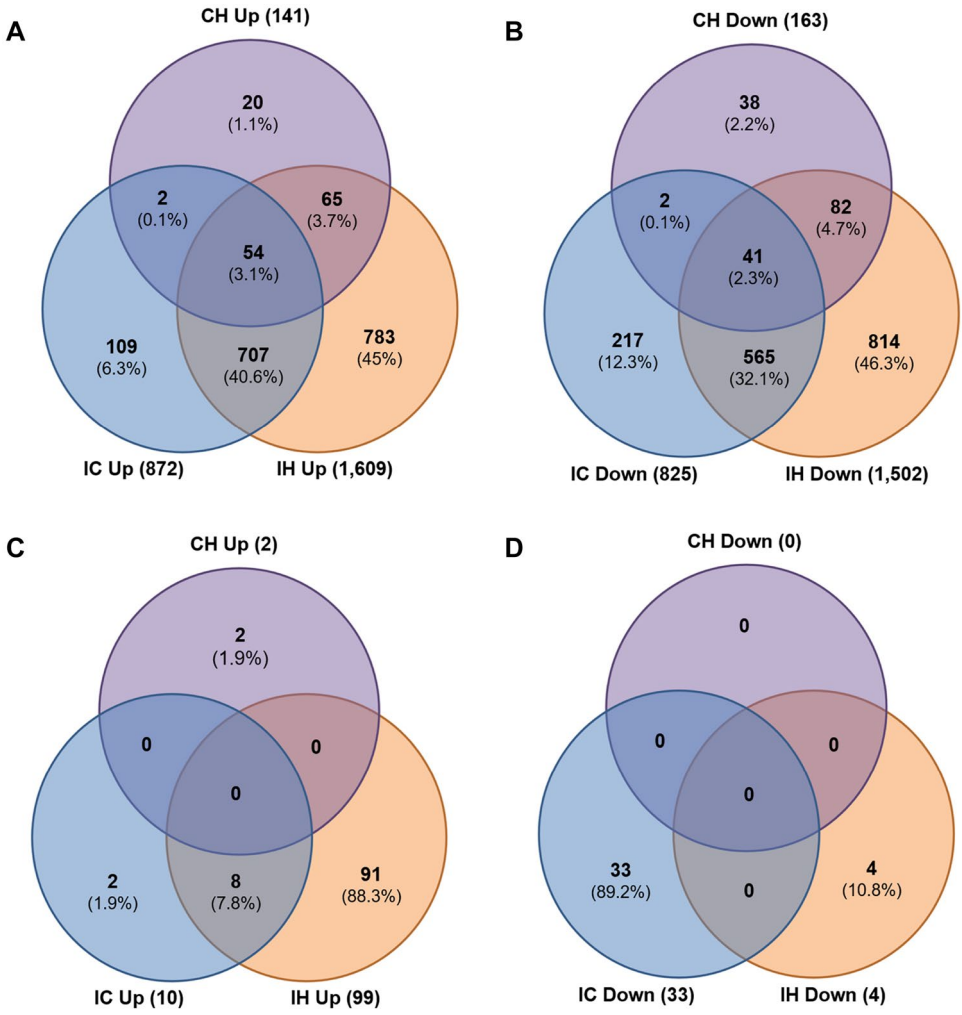

Figure 3. Venn diagrams of differentially expressed genes (DEGs) from honey bee abdomen transcriptome analysis and subsequent significant pathways from GO and KEGG enrichment analysis. Upregulated (**A**) or downregulated (**B**) genes of each group were obtained by comparison with the control group. Up- (**C**) or downregulated pathways (**D**) with FDR < 0.05 were considered to be significant. The values in parentheses indicate the proportion of the DEGs or pathways to the total number of DEGs or pathways from the three groups

in the IH group were mainly related to protein synthesis and modification, olfactory sensing, and overall cell signaling along with the thermogenesis pathway.

To investigate any synergistic effect of IMD under high-temperature conditions, KEGG pathway enrichment analysis was performed with DEGs from the comparisons of CH vs. IH and IC vs. IH. A total of 52 KEGG pathways were upregulated, and one pathway was downregulated in the CH vs. IH comparison. In contrast, only three KEGG pathways were upregulated in the IC vs. IH comparison (Supplementary Table 3). The top 20 upregulated pathways are presented in Figure 4. Several pathways upregulated in the CC vs. IH comparison (e.g., insulin signaling pathway, axon guidance, Hippo signaling pathway, Rap1 signaling pathway, and regulation of actin cytoskeleton) were also observed to be significantly upregulated in the CH vs. IH group comparison.

