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Posted Date: 31 October 2023

doi: 10.20944/preprints202310.1937.v1

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Article

Effects of Clothianidin Pesticide Application on Honey Bees Colony Strength and Stress-Related Genes in the Vicinity of Rice Fields

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Abstract: Honey bees are vital organisms that provide ecological and economic value to humans. However, in recent years, the increase in honey bee colony losses due to various environmental factors, including pesticides, has become a growing concern. In Europe, neonicotinoid pesticides that are banned are being used without restrictions in the domestic setting, leading to ongoing damages. Ongoing research is continually being conducted to demonstrate the risks associated with neonicotinoid pesticides. However, validation of the actual damages and impact in the field remains unknown. Therefore, in this study, we observed changes in honey bee (*Apis mellifera*) colonies located near rice cultivation areas as they progressed beyond the rice pesticide application period. Furthermore, we collected honey bees exposed to the clothianidin and analyzed their stress-related gene expression. The results showed that the foraging behavior of honey bee colonies located near rice cultivation areas did not exhibit significant differences between the treatment site (Cheongyang and Gimje) and the control site (Wanju) during the experimental period. However, it was observed that the expression levels of stress-related genes in honey bees collected from the treatment group were significantly higher than those in the control. Most of the stress-related genes were associated with detoxification processes in response to pesticides. As a result, pesticide treatment in proximity to rice cultivation areas did not cause direct damage to honey bees but had an indirect impact, suggesting the potential for ongoing chronic damage.

Keywords: neonicotinoids; pollinator; rice field; qRT PCR; toxicity stress-genes

1. Introduction

Animal pollinators, including honey bees (*Apis mellifera*), play a crucial role in the pollination of the majority of angiosperms in the natural ecosystem [1], and they exert a significant influence on the productivity of crops essential for human food [2–4]. Honey bees are the most economically valuable managed pollinators not only for their ecological significance but also within the industrial insect market [5–7]. However, recent issues, such as colony collapse disorder (CCD) and unexplained disappearances of honey bees during overwintering, have highlighted challenges in identifying their underlying causes [8–10]. Researchers suggest that these issues are caused by a combination of various factors that are collectively influencing the phenomenon [11–13]. Factors threatening honey bee colonies include abnormal climate, pesticides, and pests. Abnormal climate is a significant factor in the reduction of honeybee populations, causing issues such as bees not returning to the hive due to sudden temperature changes during foraging activities [14,15]. In the case of pests, there are various parasitic insects, including varroa mites (*Varroa destructor*), which infest honey bee larvae, acting as vectors for pathogens and viruses, thereby affecting growth and development while

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reducing colony strength [16,17]. Furthermore, predatory hornets (*Vespa* sp.) target honey bees for foraging, posing a significant concern for beekeepers in terms of mass predation [18,19]. Beekeepers utilize various pesticides to eradicate pests such as varroa mites in apiaries. Naturally, these pesticides also affect honey bees, causing various toxicity issues [20,21]. Lastly, pesticides could be a primary factor leading to the collapse of honey bee colonies. Specifically, insecticides used in the cultivation of crops near apiaries can have adverse effects on honey bees [22–24].

Neonicotinoid pesticides are systemic insecticides that exist in absorbed form within plants, potentially being transmitted to honey bees through nectar and pollen [25]. Neonicotinoid-based pesticides have a high potential for long-term persistence, leading to a likelihood of causing chronic toxicity to honey bees [26]. For these reasons, in order to protect natural pollinators, including wild pollinators [27,28] neonicotinoid-based pesticides have been banned for use in Europe. However, in the domestic context, neonicotinoid-based pesticides are still being used extensively across a wide range of agricultural sectors. Among neonicotinoid-based pesticides, clothianidin is primarily applied as seed treatment [29] and, in the domestic context, it is used in rice cultivation through drone aerial spraying [30,31]. The risks of clothianidin to honey bees have already been demonstrated in numerous studies. According to Prisco et al. [32] it is known to affect the immune function of honey bee and promote the development of viral pathogens (diseases). Furthermore, it may also reduce the number of worker bees returning to the colony after foraging due to lost of memory [33]. Thus, there is a substantial risk of clothianidin pesticide exposure for domestic beekeepers around croplands, including rice cultivation. Therefore, there is ample need for research on exposure control and alternative pesticides around croplands within the vicinity of apiaries.

