Doctoral Dissertation (Censored)

博士論文（要約）

# Identification and analysis of target genes of ecdysone receptor in worker honey bee brain

# （ミツバチ働きバチの脳におけるエクダイソン受容体の標的遺伝子の同定と解析）

A Dissertation Submitted for the Degree of Doctor of Philosophy

December 2021

令和３年１２月博士（理学）申請

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## Abstract

Social bees such as honey bees and bumble bees live in a colony comprising a reproductive queen, and males and non-reproductive female workers. European honey bee (*Apis mellifera* L.) workers exhibit a division of labor according to their age after eclosion, in which younger bees engage in nursing their brood (nurse bees) and older bees engage in foraging for foods outside the hive (foragers). The brain transcriptome changes are suggested to correspond with the behavioral changes, but the neural and molecular mechanisms underlying each labor are not yet clear.

It is noteworthy that, the workers of social bee species, share some behavioral elements of foraging behavior: searching for food, collecting nectar and pollen, remembering the location of food sources, and bringing them to the hives, regardless of the different form of the division of labors of workers; for example, the honey bee workers shift their labors associated to their age whereas bumble bee workers are engaged in different tasks depending on their body size. The molecular and neural basis that underlie the conserved behavioral elements of foraging behavior of the social bees are likely to play an important role in the control of the foraging behavior. Preceding studies used immediate early genes (IEGs), which are transiently expressed in activated neurons, as markers to detect neural activities and elucidate the molecular and neural mechanisms underlying certain behaviors in various animal species. In the honey bee, expression of IEGs and secondary upregulated genes involved in ecdysone-signaling, including *ecdysone receptor* (*EcR*), are reported to be induced in some specific brain regions during foraging flight of the worker honey bee, suggesting that some neural activities are induced in the brains of worker honey bees by the foraging flight. In my doctoral course study, I intended to identify molecular and neural bases that underly the conserved behavioral elements of the foraging behavior of social bee workers.

In Chapter 1, I investigated whether the induction of some IEGs and secondarily upregulated genes during foraging behavior is a cross-species phenomenon between the honey

bee and the bumble bee. I showed that bumble bee homologs of IEGs were also upregulated during foraging flight and highly expressed in a higher-order center of the insect brain, called the mushroom bodies. In addition, I found that the expression of bumble bee *EcR* was induced after IEG induction during foraging behavior as in the honey bee. These findings suggested that neurons activated during the foraging flight and functions of secondarily induced EcR in the brain are conserved among these two social bee species.

In Chapter 2, I focused on the role of EcR, which is upregulated in the worker honey bee brain by foraging behavior. EcR is a well-known nuclear hormone receptor for the molting hormone ecdysone and regulates metamorphosis in holometabolous insects and oogenesis in female insect ovaries. The function of EcR expressed in the adult worker honey bee brain, however, has not been analyzed. Thus, I used chromatin immunoprecipitation-sequencing analysis of the honey bee worker brains to identify EcR target genes whose expression is induced by the foraging behavior. These results are the first to suggest that at least a part of the canonical ecdysone signaling that functions during metamorphosis is also regulated by EcR in the brain during foraging behavior of honey bee workers, suggesting a novel role of ecdysone signaling in the foraging behavior.

## Abbreviations

*Am* : *Apis mellifera*

*Bi* : *Bombus ignitus*

*HR38* : *hormone receptor like 38*

*Egr1*: *early growth response 1*

*EcR* : *ecdysone receptor*

IEG: immediate early gene

MB: mushroom body

KC: Kenyon cell

20E: 20 hydroxyecdysone

USP: Ultraspiracle

EcRE: ecdysone response element

TBS: Tris-buffered saline

EDTA: ethylenediaminetetraacetic acid

MNase: micrococcal nuclease

ChIP-seq: chromatin immunoprecipitation-sequencing

RNA-seq: RNA-sequencing

qRT-PCR: quantitative reverse transcription-polymerase chain reaction

DEG: differentially expressed gene

*E74 (75, 78C, 93)*: *ecdysone-induced protein 74 (75, 78C, 93)*

*Br-C: broad-complex C*

*Ftz-f1*:*ftz transcription factor 1*

*msi*: *musashi*

*RAF2*: *RING-associated factor 2*

*SORL1*: *sortilin related receptor 1*

*JHDM2*: *JmjC domain-containing histone demethylase 2*

*mgl*: *megalin*

# General Introduction

Many animal species form societies. Among them, social insects, such as ants, termites, and some bee species, form societies with behavioral and physiological differentiation among individuals, termed division of labor, which has attracted great attention from the viewpoint of physiological polymorphisms resulting from gene expression changes but not to genomic differences (Evans & Wheeler, 2001; Glastad et al., 2015). Eusociality, known as the most advanced form of society, is characterized by the overlap of multiple generations, cooperative brood care, and reproductive division of labor, termed castes (Michener, 1969). Hymenoptera is an insect order that includes sawflies, wasps, ants, and bees, and has been widely studied to elucidate the mechanisms and evolution underlying social behaviors because it contains many species that exhibit different levels of social organization, from the primitive solitary behaviors of sawflies to the highly sophisticated behaviors of eusocial species like honey bees and bumble bees (Kocher & Paxton, 2014; Wheeler, 1986).

In some eusocial hymenopteran insects, there is not only reproductive division of labor between queen(s) and workers, but also a division of labors among workers. For example, workers of the European honey bee (*Apis mellifera*), a model organism for studies of eusociality, change their tasks in association with their age after eclosion; young bees clean the hive and take care of the eggs or larvae in the dark hive, and older bees guard against intruders in front of the hive and forage for nectar and pollen outside the hive (Seeley, 1995).

The molecular and neural mechanisms that enable social bee workers to exhibit the division of labor have remained a mystery. Recent studies revealed differences in gene expression patterns in the brain among honey bee workers executing different tasks (Lutz et al., 2012; Whitfield et al., 2003, 2006). The whole genome of the honey bee was read in 2006 (Weinstock et al., 2006), allowing for next-generation sequencing studies of expressional and epigenetic regulation of whole genes that have contributed to elucidate the molecular mechanisms

underlying the division of labors of workers associated with age (Foret et al., 2012; Guan et al., 2013; Herb et al., 2012; Liu et al., 2019). Though these studies have deepened our knowledge about the molecular and genomic traits associated with the differentiation of the division of labors of workers, the molecular mechanisms involved in the regulation of individual behaviors remain to be elucidated.

To address this question, it is useful to identify neurons activated in a behavior-dependent manner in the brain. Recent studies used several immediate early genes (IEGs) as tools to detect neural activity in the honey bee brain associated with certain behaviors, such as orientation flight (Lutz & Robinson, 2013), the alarm pheromone response (Alaux & Robinson, 2007), and foraging (Kiya et al., 2007). In addition to IEGs, some other genes are also induced in a behavior-dependent manner in the honey bee brain (Shpigler et al., 2017; Singh et al., 2018). Because these genes are induced in response to certain behaviors, analysis of the regulation and functions of these genes could be effective to investigate the molecular and neural mechanisms regulating the corresponding behaviors (Sommerlandt et al., 2019). Therefore, I intended to use IEGs to search for molecular and neural mechanisms that underlie the foraging behavior of social bee workers, which would allow me to explore the regulatory system of gene expression associated with the foraging behavior.

In my graduate school studies, I focused on the foraging behavior of two social bee species: the honey bee and the bumble bee, the latter of which is a close relative of the honey bee (Porto & Almeida, 2021). Although their divisions of labor are based on different physiological states: age after eclosion in the honey bee and body size in the bumble bee (Goulson et al., 2002), the foragers of these species share some behavioral elements, such as searching for food, collecting nectar or pollen, remembering the location of food sources, and returning to the hive. Therefore, if the response of certain genes and/or neurons associated with foraging behaviors are conserved among these two species, they could be important molecular and neural mechanisms underlying foraging behavior. To better understand the mechanisms underlying the foraging

behavior of social bees, I began with two questions: First, are the same genes upregulated by foraging flight in the honey bee brain also upregulated by foraging flight in the brain of another Apidae species, the bumble bee? Second, if this is the case, what is the role of these genes in the regulation of foraging behavior in worker bees?

My doctoral thesis consists of two chapters. In Chapter 1, to address the first question, I examined the expression levels of genes that are known to be upregulated in the honey bee brain by foraging flight in the brain of bumble bee forager. My findings revealed that upregulation of IEGs and secondary upregulation of *ecdysone receptor* (*EcR)* after foraging flight were common to the foragers of both species, and that the genes examined in my experiments were highly expressed in the mushroom bodies, a higher-order center of the insect brain in both species. In Chapter 2, based on the results of Chapter 1, I explored the downstream target genes of EcR to reveal the role of ecdysone signaling in the brains of honey bee workers associated with foraging behavior. The results of my studies suggested that genes expressed in a certain type of Kenyon cells, intrinsic neurons of the mushroom bodies, are involved in foraging behavior, and that at least some of the downstream signaling pathways induced by one of these genes, *EcR*, are involved in metabolic control.

# Chapter 1

## Expression analysis of the genes upregulated during foraging flight in the brains of both the honey bee and bumble bee

## Introduction

Eusocial bees, including the honey bee and bumble bee, exhibit highly sophisticated sociality as represented by their caste differentiation and division of labor of workers (Figs. 1 and 2). Their social behaviors, however, show some species-specific differences. The division of labor of workers in honey bees is based on their age after eclosion (Seeley, 1995; von Frisch et al., 1967); younger nurse bees take care of the brood and older foragers convey information to their nestmates about the location of a food source by performing a unique dance called the "waggle dance" (Fig. 2A). On the other hand, the division of labor of workers in bumble bees is based on their body size; smaller workers remain in the hive to engage in nursing the brood (Cameron, 1989; Goulson et al., 2002) whereas larger workers are engaged in foraging outside the hive within a few days after eclosion (Riveros & Gronenberg, 2009) (Fig. 2B). In contrast to honey bee foragers, bumble bee foragers do not communicate the location of their foraging success to their nestmates; successful foragers simply alert their nestmates to the presence of a food source by running about in the hive (Barron & Plath, 2017; Dornhaus & Chittka, 2001). Although the molecular and neural mechanisms of the foraging behaviors of honey bee workers have been intensively investigated, they have been poorly clarified in bumble bees (Kodaira et al., 2009; Shpigler et al., 2016; Tobback et al., 2011).

Involvement of the mushroom bodies (MBs), a higher-order center involved in learning and memory as well as in the integration of multimodal sensory information in the insect brain (Heisenberg, 1998), is implicated in the foraging behavior of the European honey bee (*Apis mellifera, Am*)(Dobrin et al., 2011; Fahrbach et al., 1998; Farris et al., 2001). Honey bee MBs comprise intrinsic neurons termed Kenyon cells (KCs) that are classified into four subtypes: class I large (l)-, middle (m)-, small (s)-type KCs, and class II KCs, based on the size and position of their somata and their gene expression profiles (Kamikouchi et al., 2000; Kaneko et al., 2013, 2016; Paul et al., 2006; Takeuchi et al., 2001, 2004; Yamazaki et al., 2006). The KC subtypes are

thought to be conserved among Aculeate Hymenopteran species that exhibit nest-building behaviors (Oya et al., 2017).

Earlier studies used immediate early genes (IEGs), whose expression is rapidly upregulated in certain neurons after neuronal activation, to identify the brain regions related to certain behaviors (Sommerlandt et al., 2019). Findings from these studies using a battery of IEGs, such as *kakusei* (noncoding RNA identified from *A. mellifera*)(Kaneko et al., 2013; Kiya et al., 2007), *early growth response gene-1* (*Egr1*, also known as *NGFI-A*, *Krox24*, *zif268*, and *zenk*) (Klein et al., 2010; Mello et al., 1992; Milbrandt, 1987; Ugajin et al., 2013), and *hormone receptor-like 38* (*HR38*), a member of the subfamily of nuclear receptor 4A) (Fujita et al., 2013; Maxwell & Muscat, 2006; Velarde et al., 2006; Yamazaki et al., 2006), suggested a possible role of the sKCs and some mKCs in the MBs in sensory processing during foraging flight of the honey bee (Kaneko et al., 2016). In addition to *HR38* (Baker et al., 2003), *ecdysone receptor* (*EcR*) and other ecdysone signaling-related genes suggested to be upregulated by *Egr1* in the brains of honey bee foragers (Khamis et al., 2015), such as *dopamine/ecdysteroid receptor* (*DopEcR*) and *dopa decarboxylase* (*Ddc*), are also reported to be upregulated during the foraging flight in honey bees, raising the possibility that ecdysone signaling in the honey bee brain is involved in foraging behavior (Khamis et al., 2015; Singh et al., 2018). Neural activities or gene upregulation as represented above have not been analyzed in the brains of bee species other than the honey bee.

