A simple method for ex vivo honey bee cell culture capable of in vitro gene expression analysis

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Abstract

Cultured cells are a very powerful tool for investigating biological events in vitro; therefore, cell lines have been established not only in model insect species, but also in non-model species. However, there are few reports on the establishment of stable cell lines and development of systems to introduce genes into the cultured cells of the honey bee (Apis mellifera). We describe a simple ex vivo cell culture system for the honey bee. Hemocyte cells obtained from third and fourth instar larvae were cultured in commercial Grace’s insect medium or MGM-450 insect medium for more than two weeks maintaining a normal morphology without deterioration. After an expression plasmid vector bearing the enhanced green fluorescent protein (egfp) gene driven by the immediate early 2 (IE2) viral promoter was transfected into cells, EGFP fluorescence was detected in cells for more than one week from one day after transfection. Furthermore, double-stranded RNA corresponding to a part of the egfp gene was successfully introduced into cells and interfered with egfp gene expression. A convenient and reproducible method for an ex vivo cell culture that is fully practicable for gene expression assays was established for the honey bee.

Introduction

Controlled in vitro cell systems, of both established cell lines and primary cultures, are valuable tools for understanding basic biological events by simplified host environment. Efforts to produce insect cell lines have resulted in the establishment of more than 1000 continuous cell lines from various insect species [1]. These cell lines are utilized for a broad range of biological research areas and the industrial mass production of recombinant proteins [2–4]. Nevertheless, cultured insect cells suitable for a specific research objective are not always available. In most cases, researchers use cell lines derived from closely related or commercially available species as an alternative or develop their own cell cultures [5].
The honey bee, *Apis mellifera* is one of the most important insects not only for honey production and its role as a pollinator of cultivated and natural plants, but also as a model insect for biological research, particularly on social behavior and learning. Decreases have recently been reported in managed honey bee colonies, and colony declines represent an ongoing concern that threatens the stable supply of pollinators worldwide [6–8]. One of the causes of colony declines is infections by viral pathogens [9–11]. *In vitro* systems, namely, primary cell cultures and cell lines, are valuable tools for investigating the mechanisms underlying interactions between viruses and host cells [2, 12]. Therefore, the development of honey bee cell cultures has been attempted, resulting in several primary cell cultures derived from a number of tissues and suitable culture media [13–15]. Only two stable cell lines have been established in the honey bee. One is the spontaneously immortalized cell line, AmE-711 [16] which has been utilized to investigate viral infection dynamics [17]. The other is the transformed cell line (MYN9) immortalized by the introduction of the human *c-myc* proto-oncogene [18]. However, these cell lines were infected with viruses [15, 17, 18].

Increased interest in the genetic regulatory mechanisms of the honey bee, *A. mellifera* has led to whole genome sequencing [19, 20] and the use of *in vivo* systems, such as gene knockdown by RNA interference (RNAi) [21], transposon-mediated germline transformation [22], and targeted mutagenesis (gene knockout) by genome editing [23–25]. Nevertheless, difficulties have been associated with experiments involving the individuals of eusocial species. Only a few studies have reported *in vitro* systems to induce gene expression through the introduction of expression vectors in honey bee cultured cells [18, 26]. To obtain a more detailed understanding of gene functions, the need for *in vitro* systems that enable examinations of gene interactions and their regulatory signal transduction pathways is increasing.

In the present study, we describe a simple, convenient, and reproducible method for an *ex vivo* honey bee hemocyte cell culture derived from the larval hemolymph. Commercial culture media, transfection reagents and expression vectors were compatible with cultivation, the introduction of exogenous DNA, and induction of gene expression in cultured cells. Moreover, the application of double-stranded RNA (dsRNA) to cultured cells successfully interfered with the expression of the target gene. This *ex vivo* cell culture system will provide new options for the study of honey bee molecular biology.

### Materials and methods

#### Insects

Embryos, larvae, and pupae of the Italian honey bee, *Apis mellifera ligustica* were collected from colonies maintained in the research apiary of the Institute of Livestock and Grassland Science, NARO (Tsukuba, Japan).

#### Culture medium and supplements

Commercially available Grace’s insect medium, Supplemented (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% heat-treated fetal bovine serum (FBS, Corning, Corning, NY, USA) and MGM-450 insect medium [27] containing 20% FBS were used. Both culture media were supplemented with 10% antibiotics (Antibiotic-Antimycotic, Thermo Fisher Scientific).

#### Preparation of honey bee cells and culture conditions

The honey bee cells to be cultured were obtained from the hemolymph, embryos and fat bodies. To collect hemolymph cells, third or fourth instar larvae were carefully removed from
comb cells using ring tip forceps, surface-sterilized with 70% ethanol for 10 min, and air-dried on a sterilized paper towel in a laminar flow hood. The dorsal abdomen was torn with a pair of forceps under a stereomicroscope, and one to two droplets of the hemolymph per individual were recovered in a 1.5-ml microcentrifuge tube filled with 500 μl of culture medium (Fig 1). The medium containing hemolymph cells was filtrated using a cell strainer (pluriStrainer Mini 10 μm, pluriSelect, Leipzig, Germany) to remove fat bodies and other debris. The filtrate was mixed with approximately the same volume of medium and centrifuged at 500 x g for 15 min to collect cells. Cells were then suspended in 1 ml of culture medium and centrifuged at 300 x g for 5 min. This centrifugation step was repeated once more. The cells of 15 larval hemolymph equivalents were suspended in 100 μl of culture medium.

Embryos (approximately 100 on average per preparation) of various stages (66–72 hours after oviposition) were removed from comb cells using thin forceps, surface-sterilized with 70% ethanol for 5 min, and air-dried on sterilized filter paper in a laminar flow hood. They were transferred to a drop of culture medium and chorions were removed with forceps. Dechorionated embryos were transferred into a 1.5-ml microcentrifuge tube filled with 50 μl of culture medium and gently homogenized with a plastic pestle. The homogenate was filtrated using a 40-μm cell strainer (pluriStrainer Mini 40 μm, pluriSelect).

Pupae were removed from comb cells using ring tip forceps. Fat bodies were dissected from the sterilized pupal abdomen, transferred to a 1.5-ml microcentrifuge tube filled with 1 ml of medium, and tissue fragments were rinsed in culture medium. Recovered cells in 100 μl of culture medium were dispensed into a well of a 96-well tissue culture plate (Sumitomo Bakelite, Tokyo, Japan). Each 96-well tissue culture plate was sealed with vinyl tape and incubated at 34˚C.