While there have extensive laboratory-based research to establish the risks posed by clothianidin to honey bees, knowledge on its impact to honey bees associated with field conditions is scanty. Therefore, in this study, we investigated the influence of neonicotinoid pesticide application in rice cultivation areas on the strength of nearby honey bee colonies and their stress-related genes. To accomplish this, we monitored the strength of colonies located near rice cultivation areas and collected worker bees on days of neonicotinoid pesticide treatment and one month later for the analysis of stress-related gene expression levels.

2. Materials and Methods

1. Study site

The test species used in the experiment were honey bees (*Apis mellifera*) reared at the experimental apiary of the National Institute of Agricultural Sciences in Wanju, Jeollabuk-do, Republic of Korea. The experiment was conducted in three different locations, including the control group in Wanju, Jeollabuk-do (35°49'41.1"N 127°02'47.6"E), and the treatment groups in Gimje, Jeollabuk-do (35°46'17.8"N 126°58'59.6"E) and Cheongyang, Chungcheongnam-do (36°23'27.0"N 126°46'11.7"E) (Figure 1). All experimental locations were situated in proximity to rice (*Oryza sativa*) cultivation areas, with a total of 18 honey bee colonies, six in each study site. The treatment site is a region where rice cultivation is traditionally practiced. Pesticide application was typically conducted in two stages: the first application was carried out just before the rice flowering stage (Gimje: 8th August, Cheongyang: 14th August), and the second application took place when the rice flowers were in bloom, with more than 50% of the flowers bloomed (Gimje: 21st August, Cheongyang: 28th August). The pesticide treatment involved aerial spraying using a drone, with a solution of 2% clothianidin and 4% flubendiamide suspension concentrate, diluted to 0.8 ℓ/10 a. The control site did not undergo any pesticide application.

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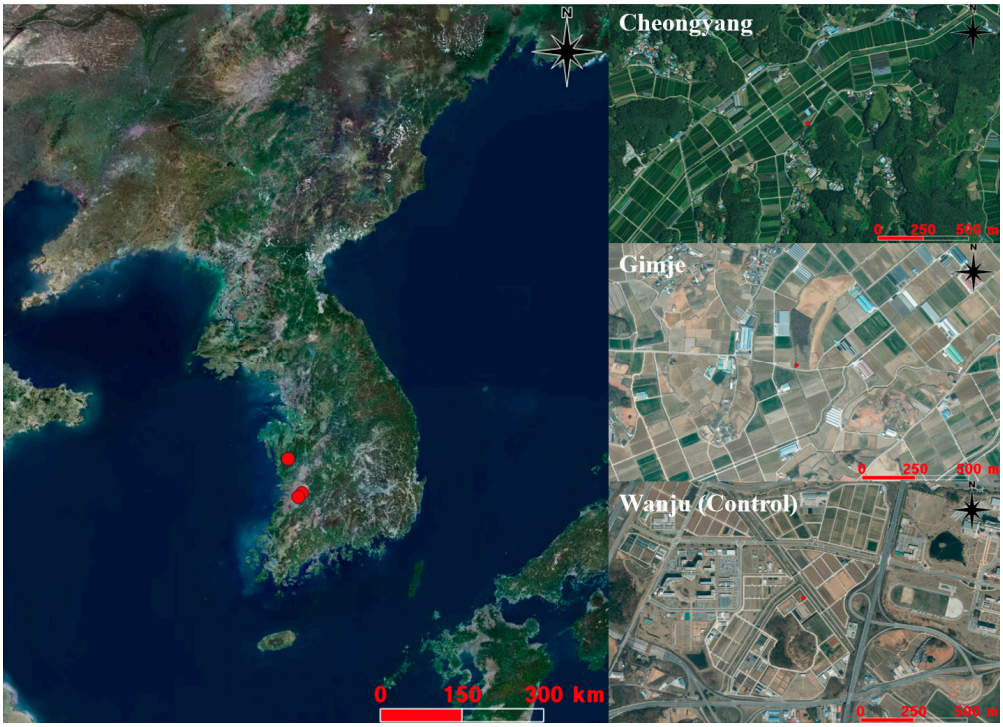

Figure 1. Geographical location of experimental sites. Treatment sites include Gimje and Cheongyang, while the control site is in Wanju. The red circles represent the locations where honey bee (*Apis mellifera*) hives were installed in each site.

2. Landscape analysis

To assess the regional landscape factors, a landscape analysis was conducted. The landscape within a 2 km radius around each colony installation site was analyzed. The landscape was categorized into a total of 8 factors (forest, grassland, crop, rice field, facility, bare land, road, water). Map data were obtained and utilized from the Environmental Geographic Information service provided by the Ministry of Environment, Republic of Korea [34]. Calculation of landscape factors was conducted using QGIS 3.28 [35].

