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Immediate early genes *Egr-1*, *Hr-38* and *Kakusei* function throughout the entire foraging period: An update study on honeybees

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Abstract

Honeybee foraging is a well-organized behavior that has been explored extensively. During foraging, bees search for food, taste quality, memorize the source, interact and communicate with other members of the colony, and collect food and stores it for the colony. This level of intelligence and typically well discipline behavior of these tiny insects can be easily observed during the foraging and thereby has attracted several researchers to uncover the mysterious behavior of honeybees and underlying regulatory mechanisms. Notably, a piece of study that interpreted honeybee dance language, a behavior used by honeybees used for communication among other members of the colony, during foraging, was Nobel prize winning research. However, only a little knowledge has been accumulated at the molecular and cellular levels toward understanding the mechanisms underlying of these sophistically regulated behaviors. Towards this approach, immediate early genes (IEGs) have been repeatedly shown to be promising tools in the cellular and molecular studies for understanding the regulatory mechanisms. Many studies have revealed that IEGs are genetic markers in honeybee brain functions and behaviors. Our recent studies have shown that three IEGs *Egr-1*, *Hr38* and *Kakusei* have potential roles in honeybee foraging as well as learning and memory (Singh et al., 2018; Singh & Takhellambam, 2021; Singh et al., 2020). Further understanding, how long these genes remain upregulated during foraging, can reveal us the period of these genes' participation during foraging. This piece of work is an extension of the earlier study to investigate whether gene expression continue to increase for longer time during the food reward foraging. Our study showed *Egr-1* (09:00 - 14:00 hr./ 14:00 - 14:15 hr./ 14:15 - 14:30 hr./ 14:30 - 14:45 hr./ 14:45 -15:00 hr./ 15:00 - 15:15 hr./ 15:00 - 15:30 hr.: P=<0.0001) and *Hr-38* (09:00 - 14:00 hr./ 14:15 - 14:30 hr./ 14:30 - 14:45 hr./ 14:45 - 15:00 hr.: p=<0.0001; 15:00 - 15:15 hr.: p=0.0003; 15:15 - 15:30 hr.: p=0.0008) expression continue to increase up to 15:30 hr., while *Kakusei* continue to increase up to 14:45 hr. (09:00 - 14:00 hr./ 14:00 - 14:15 hr./ 14:15 - 14:30 hr./ 14:30 - 14:45 hr./ 14:45 - 15:00 hr.: p=<0.0001), from the start of foraging or before foraging. The expression levels of all the three genes at 17:00 hr. is significantly higher than the 18:00 hr. (p=<0.0001) which is after foraging. We found that the gene expression level continued to remain upregulated throughout the entire 3 hours of foraging. This reveals that all the three genes *Egr-1*, *Hr38*, *Kakusei,* have a role to play from the beginning until the end of food reward foraging.

Key words: Honeybee; Foraging; Learning; Interaction; Communication; Immediate Early Genes; *Egr-1*; *Hr38*; *Kakusei*

1. Introduction

Honeybees are one of the most vastly studied insects due to their unique well-orchestrated social behaviors. A bee colony demonstrates like a human kingdom ruled by a king or a queen in which citizens of the monarch strictly obey and performed different tasks with discipline. A honeybee colony has division of labor among the worker bees based on age difference (Johnson, 2010; Sagili et al., 2011; Siegel et al., 2013). Thanks to the various researchers who have

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wholeheartedly dedicated in honeybee research to discover and understand this intelligent and mysteriously beautiful social lifestyle of honeybees. Since time immemorial, the role of honeybees in human lives is indispensable. They serve as one of the major pollinators required for crop yielding, and manufacture honey (in their tiny bodies) which is used as a delicious food item/ingredient or medicine (Singh & Chanu, 2021). Over the past several decades, foraging of honeybees has been extensively studied to understand the various intelligent characters of honeybee behavior. In 1973, Karl von Frisch was awarded Nobel Prize in Physiology or Medicine in recognition of his exceptional contribution in behavioral research in honeybees. In that he interpreted that waggle dance which looks like the alphabet number 8, a unique movement of honeybees as a symbolic language to communicate with other members of the colony for location of food source and quality of food etc. (Michelsen, 2003; Singh & Takhellambam, 2021).

One of the most fascinating part in honeybee foraging is the variety of dynamic behavioral components that comprise of food search, identification and memorization location of food source, taste for food quality, interaction and communication among the foraging bees, recruitment of foragers, carry and store food in the hive (Frisch, 1965; Seeley, 1995). These are also the common intelligent behaviors of much larger size social animals and humans. Therefore, honeybee can be one of the best choice models to study social behaviors and understand the regulatory mechanisms. Interestingly, one can observe all these behaviors by just placing sugar solution on a plate and allow the honeybees to come and drink. Taking this advantage, we investigated few IEGs to examine their roles in the foraging of honeybees. Our previous studies have demonstrated potential roles of three IEGs *Egr-1*, *Hr-38* and *Kakusei* during the daily foraging of honeybee (Shah et al., 2018; Singh, 2019; Singh & Chanu, 2024; Singh et al., 2018; Singh & Takhellambam, 2021; Singh et al., 2020). In the present study we aim to examine the role of these genes in the longer duration of foraging by collecting extending 1 hour more than the previous 2 hours of collection.