1. Surface-sterilize third or fourth instar larvae with 70% ethanol for 10 min and air-dry.
2. Tear the dorsal abdomen with a pair of forceps and recover one to two droplets of the hemolymph per individual (a total of 15 individuals) in a 1.5-ml tube filled with 500 μl of Grace’s insect medium containing 10% FBS and 10% antibiotics.
3. Filtrate the medium containing hemocytes using a 10-μm cell strainer and centrifuge three times with fresh culture medium.
4. Suspend hemocytes in 100 μl of culture medium and dispense into a well of a 96-well tissue culture plate, seal with vinyl tape, and incubate at 34˚C.

Microscopy
Cell viability was examined using the trypan blue exclusion assay as previously described [28] with slight modifications. Cells were re-suspended in culture medium (100 μl) in a well, and a small portion (3 μl) was mixed with an equal volume of 0.4% trypan blue solution (Fujifilm Wako Pure Chemical, Osaka, Japan) in a 1.5-ml microcentrifuge tube. The mixture (6 μl) was then transferred to a hemocytometer (Watson, Tokyo, Japan) and the number of viable (unstained) and dead (stained) cells was counted under a microscope (IX70, Olympus, Tokyo, Japan). Three replications (three wells) were made for each incubation period, and the wells from which cells were taken were sacrificed after each count. Cell viability was calculated by the ratio of viable cells to total cells.

Enhanced green fluorescent protein (EGFP) fluorescence was observed under a fluorescent microscope equipped with a GFP filter (BZ-8100, Keyence, Osaka, Japan). The EGFP-positive and -negative cells observed within randomly selected areas of 200 μm x 200 μm squares under a fluorescent microscope were photographed and counted to estimate...
transfection efficiency (EGFP-positive cells among viable cells). The number of cells in three square areas per well was counted and three replications (for three wells) were made for each measurement.
Polymerase Chain Reactions (PCR) and sequence analysis

To identify the origin of cultured cells, a fragment of the *A. mellifera* mitochondrial cytochrome-c oxidase subunit I (COI) gene (GenBank: NM_001177490) was amplified using KOD One DNA polymerase (Toyobo, Osaka, Japan) with the universal primer set [29] (Table 1) and genomic DNA as the template. Genomic DNA was extracted from cultured cells and adult workers using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) according to the supplier’s protocol. PCR conditions were as follows: 30 cycles at 94˚C for 10 sec, 55˚C for 5 sec, 68˚C for 5 sec, and final extension at 68˚C for 10 min. PCR products were examined by agarose gel electrophoresis and each PCR product was cloned into the pCR4Blunt-TOPO vector using the Zero Blunt TOPO PCR Cloning kit for Sequencing (Invitrogen, Thermo Fisher Scientific). The nucleotide sequences of cloned DNA fragments were determined by dye-labeled cycle sequencing using the BigDye Terminator Cycle Sequencing kit v3.1 (Applied Biosystems, Foster City, CA, USA) and analyzed with a DNA sequencer (ABI 3130x1, Applied Biosystems).

Reverse Transcription PCR (RT-PCR). RT-PCR was performed to examine gene expression. Total RNA was extracted from cultured cells using the RNeasy Mini kit (Qiagen) according to the supplier’s protocol. Short fragments from transcripts of the *egfp* gene (656 bp) and the *elongation factor-1 α* gene of *A. mellifera* (GenBank: XM_006569890) (315 bp) were amplified using the QIAquick RT-PCR kit using primer sets (Table 1) and total RNA (20 ng/ 20 μl reaction) as the template. PCR conditions were as follows: 50˚C for 30 min, 95˚C for 15 min, 35 cycles at 94˚C for 45 sec, 65˚C for 30 sec, and 72˚C for 1 min followed by final extension at 72˚C for 10 min. PCR products were examined by agarose gel electrophoresis.

Plasmid construction and transfection

Three types of expression vector plasmids were used for transfection. An expression vector carrying the *egfp* gene under the control of the immediate early 2 (IE2) viral promoter (IE2::EGFP) was previously constructed [30]. The plasmid pIZT-EGFP has the pIZT/V5-His backbone (Thermo Fisher Scientific, Waltham, MA, USA) and the fragment coding the *egfp* ORF was inserted downstream of the IE2 promoter. The plasmid carrying the *egfp* gene driven by the *Bombyx mori actin 3* gene promoter (BmA3::EGFP) was previously constructed [30]. The plasmid carrying the *egfp* gene driven by the *B. mori actin 3* and *Drosophila melanogaster heat-

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**Table 1. List of primers.**

| Primers | sequence (5’ - 3’) |
|---------|-------------------|
| **COI universal primers** | |
| LCO 1490 | GGTCAACAAATCATAAAGATATTGG |
| HCO 2198 | TAAACTTCAAGGGTGACCAAAAAATCA |
| **Gene-specific primers** | |
| EGFP L | ACGTAAAACGGCCACAAGTTCC |
| EGFP R | CGCCCGCTTACTTGTACAGCTC |
| Am ef-1α L | GTGTGTAACAAATTGATTGTTGGTG |
| Am ef-1α R | AAGACGGAGAGCCTGCTGTGTA |
| **T7-flanking primers for dsRNA synthesis** | |
| EGFP dsRNA L | TAATACGACTCACTATAGGGGCGTGAACACCTACCTAC |
| EGFP dsRNA R | TAATACGACTCACTATAGGGGCGTGAACACCTACCTAC |
| Am kmo dsRNA F | TAATACGACTCACTATAGGGGCGTGAACACCTACCTAC |
| Am kmo dsRNA R | TAATACGACTCACTATAGGGGCGTGAACACCTACCTAC |

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shock protein 70 gene promoters in tandem (BmA3::EGFP/Dmhsp70::EGFP) was a transformation vector for the sawfly, Athalia rosae [31]. These vector plasmids were proven to induce egfp gene expression in the cultured cells of lepidopteran species (IE2::EGFP and BmA3::EGFP) [30] and D. melanogaster (BmA3::EGFP/Dmhsp70::EGFP) [32]. Vector plasmids were extracted and purified from Escherichia coli host cells (DH5α) using the QIAGEN Plasmid Mini kit (Qiagen, Hilden, Germany) or Mini Plus Plasmid Extraction kit (Viogene, New Taipei City, Taiwan).