Table I List of differentially expressed genes (DEGs) in the control/heat-shocked (CH), imidacloprid/control (IC), and imidacloprid/high temperature (IH) groups compared to the control/control (CC) group. Among significant DEGs ($p_{adj} < 0.05$) whose base means (an average of DEG transcript levels from the four groups) of transcription levels were above the averages of total transcripts, 10 DEGs with the highest fold changes are displayed from up- and downregulated DEGs of each group

Group	Upregulated			Downregulated		
	Gene ID	Description	Log_2FC	Gene ID	Description	Log_2FC
CH	724488	Protein lethal(2)essential for life	2.43	409261	Glycerol kinase	−2.51
	102655100	Protein Cep78 homolog	2.20	552303	RNA-binding protein cabeza	−1.45
	412526	Protein disulfide-isomerase A6 homolog	1.90	413697	Uncharacterized protein	−1.38
	724367	Protein lethal(2)essential for life	1.87	410614	Tubulin alpha chain	−1.25
	725967	D-3-phosphoglycerate dehydrogenase	1.78	410852	Protein Fe65 homolog	−1.20
	406088	Vitellogenin precursor	1.69	726418	Flavin-containing monooxygenase FMO GS-OX-like 4	−1.12
	552531	10 kDa heat shock protein, mitochondrial	1.57	72165	Aquaporin AQPAe.a	−1.09
	409384	Heat shock protein 60A	1.53	410567	Zinc transporter ZIP11	−1.05
	412150	Endoplasmin	1.43	100576271	Uncharacterized protein	−1.04
	412287	FK506-binding protein 59	1.29	409789	Uncharacterized peptidase C1-like protein F26E4.3	−1.03
IC	724239	Kynurenine/alpha-aminoadipate aminotransferase, mitochondrial	2.58	102654766	Leucine-rich repeat-containing protein 40-like	−3.36
	725967	D-3-phosphoglycerate dehydrogenase	2.34	406088	Vitellogenin precursor	−2.83
	727622	Probable aspartate aminotransferase, cytoplasmic	1.89	807698	NADH dehydrogenase subunit 5	−2.65
	408286	2-oxoglutarate dehydrogenase, mitochondrial	1.71	409801	Esterase E4	−2.62
	413164	Membrane-associated progesterone receptor component 1	1.52	807690	Cytochrome c oxidase subunit III	−2.58
	408733	Protein pinocchio	1.37	807692	NADH dehydrogenase subunit 1	−2.51
	412876	Pyruvate carboxylase, mitochondrial	1.36	409261	Glycerol kinase	−2.41
	409366	Integral membrane protein 2C	1.24	807702	NADH dehydrogenase subunit 4	−2.41
	410087	Uncharacterized protein	1.19	807696	ATP synthase F0 subunit 6	−2.23
	100578660	Eukaryotic translation initiation factor 2 subunit 2	1.18	724187	Leucine-rich repeat neuronal protein 3	−1.87

Table I (continued)

Group	Upregulated			Downregulated		
	Gene ID	Description	Log_2FC	Gene ID	Description	Log_2FC
IH	412876	Pyruvate carboxylase, mitochondrial	2.32	807692	NADH dehydrogenase subunit 1	−2.53
	727622	Probable aspartate aminotransferase, cytoplasmic	2.19	409261	Glycerol kinase	−2.50
	408286	2-oxoglutarate dehydrogenase, mitochondrial	2.18	552303	RNA-binding protein cabeza	−2.40
	552259	Staphylococcal nuclease domain-containing protein 1	2.15	107963970	Uncharacterized protein	−2.19
	409384	Heat shock protein 60A	1.97	807702	NADH dehydrogenase subunit 4	−1.94
	413164	Membrane-associated progesterone receptor component 1	1.96	409849	Fasciclin-2	−1.90
	724293	Protein yellow	1.90	726418	Flavin-containing monooxygenase FMO GS-OX-like 4	−1.90
	408706	Heat shock 70 kDa protein 4	1.86	807698	NADH dehydrogenase subunit 5	−1.89
	412111	Vigilin	1.81	413574	26S proteasome non-ATPase regulatory subunit 2	−1.89
	102655100	Protein Cep78 homolog [Apis mellifera]	1.77	807690	Cytochrome c oxidase subunit III	−1.83