The IEGs are the genes which are transcribed rapidly and transiently within minutes following activation by a stimulus such as depolarization, growth factor, or neurotransmitter (Dampney & Horiuchi, 2003; Morgan & Curran, 1989, 1991; Sheng & Greenberg, 1990). It may be noted that IEGs have persistent roles from the first stages of brain development unto the adulthood and indicates possible inherent features in everyday functions of brain (Loebrich & Nedivi, 2009; Perez-Cadahia et al., 2011). They also play an essential role in short- or long-lasting phenotypic changes that occur in neurons in response to different stimuli and cellular circumstances (Dijkmans et al., 2009; Perez-Cadahia et al., 2011; Singh & Takhellambam, 2021). Following a stimulation, the early response neurons reacted within milliseconds/minutes, whereases late response may continue for hours to days even leading to permanent changes that requires changes in gene expression (Clayton, 2013; Hughes & Dragunow, 1995). Moreover, late response is linked to learning, memory and sensitization processes and even to drug tolerance habits etc. (Clayton, 2013; Clayton et al., 2020) and involvement of IEGs in the regulation of neurotransmitter regulated genes within neurons is documented (Hughes & Dragunow, 1995). In the process of nerve stimulation, IEGs are first activated genes linking to membrane events and nucleus and thereby considered as first part in general neuron response to a natural stimulus (Beckmann & Wilce, 1997). Moreover, depending on the stimulus type IEGs encoded proteins may be individually regulated in different regions of the brain (Beckmann & Wilce, 1997); this indicates same/different IEGs at different parts of the brain following stimulation, may pass different signals to perform different behavioral tasks depending on the type of the stimulus.

Early growth response factor 1 (*EGR1*) is a member of the EGR family, and its protein product is an important transcription factor, which contains an activation or repressive regulatory region with three Cys2-His2 subclass zinc finger motif structure (Sukhatme et al., 1988; Wang et al., 2021). In adulthood, EGR-1 is expressed widely throughout the brain, and it has several key regulatory functions in cognition, emotional response, social behavior (Beckmann & Wilce, 1997; Herdegen et al., 1995; Knapska & Kaczmarek, 2004). The homologous *Egr-1* has been widely considered as neural marker in insects and the gene has been extensively used for understanding the regulatory mechanisms of social behavior in honeybees (Sommerlandt et al., 2019). Among other IEGs *Hr-38* (Hormone receptor-like in 38) and *Kakusei* (a nuclear non-coding RNA) have been documented to have substantial roles neuromodulation and social behavior in honeybees and drosophila (Fujita et al., 2013; Kiya et al., 2012; Singh & Takhellambam, 2021; Singh et al., 2020; Takayanagi-Kiya & Kiya, 2019). Interestingly our recent findings have shown potential involvement of these three IEGs *Egr-1*, *Hr-38* and *Kakusei* in the foraging of Western honeybees *Apis Melifera*, in learning and memory (Singh et al., 2018; Singh & Takhellambam, 2021; Singh et al., 2020). Extending this work, in the present study we aim to provide the information whether the three IEGs continue to have a role until the end of food reward foraging.

2. Methods

2.1. Behavioral experiment

Behavioral experiment was conducted inside the bee house at National Centre for Biological Sciences (NCBS), Tata Institute of Fundamental Research (TIFR), Bangalore. This is an outdoor flight cage that allows to perform the behavioral tests and sample collection in semi natural environment allowing the bees to forage freely from the hive to the feeder with minimal disturbance. The honeybees (*Apis Melifera*) were given with pollen and 1 M sucrose solution every day from 14:00 hr. to 15:00 hr. About two weeks before the sample collection began, feeder was presented every day at the same time making sure that the foraging bees learned, remembered and visited the feeders every day about the same time. Based on the time we collected the samples; the study groups are categorized as bellow. About 1 to 2 bees were collected at each time points on each day of collection and each group consists of 5 bees. Further details are available in our previous reports (Singh et al., 2018; Singh & Takhellambam, 2021; Singh et al., 2020). Even though honeybee falls under the higher invertebrate category, studies on honeybees neither have required for an ethical approval nor a consideration for waiver (Singh et al., 2018; Singh & Takhellambam, 2021; Singh et al., 2020).

2.2. Sample collection

2.2.1. Before and after foraging

Before foraging group consists of honeybees collected in the morning at 9:00 hr. in the hive before they flew out to forage. After foraging group consists of honeybees collected in the hive at 18:00 hr. in the evening after bees completed foraging and remained in the hive. The collected bees were marked by pen (Uni POSCA Paint Markers, Uni Mitsubishi Pencil, UK) on the head during foraging while they were drinking sucrose solution. The marked bees on the feeder plate are shown in Figure 1. 50 mL falcon tubes with multiple holes made on it were used collecting the bees. As soon as the bees were collected and they were immediately flash frozen in liquid nitrogen, then stored at -80°C for further experiments (Singh et al., 2018; Singh & Takhellambam, 2021; Singh et al., 2020).

2.2.2. Sample collection during foraging

Samples collected during foraging are categorized into 14 groups which were collected for a long span of 3 hours duration starting at 14:00 hr. and ending at 17:00 hr. keeping 15 min intervals between adjacent time points. The time points are 14:00 hr., 14:15 hr., 14:30 hr., 14:45 hr., 15:00 hr., 15:15 hr., 15:30 hr., 15:45 hr., 16:00 hr., 16:00 hr., 16:15 hr., 16:30 hr., 16:45 hr., and 17:00 hr. respectively. The 14:00 hr. group was collected on the feeder plate before presenting the sucrose solution and the rest of the time point samples were collected after presenting sucrose solution when the bees landed at the feeder at those specific set time points. We collected 1-2 bees a day at each time point and continued collection on the subsequent days until the number reach 5 to 6 bees at each time point and thus each time point has 5/6 bees in the analysis. The detail procedures were described in our previous articles (Singh et al., 2018; Singh & Takhellambam, 2021; Singh et al., 2020).