One day after the incubation, Day 1 cells were subjected to transfection after culture medium had been replaced by antibiotic-free medium (Fig 2). Two transfection reagents, TransIT-Insect (Mirus, Bio, Corp., Madison, WI, USA) and FuGene HD (Promega, Madison, WI, USA) were used. The plasmid was mixed with 9 μl/well of serum-free culture medium and transfection reagent (transfection mixture). After an incubation at 25˚C for 30 min, the transfection mixture was applied to Day 1 cells in the wells and the plate was incubated at 34˚C.

Gene knockdown by RNA interference (RNAi)

Short cDNA fragments of the egfp gene (298 bp) and kynurenine 3-monooxygenase (kmo) gene of A. mellifera (GenBank: XM_624243) (357 bp) were PCR-amplified using a PrimeStar HS DNA polymerase (Takara Bio, Shiga, Japan), gene-specific primers to which the T7 RNA polymerase promoter sequence was incorporated at the 5’ end (Table 1). kmo dsRNA was used as the control because the kmo mutation does not affect viability in several insects including hymenopteran species [33–35]. The template DNAs used were as follows: the BmA3::EGFP/Dmhsp70::EGFP plasmid carrying egfp ORF, and cDNA synthesized from the total RNA of workers in the early pupal stage using Prime Script II (Takara Bio). Each dsRNA was synthesized using an Ambion MEGAscript T7 kit (Ambion, Thermo Fisher Scientific) and the PCR-amplified T7-flanking DNA fragment as a template. The concentration of synthesized dsRNA was adjusted to 5 μg/μl and maintained at –20˚C until used.

dsRNA (1 μg/well) was added to the transfection mixture, incubated at 25˚C for 30 min, and applied to Day 1 cells (RNAi, procedure A, Fig 2). In another experiment (RNAi, procedure B, Fig 2), dsRNA alone was applied to Day 1 cells and incubated for one day, namely cells were soaked in dsRNA-containing culture medium for one day. Day 2 cells soaked in dsRNA-

Fig 2. Manipulation timeline of the ex vivo cell culture. Days after incubation were referred to as Day 0, Day 1, Day 2, and thereafter. The cells on each day after the incubation were referred to as Day 1 cells, Day 2 cells, Day 3 cells, and thereafter. Experimental procedures for the induction or inhibition of gene expression are described below the time course. To induce gene expression, the expression vector plasmid was transfected into cells on Day 1. To inhibit gene expression (RNAi, procedure A), a mixture of the expression vector plasmid and dsRNA was transfected into cells on Day 1. In another procedure to inhibit gene expression (RNAi, procedure B), cells were incubated with dsRNA-containing culture medium on Day 1, incubated for one day, and the expression vector plasmid was then transfected into cells on Day 2.
containing culture medium were then subjected to the transfection of the expression vector plasmid with transfection reagent as described above. Cells treated with dsRNA were examined for EGFP fluorescence under a fluorescent microscope three days after transfection (Fig 2), namely Day 4 cells for procedure A and Day 5 cells for procedure B, and total RNA was then extracted for a RT-PCR analysis.

Results

Viability of ex vivo cultured honey bee cells

Among the cells prepared from the larval hemolymph, embryos and fat bodies, hemolymph-derived cells were suitable for further cultivation because they were uniform in size and adherent (Fig 3). In contrast, embryonic cells and fat bodies were both difficult to manipulate due to their overall characteristics. Embryonic cells were a mixture of cells derived from various tissues and inefficient to recover because the total number of viable cells to introduce into the culture was low. Fat body cells were aggregated and irregular in shape and size, in addition to the difficulty of recovery from pupae. Hemolymph-derived cells had the characteristics of adherent hemocytes consisting of plasmatocytes and granulocytes. Filopodia, which are required to adhere to foreign surfaces, were observed. Spreading plasmatocytes had spindles

Fig 3. Morphology of honey bee cells prepared from each tissue. A. Embryonic cells derived from whole embryos. B. Pupal fat body cells were aggregated and irregular in shape and size. C. Hemolymph-derived cells were adherent and relatively uniform in shape and size. D. A magnified image of larval hemocytes. The arrow and arrowhead indicate a plasmatocyte and granulocyte, respectively. Bars indicate 100 μm.

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(Fig 3D, arrow) and granulocytes were round in shape (Fig 3D, arrowhead) [36–38]. Hemo-
cytes fully confluent in a well of a 96-well plate (15 larval hemolymph equivalents/well, or
approximately $4.45 \times 10^5$ cells/well) were cultured at $34^\circ$C in Grace’s insect medium contain-
ing 10% FBS or MGM-450 medium containing 20% FBS supplemented with 10% antibiotics.
More than 80% of cells cultured in Grace’s insect medium and approximately 70% of those in
MGM-450 medium were viable after 14 days (Fig 4). Hemocyte cells were successfully cultured
in 33 out of 36 replicates, and cells in the three unsuccessful cases were contaminated and
eventually died. We investigated whether these cells originated from the honey bee was exam-
ined by the PCR amplification of a mitochondrial cytochrome c oxidase subunit I (COI) gene
fragment (709 bp). The sequences of 29 clones from four independent cultures were deter-
mined, and 28 completely matched that of A. mellifera, while one had a single-base substitu-
tion. These results confirmed the cells were of A. mellifera origin (Fig 5).

**Induction of gene expression**

Three types of expression vector plasmids were subjected to transfection. All plasmids carried the egfp gene as a reporter under the control of different promoters: the *immediate early 2* (IE2) viral promoter (IE2::EGFP), the *Bombyx mori actin3* gene promoter (BmA3::EGFP), and the *B. mori actin3* and *Drosophila melanogaster heat-shock protein 70* gene promoters in tandem (BmA3::EGFP/Dmhsp70::EGFP). Day 1 cells were transfected with each plasmid using transfection reagents. EGFP fluorescence was detected one day after transfection through Day 14, only when the IE2-driven egfp gene vector (IE2::EGFP) was used (Fig 6). It is noted that there was no background autofluorescence in control (untreated) cells (S1 Fig). The number of cells emitting EGFP fluorescence peaked on Day 4–5 or 3–4 days after transfection. Transfection efficiency in terms of the proportion of cells exhibiting EGFP in Day 5 cells was higher when cells were cultured in Grace’s insect medium than in MGM-450 insect medium. TransIT-Insect showed higher transfection efficiency than FuGene HD (Fig 7A).