3. Monitoring the strength of honey bee colonies

The survey was conducted weekly from July 20th, 2023, to October 4th, 2023. The strength of honey bee colonies was set at an average of 4±1.5 combs in a single brood chamber. The scale representing the strength of honey bee colonies was determined based on the number of worker bees, capped brood, and larvae cells attached to the combs. The methods for measuring each of these were conducted following the procedures outlined by Delaplane et al. [36]. The number of worker bees was calculated by measuring the ratio of worker bees covering a single comb, and it was calculated to be 2,500 when it reached 100%. The number of larvae and capped brood cells was determined by dividing one side of the comb into 32 equal sections and calculating at a rate of 100 individual cells per section.

4. Quantitative real-time PCR

To test the specificity of major biomarker genes involved in detoxification, stress response and development of honey bees, quantitative real-time PCR (qPCR) was conducted. Total RNA was extracted from ten honey bee abdomens of three biological replicates from each group using TRIzol® reagent (ambion, CA, USA) according to the manufacturer's instructions. The RNA extract was treated with DNase I (GenDEPOT, TX, USA). After DNase I treatment, the first strand cDNA was synthesized from 2.0μg of total RNA to cDNA with EcoDry™ (TAKARA Korea Biomedical Inc., Seoul, Republic of Korea) at 42◦C for 60min. qPCR was performed using QuantStudio5

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(Appliedbiosystems, MA, USA). The cycling conditions were as follows: 95°C for 5 minutes, 38 cycles of 95°C for 30 seconds, 57°C for 30 seconds, and 72°C for 30 seconds. The amplification mixtures (total volume 20 μL) for all assays contained 10 μL of AccuPower® 2X GreenStar™ qPCR Master Mix (Bioneer, Seoul, Korea) and 100 ng of Template DNA. The tested genes transcription levels and two reference genes were evaluated. The tested genes included: Cytochrome P450 9Q1 (CYP9Q1), 9Q2 (CYP9Q2), 9Q3 (CYP9Q3), 6AS3 (CYP6A3), and 336A1 (CYP336A1), carboxyl esterase (CbE), and Esterase E4 (Este4) as the phase I detoxification genes; Glutathione S-transferase (GST) D1 (Gstd1), S1 (GstS1), and S4 (GstS4) as the phase II detoxification genes; ATP-binding cassette (ABC) transporter subfamily C member Sur (Sur), subfamily D member 1 (ABCD1), and G family member 20 (ABCG2) as the phase III detoxification genes; Heat shock protein (Hsp) 70Ab-like (Hsp70) and 90 (Hsp90) as the heat stress response genes; Catalase (Cat), phospholipid hydroperoxide glutathione peroxidase (Gtpx2), Nitric oxide synthase (Nos), Superoxide dismutase (Sod), peroxiredoxin 1 (Tpx1) as the oxidative stress response genes, Juvenile hormone esterase (JhE), Serine/threonine-protein kinase mTOR (mTOR), Insulin receptor substrate 1-B (IRS), Forkhead box protein O (FoxO), and Vitellogenin (Vg) as the metabolism and development-related genes; and 40S ribosomal protein S18 (rpS18) and actin related protein 1 (Actin) as the reference genes [37]. The Primer information are listed (Table S1).

5. Data analysis

All data analysis was conducted using the software R [38]. During the monitoring period, the mean strength of honey bee colonies by region was tested using Two-way ANOVA. Throughout the entire monitoring period, the comparison of mean cumulative strength of honey bee colonies among regions was tested using One-way ANOVA and Duncan multiple range test. At each point in time of pesticide treatment, the mean changes in the strength of honey bee colonies between the treatment groups from two different regions and control were compared using the Kruskal-Wallis rank sum test. Subsequently, a post-hoc analysis was performed using the Conover-Iman test.

3. Results

1. Analysis of landscape factors within a 2 km radius of the honey bee colonies establishment sites

The results of the land cover rate (%) analysis within a 2 km radius from each survey site of the honey bee colonies establishment areas revealed that in Gimje, the treated area, the rice field cover rate was the highest at 44.2% (Figure 2). In the case of the other site, Cheongyang represent 16.4%, while in the untreated control site, Wanju exhibited 6.5%.