Figure 1 Honeybee foraging and sample collection overview. (A) honeybees feeding on 1M sucrose, (B) honeybees in the hive, (C) honeybees feeding on artificial pollen, and (D) honeybees on the honeycomb in the hive

2.3. Brain dissection

The frozen bees from the -80˚C were lyophilized for 20 min at -50˚C with vacuum condition 0.420 mBar using lyophilizer (Freeze Zone1 PlusTM 4.5-liter cascade Freeze Dry System, Labconco Corporation, Kanas City). Brain dissection was carried out in a glass chamber with 100% ethanol placed on dry ice under the light microscope using surgical instruments. The dissected whole brain was immediately transferred into 1.5 mL Eppendorf tube on dry ice, and 500 µL Trizol (Trizol Reagent, ambion RNA, life technology) was added.

2.4. RNA and cDNA preparation

The frozen brain was thawed on ice, then homogenized using electronic homogenizer (Micro-Grinder Pestle Mixer, RPI Research Products International) with pestle (Micro-Tube Sample Pestles, Research Products International). Centrifugation was carried out at 10000g for 5min at 4^0C. The upper clear portion which contained RNA was gently removed without disturbing the lower DNA, tissue debris and the protein fractions, then transferred into another tube. The total RNA was quantified, and purity was checked using a nanodrop spectrophotometer. The absorbance ratio 260/280 greater than 1.8 has been considered for further processing. Then equal amount of total RNA from each sample was used for cDNA preparation using cDNA kit SuperScript™III First-Strand Synthesis System supplied by Invitrogen (Thermo Fisher Scientific); manufacturer's protocol was followed in the preparation.

2.5. Quantitative real time PCR (qPCR)

The cDNA from each brain sample was amplified by qPCR for each target genes and three replicates were prepared for each sample, using 7900HT Fast Real Time PCR System (Applied Biosystem, Singapore). Final reaction volume of each sample was 10µl with cDNA, oligonucleotide primers (Sigma Aldrich) of the specific target genes and SYBR Green (KAPA Syber1 FAST PCR Master Mix (2X) ABI Prism1). For endogenous control *Rp49* was used. Each qPCR plate was prepared with five standard reactions of standard cDNA with five different serial dilutions (1/10, 1/100, 1/1000, 1/10000, 1/100000). We also included a negative control for each gene without cDNA and each sample was run in triplicates. We

followed the same procedures described in our previous publications (Singh et al., 2018; Singh & Takhellambam, 2021; Singh et al., 2020). The details of the primers are provided in Table 1.

Table 1 Gene locations in the chromosome, oligonucleotide primers

Gene Name	NCBI Gene ID	Chromosome No. & location	Oligonucleotide primer sequence 5′ - 3′
Egr-1	726302	LG15	F- GCTCTGAGGGTGATTTCTCG
		NC_007084.3	R- GAGAAACCGTTCTGCTGTGA
Hr-38	551592	LG13	F- GCACGAATCAATCTTCTACAACC
		NC_007082.3	R- AATCCGCCAGGGTACTACATC
Kakusei	100049563	LG2	F- TGGGTAGGGTTGGTAAGGGAA
		NC_007071.3	R- ACACGAAACCATCCTGCCAC
Rp-49	406099	LG11	F- CAGTTGGCAACATATGACGAG
		NC_007080.3	R- AAAGAGAAACTGGCGTAAACC

Note: LG stands for linkage group and NC refers to reference sequence accession number. *Egr-1*, *Hr-38*, *Kakusei* are target genes while *Rp-49* is the endogenous control gene.

2.6. Statistical analysis for relative gene expression changes

A standard curve was plotted for each gene, and it was used to quantify the unknown quantity expression level of the respective genes. Representative standard curve plots for each gene are provided in Figure 2. The expression level of each gene at each time point was calculated with the help of relative standard curve method estimated by SDS 2.4 software supplied with the 7900HT Fast Real system (Applied Biosystem, Singapore). The CT values were considered for gene expression level and *Egr-1*, *Hr-38* and *Kakusei* expression levels were normalized with the CT values of endogenous control *Rp-49*. We calculated the fold change using delta-delta (DD)^- CT values at each time point relative to the gene expression level at 14:00 hr. And the standard deviation (SD) was calculated following the protocol provided by 7900HT Fast Real system (Applied Biosystem, Singapore). All the groups were subjected to normality tests with the help of Graphpad prism and each group remain within normal/Gaussian distribution. Each group passed Shapiro-Wilk normality test as the p-values were not significant ($p>0.05$). Lognormal QQ plot is provided in Figure 2. Gene expression level across different time points was examined by one-way ANOVA with Sidak's test for correction of multiple comparison analyses, using GraphPad Prism Version 10.4.1 (Motulsky, 2016) (http://www.graphpad.com).

To further compare the gene expression level among the three genes *Egr-1*, *Hr38* and *Kakusei* across the study time points Two-way Anova with Sidak's correction for multiple comparisons was used. The statistics were carried out, analyzed and interpreted with the help of GraphPad Prism Version 10.4.1 and the Handbook of Parametric and Nonparametric Statistical Procedures by David J. Sheskin (Handbook of Parametric and Nonparametric Statistical Procedures: Third Edition IBSN: 1584884401) described in GraphPad Prism (http://www.graphpad.com). Two-way Anova formulate the percentage of the variability of gene expression among the comparing genes resulted by four components 1) interaction between the row and column factor, 2) row factor, 3) column factor and 4) remainder of the variation also called residual variation. The null hypothesis indicates no interaction between columns (data sets) and rows whereas alternate hypothesis indicates existence of interaction. The column factor p-value provides the statistical difference between means of each column while totally omitting the rows. Subsequently row factor p-values revealed the statistical difference between the means of each row while totally omitting the columns.