We then selected the optimal conditions for inducing gene expression using cells cultured
in Grace’s insect medium and transfected with the IE2::EGFP vector plasmid using the TransIT-
Insect reagent. The number of EGFP-positive cells was small when the recommended proto-
col of the TransIT-Insect reagent (0.1 μg of plasmid DNA with 0.2 μl of the TransIT-Insect

![Fig 4. Ex vivo cultured hemocyte cells and viability after incubation. A. Hemocyte cells obtained from the hemolymph of 15 larval equivalents were fully confluent (approximately $4.45 \times 10^5$ cells/well) in a well of a 96-well plate. The bar indicates 100 μm. B. Viability of cells after the incubation. Viability is the ratio of viable cells among all cells counted using a hemocytometer (three replications for each incubation period). Error bars indicate the standard error of the mean.](https://doi.org/10.1371/journal.pone.0257770.g004)
reagent) was applied to Day 1 cells for transfection (Fig 7B, left). Day 1 cells were then transfected with different amounts of plasmid DNA (1.0, 3.0, 5.0 and 10.0 μg) using 0.4 μl of the TransIT-Insect reagent (Fig 7B, right). EGFP-positive cells became detectable when the amount of Trans-IT-Insect reagent was doubled (0.4 μl) and plasmid DNA was more than 1.0 μg. The proportion of EGFP-positive cells increased when 5.0 μg of plasmid DNA was applied, while the viability of cells was not affected by the amount of the plasmid added. Plasmid DNA (5.0 μg) was then applied to Day 1 cells using different volumes (0.4, 0.8, 1.0 and 1.5 μl) of the TransIT-Insect reagent. The proportion of EGFP-positive cells was the highest when 0.8 μl of TransIT-Insect was used; however, the addition of more than 1.0 μl of the TransIT-Insect reagent reduced the proportion of EGFP-positive cells (Fig 7C). The exchange of medium on the day after the addition of the TransIT-Insect reagent promoted cell survival because an excess amount of the TransIT-Insect reagent is toxic to cells (Fig 7D). Therefore, to induce gene expression, cells incubated in Grace’s insect medium need to be transfected with 5.0 μg of the IE2::EGFP vector plasmid using 0.8 μl of the TransIT-Insect reagent. Under optimal conditions, transfection efficiency (EGFP-positive cells among viable cells) was 7.78% ± 0.49 (mean ± SE) (16 replications).

Inhibition of gene expression by RNAi
dsRNA corresponding to a part of the egfp gene was synthesized and applied to the cell culture. The IE2::EGFP vector plasmid and egfp dsRNA or control kmo dsRNA were mixed and introduced into Day 1 cells using the TransIT-insect reagent (RNAi, procedure A). The expression of the egfp gene was examined on Day 4, namely, three days after application. The egfp transcript was maintained at a lower level than in cells devoid of egfp dsRNA (Fig 8). The proportion of EGFP-positive cells also markedly declined (Fig 9). egfp transcripts and the proportion of EGFP-positive cells were unaffected by the application of kmo dsRNA. We then examined

Fig 5. Alignment of nucleotide sequences of the mitochondrial cytochrome c oxidase subunit I (COI) gene. A partial sequence of the Apis mellifera COI gene (658 bp excluding the primer sites) is shown on the top. Twenty-eight out of the 29 clones obtained from ex vivo cultured cells had the same sequences to A. mellifera COI (middle line). One clone (bottom line) had a single base substitution (arrow). Asterisks indicate identical bases.

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whether *egfp* dsRNA was taken up by cultured cells without the transfection reagent when it was added to culture medium (RNAi, procedure B). *egfp* dsRNA was added to Day 1 cells and soaked for a day, and the IE2::EGFP vector plasmid was transfected into cells using the TransIT-Insect reagent on the next day (Day 2). The level of the *egfp* gene transcript remained low and EGFP fluorescence was only detected in a small proportion of cells, similar to when the mixture of the plasmid vector and *egfp* dsRNA was transfected into cells (Fig 9).

These results indicated that gene expression in *ex vivo* cultured cells was inhibited by RNAi-mediated gene silencing regardless of the presence of a transfection reagent. The gene knockdown effect persisted for at least one week after the introduction of dsRNA.

**Discussion**

*Ex vivo* cultured hemocyte cells are capable of inducing gene mis-expression (induction and suppression) and serve as an *in vitro* system for a gene functional analysis of the honey bee (Figs 1 and 10). Commercially available culture media and transfection reagents are available for these experiments. Although continuous cell lines are desirable for *in vitro* experiments, particularly virus-cell interactions in the honey bee, only two cell lines, both derived from
embryos, have been reported to date: one is an established continuous cell line (AmE-711) [16] and the other is an engineered cell line (MYN9) by the introduction of a human nuclear proto-oncogene (c-myc) [18]. Difficulties are associated with maintaining these cell lines, which may be due to viral infection [15, 17, 18]. In contrast, primary cultured cells have been established from various tissues and organs in the honey bee, and these primary cell cultures are proven to be useful for viral studies and neuronal processes [15, 39, 40]. Although primary cell cultures and ex vivo cultured cells may only be maintained for a limited period of time under healthy conditions, these cells are available for examining gene expression and signaling pathways [2, 5]. The present results demonstrating the induction of gene mis-expression indicate that ex vivo cultured hemocyte cells will be a practical option for an in vitro system for the honey bee. Gene expression was evidently induced, and transcripts were detectable after PCR amplification; however, the proportion of the cells in which EGFP fluorescence was detected was low. Mosquito cell lines with low transfection efficiency have been used for luciferase reporter gene assays [41]. Reporter gene assays are highly sensitive, and generally feasible as

Fig 7. Selection of optimal conditions for the induction of gene expression. A. Comparison of transfection reagents. B. The optimal amount of vector plasmid DNA to be transfected was 5.0 μg/well when 0.4 μl/well of the TransIT-Insect reagent was applied. The graph on the left shows transfection efficiency when the conditions recommended by the supplier were employed. C. The optimal dose of the transfection reagent was 0.8 μl/well when 5.0 μg/well of plasmid DNA was applied. D. Effects of medium exchange after transfection on cell viability. Error bars indicate the standard error of the mean of three replications.