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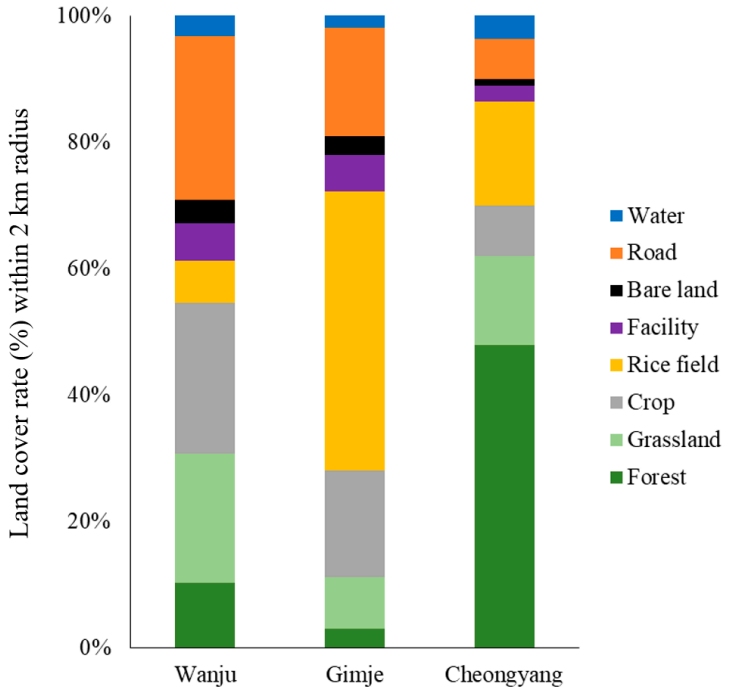

Figure 2. Landscape cover rate (%) of each beehive (control: Wanju, treatment: Gimje and Cheongyang) establishment, located within a 2 km radius.

2. Monitoring the strength of honey bee colonies in nearby rice fields

During the study period, monitoring the strength of honey bee colonies revealed no significant differences in the number of adult bees, larvae and capped brood among the three regions (Table 1; Figure 3). Throughout the monitoring period, the comparison of the overall cumulative strength of honey bee colonies showed that Cheongyang had the highest number of adult bees (Figure 4). However, no statistically significant difference was observed ($F = 0.4082$, $P = 0.6720$). Regarding the number of larvae, Wanju had the highest count, but there was no statistically significant difference ($F = 0.2959$, $P = 0.7481$). As for capped brood, Gimje had the highest count, with no statistically significant difference found ($F = 0.2333$, $P = 0.7948$). Results of the comparison between the Wanju site and each treatment group showed a significant difference ($\chi2=7.2417$, $P = 0.0268$) in the number of adult bees after the first pesticide application in Gimje, in contrast to Cheongyang and Wanju (Table 2).

Table 1. Statistical values of two-way ANOVA comparing the mean strength of honey bee (*Apis mellifera*) colonies by region during the monitoring period.

Effect	Variable*	F value	df	P value
Location	**A**	2.13		0.12
	L	1.00	2	0.37
	C	0.89		0.41
Date	**A**	1.59		0.12
	L	1.76	9	0.13
	C	1.80		0.07
Location:Date	**A**	0.47		0.97
	L	1.21	18	0.26
	C	0.54		0.94

* A: Adult bee, L: Larvae, C: Capped brood.

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Table 2. Comparison of changes in strength of honey bee (*Apis mellifera*) colonies (mean±SE) by pesticide application day in each treatment group (Cheongyang and Gimje). We used the Kruskal-Wallis rank sum test to compare the means, followed by post-hoc analysis using the Conover-Iman test.

	Wanju	Gimje	Cheongyang	χ^2	*P*
Gimje 1st spray					
Adult bee	-951.9±942.4b	1,456.7±305.1a	383.3±430.9b	7.2417	**0.0268**
Larvae	483.2±1,117.9a	650.0±656.5a	783.3±665.6a	0.1260	0.9389
Capped brood	-666.7±302.5a	216.7±675.1a	-150.0±569.0a	0.8573	0.6514
Gimje 2nd spray					
Adult bee	1,552.5±782.2a	-172.5±402.7a	325.8±657.5a	1.9084	0.3851
Larvae	-166.7±892.1a	-350.0±592.9a	1,550.0±1,506.8a	0.5743	0.7504
Capped brood	1,633.3±1,027.0a	1,200.0±703.2a	3,433.3±1,430.0a	0.7932	0.6726
Cheongyang 1st spray					
Adult bee	-651.7±788.2a	95.8±724.5a	824.2±464.3a	2.4993	0.2866
Larvae	483.3±1,117.9a	650.0±656.5a	783.3±665.6a	0.1260	0.9389
Capped brood	-666.7±302.5a	216.7±675.1a	-150.0±569.0a	0.8573	0.6514
Cheongyang 2nd spray					
Adult bee	-38.3±371.3a	-766.7±564.5a	268.3±587.4a	1.7258	0.4219
Larvae	766.74±1,163.2a	-16.7±398.2a	-2,116.7±940.3a	4.6433	0.0981
Capped brood	-516.7±784.0a	250.0±273.6a	116.7±899.2a	0.7142	0.6997