In Chapter 1, I analyzed two IEGs, *HR38* and *Egr1*, and late-upregulated *EcR* to evaluate neural activity in the bumble bee (*Bombus ignitus*, *Bi*) and the honey bee (*A. mellifera*) during foraging flight to disclose common and species-specific features of the neural activity related to foraging. First, I confirmed that both *BiHR38* and *BiEgr1* exhibit an immediate early response similar to *AmHR38* and *AmEgr1*. Next, I analyzed the expression pattern of three bumble bee genes, *HR38*, *Egr1*, and *EcR*, in the forager brain under two experimental conditions. In the first condition, the hives were set in a greenhouse partly resembling natural conditions, and in the second condition, the hives were set in a laboratory flight-cage, enabling sampling of foragers

according to the foraging time-course. I also performed *in situ* hybridization to detect the expression profiles of these genes in bumble bee forager brains. The findings indicated that both premature mRNA for *HR38* and mature mRNA for *Egr1* are induced in the bumble bee brains during foraging flight, with sparse detection of both *HR38* and *Egr1* mRNAs inside the whole MBs, while those genes are expressed preferentially in the sKCs in the honey bee brain. In addition, I showed that expression of *BiEcR* was significantly higher in forager brains than in nurse bees and expressed preferentially in the sKCs of the MBs in foragers – the same expression pattern of *AmEcR* in the honey bee brain. These results suggest that neural activity in MBs during foraging flight and the function of ecdysone signaling in the sKCs are conserved at least among these two species.

## Materials and Methods

### *Bees*

Bumble bee (*B. ignitus*) colonies at Tamagawa University (Machida-Shi, Tokyo, Japan) are usually kept under laboratory conditions (28°C, 70% humidity, 24 h dark). For the present study, two colonies were placed in a greenhouse (Fig. 3A) and three colonies were placed in a laboratory flight-cage (1 m × 50 cm × 50 cm, equipped with pollen feeding sites and absorbent cotton soaked in sugar water, Fig. 3B), and maintained under laboratory conditions (25 ± 3°C, 73 ± 5% humidity, and natural day/light hours) at Tamagawa University. The bumble bee colonies were purchased from Agrisect Inc. (Inashiki-Shi, Ibaraki, Japan). Three European honey bee (*A. mellifera*) colonies were purchased from Kumagaya Apiary (Kumagaya-Shi, Saitama, Japan) and kept outside at the University of Tokyo (Bunkyo-Ku, Tokyo, Japan) (Fig. 3C).

### *Sampling for IEG analysis*

A total of 70 *B. ignitus* workers were randomly collected from a colony and groups of 5 workers were divided into 14 insect cages (round plastic containers, 15 cm diameter and 4.5 cm high), and kept in a dark incubator at 25°C overnight. The next morning at 8:00, all workers were set under a luminescent light in the laboratory space. Anesthesia was induced in 35 workers (7 insect cages) by supplying $CO_2$ to the insect cages for seizure induction after hypoxia (Bartel et al., 1989; Rodgers et al., 2007), and 5 min later the $CO_2$ in the 6 insect cages was exchanged with fresh air. The workers were collected at each of several time-points (0, 15, 30, 60, 120, and 180 min) after the $CO_2$ was exchanged with fresh air ("$CO_2$"). Workers anesthetized continuously with $CO_2$ for 120 min were collected as a negative control ("NC"), in which I expected to detect no IEG response. Another 35 workers (7 insect cages) that were supplied with air flow instead of $CO_2$ were collected at the same time-points (10 min before the onset of the $CO_2$ supply, 0, 15, 30, 60, 120, and 180 min after the $CO_2$-fresh air exchange) as a series of positive controls ("PC"), in

which I expected to detect the induction of IEGs due to the surrounding stimuli, but not due to exposure to high levels of $CO_2$. After the bees were immediately anesthetized in ice water, the whole brains were dissected with fine tweezers and scalpels under a binocular microscope, and then frozen at −80°C for preservation.

***Sampling for foraging flight analysis***

The sampling of *B. ignitus* foragers was performed in August 2018. For sampling in the greenhouse, "Foragers" that visited flowering fruit trees (*Pouteria lucuma*) with pollen loads and "Nurse bees" that were engaged in the in-hive tasks (feeding the brood, smoothing the nest combs, or warming eggs and pupae) were captured from the hives around 14:00. For sampling from the laboratory flight-cage, the day before the sampling day, all workers outside the hives were recovered in the hives and the hive entrances were closed. The next day at 8:30, the entrances were opened and the workers emerging from the entrances were immediately captured. At the same time, nurse bees in the hives were collected. Workers that were foraging around the pollen feeder at 3–7 min (8:37) and 25–30 min (9:00) after opening the entrance were collected. After they were anesthetized in ice water, the body size from the top of the head to the bottom of the abdomen of each bee was measured using a ruler with 1-mm resolution. For quantitative reverse transcription-polymerase chain reaction (qRT-PCR), the MBs and other brain regions were dissected as depicted in Fig. 4, and frozen at -80°C for preservation.

Sampling of *A. mellifera* foragers was performed from September to October 2019. The day before the sampling day, the bees that returned to the hives with pollen loads were caught as foragers and marked on the thorax with a non-permanent marker pen. Early the next morning (6:30), marked bees were collected from inside the hives, i.e., foragers that had not yet begun foraging that day. At 8:30, the hive entrances were opened and the foragers exiting the hive were captured immediately. Foragers returning to their hives with pollen loads were collected at 9:00, 9:30, and 12:30. Nurse bees were also collected from inside the hives at 6:30, 9:30, and 12:30

based on their behaviors of plunging their heads into honeycomb cells that contained larvae more than twice as evidence of nursing their broods (Ugajin et al., 2018). Half of the foragers and nurse bees captured at 12:30 were incubated in a dark incubator till 22:30. All the bees were promptly anesthetized in ice water and their brains were dissected for qRT-PCR as described above. Because the hypopharyngeal glands, which synthesize royal jelly used as food for larvae, are well developed in nurse bees but shrunken in foragers (Huang et al., 1994), nurse bees collected were further screened under a binocular microscope to collect brains only from bees with well-developed glands (Ueno et al., 2016).

***qRT-PCR analysis***

Expression analysis by qRT-PCR was performed essentially as described previously (Ugajin et al., 2013) using TB Green *Premix Ex Taq II* (Tli RNaseH plus; Takara) and gene-specific primers (Table 1) with a Light Cycler 480 Instrument II (Roche Life Science, Indianapolis, IN, USA).

The PCR conditions were as follows: 95℃, 5 min, (95℃, 10 s; annealing temperature of each gene is shown in Table 1, 10 s; 72℃, 10 s) × 45 cycles, 65℃, 1 min; 97℃, 0 s; and 40℃, 30 s. The selectivity of all primers was verified by agarose gel electrophoresis of the RT-PCR products amplified using Ex Taq Hot Start Version (Takara) and by analyzing melting curves of the qRT-PCR products. The expression of each gene was normalized to that of *EF1α* and *Actin* because normalization with multiple housekeeping genes enables detection of reasonable relative expression levels. The relative expression was calculated using the ΔΔ Ct method (ABI user bulletin #20). The calibration samples were obtained from the brains of bees collected at 0 min after the cessation of anesthesia in the $CO_2$ group for the immediate early response validation of *B. ignitus* from the MBs of nurse bees in the greenhouse experiment of *B. ignitus*, from the MBs of nurse bees collected at 8:30 in the laboratory flight-cage experiment of *B. ignitus*, and from the MBs of nurse bees collected at 6:30 in the foraging flight experiment of *A. mellifer*a.

***In situ hybridization***

I used forager brains collected for the above-described experiment in *B. ignitus* to evaluate *BiHR38*, *BiEcR*, and *BiEgr1* expression. Whole brains embedded in Tissue-Tek O.C.T. Compound (SAKURA Finetek) were frozen and sliced into 10-μm-thick sections. The cDNA fragments, corresponding to the *BiHR38*, *BiEcR,* and *BiEgr1* coding regions, were amplified from *B. ignitus* cDNA using gene-specific primers (Table 1). *In situ* hybridization was performed with digoxigenin-labeled riboprobes essentially as described previously (Suenami et al., 2016). Images of the brain slices were obtained using an optical microscope (BX-50, Olympus) and multiple photos were merged using Adobe Photoshop (CS3 EXTENDED ver.10.0, Adobe Systems) if necessary.

***Statistical analysis***

All statistics were performed using R statistical software (ver.3.3.3). For IEG analysis, two-way ANOVA (factor 1, treatment; factor 2, time) was performed. After that, for the $CO_2$ and PC groups, one-way ANOVA and Dunnett's test ($CO_2$ groups were compared with the 0-min group, the PC groups were compared with the -10-min group). To compare the NC group with the $CO_2$ 0-min group, an F test followed by Student's t test or Welch's t test was performed. The expression of *EF1α* and *Actin* differed slightly among the groups (Tukey-Kramer test after two-way ANOVA, $CO_2$-NC: $p < 0.01$, $CO_2$-PC: $p < 0.001$). For the greenhouse sampling of *B. ignitus*, two-way ANOVA (factor 1, tissue; factor 2, bee type) was performed. After the F test, Student's t test or Welch's t test was performed to compare each tissue between bee types. The expression of *EF1α* and *Actin* did not differ significantly for each bee type, but did differ significantly for each tissue by two-way ANOVA. For the laboratory flight-cage sampling of *B. ignitus*, three-way ANOVA (factor 1, tissue; factor 2, bee type; factor 3, time) was performed, and then the Tukey-Kramer test was performed to compare between bee type and flight time for each tissue. The expression of *EF1α* differed significantly depending on the tissue ($p < 0.001$) and bee type ($p < 0.05$), and

the expression of *Actin* differed significantly depending on the bee type ($p < 0.05$). For the sampling of *A. mellifera*, three-way ANOVA (factor 1, tissue; factor 2, bee type; factor 3, time) was performed for all samples. For each tissue, the Tukey-Kramer test was used to compare the upregulation dependence on the time-course, and Student's t test or Welch's t test was performed after the F test to compare nurse bees and foragers at each time-point. The expression of *EF1α* differed significantly depending on the tissue ($p < 0.001$), bee type ($p < 0.001$), and time ($p < 0.001$), and the expression of *Actin* differed significantly depending on the bee type ($p < 0.05$). The body sizes of *B. ignitus* were compared using Student's t test and the Tukey-Kramer test.

## Results

### *Validation of the IEG response of B. ignitus genes by qRT-PCR*

I first examined whether *BiHR38*, *BiEgr1*, and *BiEcR* show an immediate early response. I also examined premature mRNA for *BiHR38* (termed pre*BiHR38*), because *HR38* is induced a little later than *Egr1* (Kendall et al., 1994). For this, *B. ignitus* workers were anesthetized with $CO_2$ and seizures were induced by awakening them from anesthesia. After $CO_2$ was supplied to the insect cages, all the bees fainted within 5 min. When the $CO_2$ was exchanged with fresh air (cessation of anesthesia), some workers began to twitch their legs within a few minutes, which is a typical movement related to $CO_2$-induced seizures (Kiya et al., 2007). Within 15 min, some bees got up and a few of them began to walk. Within 30 min, almost all workers were up, breathing with their abdomen, or grooming. Within 60 min, some workers were walking or flying, and approximately 60% of workers within 120 min and all of them within 180 min were actively walking or flying about the cage.

The qRT-PCR results indicated that the *BiHR38* expression level normalized to that of *BiEF1α* (Fig. 5A) changed depending on both the $CO_2$ treatment and time after cessation of anesthesia (factor 1, 2: $p < 0.001$, two-way ANOVA). The relative *BiHR38* expression level increased beginning 30–60 min after the cessation of $CO_2$ anesthesia and peaked at 120 min (Fig. 5A(i)). In contrast, in the NC group, which was continuously anesthetized with $CO_2$ for 120 min, the *BiHR38* expression level did not change significantly compared with that at 0 min after the cessation of anesthesia as assessed by Student's t test, indicating that the induction of *BiHR38* expression is associated with awakening from anesthesia. The *BiHR38* expression level in the PC group also changed slightly depending on the time after cessation of anesthesia. The *BiHR38* expression level in the $CO_2$ group was 22-fold higher than that in the PC group at the upregulation peak at 120 min (mean relative expression [normalized by *EF1α*], $CO_2$: 52.1, PC: 2.4). Essentially, the same results were obtained for the *BiHR38* expression level normalized with *BiActin* (Fig.