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Fig 8. Comparison of transcripts levels by RT-PCR. Short fragments of the *egfp* gene (656 bp, top panel) and *A. mellifera* elongation factor-1α gene (315 bp, bottom panel) were amplified using gene-specific primer sets and total RNA extracted from Day 5 cells as the templates. *egfp* gene transcripts were detected in cells transfected with the IE2::EGFP vector, but not in cells to which dsRNA corresponding to the *egfp* gene was applied. Each lane indicates the treatments that *ex vivo* cells received: no treatment (Control), transfection of the IE2::EGFP vector (IE2::EGFP), transfection of the IE2::EGFP vector associated with *egfp* dsRNA (IE2::EGFP + *egfp* dsRNA), and transfection of the IE2::EGFP vector associated with dsRNA corresponding to an unrelated *A. mellifera* kynurenine 3-monoxygenase (kmo) gene (IE2::EGFP + kmo dsRNA).

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Fig 9. Fluorescent micrographs showing a decrease in EGFP emissions by RNAi-based gene silencing in *ex vivo* cultured cells. Top panels (A–C) show the effects of the application of dsRNA using the transfection reagent (RNAi, procedure A), and bottom panels (E–G) show the effects of soaking with dsRNA without transfection reagent (RNAi, procedure B). *Ex vivo* cells were transfected with the IE2::EGFP vector (A and E, IE2::EGFP), transfected with the IE2::EGFP vector and the application of *egfp* dsRNA (B and F, +*egfp* dsRNA), and transfected with the IE2::EGFP vector and application of kmo dsRNA (C and G, +kmo dsRNA). D, H. The transfection of and soaking with *egfp* dsRNA both decreased the proportion of EGFP-positive cells. Bars indicate the standard error of the mean of three replications.

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long as the expression vectors may be transfected into a portion of the cultured cells. *Ex vivo* cultured hemocyte cells, even with low transfection efficiency, may be used for these assays. Possible applications of *ex vivo* cultured hemocyte cells will involve the study of factors (*cis* and *trans* elements) that influence transcriptional activity and their utilization as material for
RNA-seq after induction of conditional expression. *Ex vivo* cultured hemocyte cells may be used to examine susceptibility to chemical reagents such as insecticides, in combination with reporter gene assays. They will also be applicable to the screening of effective genome editing tools, CRISPR/Cas9, through examinations of DNA-digesting efficiency by inducing the expression of the vector bearing guide RNA and cDNA of nuclease.

One day after the incubation (Day 1), replace the culture medium with antibiotic-free medium. Induction of gene expression (left):

1. Mix 5.0 μg of the IE2::EGFP vector plasmid and 0.8 μl of the TransIT-Insect reagent in 9 μl of serum-free culture medium (transfection mixture) and incubate at 25˚C for 30 min.
2. Apply the transfection mixture to Day 1 cells in a well and incubate at 34˚C.
3. Replace culture medium with fresh medium on the next day (Day 2) and incubate at 34˚C.

Inhibition of gene expression by the application of dsRNA at the same time as transfection (middle, RNAi, procedure A):

1. Mix 5.0 μg of the IE2::EGFP vector plasmid, 0.8 μl of the TransIT-Insect reagent and 1 μg of egfp dsRNA in 9 μl of serum-free culture medium and incubate at 25˚C for 30 min.
2. Apply the mixture to Day 1 cells in a well and incubate at 34˚C.
3. Replace culture medium with fresh medium on the next day (Day 2) and incubate at 34˚C.

Inhibition of gene expression by the soaking of dsRNA prior to transfection (right, RNAi, procedure B):

1. Apply 1 μg of egfp dsRNA to Day 1 cells in a well and incubate at 34˚C for one day.
2. Mix 5.0 μg of the IE2::EGFP vector plasmid and 0.8 μl of the TransIT-Insect reagent in 9 μl of serum-free culture medium (transfection mixture) and incubate at 25˚C for 30 min.
3. Apply the transfection mixture to Day 2 cells in a well and incubate at 34˚C.
4. Replace culture medium with fresh medium on the next day (Day 3) and incubate at 34˚C.

The successful RNAi-based gene silencing of *ex vivo* cultured cells was achieved by simply soaking with dsRNA in culture medium. Gene silencing by dsRNA-mediated RNAi in cell cultures has been exclusively performed using established continuous cell lines [42]. Following the success of soaking RNAi in *Drosophila* S2 cells [43], this method has been applied to other insect cell lines, such as lepidopteran Sf21 originating from *Spodoptera frugiperda* and SL-1 from *S. litura*, Tc81 and TcA from the coleopteran *Tribolium castaneum*, and Lepd-SL1 from the coleopteran *Leptinotarsa decemlineata* [44–47]. The functional uptake of dsRNA achieving RNAi-based gene silencing occurred by the soaking method in these cell lines. Similarly, dsRNA in culture medium was taken up by *ex vivo* cultured honey bee hemocyte cells. It is important to note that insect hemocytes are more likely to be sensitive to dsRNA uptake than other tissues, even in species in which systemic RNAi is not recognized [48–51]. Previous studies demonstrated that phagocytic hemocytes in *D. melanogaster* are specialized in the uptake of dsRNA by endocytosis, which is mediated by scavenger-like pattern-recognition receptors [52, 53]. RNAi-based gene silencing in *vivo* by the injection of dsRNAs into the adult body cavity is less effective in the honey bee because tissue-dependent dsRNA uptake seems to be limited to fat body cells [54]. However, cultured cells are free from the constraints imposed in *in vivo* environments that interfere with dsRNA uptake, such as nucleases degrading dsRNA in the hemolymph [55] and macromolecules competing with scavenger-like receptors for the...
binding of dsRNA [52]; therefore, ex vivo hemocyte cells are susceptible to RNAi, which also reflects its original physiological characteristics. The transfection of dsRNA into a cell culture is an alternative to the soaking method and effectively induces intracellular RNAi in ex vivo cultured honey bee hemocyte cells, similar to the continuous cell lines of Sf21 and CiE1 from the lepidopteran Chrysodeixis includens [56, 57].

Commercially available culture media and transfection reagents are compatible with the ex vivo honey bee cell culture method described here. The viral promoter, IE2, which is often used in versatile expression vectors, induced constitutive gene expression; however, the promoters of different insect species (Bombbyx actin3 and Drosophila heat-shock protein 70) did not work in the present study. Some promoters of honey bee genes have been shown to drive conditional gene expression in the Sf21 cell line [58], and are assumed to function in ex vivo cultured honey bee cells. The manipulation of endogenous gene expression will enable analyses of the functions of promoters, cis regulatory elements, transcriptional factors, and signal transduction.