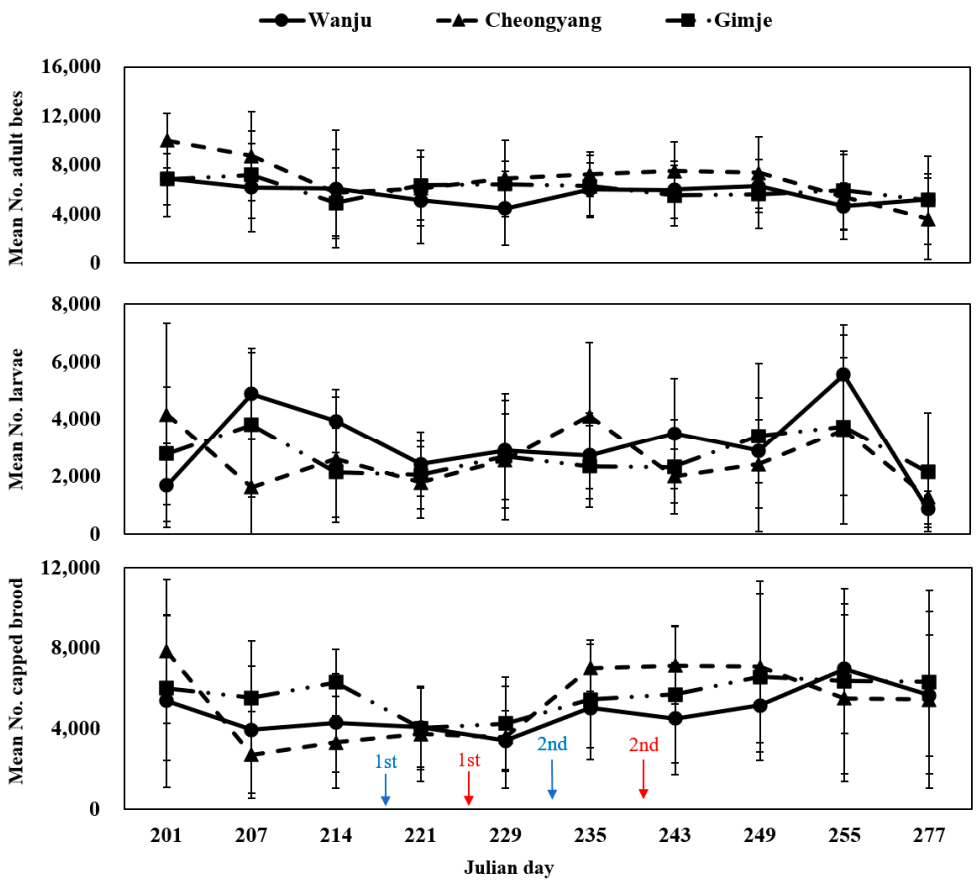

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Figure 3. Monitoring analysis of the strength of honey bee (*Apis mellifera*) colonies (Indicating the following from top to bottom: mean number of adult bees, larvae and capped brood) installed in each treatment (Gimje and Cheongyang) and control (Wanju) area from July 20th, 2023, to October 4th, 2023. Blue arrow represents the pesticide treatment day in Gimje, while red corresponds to Cheongyang. The error bars represent the standard deviation.