4. DISCUSSION

4.1. Effects of high temperature on honey bee colonies as a single stressor

Following the simulated heat wave of 41 °C for 6 h, the inner temperatures of bee hives were effectively maintained at temperatures at least 2 °C lower than outside temperatures (Figure S1), indicating that the thermoregulation of workers efficiently lowered inner hive temperature. The inner temperatures of bee hives were maintained at 33.8 to 37.0 °C in the summer when ambient temperatures rose up to 39 °C (Fahrenholz et al. 1989). Under high-temperature conditions, honey bee workers actively evaporate water and ventilate by fanning to adjust hive temperature to the range required for proper brood development (Jones and Oldroyd 2006). When inner hive temperature reaches 35 °C, honey bees start to show fanning behavior, and with higher temperatures, some of them move out to the hive entrance and begin fanning (Southwick and Moritz 1987). Honey bees also recruit spare bees as water foragers following heat shock (Bordier et al. 2017a, b). Although bees were confined inside their hives in this experiment, aggressive fanning behavior was observed at the entrances of the hives following heat treatment, which would reduce inner hive temperature and increase water evaporation.

The total number of DEGs in the CH group relative to the CC group was 5.7-fold and 10.2-fold lower than those of the IC and IH groups, respectively, which suggests that worker bees tolerated the heat wave without a large alteration in gene expression profile (Figure 3). High

Table II Functional annotation of differentially expressed genes in the honey bee abdomen following imidacloprid/high-temperature exposure

Group	Up/down regulation	Function	Source	GO term and KEGG pathway	FDR
CH	Up (2)		KEGG	Protein processing in endoplasmic reticulum	6.3E-17
				Longevity regulating pathway—multiple species	0.029
	Down (0)			No significant pathways detected	
IC	Up (10)		GO:CC	Organelle	0.025
				Intracellular organelle	0.037
				Ribosome	0.025
			KEGG	Thermogenesis	0.047
	Down (33)	Cellular respiration	GO:CC	Respirasome	8.3E-05
				Mitochondrial membrane	0.002
				Mitochondrial envelope	0.006
			GO:MF	Electron transfer activity	0.002
				Oxidoreductase activity, acting on NAD(P)H, quinone or similar compound as acceptor	0.009
				NADH dehydrogenase (ubiquinone) activity	0.009
				NAD(P)H dehydrogenase (quinone) activity	0.009
				NADH dehydrogenase (quinone) activity	0.009
				NADH dehydrogenase activity	0.016
			GO:BP	ATP synthesis coupled electron transport	0.001
				Mitochondrial ATP synthesis coupled electron transport	0.001
				Respiratory electron transport chain	0.001
				Oxidative phosphorylation	0.002
				ATP metabolic process	0.018
				Cellular respiration	0.012
IH	Up (99)	Protein synthesis and modification	GO:CC	Ribosome	6.1E-04
			GO:MF	Structural constituent of ribosome	0.008
			GO:BP	Cellular protein modification process	6.1E-04
				Protein modification process	6.1E-04
				Ribonucleoprotein complex biogenesis	0.005
				Protein phosphorylation	0.011
				Protein modification by small protein conjugation or removal	0.015
				Ribosome biogenesis	0.031
				Peptide biosynthetic process	0.041
			KEGG	Protein processing in endoplasmic reticulum	2.0E-11
				Ribosome	1.5E-07

Table II (continued)

Group	Up/down regulation	Function	Source	GO term and KEGG pathway	FDR
		Olfactory sensing	GO:MF	Olfactory receptor activity	0.011
				Odorant binding	0.011
			GO:BP	Sensory perception	0.001
				Sensory perception of chemical stimulus	0.003
				Sensory perception of smell	0.005
				Detection of stimulus	0.010
				Detection of chemical stimulus	0.011
				Detection of stimulus involved in sensory perception	0.011
				Detection of chemical stimulus involved in sensory perception	0.011
				Detection of chemical stimulus involved in sensory perception of smell	0.011
		Signaling pathway	KEGG	Insulin signaling pathway	0.006
				ErbB signaling pathway	0.007
				Growth hormone synthesis, secretion and action	0.007
				Longevity regulating pathway	0.030
				Ras signaling pathway	0.020
				AMPK signaling pathway	0.036
				Longevity regulating pathway—multiple species	0.039
				MAPK signaling pathway—fly	0.045
			KEGG	Thermogenesis	0.030
	Down (4)		KEGG	Ribosome	0.002