We selected hemocyte cells for the ex vivo culture primarily because of their adherence and ease of handling. Insect hemocytes play crucial roles in the cellular immune system [59]. Specific cellular reactions, such as phagocytosis, micro-aggregation, nodulation, and encapsulation, have been examined in detail [59, 60]; however, there are relatively few studies on the honey bee [61, 62]. The molecular mechanisms underlying insect cellular immunity remains unclear [2]. Since the in vitro system of insect hemocytes that retains their original characteristics is a requisite for examining the essential processes of cellular immune reactions, attempts to develop hemocyte primary cell cultures have been made in several insects, including the honey bee [40, 63–68]. Primary cultured honey bee hemocytes could be infected with sacbrood virus and Flock House virus, which replicate intercellularly and peak in abundance 72–96 hours after infection [40]. Our method to enable the control of gene expression in ex vivo cultured honey bee hemocyte cells is simple and reproducible. A functional analysis of candidate genes involved in the cellular immune response will be possible using ex vivo cultured honey bee hemocytes through the combination of viral infection and RNAi-based gene silencing. The method described here may be applicable to other insect species and provides an in vitro system not only for immunological examinations, but also a wide range of insect studies.

Supporting information

S1 Fig. Autofluorescence was undetectable in ex vivo cultured hemocyte cells. Top panels show phase-contrast micrographs of A. untreated (intact) ex vivo cultured hemocyte cells and C. cells transfected with expression vectors of IE2::EGFP. Bottom panels show the micrographs of B. untreated cells and D. cells transfected with expression vectors of IE2::EGFP. Background autofluorescence was detected neither in untreated (intact) cells nor egfp expression-induced cells. All cells represent Day 7 cells. Bars indicate 100 μm.
(PDF)

S2 Fig. Raw agarose gel images for Fig 8. Gel images were taken using a FUNA-BOX Imaging System (FBOX-03Sir, Funakoshi, Tokyo, Japan) equipped with a digital camera (XZ-2, Olympus, Tokyo, Japan) and UV transilluminator (M10E, UVP, Upland, CA USA). Images were cropped and labeled using Adobe Photoshop Elements 15 software.
(PDF)

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References

1. Bairoch A. The Cellosaurus, a cell line knowledge resource. J Biomol Tech. 2018; 29: 25–38. https://doi.org/10.7171/jbt.18-2902-002 PMID: 29805321

2. Smaghe G, Goodman CL, Stanley D. Insect cell culture and applications to research and pest management. In Vitro Cell Dev Biol Animal. 2009; 45: 93–105. https://doi.org/10.1007/s11626-009-9181-x PMID: 19247722

3. Thompson CM, Montes J, Aucoin MG, Kamen AA. Recombinant protein production in large-scale agitated bioreactors using the baculovirus expression vector system. In: Murhammer D, editor. Baculovirus and Insect Cell Expression Protocols, Methods in Molecular Biology. Vol 1350. New York: Humana Press; 2016. pp. 241–261. https://doi.org/10.1007/978-1-4939-3043-2_11 PMID: 26820861

4. Arunkarthick S, Asokan R, Aravindharaj R, Niveditha M, Krishna Kumar NK. A review of insect cell culture: establishment, maintenance and applications in entomological research. J Entomol Sci. 2017; 52: 261–273. https://doi.org/10.18474/JES17-02PT.1

5. Mandrioli M, Monti M, Tedeschi R. A practical guide to insect cell cultures: establishment and maintenance of primary cell cultures. Halteres. 2015; 6: 132–141. https://www.biodiversitylibrary.org/page/49900449

6. Potts SG, Biesmeijer JC, Kremen C, Neumann P, Schweiger O., Kunin, WE. Global pollinator declines: trends, impacts and drivers. Trends Ecol Evol. 2010; 25: 345–353. https://doi.org/10.1016/j.tree.2010.01.007 PMID: 20188434

7. Goulson D, Nicholls E, Botías C, Rotheray EL. Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. Science. 2015; 347: 1255957. https://doi.org/10.1126/science.1255957 PMID: 25721506

8. Manley R., Boots M. and Wilfert L. Emerging viral disease risk to pollinating insects: ecological, evolutionary and anthropogenic factors. J Appl Ecol. 2015; 52: 331–340. https://doi.org/10.1111/1365-2664.12385 PMID: 25954053

9. Chen YP, Siede R. Honey bee viruses. Adv Virus Res. 2007; 70: 33–80. https://doi.org/10.1016/S0065-3527(07)70002-7 PMID: 17765703

10. Cox-Foster DL, Conlan S, Holmes EC, Palacios G, Evans JD, Moran NA, et al. A metagenomic survey of microbes in honey bee colony collapse disorder. Science. 2007; 318: 283–287. https://doi.org/10.1126/science.1146498 PMID: 17823314

11. McMenamin AJ, Genersch E. Honey bee colony losses and associated viruses. Curr Opin Insect Sci. 2015; 8: 121–129. https://doi.org/10.1016/j.cois.2015.01.015 PMID: 32846659

12. Carrillo-Tripp J, Bonning BC, Miller WA. Challenges associated with research on RNA viruses of insects. Curr Opin Insect Sci. 2015; 8: 62–68. https://doi.org/10.1016/j.cois.2014.11.002 PMID: 32846681

13. Hunter WB. Medium for development of bee cell cultures (Apis mellifera: Hymenoptera: Apidae). In Vitro Cell Dev Biol Animal. 2010; 46: 83–86. https://doi.org/10.1007/s11626-009-9246-x PMID: 20033792

14. Genersch E, Gisder S, Hedtke K, Hunter WB, Möckel N, Müller U. Standard methods for cell cultures in Apis mellifera research. J Apicult Res. 2013; 52: 1–8. https://doi.org/10.3896/IBRA.1.52.1.02
15. Guo Y, Goodman CL, Stanley DW, Bonning BC. Cell lines for honey bee virus research. Viruses. 2020; 12: 236. https://doi.org/10.3390/v12020236 PMID: 32093360

16. Goblirsch MJ, Spivak MS, Kurtti TJ. A cell line resource derived from honey bee (Apis mellifera) embryonic tissues. PLoS ONE. 2013; 8: e69831. https://doi.org/10.1371/journal.pone.0069831 PMID: 23894551