3. Result

Our previous studies reported potential roles of IEGs *Egr-1*, *Hr-38*, *Kakusei* and few downstream genes (Singh et al., 2018; Singh & Takhellambam, 2021; Singh et al., 2020). In the previous studies, the transient overexpression of *Egr-1*, *Hr-38* and *Kakusei* during two hours of rewarded foraging was observed. Moreover the expression levels during foraging remain significanly higher than the levels at before foraging, after foraging and the start of foraging. We are further interested in knowing whether the expression levels of these three genes remained overexpressed beyond two hours if we continue to reward food. Therefore, in this study we extended collection time from 2 to 3 hours. This can reveal us whether the roles these genes contained only a limited period during foraging or over the entire period of foraging. As we already have the previous 2 hours collection data as well as before and after foraging, in this study we collected

samples from 16:15 hr. to 17:00 hr. with the intervals of 15 min. It may be noted that about 17:00 hr. bees also stopped foraging as the winter sun set began (we did this experiment during December like the previous studies). In our observation, a bee made about 20 trips in the bee house set up at NBCS campus, during the entire time of foraging of the day and the trip became slower and slower as the bee continued foraging. We combine the data of previous study of during (14:00 hr. to 16:00 hr.), before and after foraging with the present data of 16:15 hr. to 17:00 hr. of during foraging. Thus, in this study, we combined the data and analyzed and provide the gene expression profile of *Egr-1*, *Hr-38* and *Kakusei* during the three-hour food-reward foraging (14:00 hr. to 17:00 hr.) along with before and after foraging. And the results indicate a prolong upregulation of *Egr-1*, *Hr-38* and *Kakusei* during three-hour food-reward foraging period. This reveals that the participation of these genes to be indispensable throughout the entire period of foraging. The results of One-way ANOVA are summarized in Figure 4 (A, B and C) and Table 2. We further examined whether there is significant difference in the expression level among these genes across the different time points. We observed that the level of *Egr-1* is much higher than the *Hr-38* and *Kakusei* throughout the entire duration of foraging, while the level of *Kakusei* is lower than the *Hr-38.* However, after two hours, *Kakusei* and *Egr-1* level came very close, but significantly lower than the *Hr-38* level. Further detail of the result of Two-way ANOVA is provided in Figure 4D and Table 3.

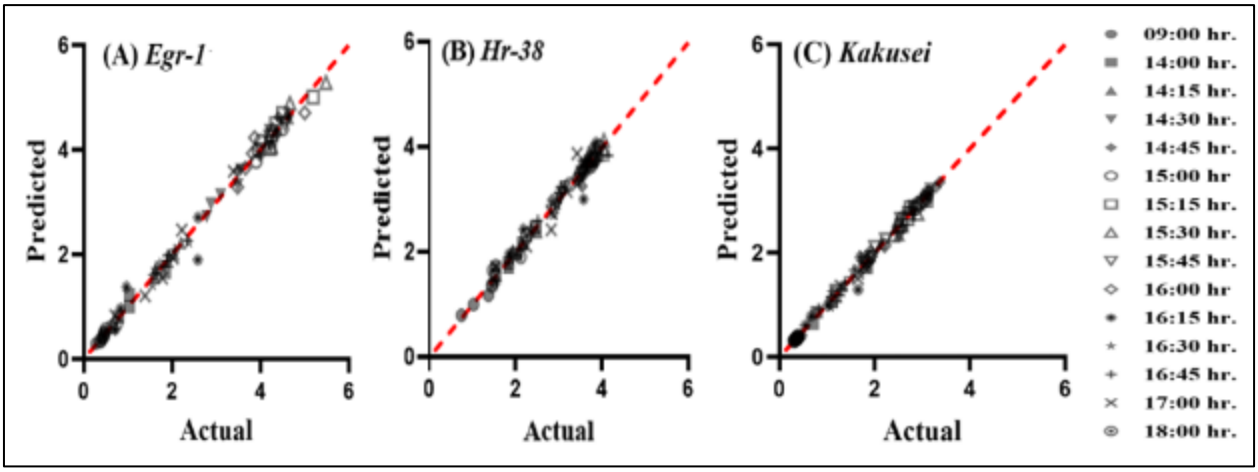

Figure 2 Lognormal distribution test using Shapiro-Wilk normality statistics

All the groups passed the Shapiro-Wilk normality test and confirms to have normal distribution of the samples, as the p-values of each group at each time point are greater than 0.05. Figures (A), (B) and (C) represent the lognormality QQ plot for *Egr-1*, *Hr-38* and *Kakusei* respectively.