temperature tolerance appears to be due to the highly developed heat shock response (HSR) system of honey bees (Elekonich 2009; Bach et al. 2021). Heat shock proteins (Hsps) are representative proteins in the HSR pathway that function as chaperones to prevent protein misfolding under stress conditions (Whitley et al. 1999). It has been reported that *Hsp20s*, *Hsp40*, *Hsp60*, *Hsp70*, *Grp78*, *Hsp82*, and *Hsp90* are highly upregulated in honey bees in response to heat shock (Koo et al. 2015; Kim et al. 2019; Bach et al. 2021; Shih et al. 2021). With their roles as regulators of general stress responses (Santoro 2000; King and MacRae 2015), the transcription levels of *Hsps* have been commonly used to estimate the stress level of honey bees (Even et al. 2012; Koo et al. 2015; Kim et al. 2019). Although heat shock-related pathways were

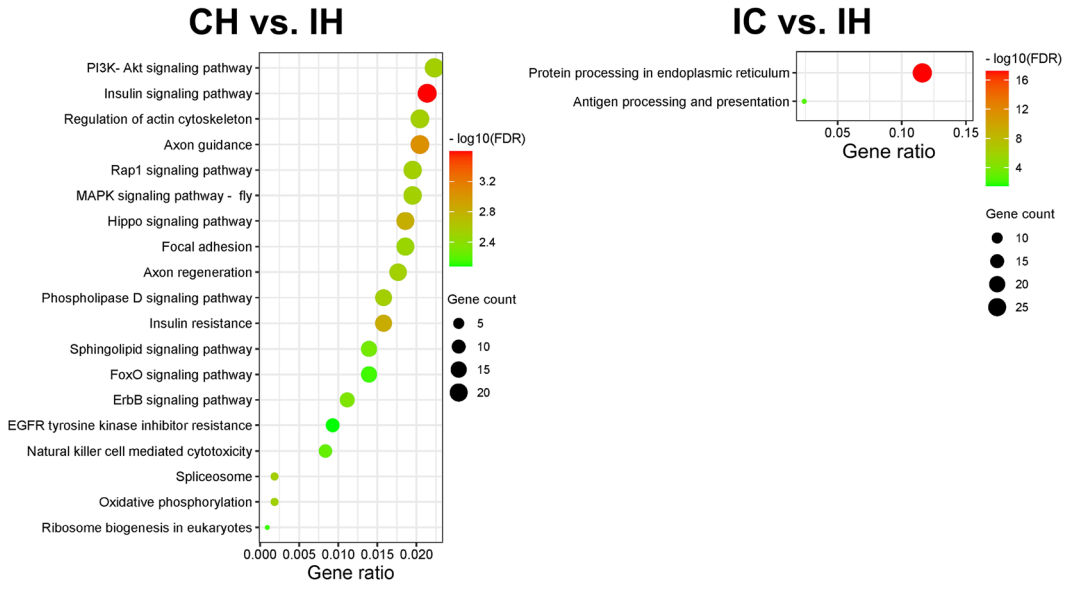

Figure 4. Upregulated KEGG pathways from the enrichment analysis of the CH vs. IH and IC vs. IH comparisons. The top 20 significantly upregulated pathways excluding human disease-related pathways are represented

not enriched, extensive upregulation of *Hsps* in transcriptome analysis and *Hsp70* and *Hsp90* in qPCR analysis following heat shock indicated that the HSR system of honey bees actively defends against heat stress without extensively altering the expression profiles of other genes (Table I, Table II, and Figure 2). The "protein processing in endoplasmic reticulum" pathway, upregulated in both the CH and IH groups under heat-treated conditions, is related to the HSR that enables proteins to fold properly under the oxidative conditions induced by heat shock (Kalmar and Greensmith 2009). Its upregulation was previously reported in the digestive tracts of heat-shocked honey bees (Bach et al. 2021). A high transcription level of *Vg*, which is known to protect honey bees from oxidative stress resulting from heat shock (Seehuus et al. 2006; Bordier et al. 2017a, b; Kim et al. 2019), was also observed under high temperature conditions in both transcriptome and qPCR data (Table I and Figure 2). Another upregulated pathway, "the longevity regulating pathway—multiple species," expresses *Hsp*s and stress resistance genes at the end of the HSR cascade to protect organisms from stressful conditions (Uno and Nishida 2016). It has been reported that antiviral and regenerative signaling pathways were altered in honey bees individually exposed to heat shock (McMenamin et al. 2020; Bach et al. 2021). Considering this, the observation of only two enriched pathways related to the stress response indicates that honey bees are capable of regulating heat stress in a selective and efficient way as a superorganism.