17. Carrillo-Tripp J, Dolezal AG, Goblirsch MJ, Miller WA, Toth AL, Bonning BC. In vivo and in vitro infection dynamics of honey bee viruses. Sci Rep. 2016; 6: 22265. https://doi.org/10.1038/srep22265 PMID: 26923109

18. Kitagishi Y, Okumura N, Yoshida H, Nishimura Y, Takahashi J, Matsuda S. Long-term cultivation of in vitro Apis mellifera cells by gene transfer of human c-myc proto-oncogene. In Vitro Cell Dev Biol Animal. 2011; 47: 451–453. https://doi.org/10.1007/s11626-011-9431-6 PMID: 21688083

19. Honey bee genome sequencing consortium. Insights into social insects from the genome of the honeybee Apis mellifera. Nature. 2006; 443: 931–949. https://doi.org/10.1038/nature05260 PMID: 17073008

20. Elskig CG, Worley KC, Bennett AK, et al. Finding the missing honey bee genes: lessons learned from a genome upgrade. BMC Genomics. 2014; 15: 86. https://doi.org/10.1186/1471-2164-15-86 PMID: 24479613

21. Beye M, Härte1 S, Hagen A, Hasselmann M, Omholt SW. Specific developmental gene silencing in the honey bee using a homeobox motif. Insect Mol Biol. 2002; 11: 527–532. https://doi.org/10.1046/j.1365-2583.2002.00361.x PMID: 12421410

22. Schulte C, Thielenberg E, Müller-Börg M, Gempe T, Beye M. Highly efficient integration and expression of piggyBac-derived cassettes in the honeybee (Apis mellifera). Proc Natl Acad Sci USA. 2014; 111: 9003–9008. https://doi.org/10.1073/pnas.1402341111 PMID: 24821811

23. Kohno H, Suenami S, Takeuchi H, Sasaki T, Kubo T. Production of knockout mutants by CRISPR/Cas9 in the European honeybee, Apis mellifera L. Zool Sci. 2016; 33: 505–512. https://doi.org/10.2108/zs160043 PMID: 27715425

24. Kohno H, Kubo T. mKast is dispensable for normal development and sexual maturation of the male European honeybee. Sci Rep. 2018; 8: 11877. https://doi.org/10.1038/s41598-018-30380-2 PMID: 30131569

25. Hu XF, Zhang B, Liao CH, Zeng ZJ. High-efficiency CRISPR/Cas9-mediated gene editing in honeybee (Apis mellifera) embryos. Genes Genomes Genet. 2019; 9: 1759–1766. https://doi.org/10.1534/g3.119.400130 PMID: 30948423

26. Chan MMY, Choi SYC, Chan QWT, Li P, Guarna MM, Foster LJ. Proteome profile and lentiviral transduction of cultures honey bee (Apis mellifera L.) cells. Insect Mol Biol. 2010; 19: 653–658. https://doi.org/10.1111/j.1365-2583.2010.01022.x PMID: 20546039

27. Mitsuhashi J. Invertebrate tissue culture methods. Tokyo: Springer; 2002. https://doi.org/10.1007/978-4-431-67875-5

28. Mitsuhashi J. Invertebrate tissue culture methods. Tokyo: Springer; 2002. https://doi.org/10.1007/978-4-431-67875-5

29. Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoeck R. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol. 1994; 3: 294–299. https://pubmed.ncbi.nlm.nih.gov/7881515/ PMID: 7881515

30. Watanabe K, Kobayashi I, Hatakeyama M, Kayukawa T, Akiduki G. Establishment and characterization of novel cell lines derived from six lepidopteran insects collected in the field. In Vitro Cell Dev Biol Animal. 2011; 56: 425–429. https://doi.org/10.1111/j.1365-2583.2010.01022.x PMID: 20546039

31. Sumitani M, Yamamoto DS, Oishi K, Lee JM, Hatakeyama M. Germline transformation of the sawfly, Athalia roase (Hymenoptera: Symphyta), mediated by a piggyBac-derived vector. Insect Biochem Mol Biol. 2003; 33: 449–458. https://doi.org/10.1016/s0965-1748(03)00009-2 PMID: 12650693

32. Uetake H, Oka K, Niki Y. Stable transformation and cloning mediated by piggyBac vector and RNA interference knockdown of Drosophila ovarian cell line. In Vitro Cell Dev Biol Animal. 2011; 47: 689–694. https://doi.org/10.1007/s11626-011-9463-y PMID: 22083624

33. Sun D, Guo Z, Liu Y, Zhang Y. Progress and prospects of CRISPR/Cas systems in insects and other Arthropods. Front Physiol. 2017; 8: 608. https://doi.org/10.3389/fphys.2017.00608 PMID: 28932198

34. Werren JH, Loehlin DW. The parasitoid wasp Nasonia: an emerging model system with haploid male genetics. Cold Spring Harb Protoc. 2009; pdb.emo134. https://doi.org/10.1101/pdb.emo134 PMID: 20147035

35. Li M, Au LYC, Douglas D, Chong A, White BJ, Ferree PM, et al. Generation of heritable germline mutations in the jewel wasp Nasonia vitripennis using CRISPR/Cas9. Sci Rep. 2017; 7: 901. https://doi.org/10.1038/s41598-017-00990-3 PMID: 28424460
36. Richardson RT, Ballinger MN, Qian F, Christman JW, Johnson RM. Morphological and functional characterization of honey bee, *Apis mellifera*, hemocyte cell communities. Apidologie. 2018; 49: 397–410. https://doi.org/10.1007/s13592-018-0566-2

37. Kamezaki M, Yoko K, Miura K. RNA interference mediated knockdown of an inhibitor of apoptosis protein induces apoptosis in *Mythimna separata* (Lepidoptera: Noctuidae), *Eur J Entomol*. 2018; 115: 223–231. https://doi.org/10.14411/eje.2018.021

38. Lavine MD, Strand MR. Insect hemocytes and their role in immunity. *Insect Biochem Mol Biol*. 2002; 32: 1295–1309. https://doi.org/10.1016/s0965-1748(02)00992-9 PMID: 1225920

39. Wu Y, Yuan X, Li J, Kadowaki T. DWV infection in *in vivo* using honey bee pupal tissue. *Front Microbiol*. 2021; 12: 631889. https://doi.org/10.3389/fmicb.2021.631889 PMID: 3364326