5B(i)). The expression of pre*BiHR38* in the $CO_2$ group also tended to increase for 15–60 min, and then rapidly decreased at 120 min after the cessation of anesthesia (Fig. 5A(ii)). Although pre*BiHR38* expression did not differ significantly between the 15–60-min time-points and that at 0 min in the result normalized with *EF1α* (Fig. 5A(ii)), the expression normalized with *Actin* was significantly different between 0 min and 60 min (Fig. 5B(ii), $p < 0.05$, Dunnett's test). This finding suggests that *BiHR38* is an IEG induced in the brain by seizures. The expression of *BiEgr1* changed more rapidly than that of *BiHR38* (Fig. 5A(iii) and Fig. 5B(iii)). The expression of *BiEgr1* increased beginning at 15–30 min, peaked at 60 min, and then decreased at 120 and 180 min. The expression changed depending on the treatment (factor 1: $p < 0.001$, two-way ANOVA) and time after the cessation of anesthesia (factor 2: $p < 0.001$). The *BiEcR* expression level in the $CO_2$ group differed significantly compared with that in the other two control groups ($CO_2$-NC: $p < 0.01$, $CO_2$- PC: $p < 0.001$, post hoc Tukey-Kramer test), as was also the case for *BiHR38*. In contrast, *BiEcR* expression increased only slightly (~2-fold) by 180 min after the cessation of anesthesia (mean of the relative expression in the $CO_2$ group at 180 min compared with that at 0 min: 2.1 in Fig. 5A(iv) and 1.8 in Fig. 5B(iv)). The change in gene expression, however, was independent of both the treatment and time after the cessation of anesthesia (factor 1: $p = 0.16$, factor 2: $p = 0.42$, two-way ANOVA). These findings indicated that both *BiHR38* and *BiEgr1*, but not *BiEcR*, exhibit an immediate early response.

***Analysis of gene expression of B. ignitus during foraging flight by qRT-PCR***

First, I analyzed the expression levels of *BiHR38*, *BiEgr1*, and *BiEcR* of *B. ignitus* between nurse bees and foragers captured in the greenhouse as a natural foraging condition. I examined not only the MBs but also other brain regions to explore the major brain regions that are active during the foraging flight. The gene expression level was normalized with that of either *BiEF1α* or *BiActin* (Fig. 6A and Table 2). The expression of *BiHR38* was significantly higher in foragers than in nurse bees in both the MBs (~2.0-fold) and the other brain regions (~2.7-fold; $p < 0.05$, Student's t test

in Fig. 6A(i); expression fold calculated from the mean of each group in Table 2A). The expression of *BiEgr1*, whose honey bee homolog notably increases during the foraging flight (Lutz & Robinson, 2013; Singh et al., 2018; Ugajin et al., 2013), was also significantly higher in foragers than in nurse bees in MBs (~3.8-fold) as well as in other brain regions (~2.6-fold). The expression of *BiEcR* was also slightly but significantly higher in foragers than in nurse bees in the MBs (~1.5-fold) and other brain regions (~1.4-fold). Moreover, the expression levels of *BiHR38*, *BiEgr1*, and *BiEcR* differed significantly between the MBs and other brain regions (factor 1: $p < 0.05$, two-way ANOVA on each gene). Interestingly, however, whereas the expression of *BiHR38* and *BiEgr1* was higher in the MBs than in the other brain regions, *BiEcR* expression was higher in brain regions other than the MBs. Essentially, the same results were obtained when the gene expression level was normalized with *BiActin* (Fig.6A(ii) and Table 2B).

Next, I analyzed whether the expression levels of *BiHR38*, *BiEgr1*, and *BiEcR* in the MBs and other brain regions of foragers change during the foraging flight. *B. ignitus* foragers kept in the laboratory flight-cage exited the bee hive as soon as the hive entrance was open at 8:30. Because the feeder was so close to the hive entrance, it took less than 30 min to complete a single foraging flight (i.e., searching for the feeder, obtaining pollen loads, and returning to the hive). The gene expression level was normalized with that of either *BiEF1α* or *BiActin* (Fig. 6B and Table 3). The expression level of *BiHR38* normalized with that of *BiEF1α* changed depending on the tissue and bee type, but did not change depending on the flight time (factor 1, 2: $p < 0.001$, factor 3: $p = 0.57$, three-way ANOVA). *BiHR38* expression in both the MBs and other brain regions was significantly higher (~2.4–2.8-fold in the MBs and 1.9–2.3-fold in other brain regions) in foragers than in nurse bees at any time-point after the onset of foraging, while no significant change in *BiHR38* expression was detected in relation to the time after the onset of a foraging flight ($p > 0.05$, Tukey-Kramer [Fig. 6B(i)]; expression fold calculated from the mean of each group in Table 3A). Significant upregulation of pre*BiHR38* was observed in the MBs at 30 min after the onset of a foraging flight ($p < 0.05$, Tukey-Kramer test). The expression level of

*BiEgr1* changed depending on the tissue and flight time, but did not change in relation to the bee type (factor 1, 3: $p < 0.001$, factor 2: $p = 0.10$, three-way ANOVA). *BiEgr1* expression in the MBs and other brain regions was significantly higher in foragers at 30 min after the onset of foraging (~2.7-fold in the MBs and 1.3-fold in other brain regions compared with foragers at 8:30and those at 9:00 in Table 3). Whereas the expression level of *BiEcR* changed depending on the tissue and bee type (factor 1, 2: $p < 0.001$, factor 3: $p = 0.22$, three-way ANOVA), significant upregulation was not observed in either tissue at any time-point after the onset of the foraging flight (Fig. 6B(i)). Additionally, the *BiEcR* expression level in the MBs was higher (~1.6-fold) in foragers at 9:00 than in nurse bees, and that in the other brain regions was higher (~1.3-fold) in foragers at 9:00 than in nurse bees (Table 3A). Essentially, the same results were obtained for gene expression levels normalized with that of *BiActin* (Fig. 6B(ii) and Table 3B).

Taken together, these findings indicated that the expression of both pre*BiHR38* and *BiEgr1*, but not *BiEcR*, significantly increased in association with the foraging flight for 30 min. Moreover, the expression level of *BiHR38* and *BiEgr1* was higher in the MBs than in other brain regions, whereas the expression level of *BiEcR* was higher in brain regions other than the MBs.

The body sizes of the foragers were significantly larger than those of nurse bees in both the greenhouse and laboratory experiments (Fig. 7, $p < 0.05$, Student's t test and the Tukey-Kramer test), which is consistent with a previous observation that relatively larger bumble bee workers tend to be engaged in foraging and smaller workers tend to engage in the in-hive tasks.

***Reexamination of gene expression in A. mellifera during foraging flight by qRT-PCR***

To compare the gene expression patterns in *B. ignitus* with those in *A. mellifera*, I reexamined the neural activity of *A. mellifera* during foraging flight using the same sampling protocol. As seen in the laboratory flight-cage experiment for *B. ignitus*, *A. mellifera* workers came out from the open-air hive as soon as the hive entrance was open at 8:30. The expression level of each gene was normalized with that of either *AmEF1α* or *AmActin* (Fig. 8 and Table 4). The relative

expression levels of *AmHR38* normalized with that of *AmEF1α* changed depending on the tissue (MBs or the other brain regions), bee type (nurse bees or foragers), and time (factor 1, 2, 3: $p < 0.001$, three-way ANOVA). The expression level of *AmHR38* in both brain tissues of the forager was not significantly different until 60 min after the onset of the foraging flight (9:30), and was significantly higher in active foragers at 12:30 (Figs. 8A,B). The expression of *AmHR38* in foragers was significantly different from that of nurse bees at 60 min (9:30) and 4 h (12:30) after the onset of the foraging flight (Fig. 8A, ~4.9-fold in the MBs and 4.5-fold in other brain regions, calculated with the data in Table 4A). The expression decreased at 22:30 in foragers that were captured after the foraging flight and kept in a dark incubator for 10 h. pre*AmHR38* was markedly upregulated from 30–60 min after the onset of the foraging flight and the expression level was maintained in foragers at 12:30 and again decreased in foragers at 22:30 (Fig. 8A). Together, these findings indicate that *AmHR38* was induced by the foraging flight. The change in *AmEgr1* expression also depended on the bee type and flight time (factor 2, 3: $p < 0.01$, factor 1: $p = 0.61$, three-way ANOVA). The expression level of *AmEgr1* at 12:30 was significantly higher in foragers than in nurse bees in both the MBs and other brain regions (Fig. 8A, ~5.7-fold in the MBs and 3.5-fold in other brain regions), and again decreased in foragers at 22:30. *AmEcR* expression in the MBs was also significantly higher (~2.6-fold) in foragers than in nurse bees at 12:30, but there was no significant difference in the *AmEcR* expression levels in the other brain regions between nurse bees and foragers at 12:30 (Fig. 8A, same trend as shown in Fig. 5B). Although gene expression levels in nurse bees also changed significantly at some sampling points (*AmHR38* in the MBs at 22:30 in Fig. 8A, *AmEgr1* in the other brain regions at 12:30 in Fig. 8B), significant differences were not consistently detected for the expression levels normalized with that of either *AmEF1α* or *AmActin*.

Taken together, these results suggested that both *HR38* and *Egr1* were significantly upregulated by foraging flight in both *B. ignitus* and *A. mellifera*, and a slight upregulation was also observed for late-induced gene *EcR*.

***Detection of activated cells by in situ hybridization***

I performed *in situ* hybridization analysis using brain sections of *B. ignitus* foragers to detect the cells activated in the forager brains. Both *BiHR38* and *BiEgr1* were strongly expressed in the MBs (Figs. 9A–D and E–G, respectively). Cells activated by *BiHR38* and *BiEgr1* were detected sparsely in the whole KCs (Figs. 9C, D, G). On the other hand, *BiEcR* was detected preferentially and locally in the small-type KCs in the MBs (Figs. 9H–J). *BiEcR* was also weakly detected in the whole brain cortex (Fig. 9H). Thus, consistent with qRT-PCR analysis, these findings suggested that both *BiHR38* and *BiEgr1* were induced mainly in the MBs, and that *BiEcR* was expressed both in the sKCs and other brain regions, as previously reported in *A. mellifera* (Takeuchi et al., 2007; Ugajin et al., 2013; Yamazaki et al., 2006).

## Discussion

After awakening from $CO_2$ anesthesia, the expression of pre*BiHR38*, *BiHR38*, and *BiEgr1* was prominently induced, indicating that both *HR38* and *Egr1* exhibit an immediate early response in the brains of *B. ignitus*. *BiEgr1* was induced earlier (~30 min after the cessation of anesthesia) than *BiHR38* (~60 min; Fig. 5), which is consistent with previous studies in moths (Fujita et al., 2013), flies (Fujita et al., 2013), honey bees (Ugajin et al., 2018), and mammals (Kendall et al., 1994). Expression of *BiHR38* and *BiEgr1* was very low, but significantly upregulated in the PC group, suggesting that novel surroundings, such as light exposure or unfamiliar visual objects, stimulated the bees (Lutz & Robinson, 2013; Sommerlandt et al., 2017). Expression of pre*BiHR38* was also induced transiently within 30 min, earlier than *BiHR38* expression (Fig. 5), which likely reflects the time needed for premature mRNA to be processed into mature mRNA. A previous study showed that induction of the expression of honey bee IEGs such as *kakusei* and *AmEgr1* is much more rapid –within 15–30 min after the cessation of anesthesia (Kiya et al., 2007; Ugajin et al., 2013). In contrast, the present results showed that the increased expression of both pre*BiHR38* and *BiEgr1* was significant and prominent within 30 min after the cessation of $CO_2$ anesthesia (Fig. 5). These findings give rise to the possibility that $CO_2$ treatment (~5 min), which was long enough to fully anesthetize the large *B. ignitus* workers, caused deep anesthesia and that it took a relatively long time for the workers to awake from the anesthesia, which resulted in a time-lag between the cessation of anesthesia and IEG expression. On the other hand, *BiEcR* expression was not significantly induced till 120 min (Fig. 5), and then expressed higher at 180 min after the awakening from anesthesia, indicating that *BiEcR* did not exhibit an immediate early response. Unexpectedly, only low expression of *BiHR38* and *BiEgr1* was induced in bees that were exposed continuously to the same luminescent light without $CO_2$ treatment, which I originally had expected to act as a positive control. It is possible that bees were exposed to some light while being kept in a dark incubator, so that the luminescent light might not have been

sufficient stimulus for activating IEGs in this study.