4.2. Effects of IMD on honey bee colonies as a single stressor

Despite no apparent changes in phenotypes, in-hive temperatures, or RH following chronic exposure of honey bees to 20 ppb IMD for 14 days (Figure S1), honey bee physiology appeared to be severely affected by IMD treatment from the perspective of the gene transcription profile in that a large number of pathways were affected in the IC group (Figure 3). The notable feature in the enriched pathways identified following IMD exposure was the general

downregulation of cellular respiration-related pathways (Table II). It has been reported that IMD negatively affects mitochondrial respiration in honey bees (Nicodemo et al. 2014). IMD is known to show biphasic effects on the physiology of honey bees depending on concentration (Cook 2019; Kim et al. 2022b). Chronic exposure to low concentrations of IMD leads to an increase in the metabolic rate of honey bees. In contrast, chronic exposure to high concentrations of IMD results in a reduction in metabolic rate (Meikle et al. 2018; Gooley and Gooley 2020; Kim et al. 2022b), which resembles the case of the IC group in this study.

Downregulation of pathways in the electron transfer system of mitochondrial respiration (e.g., "electron transfer activity," "NADH dehydrogenase activity," "ATP synthesis coupled electron transport," and "oxidative phosphorylation") was the most apparent feature in the DEG profiles and enriched pathway analysis for the IC group. In contrast, several genes related to gluconeogenesis (e.g., aspartate aminotransferase, 2-oxoglutarate dehydrogenase, and pyruvate carboxylase) (DeRosa and Swick 1975; Smith et al. 1992; Kumashiro et al. 2013) were significantly overexpressed in the IC group (Tables I and II). It was previously reported that chronic exposure of honey bees to low IMD concentrations (5 and 20 ppb) induced diabetic symptoms (e.g., downregulation of insulin signaling pathway and reduction of body weight) (Kim et al. 2022b). The energy metabolism disorder in diabetes is known to increase the rate of gluconeogenesis (Magnusson et al. 1992). Considering this, the upregulation of gluconeogenesis in the IC group might be a compensating response to the shortage of chemical energy due to the induced diabetic symptoms and reduced cellular respiration.

4.3. Synergistic effects of IMD and high temperature on honey bee colonies as combined stressors

Hsps were highly overtranscribed following high-temperature treatment, as discussed in Sect. 4.2, but the transcriptional responses of *Hsps* to IMD exposure in honey bees are known to vary depending on the concentration, duration, and condition of the treatments (Koo et al. 2015; Kim et al. 2019; Manzi et al. 2020). In this study, the transcription levels of *Hsp70* and *Hsp90* were not affected by IMD treatment alone (i.e., IC), but they were significantly higher following IMD and high temperature combined treatment (i.e., IH) compared to high temperature treatment alone (i.e., CH) (Figure 2), indicating that both stressors interact with each other, producing synergistic adverse effects on honey bees.

Additional evidence for synergism of IMD and high temperature was observed from the transcriptome analysis. The expression profiles of a much larger number of genes (783 upregulated and 814 downregulated genes) were altered in the IH group following the combined treatment, and most of their enriched pathways were not shared by either the CH or IC groups (Figure 3). The GO term "ribosome" upregulated in the IC group was more significantly upregulated in the IH group (FDR 0.025 vs. 0.00083). The upregulation of other pathways was related to protein synthesis and modification, which appear to be induced by the reactive oxygen species generated by both IMD and high temperature treatments (He et al. 2021; Zhao et al. 2021). Although cellular respiration-related pathways, which were downregulated following IMD treatment alone in the IC group, were not enriched in the IH group, the inclusion of NADH dehydrogenase subunits 1, 4, and 5 and cytochrome c oxidase subunit III in the top 10 list of downregulated genes (Table I) indicates that cellular respiration is still substantially affected following the combined treatment of IMD and high temperature.