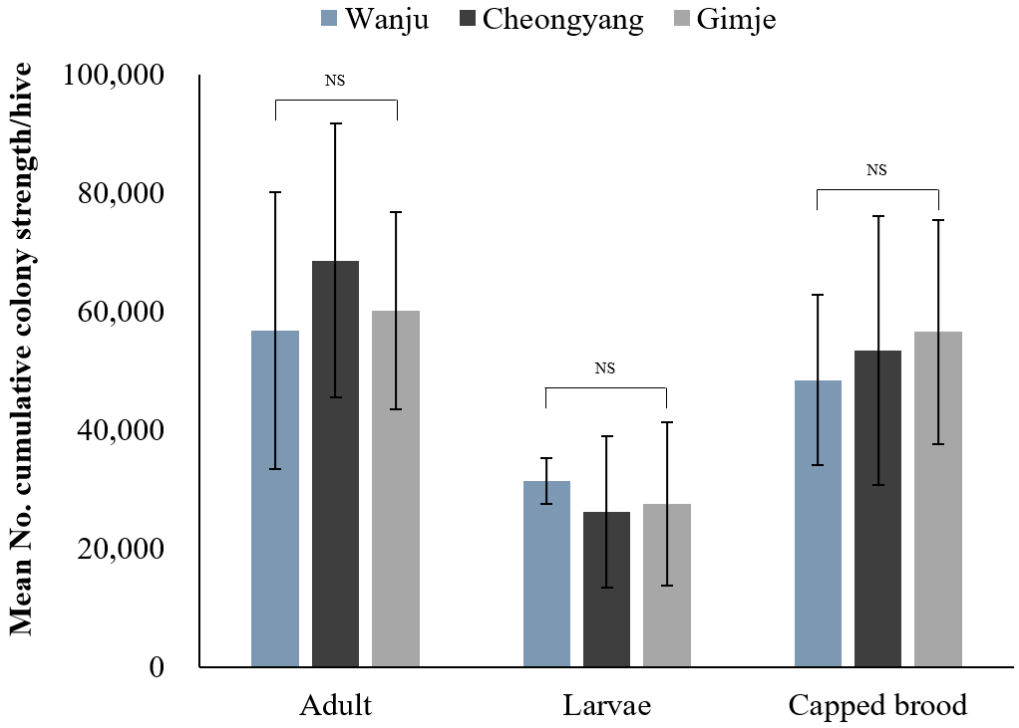

Figure 4. Mean number of cumulative strength of honey bee (*Apis mellifera*) colonies established in treated (Cheongyang and Gimje) and control (Wanju) sites from July 20th to October 4th, 2023. The error bars represent the standard deviation. Means of the three regions were compared through one-way ANOVA ($P<0.05$).

3. Genomic analysis of honey bee stress responses

On the day of pesticide application, the Cheongyang and Gimje sites displayed lower expression levels than Wanju site (Figure 5). The transcription levels of ABCG20, HSP70, Catalase, Nos, Sod, JhE, and Vg increased. The highest expressions were observed in Catalase, Juvenile hormone esterase (JhE) and Superoxide dismutase (Sod). In detoxification phases (phase Ⅰ, Ⅱ and Ⅲ), transcription of detoxification phase Ⅰ (CYP336A1, CYP6AS3, CYP9Q1, CYP9Q2, CYP9Q3, CbE, Este4) showed lower expressions.

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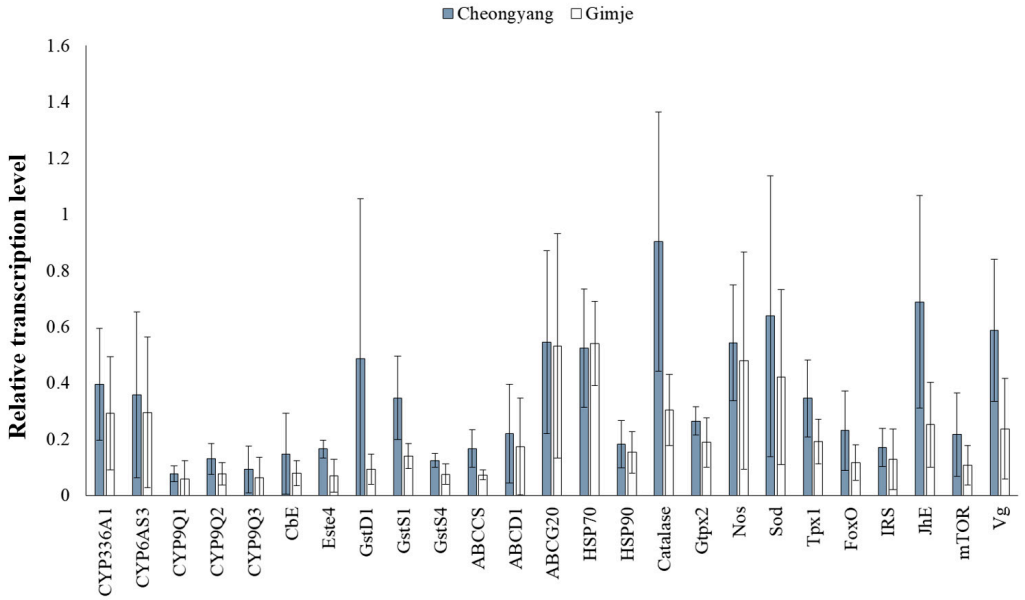

Figure 5. qPCR results of genes related to detoxification, the heat shock response, the oxidative stress response and the development of honey bees (*Apis mellifera*) on the day of pesticide application. The error bars represent the standard deviation.