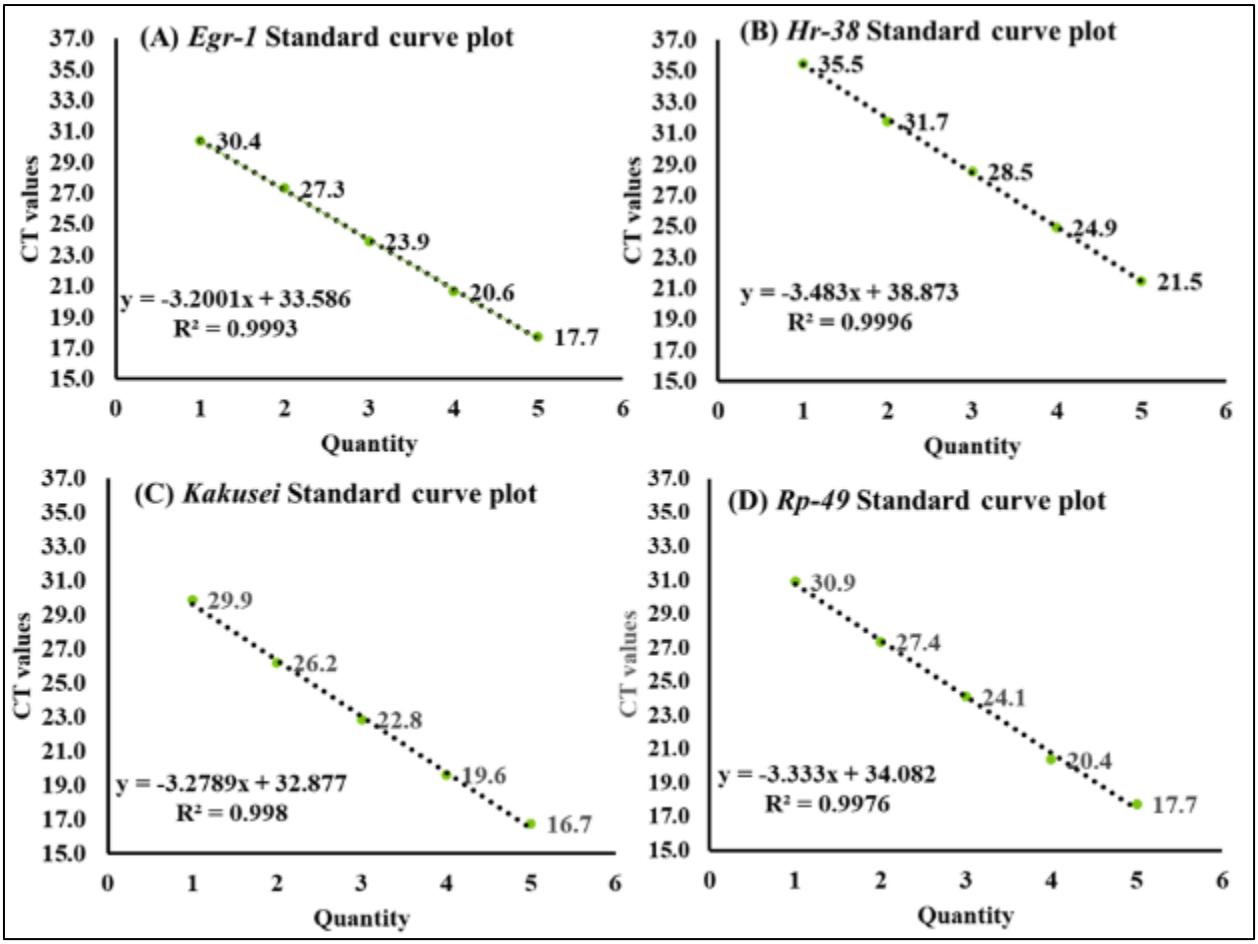

Figure 3 Standard curve plots for IEGs and endogenous control

(A) represents standard curve for *Egr-1*, (B) represents standard curve for *Hr-38*, (C) represents standard curve for *Kakusei*, (D) represents standard curve for endogenous control *Rp-49.* The coefficient of determination (R^2) measures how strong is the linear relationship between the standard CT values (Y-axis) and quantity (X-axis). In general, $R^2 > 0.9$ is considered to have a strong relationship between X-axis and Y-axis in a linear model. Respective standard curve equations that have been used for calculating the unknown quantities are also provided in each figure.