Upregulation of various signaling pathways was more obvious in the functional annotation of DEGs when comparing the IH group to the CH group, which demonstrates the large synergistic adverse effects of IMD on honey bees exposed to high temperature (Figure 4). Energy metabolism pathways such as the "insulin signaling pathway" and "insulin resistance," which were not identified in the functional annotations of IMD-treated honey bees either in this (IC group) or a previous

study (Kim et al. 2022b), were significantly upregulated when honey bees were exposed to both stressors simultaneously. This finding indicates that exposure to high temperature conditions can aggravate disorders in the insulin signaling pathway potentially induced by IMD, which eventually disrupt the energy metabolism of worker bees (Cook 2019). Considering that disrupted energy metabolism most likely impairs the in-hive temperature regulation system of fanning and ventilation and that in the long term, insulin resistance can disturb foraging activity and labor division of worker bees (Ament et al. 2008; Kim et al. 2022b), the combined effects of IMD and high temperature can deteriorate the overall health of honey bee colonies in a synergistic way. This notion is supported by the fact that both IMD and high temperature are known to induce precocious onset of foraging (Medina et al. 2018; Colin et al. 2019). One of the possible mechanisms for the precocious foraging under high temperature and IMD exposure may be the low nutritional status caused by the disruption of the energy metabolism (Cho et al. 2022; Kim et al. 2022b), since the nutritional status of bees is known to influence influence foraging onset independently from the social regulations (Toth et al. 2005). The precocious foraging of bees induced by stresses could accelerate failure of honey bee colonies through the recruitment of young foragers and therefore the reduction of the adult population (Perry et al. 2015). The disruption of insulin pathway is also contributable considering the importance of the pathway in labor division of honey bees (Ament et al. 2008). The upregulation of "regulation of actin cytoskeleton," "axon guidance," "focal adhesion," and "axon regeneration" appears to be related to the regeneration of damaged tissues, as reported for the Hippo signaling pathway involved in the regeneration of honey bee digestive tracts under heat shock (Bach et al. 2021).

In contrast to the comparison of CH vs. IH, only two KEGG pathways were upregulated in the comparison of IC vs. IH (Figure 4), which is similar to the comparison of CC vs. CH, as discussed in Sect. 4.1. Taken together, this finding suggests that the relative synergistic effects of IMD on heat-stressed honey bees (i.e., CH vs. IH) are larger than those of high temperature on IMD-exposed honey bees (i.e., IC vs. IH).

Along with the changes in gene expression profiles, a failure of thermoregulation following the combined treatment of IMD and high temperature was observed in the 3rd experiment (Figure S1), with the inner temperatures of the hives reaching up to 41 °C. In addition to the potential synergistic negative effects in diverse physiologies, such high temperatures can damage the overall fitness of honey bee colonies. Heat stress up to 40 °C during the pupal stage leads to precocious foraging behavior and forewing length reduction in honey bee workers (Medina et al. 2018). Pupae developing at 37 °C exhibit brain plasticity disorder (Groh et al. 2004), and the body weights of worker bees maturing at 35 °C were lower than those of bees maturing at 25 and 30 °C and field bees (Cho et al. 2022). All of these results suggest that thermoregulation failure of IMD-treated honey bees under heat stress conditions, along with the synergistic negative effects on honey bee metabolic pathways, likely results in developmental disorders of honey bees.

5. CONCLUSIONS

Honey bees, as a colony, coped well with the heat stress through the active thermoregulation and upregulation of heat stress response pathways. However, transcriptional analysis suggested that a pre-exposure to IMD could influence the heat stress response of honey bees, aggravating the adverse effects of IMD on honey bee physiology such as energy metabolic pathway. Large-scale tracking of in-hive temperatures and colony development following IMD exposure and heat stress should be performed to investigate the possible synergistic effects of the two stress factors on honey bee colony thermoregulation and performances. Such studies will be important for further exploring the effects of environmental pollution and climate change on honey bees.

SUPPLEMENTARY INFORMATION

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AUTHOR CONTRIBUTION

Sanghyeon Kim: conceptualization, formal analysis, funding acquisition, investigation, visualization, writing—original draft. Susie Cho: investigation. Si Hyeock Lee: project administration, supervision, funding acquisition, writing—review and editing. All authors read and approved the final manuscript.

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DATA AVAILABILITY

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

CODE AVAILABILITY

Not applicable.

DECLARATIONS

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent to publish Not applicable.

Conflict of interest The authors declare no competing interests.

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