For gene expression analysis during the foraging flight, I set colonies in a greenhouse and in a laboratory flight-cage for the sampling of bumble bee workers, but I used colonies maintained outside for the sampling of honey bees. This was because honey bee workers tended to gather around the fluorescent lamp, making it difficult for them to forage normally in the restricted laboratory space. In contrast, the foraging of bumble bees did not seem to be affected in the greenhouse or in the laboratory flight-cage. In the small flight-cage, bumble bees tended to complete one foraging flight in less than 30 min, and therefore I collected workers at three time-points within 30 min after the onset of the foraging flight. I found that expression of pre*BiHR38* and *BiEgr1* was upregulated 30 min after the onset of the foraging flight, and was more prominent in the MBs than in the other brain regions, suggesting that the MB neural activity increased in the bumble bee during the foraging flight. It is plausible that induction of pre*BiHR38* preceded that of *BiHR38* and eventually resulted in the increased expression of *BiHR38* in foragers at 14:00 (Fig. 6A). In contrast, *BiEcR* was not significantly upregulated by the foraging flight at 30 min (Fig. 6B), which is consistent with my previous finding that *BiEcR* did not show an immediate early response until 120 min after awakening from anesthesia (Fig. 5). Like *BiHR38* and *BiEgr1*, however, the expression level of *BiEcR* was also higher in foragers than in nurse bees collected at 14:00 (Fig. 6A). This might be explained by the fact that the expression level of *BiEcR* was slightly, but significantly, higher in the $CO_2$-treated group 180 min after awakening from anesthesia (Fig. 5). It might also be that *BiEcR* was upregulated later by the foraging experience, as reported previously (Singh et al., 2018).

Although I first expected that *AmHR38*, *AmEgr1*, and *AmEcR* were upregulated in the brain within 30 min from the onset of foraging based on a previous report, they were not upregulated even at the end of a foraging bout, except for pre*AmHR38* (Fig. 8). The expression of *AmHR38* did not change for 30 min after the onset of foraging when pre*AmHR38* was already upregulated (Fig. 8), suggesting that *AmHR38* could be induced by a single foraging flight longer

than 30 min. This could account for the finding that the *AmHR38* expression level was higher in foragers than in nurse bees collected at 12:30(Fig. 8). Also, neither *AmEgr1* nor *AmEcR* was upregulated in the MBs within 30 min after the onset of the foraging flight, but the expression of both was higher in foragers than in nurse bees collected at 12:30 and downregulated in foragers captured and kept in a dark incubator for 10 h (Fig. 8). It might be that *AmEgr1* induction could not be detected 30 min after the onset of the foraging flight because I did not analyze the foraging flight time of individual workers: I might have collected workers that had been engaged in foraging for less than 30 min, even 30 min after the entrance was opened to allow them to forage freely.

Also, in the present study, *BiHR38* and *BiEgr1* expression levels were high (~2-3-fold) in foragers at 8:30 before the onset of the foraging flight compared with that in nurse bees at the same time, and these genes were preferentially expressed in the MBs over the other brain regions (Fig. 6). This was also at least partly true for the honey bees; the *AmHR38* expression level was higher in foragers than in nurse bees at 6:30 (~2-fold; Fig. 8). I assume that the MB neural activity of foragers at that time was already upregulated, although the foragers inside the hive had not yet engaged in foraging. Eusocial bee foragers (*A. mellifera* and *B. terrestris*) have a steady circadian rhythm as they work outside in daylight and are influenced by temperature, whereas the circadian rhythm in nurse bees is attenuated as they work all day in a dark hive with a constant temperature (Bloch, 2010; Bloch et al., 2001; Nagari et al., 2017; Yerushalmi et al., 2006). Bumble bee (*B. terrestris*) foragers in the wild become active at almost 6:00 when they are turning out from the hive by ones and twos (Stelzer & Chittka, 2010). In the present study, the hive entrance was shut until 8:30, which is the usual start time for bumble bees to forage. I assume that the foragers recalled the start time for foraging and their foraging-related neural activity was induced, as previously reported (Naeger & Robinson, 2016; Singh et al., 2018; Singh et al., 2020). *Egr1* is suggested to be an IEG that is upregulated before the onset of foraging by the reward learning associated with time (Shah et al., 2018). Social bee foragers must deal with changes in the good

flowering locations according to the time of day. At the end of the day, the foragers must remember the best feeding locations at the different times to be ready for the next day. Thus, for effective foraging, the induction of *HR38* in the MBs – the higher center for memory and learning – in the early morning may reflect neural activity needed to recall their foraging experience.

In the *B. ignitus* brain, both *BiHR38* and *BiEgr1* were expressed sparsely in the entire MBs of the foragers captured at 14:00 in the greenhouse, contrary to a previous report that both genes were expressed preferentially in the sKCs in the brains of honey bee foragers (Ugajin et al., 2013; Yamazaki et al., 2006) (Fig. 9D, G). It is unclear whether those differences in the expression patterns of IEGs in the MBs are related to species-specific traits of foraging behavior between honey bees and bumble bees, or simply reflect differences in the experimental conditions set for the honey bees and bumble bees (e.g., flight distance, flight speed, or feeder). Further studies are needed to discriminate these two possibilities.

It is noteworthy that *EcR* was expressed preferentially in the sKCs in the MBs in both *B. ignitus* and *A. mellifera* (Fig. 9J). Ecdysone signaling is suggested to be involved not only in molting or metamorphosis, but also in various social behaviors in insects (Pandey & Bloch, 2015). Although three types of class I KCs in the MBs are reported to be conserved among Aculeate insects, EcR protein is distributed in the whole MBs in *Camponotus japonicus* (Nemoto & Hara, 2007). It might be that the functions of EcR in the brain differ between Formicidae and Apoidea, and are conserved among social Apidae species.

In conclusion, these findings suggested that the brain neural activity evoked by foraging flight are at least partly conserved among two social bee species: *A.mellifera* and *B.ignitus* (Table 5). Especially, there is high possibility that the functions of late-upregulated *EcR* in the sKCs are commonly conserved in foraging bees in both species.

## Chapter 2

## Identification and analysis of target genes of ecdysone receptor in the worker honey bee brain

本章については、5 年以内に雑誌等で刊行予定のため、非公開。

## General Discussion

Through my doctoral course studies presented above, I obtained at least partial answers to the questions I posed regarding the mechanisms of foraging behavior in social bees. First, the foragers of social bees, honey bees and bumble bees, share a common mechanism in which gene expression levels of IEGs and EcR are upregulated in the brain by the foraging behavior. The cells expressing each gene were also found to be, at least partially, conserved among these species. These findings indicate that the neural mechanisms associated with the foraging behavior of social bees are independent of the style of division of labor and species. Next, to understand the downstream pathways induced during foraging behavior, I searched for direct target genes of EcR, which is induced later by foraging flight and exhibits highly conserved expression patterns between species. As a result, *Am*EcR was suggested to regulate its target genes, including canonical ecdysone signaling, by recognizing EcR-USP complex motifs, as seen in metamorphosis, and the expression of some of the target genes is induced during foraging behavior. Although roles for ecdysone and ecdysone signaling in social bees have been demonstrated in the induction of ovarian development and the initiation of reproductive caste differentiation, their roles in the division of labor among workers and the role of EcR expressed in the worker brain have remained a mystery (Pandey & Bloch, 2015). Based on the estimated functions of EcR target genes identified in this study, it is possible that ecdysone signaling acts as a metabolic regulator in the worker honey bee brain during foraging behavior.

It may be that the function of EcR in the regulation of metabolic pathways induced by EcR during foraging behavior is conserved in insects other than honey bees. For example, in bumble bees, which have a different style of division of labor of workers, the expression of EcR is upregulated during foraging behavior as described in Chapter 1, suggesting that the functions of EcR in the bumble bee are similar to those in the honey bee. Furthermore, if EcR regulates metabolic pathways during foraging behavior in the adult bee brain, it is likely that such metabolic

control systems are also at work in the brains of other Aculeata species (e.g., social wasps) as they must also cope with the long-distance foraging flight to return to their hives.

The molecular mechanisms underlying the increased expression *AmEcR* in the worker brain by foraging behavior are unknown, but previous studies suggested that IEGs directly regulate the expression of EcR. Khamis *et al.* (2015) suggested that five transcription factors are involved in the upregulation of many genes in the brain by foraging behavior; one of these transcription factors is *Egr1*, whose binding motif exists in the *EcR* enhancer region, suggesting that EcR may be induced downstream of IEGs (Khamis et al., 2015). Subsequently, Singh *et al.* (2018) showed that several candidate genes downstream of *Egr1*, including *EcR*, are upregulated by foraging behavior (Singh et al., 2018). Consistent with these previous studies, in Chapter 1, I observed that *AmEcR* expression was increased after the upregulation of IEGs caused by seizure induction in the bumble bee brain. On the other hand, although both IEGs and EcR are preferentially expressed in sKCs in the MBs of the honey bee forager brain (Kiya et al., 2007; Yamazaki et al., 2006), the expression patterns of IEGs were not preferential to sKCs, where EcR is selectively expressed, in the bumble bee brains (Fig. 9). This suggests that EcR may be promoted in some activated neurons in the bumble bee brain, though there may be other conditions for EcR upregulation not driven by IEG.

Because *AmEcR* is preferentially expressed in sKCs in the MBs in the worker brain, it is likely that EcR target genes are also expressed in sKCs. The results obtained in Chapter 2 partially support this hypothesis: some of the genes identified as *Am*EcR target genes, such as *E75* (Paul et al., 2006), *E74* (Paul et al., 2005), *USP* (Yamazaki et al., 2006), and *kakusei* (Kiya et al., 2007) are preferentially expressed in sKCs. In contrast, *E93* and *Br-C*, which are preferentially expressed in lKCs (Paul et al., 2006; Takeuchi et al., 2001) were not detected as *Am*EcR targets. These findings suggest that only a part of the canonical EcR signaling pathway functions in the sKCs of the MBs in the honey bee.

In summary, it may be that sKCs in MBs of social bees are activated to promote the

expression of IEGs during foraging flight, followed by the upregulation of EcR, which results in the regulation of metabolic pathways. These findings lead to a novel role of sKCs, which were previously suggested to be involved in sensory information processing during foraging flight based on the foraging behavior-dependent upregulation of IEGs (Kaneko et al., 2016; Kiya et al., 2007). To test this hypothesis, further studies are needed to clarify the function of individual EcR target genes and their localization in EcR-expressing cells in the brains of worker bees. In addition, analysis focusing on gene expression at the individual neuron level will lead to a better understanding of the signal transduction systems induced in the brain.

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# Figures & Tables

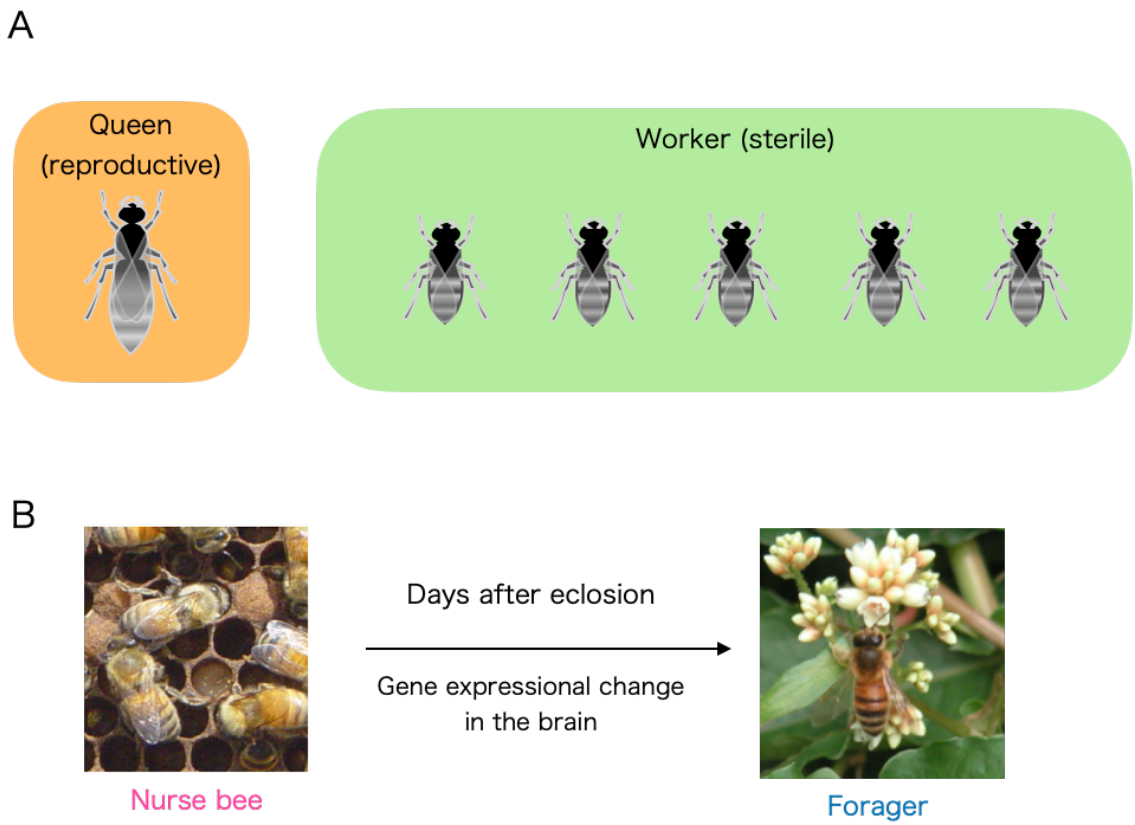



**Figure 1 Society of the honey bee.**

(A) Honeybees have female castes comprising of a reproductive queen and sterile workers. (B) Division of labor of workers. Younger nurse bees are engaged in taking care of the brood, while older foragers forage for pollen and nectar to bring them to the hive.