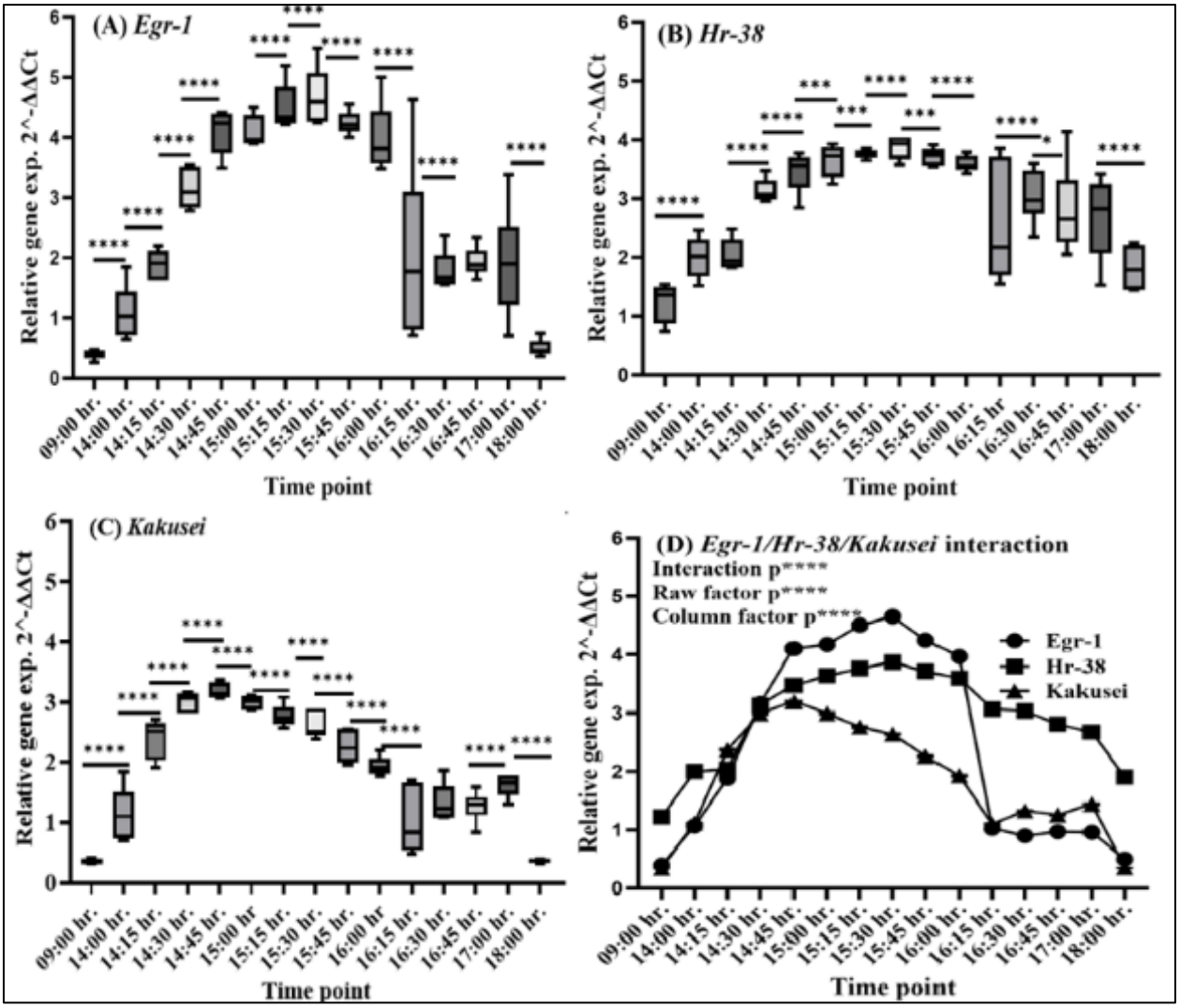

Figure 4 Summary of genetic profiling of IEGs

(A) represents *Egr-1* (B) represents *Hr38* (C) represents *Kakusei* (D) represents *Egr-1*, *Hr38* and *Kakusei* interaction summery. Data are shown as fold changes with respect to 14:00 hr., the time point at which the bees began foraging. 09:00 hr. is the time of collection in the hive before foraging and 18:00 hr. is the time after foraging respectively while the rests are the different times of collection with 15 min intervals during food regard foraging. Figures (A) *Egr-1*, (B) *Hr-38* and (C) *Kakusei* are graphical representations of the relative fall change ($\Delta\Delta^{-}CT$) at different time points. Statistical difference between adjacent time points were analyzed using One-way ANOVA with Sidak's multiple comparisons test. Figure (D) represents the graphical view of interaction among the three genes across the time points, and the interaction analysis was carried out using Two-way ANOVA with Sidak's multiple comparisons test. The p values greater than 0.05 are considered statically not significant and * indicates P< 0.05, ** indicates P< 0.01, *** indicates P < 0.001, **** indicates P < 0.0001.

Table 2 One-way Anova with Sadak's multiple comparisons test for examining statistical difference between consecutive time points for *Egr-1*, *Hr38* and *Kakusei*, showing adjusted P-values

Comparing groups	***Egr-1***		***Hr-38***		***Kakusei***	
	P-value	Effect size (95%CI)	P-value	Effect size (95%CI)	P-value	Effect size (95%CI)
14:00 vs. 09:00 hr.	<0.0001	0.669 (0.583 - 0.755)	<0.0001	0.774 (0.695 - 0.853)	<0.0001	0.771 (0.723 - 0.819)
14:15 vs. 14:00 hr.	<0.0001	0.813 (0.727 - 0.899)	0.7449	0.046 (-0.033 - 0.125)	<0.0001	1.249 (1.201 - 1.297)
14:30 vs. 14:15 hr.	<0.0001	1.275 (1.189 - 1.361)	<0.0001	1.090(1.011 - 1.169)	<0.0001	0.623 (0.575 - 0.671)