One month after treatment, it exhibited higher expression levels than the Wanju site (Figure 6). The transcription levels of all oxidative stress, metabolism and development related genes were highest in the Gimje as was the case with GstS1. The site in Gimje displayed higher expression levels than Wanju. The highest expression was observed in Vitellogenin (Vg), peroxiredoxin 1 (Tpx1) and Insulin receptor substrate 1-B (IRS). Also, Cheongyang displayed Glutathione S-transferase (GST) S1 (GstS1), peroxiredoxin 1 (Tpx1) and Superoxide dismutase (Sod). In this study, toxic stress conditions affected the transcription of detoxification, stress response and development in honey bees colonies.

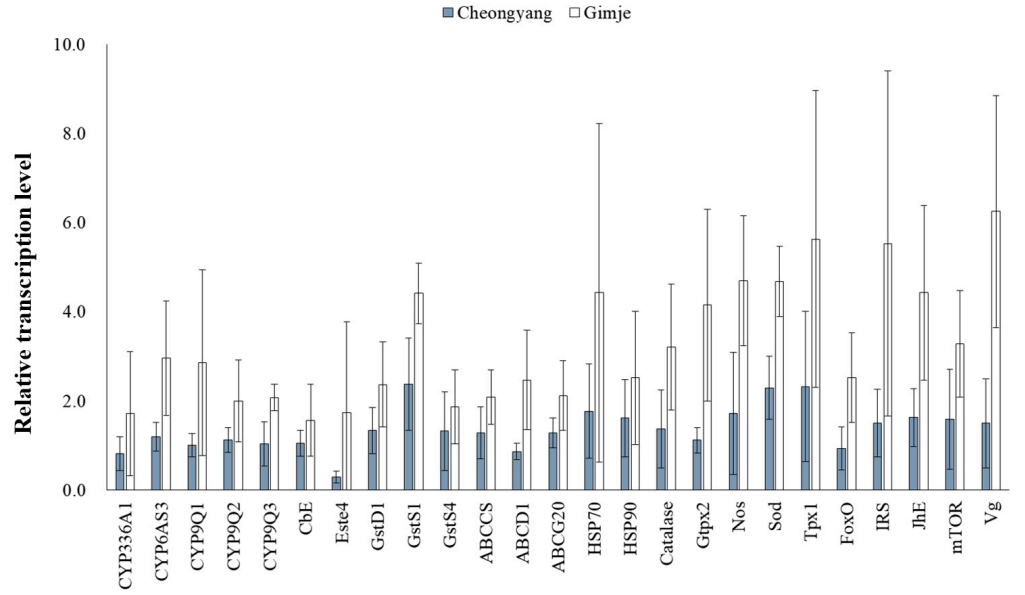

Figure 6. qPCR results of genes related to detoxification, the heat shock response, the oxidative stress response and the development of honey bees (*Apis mellifera*) in one month after pesticide application. The error bars represent the standard deviation.

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4. Discussion

In proximity to the rice cultivation area, beehives for honey bees were installed, and the strength of honey bee colonies was monitored from July 20th to October 4th in 2023. In two treatment areas (Cheongyang and Gimje), the neonicotinoid pesticide Clothianidin was conventionally applied, and a comparison was conducted between the control area (Wanju) on the strength of honey bee colonies. As a result, there was no significant difference in the strength of the honey bee colonies. According to Yao et al. [26] experiments have shown that treatment with clothianidin has minimal impact on the survival rate of adult bees. Orcic et al. [39] also suggested a tendency for chronic toxic responses in honey bees, such as weakening their immune system, rather than acute toxicity. However, clothianidin is relatively well-known for its high toxicity, with a 24-hour lethal dose 50% (LD50) of 22 ng bee^{-1}, even among neonicotinoid pesticides [40]. This indicates that some level of control can be achieved through low dose clothianidin treatment. The clothianidin used in this study was applied at a rate of approximately 5 g /10 a, a level that is considered not to significantly impact honey bee survival rates compared to previous studies [10,26,41]. Additionally, since this study did not involve direct treatment of honey bees with pesticides, the acute toxicity risk is expected to be low.