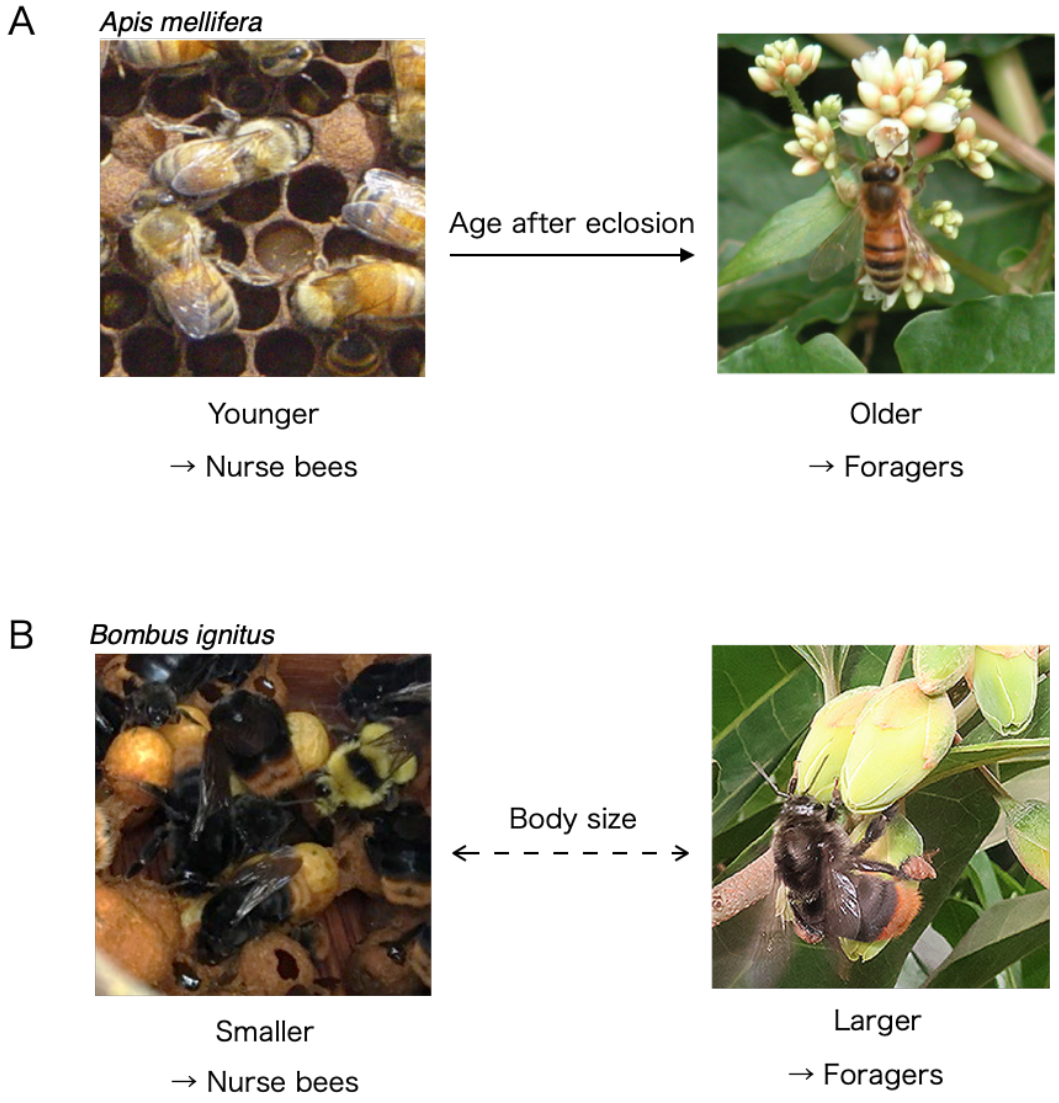



**Figure 2 Division of labors of worker honey bees and bumble bees.**

*A. mellifera* workers show a division of labor according to their age after eclosion (A) and *B. ignitus* workers show a division of labor based on their body size (B).

A

B

C

**Figure 3 Experimental set-up for bee worker sampling.**

(A) *B. ignitus* colonies were set in a greenhouse. A hive (white box in the center) was set on a block and covered with a screen to shade the hive from direct sunlight. (B) The *B. ignitus* colony was set in a laboratory flight-cage. A hive with its entrance (wooden box on the left), a small dish supplied with pollen on a blue flower-motif paper, and three brushes attached with pollen as feeders are shown. (C) *A. mellifera* colonies were set at the rooftop of Univ. of Tokyo Faculty of Science Bldg. 2. Two hive boxes made of wood are shown.

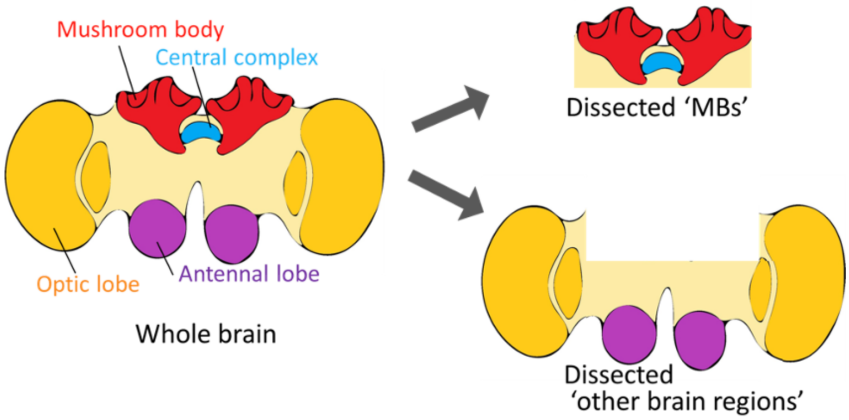



**Figure 4 Bee brain dissection for qRT-PCR analysis.**

Dissected "MBs" mainly include the MBs and central complex and dissected "other brain regions" include the antennal lobes and optic lobes.

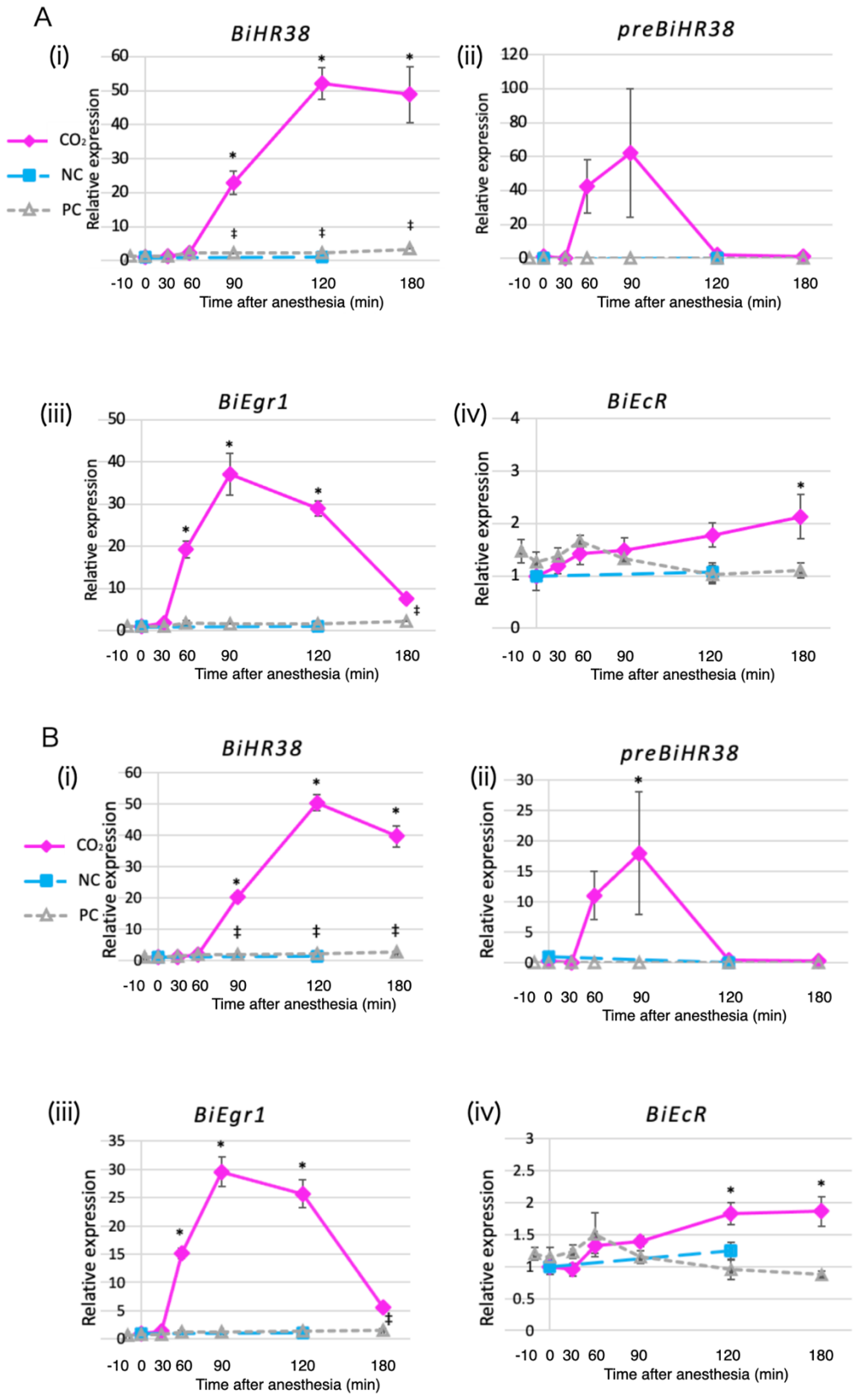
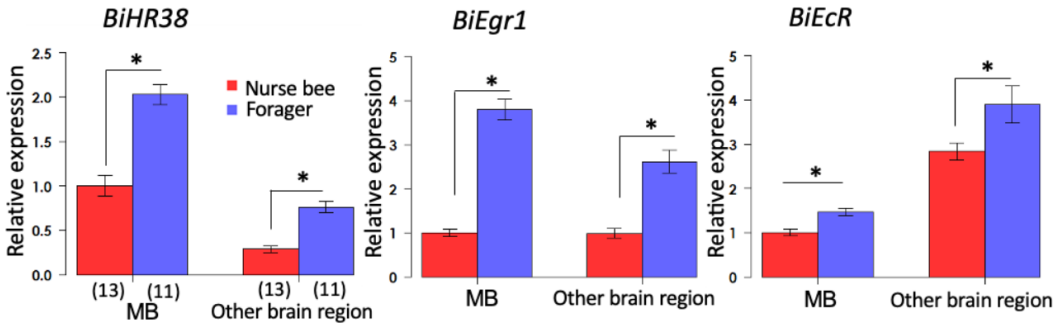

A
(i)
BiHR38
CO₂
NC
PC
Relative expression
Time after anesthesia (min)
(ii)
preBiHR38
(iii)
BiEgr1
(iv)
BiEcR
B
(i)
BiHR38
(ii)
preBiHR38
(iii)
BiEgr1
(iv)
BiEcR

**Figure 5 Analysis of *BiHR38*, pre*BiHR38*, *BiEgr1*, and *BiEcR* expression levels after seizure induction.**

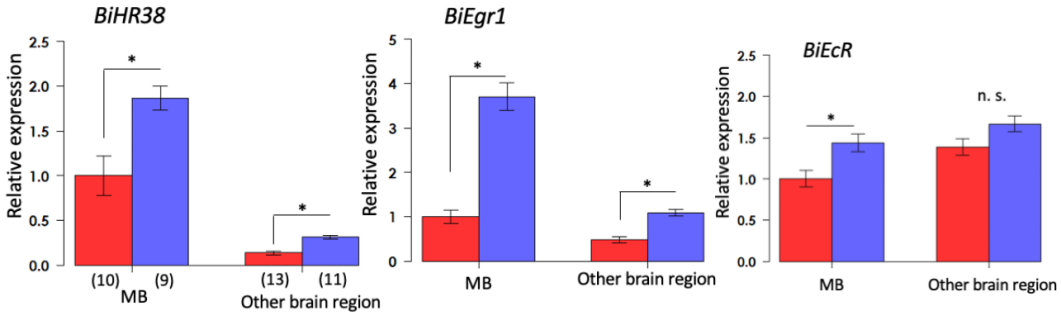
Time-course of the expression of *BiHR38*(i), pre*BiHR38*(ii), *BiEgr1*(iii) and *BiEcR*(iv) after awakening from $CO_2$ anesthesia. The expression level of each gene was normalized with that of *BiEF1α*(A) and *BiActin*(B). Magenta lines indicate the group anesthetized with $CO_2$("$CO_2$"), light blue dashed lines indicate the negative control("NC", continuously anesthetized with $CO_2$ for 120 min) and gray dotted lines indicate the positive control("PC", exposed to only fresh air flow). All data indicate means ± SEM. Significant differences on the basis of Dunnett's test after the ANOVA are indicated (*: $p<0.05$ for $CO_2$ group, ‡: $p<0.05$ for PC group). Student's t test and Welch's t test revealed no significant difference between the NC group and $CO_2$ 0-min group. Some errors were so low that it is difficult to see the error bars in the graph. n=5 for each sample.