14:45 vs. 14:30 hr.	<0.0001	0.946 (0.860 - 1.032)	<0.0001	0.338 (0.258 - 0.417)	<0.0001	0.201(0.153 - 0.249)
15:00 vs. 14:45 hr.	<0.0001	0.069 (0.017 - 0.155)	<0.0001	0.170(0.091 - 0.250)	<0.0001	-0.198 -(0.246 - 0.150)
15:15 vs. 15:00 hr.	<0.0001	0.332 (0.246 - 0.418)	0.0003	0.118(0.039 - 0.197)	<0.0001	-0.232 -(0.280 - 0.184)
15:30 vs. 15:15 hr.	<0.0001	0.150 (0.064 - 0.236)	0.0008	0.111(0.032 - 0.190)	<0.0001	-0.125 -(0.173 - 0.077)
15:45 vs. 15:30 hr.	<0.0001	-0.408 -(0.494 - 0.322)	<0.0001	-0.161 -(0.240 - 0.082)	<0.0001	-0.378 -(0.426 - 0.330)
16:00 vs. 15:45 hr.	<0.0001	-0.280 -(0.366 - 0.194)	0.0006	-0.113 - (0.192 - 0.034)	<0.0001	-0.331 -(0.379 - 0.283)
16:15 vs. 16:00 hr.	<0.0001	-2.939 -(3.022 - 2.856)	<0.0001	-0.533 -(0.638 - 0.428)	<0.0001	-0.827 -(0.890 - 0.764)
16:30 vs. 16:15 hr.	<0.0001	-0.130 -(0.209 - 0.051)	>0.9999	-0.030 (-0.156 - 0.095)	<0.0001	0.229 (0.154 - 0.304)
16:45 vs. 16:30 hr.	0.0827	0.073 (-0.006 - 0.1518)	0.5005	-0.223 - (0.348 - 0.097)	0.0827	-0.071 (0.146 - 0.004)
17:00 vs. 16:45 hr.	<0.0001	-0.010(0.089 - 0.069)	0.0181	-0.139 -(0.265 - 0.013)	<0.0001	0.182(0.107 - 0.257)
18:00 vs. 17:00 hr.	<0.0001	-0.457 -(0.523 - 0.391)	<0.0001	-0.768 -(0.873 - 0.663)	<0.0001	-1.085 -(1.148 - 1.022)
09:00 vs. 18:00 hr.	>0.9999	0.103(0.033 - 0.174)	<0.0001	0.680 (0.601 - 0.759)	>0.9999	0.008 (-0.040 - 0.056)

Note: The P-values less than 0.05 is considered statically significant. 09:00 hr. is the time before foraging, and 18:00 hr. is the time after foraging, and the rest of the time points are during foraging. Effect size represents the mean difference, and CI stands for confidence interval.

Table 3 Two-way Anova Sadak's multiple comparisons test for examining interaction among *Egr-1*, *Hr38* and *Kakusei*, showing adjusted P values at each comparing time points.

Time points	***Egr-1* vs. *Hr-38***		***Egr-1* vs. *Kakusei***		***Hr-38* vs. *Kakusei***	
	P-value	Effect size (95% CI)	P-value	Effect size (95% CI)	P-value	Effect size (95% CI)
09:00 hr.	<0.0001	-0.826 -(0.889 - 0.763)	0.1541	0.051 (-0.012 - 0.114)	<0.0001	0.877 (0.814 - 0.940)
14:00 hr.	<0.0001	-0.931 -(0.994 - 0.868)	<0.0001	-0.051 (-0.114 - 0.012)	<0.0001	0.880 (0.817 - 0.943)
14:15 hr.	<0.0001	-0.164 -(0.227 - 0.101)	0.1541	-0.487 -(0.550 - 0.424)	<0.0001	-0.323 -(0.386 - 0.260)
14:30 hr.	0.8122	0.021 (-0.042 - 0.084)	<0.0001	0.165 (0.102 - 0.228)	<0.0001	0.144 (0.081 - 0.207)
14:45 hr.	<0.0001	0.629 (0.566 - 0.692)	<0.0001	0.910 (0.847 - 0.973)	<0.0001	0.281 (0.218 - 0.344)
15:00 hr.	<0.0001	0.528 (0.465 - 0.591)	<0.0001	1.177 (1.114 - 1.240)	<0.0001	0.649 (0.586 - 0.712)
15:15 hr.	<0.0001	0.742 (0.679 - 0.805)	<0.0001	1.741 (1.678 - 1.804)	<0.0001	0.999 (0.936 - 1.062)
15:30 hr.	<0.0001	0.781 (0.718 - 0.844)	<0.0001	2.016 (1.953 - 2.079)	<0.0001	1.235 (1.172 - 1.298)
15:45 hr.	<0.0001	0.534 (0.471 - 0.597)	<0.0001	1.986 (1.923 - 2.049)	<0.0001	1.452 (1.389 - 1.515)
16:00 hr.	<0.0001	0.367 (0.304 - 0.430)	<0.0001	2.037 (1.974 - 2.100)	<0.0001	1.670 (1.607 - 1.733)

16:15 hr.	<0.0001	-2.039 -(2.139 -1.939)	0.2043	-0.075 (-0.175 - 0.025)	<0.0001	1.964 (1.864 - 2.064)
16:30 hr.	<0.0001	-2.139 -(2.239 -2.039)	<0.0003	-0.434 -(0.534 - 0.334)	<0.0001	1.705 (1.605 - 1.805)
16:45 hr.	<0.0001	-1.843 -(1.943 -1.743)	<0.0001	-0.29 -(0.390 - 0.190)	<0.0001	1.553 (1.453 - 1.653)
17:00 hr.	<0.0001	-1.714 -(1.814 -1.614)	0.0001	-0.482 -(0.582 - 0.382)	<0.0001	1.232 (1.132 - 1.332)
18:00 hr.	<0.0001	-1.403 -(1.466 -1.340)	0.0023	0.146 (0.083 - 0.210)	<0.0001	1.549 (1.486 - 1.612)

Note: The P-values less than 0.05 is considered statically significant. 09:00 hr. is the time before foraging, and 18:00 hr. is the time after foraging, and the rest of the time points are during foraging. Effect size represents the mean difference, and CI stands for confidence interval.