The primary risk factor associated with neonicotinoid pesticides is chronic toxicity, and this underscores the chronic toxicity risk to honey bees [42–44]. When exposed to neonicotinoid pesticides, honey bees experience a range of complex issues, with the most immediate impact being on the flight activity of adult bees [45]. If the wings fail to function properly, it can lead to issues with temperature regulation within the hive and foraging activities [46,47]. Particularly, when difficulties in temperature regulation arise during the winter season, the likelihood of colony collapse is higher [48]. The use of neonicotinoid pesticides can also have a significant impact on honey bee queens. According to Williams et al. [42] neonicotinoid pesticides, when exposed to honey bee queens, can cause damage to the stored sperms in the queen's spermatheca, ultimately significantly impacting the oviposition rate. As a result, there may be a deficiency of worker bees, leading to challenges in maintaining the colony. Based on these findings, it is necessary to expand the discussion of neonicotinoid pesticide exposure in honey bees to a more chronic context.

We established honeybee hives in proximity to rice cultivation areas and subjected the treatment group to two rounds of clothianidin pesticide treatment. On the day of the pesticide application, we observed a decrease in the transcription levels of Hsp90 and mTOR. In light of these results, it is worth noting that a reduction in mTOR levels through RNA interference leads to an increased sensitivity to heat shock. This effect was also associated with a diminished capacity to synthesize heat shock proteins (HSPs), including Hsp70, Hsp90, and Hsp11 [49]. Conversely, we observed an increase in the transcription levels of ABCG20, HSP70, Catalase, Nos, Sod, JhE, and Vg—genes known to trigger the heat shock response (HSR). Hsps, which are involved in the HSR, can serve as stress markers for the detection and quantification of cellular stress [50]. Furthermore, Vitellogenin (Vg) has been suggested as a plausible candidate for a stress marker, as it plays a protective role against oxidative stress and is regulated by the juvenile hormone, which is also considered to be associated with stress responses [51,52].

One month after treatment, there was a significant up-regulation in catalase activity in Gimje, where a higher concentration of imidacloprid (20 ppb) was applied. In contrast, Sod2 was down-regulated in the same area. Furthermore, on day 20 of the feeding process, only two antioxidant genes were observed to be down-regulated, namely Cat and TrxR1. Catalase plays an active role in protecting cells from oxidative damage caused by reactive oxygen species (ROS) [53]. Additionally, heat shock and brood rearing suppression led to the over-transcription of Vg [54], and Vg has been proposed as a potential molecular stress marker [55]. In particular, some cellular stress responses, such as the expression levels of heat shock proteins (Hsps) and corticotropin-releasing hormone-binding protein (CRH-BP), have more recently been utilized to assess stress in honey bees exposed to various stressors, including capture, transport, confinement, cold, heat, and UV light [56].

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5. Conclusions

In this study, we analyzed the impact of the neonicotinoid pesticide, clothianidin, on strength of honey bee colonies residing in the vicinity of rice cultivation areas. The results indicate that the direct harm inflicted on honey bee colonies by rice pesticide treatment appears to be low. However, the analysis of honey bee genes revealed that bees in the treatment area generally exhibited higher expression levels of stress-related genes. Furthermore, one month later, there was a great increase, indicating the potential progression towards chronic effects. Further research is needed to investigate the actual routes of pesticide intrusion into honey bee hives or their contact with honey bees. The findings of this study may serve as a basis tool for regulating the use of neonicotinoid pesticides.

Author Contributions: Conceptualization, M.S., J.K. and B-S.P.; Methodology, M.S., J.K. and B-S.P.; Software, B-S.P. and Y-S.C.; Validation, C-H.L. and D.K.; Formal Analysis, M.S. and P.N.A.; Investigation, M.S., J.K. and B-S.P.; Resources, D.K. and Y-S.C.; Data Curation, D.O. and B-S.P.; Writing – Review & Editing, M.S., J.K., P.N.A. and B-S.P.; Visualization, M.S., J.K. and D.O.; Supervision, P.N.A.; Project Administration, M.S. Y-S.C. and B-S.P; Funding Acquisition, M.S. Y-S.C. and B-S.P All authors have read and approved the final manuscript.

Funding: This study was conducted with support from a project (Project No. PJ015778) by the Rural Development Administration (RDA), National Institute of Agricultural Sciences (NIAS), Republic of Korea.

Data Availability Statement: The data presented in this study are within the manuscript and available on request from the corresponding author.

Acknowledgments: We are grateful to the bee breeding laboratory, NIAS for their collaboration during this study. We appreciate the technical assistance of beekeepers in the bee breeding laboratory at RDA.

Conflicts of Interest: The authors declare no conflict of interests. The sponsors had no role in the design, execution, interpretation, or writing of the study, or in the decision to publish the results.

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