A

(i)


BiHR38
BiEgr1
BiEcR
Relative expression
Nurse bee
Forager
(13) (11)
MB
Other brain region
*
n. s.

(ii)


BiHR38
BiEgr1
BiEcR
Relative expression
(10) (9)
(13) (11)
MB
Other brain region
*
n. s.

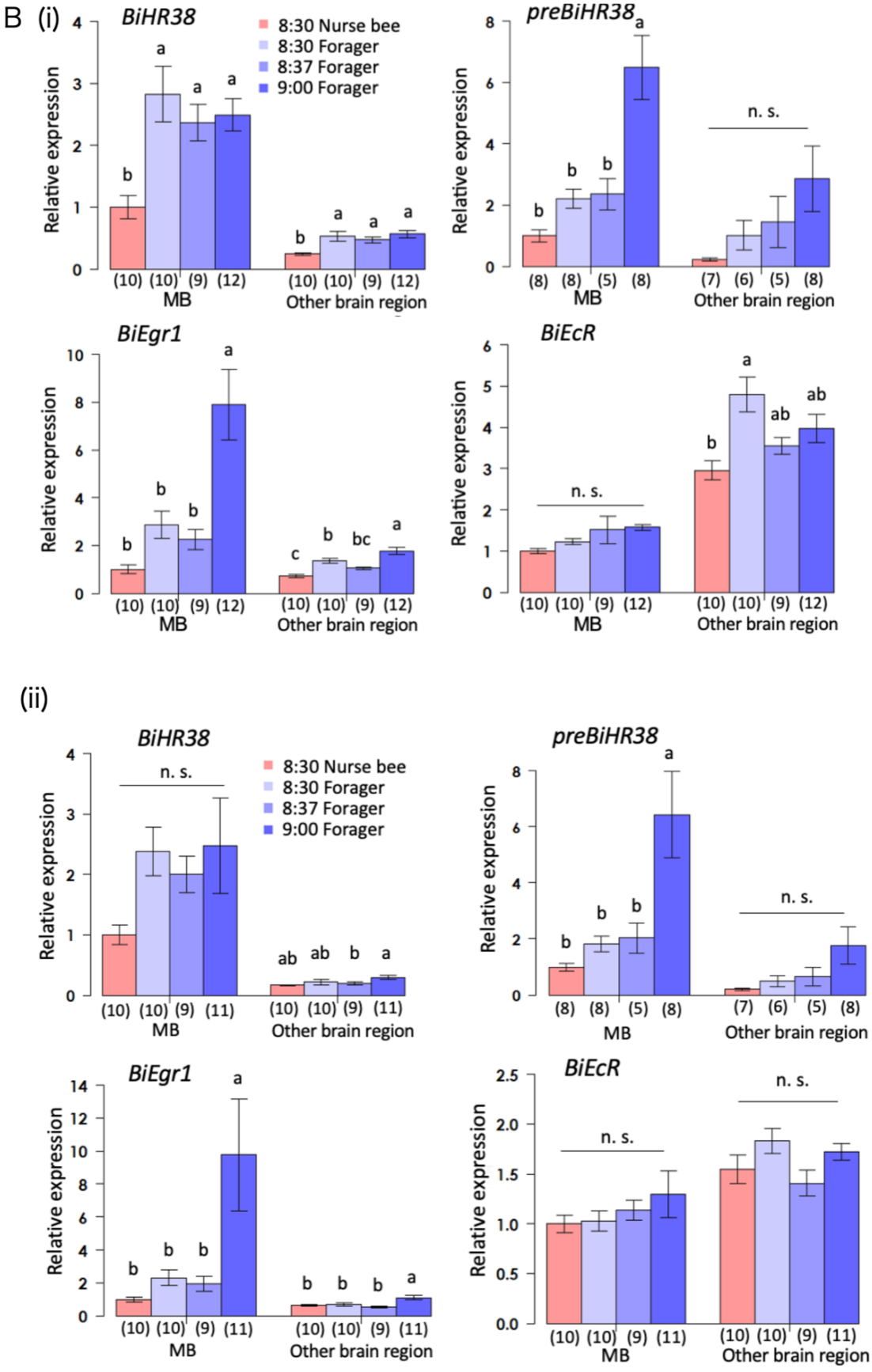

B (i)
BiHR38
8:30 Nurse bee
8:30 Forager
8:37 Forager
9:00 Forager
Relative expression
MB
Other brain region
preBiHR38
n. s.
BiEgr1
BiEcR
(ii)
BiHR38
preBiHR38
BiEgr1
BiEcR

**Figure 6 qRT-PCR analysis of *BiHR38*, pre*BiHR38*, *BiEgr1*, and *BiEcR* expression during foraging flight.**

Expression analysis for the greenhouse experiment (A) and the laboratory flight-cage experiment (B). The expression level of each gene was analyzed by qRT-PCR and normalized with that of *BiEF1α* (i) and *BiActin* (ii). Each bar represents the mean ± SEM. Significant differences are indicated by asterisks ($p<0.05$, Student's t test or Welch's t test after the F test) on the error bars in (A), or different letters ($p<0.05$, Tukey-Kramer test in (B), respectively). The sample size is indicated by the number in parentheses below the horizontal axis. n.s., not significant.

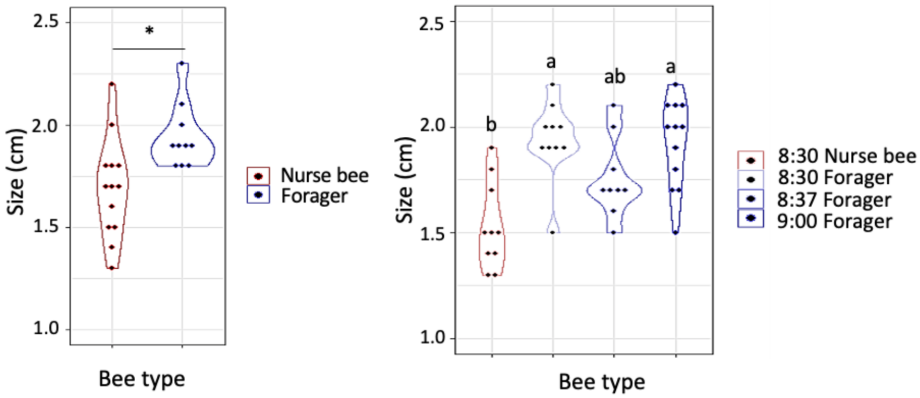
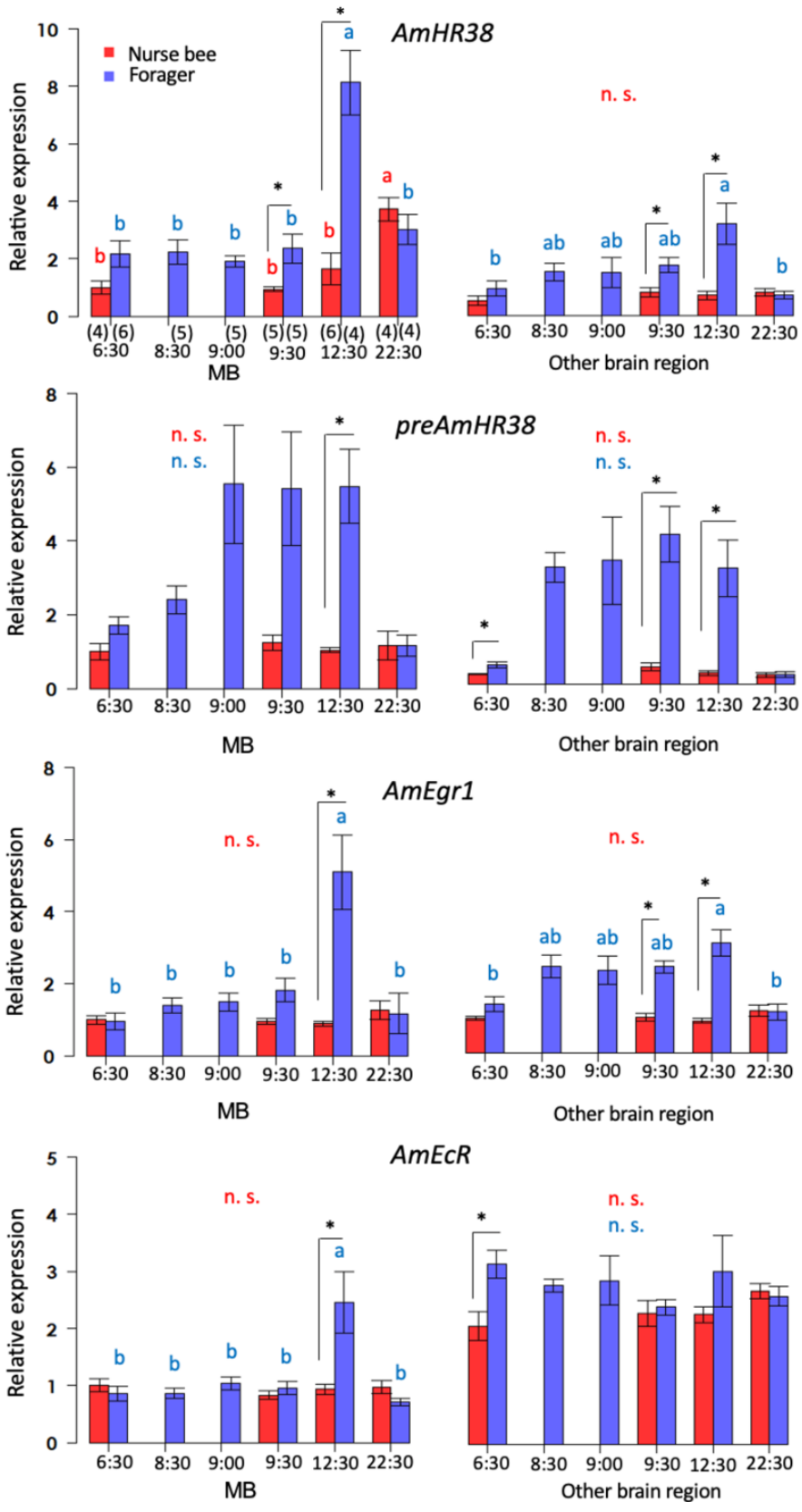



**Figure 7 Body sizes of the bumble bee workers.**

The body sizes of *B. ignitus* workers captured in the greenhouse (left) and those from the flight-cage (right). Significant differences are indicated by asterisks in the left panel ($p<0.05$, Student's t test), and different letters in the right panel ($p<0.05$, Tukey-Kramer test).