4. Discussion

Involvement of IEGs in honeybee foraging has been documented in many research reports. As the IEGs immediately response to environmental stimuli in the central nervous system (CNS), they serve as potential indicators for neuronal activity in the CNS. Our recent studies have revealed that IEGs such as *Egr-1*, *Hr-38* and *Kakusei* are involved in honeybee foraging and moreover their possible roles in associative learning and memory during foraging have also been suggested (Singh et al., 2018; Singh & Takhellambam, 2021; Singh et al., 2020). Further, the immediate expression of *Egr-1*, *Hr-38* and *Kakusei* during foraging was induced by food reward but not by random search for food and the response of these genes were independent of the time of foraging, whether morning, noon or evening. Hence, food needs to be rewarded to study the roles of these genes during foraging. Interestingly, these genes not only response immediately, but their expression level also remain significantly higher than the basal level during the two hours of foraging (Singh et al., 2018; Singh et al., 2020). It is highly possible that these genes may continue to upregulate during the entire duration of foraging, thereby their possible role throughout the entire duration of foraging. Considering the importance of *Egr-1*, *Hr-38* and *Kakusei* in the regulation of foraging, we extended one more hour to our previous experiment and examined the expression profile of these genes in three hours of food-reward foraging. It may be reminded that a honeybee makes about 20 trips to and fro from the hive to the feeder, then stop food collection for the day. And the 20 trips used to be completed within the three hours, as observed in our bee house. Therefore, we assume that the three hours could be the time which a honeybee could invest in foraging in one day.

The result in this study indicates that, all the three genes may involve during the entire duration of food-reward foraging. Because the expression level of these genes during foraging were significantly higher than the levels of before and after foraging (Figure 4A, 4B, 4C). This reveals that *Egr-1*, *Hr-38* and *Kakusei* not only may initiate or motive the continues foraging, but they may also play a potential role throughout the entire duration of food-reward foraging. We also observed that the expression levels of the three genes varied across the duration of the foraging of the bees, suggesting that degree of involvement of these genes in foraging may vary. Because pick level of *Egr-1* is higher than *Hr-38* and *Kakusei* (Figure 4D). Again, after two hours, *Egr-1* level sharply dropped but not for *Hr-38*, suggests that *Hr-38* function remain to be stronger than *Egr-1* and *Kakusei*: the level of *Kakusei* sharply dropped after 14:45 hours. In this regard, earlier studies that showed upregulation of different IEGs at different times within the same or different regions of the brain may be mentioned (Hansson & Fuxe, 2008; Kato et al., 1997; Vazdarjanova et al., 2002). The limitation of our study is that all our interpretation is based on the genetic profiles only. Further studies using the proteins coded by these genes will further validate our finding, but not for *Kakusei* as it is a non-coding RNA. Moreover, the sample size at each time point is only 5/6, further studies with more larger sample size is warranted. Additionally, the experimental condition for behavior is not fully natural as the bees can only fly within the bee house. Apart from this, the bees have full access to the natural environmental conditions, air, sunlight, plants etc. We believe such experiments performing in the semi natural conditions are rare and thereby the results will be more application to the natural conditions.

Although our previous studies have already evidenced the role of *Egr-1*, *Hr-38* and *Kakusei* during foraging of honeybees, this study further added to the previous knowledge by showing that roles might be indispensable during the entire period of food-reward foraging. It may be noted that, *Egr-1* downstream genes like ecdysone receptor (*EcR*), dopamine/ecdysteroid receptor (*DopEcR*), dopamine decarboxylase and dopamine receptor 2 (*DopR2*), which are components of ecdysteroid signaling pathway, are involved in honeybee foraging (Singh et al., 2018). This indicates possible involvement of *Egr-1* regulatory pathway that may probably involve in learning and memory processes in honeybee foraging. Meanwhile role of *Egr-1* in learning, memory and psychiatric disorders have been well documented (Gallo et al., 2018). Furthermore, studies in vertebrates also showed that the products of IEGs regulate the expression

of downstream genes that are involved in neural homeostasis and synaptic plasticity (Beckmann & Wilce, 1997; Clayton, 2000; Loebrich & Nedivi, 2009). Additionally, response of different IEGs to a stimulus at different times within the same or different regions of the brain (Hansson & Fuxe, 2008; Kato et al., 1997; Vazdarjanova et al., 2002) may regulate different behavioral functions. Therefore, there is possibility that *Egr-1*, *Hr-38* and *Kakusei* may have different roles during foraging keeping in mind that honeybee foraging is comprise of various behavioral features. Our present study does not show any specific roles of any of these genes during foraging. Gene knockout or knockdown will be required to examine their specific roles, and this may be done using other experimental model systems such as drosophila melanogaster, but will be difficult to do in honeybees because, foraging bees cannot survive in isolation. Understanding the details of regional wise IEG expression pattern in the brain will also provide information in finding various cellular and molecular paths that link to specific behavioral features, more precisely, because different brain regions control different behavioral tasks. It has been repeatedly shown that IEGs are powerful tools for finding neuronal pathways link to behaviors in insects as well as vertebrates (Singh, 2019; Sommerlandt et al., 2019; Singh, 2014; Singh and Chanu, 2025). Our finding in this study further strengthens the importance of *Egr-1*, *Hr-38* and *Kakusei* not only in honeybee foraging but also in further research of understanding complex regulatory mechanisms of different specific behavioral characteristics in which these IEGs may be used as a potential search tool, across insects to mammals.

5. Conclusion

Dynamic roles of Immediate early genes (IEGs) in honeybee foraging have been consistently demonstrated in our previus studies. This study further reveals that (IEGs) *Egr-1*, *Hr-38* and *Kakusei* strongly involves during the entire time of food reward foraging. Therefore, *Egr-1*, *Hr-38* and *Kakusei* may be considerered as promising target genes to explore deeper understanding of honeybee foraging biology.

Compliance with ethical standards

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Disclosure of conflict of interest

Authors declare no conflict of interest.

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