A


AmHR38
Nurse bee
Forager
Relative expression
MB
Other brain region
n. s.
preAmHR38
AmEgr1
AmEcR
6:30
8:30
9:00
9:30
12:30
22:30

B

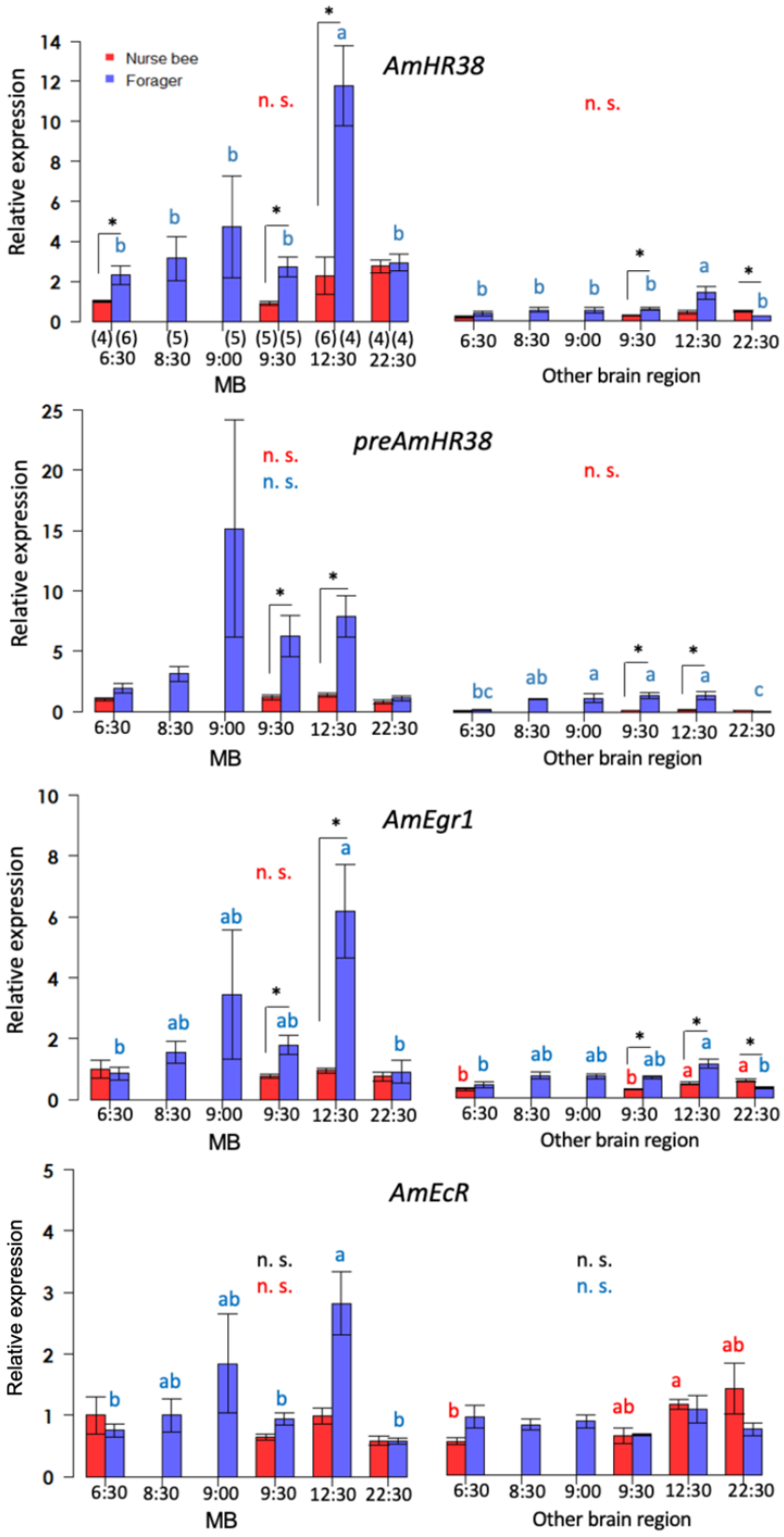

Nurse bee
Forager
AmHR38
preAmHR38
AmEgr1
AmEcR
Relative expression
MB
Other brain region
6:30
8:30
9:00
9:30
12:30
22:30
n. s.

**Figure 8 qRT-PCR analysis of *AmHR38*, pre*AmHR38*, *AmEgr1*, and *AmEcR* expression during foraging flight.**

Expression levels of *AmHR38*, pre*AmHR38*, *AmEgr1*, and *AmEcR* were analyzed by qRT-PCR and normalized with that of *AmEF1α* (A) *and AmActin* (B). Each bar represents the mean ± SEM. Significant differences are indicated using different letters ($p<0.05$, Tukey-Kramer test for nurse bees [red] and for foragers [blue] during the time-course, in each brain tissue respectively) or asterisks ($p<0.05$, Student's t test or Welch's t test after the F test) on the error bars (black). The sample size is shown below the horizontal axis in parentheses. n.s., not significant.

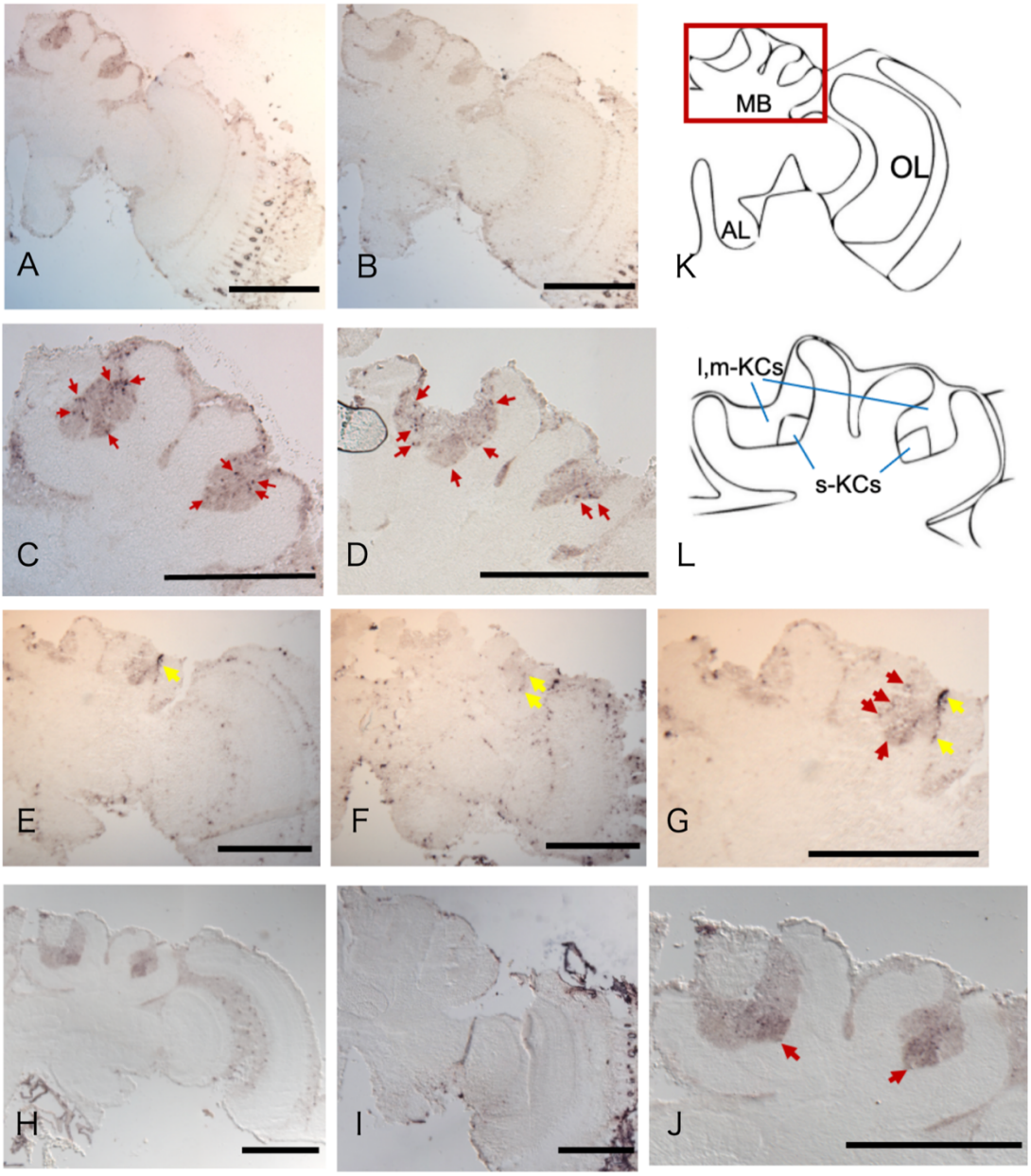



**Figure 9 *In situ* hybridization of *BiHR38*, *BiEcR*, and *BiEgr1* in forager brain sections**

Expression of *BiHR38* (A-D), *BiEgr1* (E-G), and *BiEcR* (H-J) was analyzed by *in situ* hybridization. Sections of brain hemispheres hybridized with antisense (A, E, and H) or sense probes (B, F and I) are shown. Panel (K) indicates schematic drawing of the brain hemisphere and panel (L) indicates the magnified view of the MB enclosed by the red square in panel (K). Panels (C, D, G, and J) indicate magnified views of the MB area in panels (A, E, and H),

respectively. (A-C) forager collected at 9:00 in the laboratory flight-cage; (D-J) forager collected as “Forager” in the greenhouse. Representative signals are indicated by red arrows. Processing-induced damage to the tissue is indicated by yellow arrows (E-G, respectively). The s (l, m)-KCs: small-type (large-, middle-) Kenyon cells, AL: antennal lobe, OL: optic lobe. Scale bars = 500 $\mu$m.

| Animal (analysis) | Gene name | Gene ID | Primer sequence | Size (bp) | Temp. (℃) |
|---|---|---|---|---|---|
| ***A. mellifera*** **(qRT-PCR)** | *HR38* | 551592 | 5'-CGATTGGCTCCACAGTATTC-3' and 5'-CTCCATGCGATGAGGCTCC-3' | 136 | 58 |
| | pre*HR38* | 551592 | 5'-TTATGTATGGACGTGCAGAC -3' and 5'-ATCGGATACACGTCGATTAG-3' | 125 | 52 |
| | *EcR* | 406084 | 5'-TACCACTACAACGCGCTCAC-3' and 5'-CCTCATGTACATGTCGATCT-3' | 120 | 56 |
| | *Egr1* | 726302 | 5'-CCTCACCACCCACGTGAGAA -3' and 5'-TGCTTGAGGTGGACTTTGGC -3' | 117 | 58 |
| | *EF1α* | 408385 | 5'-TTGTGCCGTGTTAATAGTCG-3' and 5'-GATCGGTCATGTCCATCTTG-3' | 149 | 56 |
| | *Actin* | 406122 | 5'-TCCCCGAATCCCGAAAG-3' and 5'-CGGAGGAACCAAAGGACAA-3' | 89 | 55 |
| ***B. ignitus*** **(qRT-PCR)** | *HR38* | 100642535 | 5'-CGATTGGCTCCACAGTATCC-3' and 5'-CTCCATGCGATGAGGTTCC-3' | 136 | 58 |
| | pre*HR38* | 100642535 | 5'-TGACGAGCCTACGACATGTC-3' and 5'-TGAATCGTGGAAGGCGAGTT-3' | 139 | 58 |
| | *EcR* | 100646757 | 5'-TATCACTACAACGCACTGAC-3' and 5'-CCGCATGTACATATCGATCT-3' | 120 | 55 |
| | *Egr1* | 100651542 | 5'-CTTAACCACTCACGTGAGAA-3' and 5'-TGTTTCAAGTGAACTTTCGC-3' | 117 | 56 |
| | *EF1α* | 100631080 | 5'-TTGTGCCGTGTTAATAGTGG-3' and 5'-GATCGGTCATGTCCATCTTG-3' | 149 | 56 |
| | *Actin* | 100646910 | 5'-GTCTCGTTTCTCGACCATAG-3' and 5'-ACTGATCTTCGAATGCCTAAA-3' | 93 | 55 |
| ***B. ignitus*** **(*in situ* hybridization)** | *HR38* | 100642535 | 5'-CAATCTTCTCACTACGTCCA -3' and 5'-GGGATAGATAGTGCGCTTTC-3' | 440 | - |
| | *EcR* | 100646757 | 5'-CACTAATCAGCCCTCAGAAG-3' and 5'-TCAAACTGAAGCACATCTCG-3' | 566 | - |
| | *Egr1* | 100651542 | 5'-GAATCTCCTGTCCCATCATC-3' and 5'-TGTTTCAAGTGAACTTTCGC-3' | 573 | - |

**Table 1 Gene-specific primers**

NCBI, National Center for Biotechnology Information; Size, PCR product size; Temp., annealing temperature setting at Light Cycler.

A

| Mean ± SEM | MB | | Other brain region | |
|---|---|---|---|---|
| | Nurse bee | Forager | Nurse bee | Forager |
| ***BiHR38*** | 1±0.12 | 2.03±0.11 | 0.28±0.04 | 0.76±0.06 |
| ***BiEgr1*** | 1±0.08 | 3.80±0.23 | 0.99±0.10 | 2.62±0.26 |
| ***BiEcR*** | 1±0.08 | 1.47±0.08 | 2.83±0.19 | 3.89±0.41 |

B

| Mean ± SEM | MB | | Other brain region | |
|---|---|---|---|---|
| | Nurse bee | Forager | Nurse bee | Forager |
| ***BiHR38*** | 1±0.22 | 1.86±0.13 | 0.13±0.02 | 0.31±0.02 |
| ***BiEgr1*** | 1±0.14 | 3.70±0.31 | 0.48±0.07 | 1.09±0.07 |
| ***BiEcR*** | 1±0.10 | 1.44±0.11 | 1.39±0.10 | 1.67±0.09 |

**Table 2 Gene expression analysis of *B. ignitus* workers collected in the greenhouse.**

Relative gene expression levels of each gene in each brain tissue of *B. ignitus* workers collected in the greenhouse normalized with those of *BiEF1α* (A) and *BiActin* (B) and calculated by taking that of each gene in the nurse bee MBs as 1.

A

| Mean ± SEM | MB | | | | Other brain region | | | |
|---|---|---|---|---|---|---|---|---|
| | 8:30 | | 8:37 | 9:00 | 8:30 | | 8:37 | 9:00 |
| | Nurse bee | Forager | Forager | Forager | Nurse bee | Forager | Forager | Forager |
| ***BiHR38*** | 1±0.19 | 2.83±0.45 | 2.37±0.29 | 2.49±0.26 | 0.25±0.02 | 0.53±0.08 | 0.48±0.05 | 0.57±0.06 |
| **pre*BiHR38*** | 1±0.20 | 2.21±0.32 | 2.36±0.51 | 6.48±1.05 | 0.21±0.05 | 1.02±0.48 | 1.46±0.83 | 2.85±1.07 |
| ***BiEgr1*** | 1±0.19 | 2.87±0.56 | 2.25±0.43 | 7.90±1.46 | 0.73±0.06 | 1.35±0.10 | 1.05±0.06 | 1.78±0.14 |
| ***BiEcR*** | 1±0.06 | 1.22±0.07 | 1.51±0.33 | 1.57±0.07 | 2.95±0.23 | 4.80±0.42 | 3.54±0.20 | 3.97±0.34 |

B

| Mean ± SEM | MB | | | | Other brain region | | | |
|---|---|---|---|---|---|---|---|---|
| | 8:30 | | 8:37 | 9:00 | 8:30 | | 8:37 | 9:00 |
| | Nurse bee | Forager | Forager | Forager | Nurse bee | Forager | Forager | Forager |
| ***BiHR38*** | 1±0.16 | 2.38±0.40 | 2.00±0.31 | 2.47±0.80 | 0.16±0.01 | 0.22±0.05 | 0.19±0.02 | 0.30±0.03 |
| **pre*BiHR38*** | 1±0.14 | 1.82±0.28 | 2.03±0.54 | 6.44±1.54 | 0.20±0.04 | 0.49±0.19 | 0.65±0.34 | 1.76±0.67 |
| ***BiEgr1*** | 1±0.15 | 2.30±0.48 | 1.94±0.44 | 9.76±3.38 | 0.65±0.06 | 0.70±0.10 | 0.53±0.05 | 1.10±0.12 |
| ***BiEcR*** | 1±0.09 | 1.03±0.10 | 1.14±0.10 | 1.29±0.23 | 1.55±0.14 | 1.83±0.12 | 1.41±0.13 | 1.72±0.08 |

**Table 3 Gene expression of *B. ignitus* workers collected in the laboratory flight-cage sampling**

Relative gene expression level of each gene in each brain tissue of *B. ignitus* workers collected in the laboratory flight-cage normalized with those of *BiEF1α* (A) and *BiActin* (B) calculated by taking that of each gene in the MB of nurse bee at 8:30 as 1.

A

| Mean ± SEM | MB | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 6:30 | | 8:30 | 9:00 | 9:30 | | 12:30 | | 22:30 | |
| | Nurse bee | Forager | Forager | Forager | Nurse bee | Forager | Nurse bee | Forager | Nurse bee | Forager |
| ***AmHR38*** | 1±0.23 | 2.17±0.47 | 2.25±0.42 | 1.91±0.20 | 0.95±0.08 | 2.36±0.50 | 1.65±0.55 | 8.13±1.13 | 3.73±0.40 | 3.01±0.52 |
| **pre*AmHR38*** | 1±0.23 | 1.71±0.24 | 2.40±0.37 | 5.54±1.60 | 1.25±0.20 | 5.41±1.54 | 1.04±0.07 | 5.48±1.01 | 1.15±0.39 | 1.16±0.30 |
| ***AmEgr1*** | 1±0.12 | 0.98±0.25 | 1.40±0.22 | 1.50±0.25 | 0.95±0.07 | 1.82±0.32 | 0.90±0.06 | 5.11±1.03 | 1.27±0.26 | 1.17±0.56 |
| ***AmEcR*** | 1±0.11 | 0.86±0.13 | 0.86±0.09 | 1.04±0.12 | 0.83±0.07 | 0.96±0.11 | 0.93±0.09 | 2.46±0.54 | 0.97±0.12 | 0.71±0.06 |

| Mean ± SEM | Other brain region | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 6:30 | | 8:30 | 9:00 | 9:30 | | 12:30 | | 22:30 | |
| | Nurse bee | Forager | Forager | Forager | Nurse bee | Forager | Nurse bee | Forager | Nurse bee | Forager |
| ***AmHR38*** | 0.50±0.16 | 0.92±0.26 | 1.51±0.31 | 1.50±0.52 | 0.80±0.16 | 1.75±0.28 | 0.70±0.15 | 3.17±0.72 | 0.81±0.13 | 0.70±0.13 |
| **pre*AmHR38*** | 0.26±0.02 | 0.51±0.07 | 3.17±0.40 | 3.35±1.18 | 0.45±0.10 | 4.07±0.76 | 0.29±0.07 | 3.14±0.78 | 0.24±0.06 | 0.24±0.08 |
| ***AmEgr1*** | 0.95±0.06 | 1.34±0.21 | 2.39±0.32 | 2.29±0.40 | 0.97±0.11 | 2.38±0.17 | 0.88±0.06 | 3.04±0.37 | 1.16±0.15 | 1.13±0.21 |
| ***AmEcR*** | 2.07±0.26 | 3.15±0.25 | 2.78±0.11 | 2.87±0.44 | 2.29±0.23 | 2.40±0.13 | 2.27±0.14 | 3.03±0.62 | 2.68±0.13 | 2.60±0.17 |

B

| Mean ± SEM | MB | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 6:30 | | 8:30 | 9:00 | 9:30 | | 12:30 | | 22:30 | |
| | Nurse bee | Forager | Forager | Forager | Nurse bee | Forager | Nurse bee | Forager | Nurse bee | Forager |
| ***AmHR38*** | 1±0.06 | 2.31±0.48 | 3.15±1.11 | 4.71±2.55 | 0.91±0.10 | 2.74±0.49 | 2.28±0.93 | 11.79±2.01 | 2.75±0.33 | 2.93±0.41 |
| **pre*AmHR38*** | 1±0.10 | 1.93±0.43 | 3.14±0.60 | 15.17±9.01 | 1.17±0.21 | 6.26±1.71 | 1.32±0.15 | 7.88±1.74 | 0.76±0.17 | 1.08±0.21 |
| ***AmEgr1*** | 1±0.29 | 0.86±0.21 | 1.54±0.36 | 3.46±2.12 | 0.76±0.06 | 1.79±0.31 | 0.96±0.09 | 6.18±1.53 | 0.76±0.14 | 0.90±0.37 |
| ***AmEcR*** | 1±0.30 | 0.75±0.11 | 1.00±0.27 | 1.84±0.81 | 0.65±0.05 | 0.94±0.10 | 0.99±0.13 | 2.82±0.51 | 0.59±0.08 | 0.58±0.05 |

| Mean ± SEM | Other brain region | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 6:30 | | 8:30 | 9:00 | 9:30 | | 12:30 | | 22:30 | |
| | Nurse bee | Forager | Forager | Forager | Nurse bee | Forager | Nurse bee | Forager | Nurse bee | Forager |
| ***AmHR38*** | 0.17±0.06 | 0.36±0.13 | 0.54±0.12 | 0.53±0.15 | 0.24±0.04 | 0.57±0.08 | 0.43±0.10 | 1.39±0.33 | 0.45±0.05 | 0.23±0.00 |
| **pre*AmHR38*** | 0.09±0.01 | 0.18±0.03 | 1.07±0.05 | 1.14±0.35 | 0.14±0.03 | 1.34±0.25 | 0.18±0.05 | 1.34±0.35 | 0.13±0.03 | 0.07±0.01 |
| ***AmEgr1*** | 0.27±0.04 | 0.42±0.10 | 0.72±0.12 | 0.70±0.08 | 0.26±0.02 | 0.66±0.05 | 0.46±0.04 | 1.10±0.15 | 0.55±0.06 | 0.31±0.03 |
| ***AmEcR*** | 0.55±0.06 | 0.96±0.18 | 0.83±0.09 | 0.88±0.10 | 0.65±0.13 | 0.66±0.02 | 1.17±0.08 | 1.09±0.23 | 1.43±0.42 | 0.75±0.10 |

**Table 4 Gene expression of *A. mellifera* workers collected during foraging flight.**

Relative gene expression level of each gene in each brain tissue of *A. mellifera* workers collected over time-course normalized with those of *AmEF1α* (A) and *AmActin* (B) calculated by taking that of each gene in the MB of nurse bee at 6:30 as 1.

| | ***Apis mellifera*** | ***Bombus ignitus*** |
|---|---|---|
| ***HR38*** | ・IEG (Ugajin *et al.* 2017)<br><br>・Upregulated by foraging flight (Yamazaki *et al.* 2006, Singh *et al.* 2018)<br><br>・Preferentially expressed in sKCs in the forager brain(Yamazaki *et al.* 2006) | ・IEG<br><br>・Upregulated by foraging flight<br><br>・Sparsely expressed in whole MB in the forager brain |
| ***Egr1*** | ・IEG (Ugajin *et al*. 2013)<br><br>・Upregulated by foraging flight (Ugajin *et al.* 2017, Singh *et al.* 2018)<br><br>・Preferentially expressed in sKCs in the forager brain (Ugajin *et al.* 2013)<br>・Expressed in whole MBs by orientation flight (Lutz *et al.* 2011) | ・IEG<br><br>・Upregulated by foraging flight<br><br>・Sparsely expressed in whole MB in the forager brain |
| ***EcR*** | ・No data about IEG response<br><br>・Upregulated by foraging flight (Singh *et al.* 2018)<br><br>・Preferentially expressed in sKCs in the worker brain (Takeuchi *et al.* 2007) | ・No IEG response<br><br>・Upregulated by foraging flight<br><br>・Preferentially expressed in sKCs in the forager brain |

**Table 5 Summary of gene expression analysis in the bumble bee and honey bee brains.**

Characteristics of *HR38*, *Egr1* and *EcR* expression patterns in the brain of *A*. *mellifera* and *B*. *ignitus* in seizure induction and during foraging flight in the brain and the cell-types in which each gene is expressed preferentially in the MBs, are shown. Findings in the present study are indicated in red.

## Acknowledgements

First, I would like to express my deepest gratitude to Professor Takeo Kubo who always provide me, great research opportunity, warm encouragement, valuable proposals, and continuous discussions. I would also like to express my gratitude to Assistant Professor Hiroki Kohno who taught me experimental skills for the very beginning and gave me valuable and critical suggestions.

I sincerely thank Professor Masato Ono, Professor Shinichi Asada, and Dr. Masakazu Nishimura (Graduate School of Agriculture, Tamagawa University) for their willingness to help and guide me in the sampling for bumble bees and for providing me with the greenhouse and foraging flight-cage.

I would like to thank Dr. Yurika Shiota for giving me vectors and primers of some bumble bee genes.

I deeply appreciate Professor Tetsuji Kakutani and Ms. Satoyo Oya, who kindly collaborate me in *Am*EcR ChIP-seq analysis, for their help with DNA library preparation, sequencing, and guidance for data analysis methods.

I am grateful to my laboratory members for providing me fresh and precious comments during my graduate research program.

Finally, I would like to thank my family for understanding my decision to study in the doctoral program, for supporting me financially, and for always looking after me warmly.