40. McMenamin AJ, Parekh F, Lawrence V, Flenniken ML. Investigating virus–host interactions in cultured primary honey bee cells. *Insects*. 2021; 12: 653. https://doi.org/10.3390/insects12070653 PMID: 34357313

41. Zhao YG, Eggleston P. Comparative analysis of promoters for transient gene expression in cultured mosquito cells. *Insect Mol Biol*. 1999; 8: 31–38. https://doi.org/10.1046/j.1365-2583.1999.810031.x PMID: 9921712

42. Yu N, Christiansen O, Liu J, Niu J, Caccia S, et al. Delivery of dsRNA for RNAi in insects: an overview and future directions. *Insect Sci*. 2013; 20: 4–14. https://doi.org/10.1111/j.1744-7917.2012.01534.x PMID: 23955821

43. Clemens JC, Worby CA, Simonson-Leff N, Muda M, Maehama T, Hemmings BA., et al. Use of double-stranded RNA interference in *Drosophila* cell lines to dissect signal transduction pathways. *Proc Natl Acad Sci USA*. 2000; 97: 6499–6503. https://doi.org/10.1073/pnas.110149597 PMID: 10823906

44. Sivakumar S, Rajagopal R, Venkatesh GR, Srivastava A, Bhatnagar RK. Knockdown of aminopeptidase-N from *Helicoverpa armigera* larvae and in transfected Sf21 cells by RNA interference reveals its functional interaction with *Bacillus thuringiensis* insecticidal protein Cry1Ac. *J Biol Chem*. 2007; 282: 7312–7319. https://doi.org/10.1074/jbc.M607442200 PMID: 17213205

45. Kayukawa T, Tateishi K, Shinoda T. Establishment of a versatile cell line for juvenile hormone signaling analysis in *Tribolium castaneum*. *Sci. Rep.* 2013; 3: 1570. https://doi.org/10.1038/srep01570 PMID: 23535851

46. Shukla JN, Kalsi M, Sethi A, Narva KE, Fishilevich E, Singh S, et al. Reduced stability and intracellular transport of dsRNA contribute to poor RNAi response in lepidopteran insects. *RNA Biol.* 2016; 13: 656–669. https://doi.org/10.1080/15476286.2016.1191728 PMID: 27245473

47. Zhao H, Yi X, Hu Z, Hu M, Chen S, Muhammad RH, et al. RNAi-mediated knockdown of catalase causes cell cycle arrest in SL-1 cells and results in low survival rate of *Spodoptera litura* (Fabricius). *PLoS One*. 2016; 8: e59527. https://doi.org/10.1371/journal.pone.0059527 PMID: 23555693

48. Eleftherianos I, Gökcen F, Felföldi G, Millichap PJ, Trenčzek TE, Ffrench-Constat RH, et al. The immunoglobulin family protein Hemolin mediates cellular immune responses to bacteria in the insect *Manduca sexta*. *Cell Microbiol*. 2007; 9: 1137–1147. https://doi.org/10.1111/j.1462-5822.2006.00855.x PMID: 17166232

49. Miller SC, Brown SJ, Tomoyasu Y. Larval RNAi in *Drosophila*? *Dev Genes Evol.* 2008: 218: 505–510. https://doi.org/10.1007/s00427-008-0238-8 PMID: 18663472

50. Terebus H, Papanicolaou A, Garbutt JS, Eleftherianos I, Huvenne H, Kanginakudru S, et al. RNA interference in Lepidoptera: An overview of successful and unsuccessful studies and implications for experimental design. *J Insect Physiol.* 2011; 57: 231–245. https://doi.org/10.1016/j.jinsphys.2010.11.006 PMID: 21078327

51. Yoko K, Kamezaki M, Yoshida T, Tanaka T, Miura K. Sensitivity to RNA interference-mediated gene silencing in intact and ligated larvae of the armyworm, *Mythimna separata* (Lepidoptera: Noctuidae). *Appl Entomol Zool*. 2013; 48: 431–439. https://doi.org/10.1007/s13355-013-0201-7

52. Saleh MC, van Rij R, Hekele A, Gillis A, Foley AE, O’Farrell PH, et al. The endocytic pathway mediates cell entry of dsRNA to induce RNAi silencing. *Nat Cell Biol*. 2006; 8: 793–802. https://doi.org/10.1038/ncb1439 PMID: 16682164

53. Tassetto M, Kunitomi M, Andino R. Circulating immune cells mediate a systemic RNAi-based adaptive antiviral response in *Drosophila*. *Cell*. 2017; 169: 314–325. https://doi.org/10.1016/j.cell.2017.03.033 PMID: 28388413

54. Jarosch A, Moritz RFA. Systemic RNA-interference in the honeybee *Apis mellifera*: tissue dependent uptake of fluorescent siRNA after intra-abdominal application observed by laser-scanning microscopy. *J Insect Physiol.* 2011; 57: 851–857. https://doi.org/10.1016/j.jinsphys.2011.03.013 PMID: 21439290
55. Singh IK, Singh S, Mogilicherla K, Shukla JN, Palli SR. Comparative analysis of double-stranded RNA degradation and processing in insects. Sci Rep. 2017; 7: 17059. https://doi.org/10.1038/s41598-017-17134-2 PMID: 29213068

56. Valdes VJ, Sampieri A, Sepulveda J, Vaca L. Using double-stranded RNA to prevent in vitro and in vivo viral infections by recombinant baculovirus. J Biol Chem. 2003; 278: 19317–19324. https://doi.org/10.1074/jbc.M212039200 PMID: 23646552

57. Johnson JA, Bitra K, Zhang S, Wang L, Lynn DE, Strand MR. The UGA-CIE1 cell line from Chrysodeixis includens exhibits characteristics of granulocytes and is permissive to infection by two viruses. Insect Biochem Mol Biol. 2010; 40: 394–404. https://doi.org/10.1016/j.ibmb.2010.03.005 PMID: 20381615

58. Schulte C, Lebougle G, Otte M, Grünwald B, Gehne N, Beye M. Honey bee promoter sequences for targeted gene expression. Insect Mol Biol. 2013; 22: 399–410. https://doi.org/10.1111/imb.12031 PMID: 23668189

59. Strand MR. Insect Hemocytes and their Role in Immunity. In: Beckage NE, editor. Insect Immunology. Academic Press; 2008. pp. 25–47. https://doi.org/10.1016/B978-012373976-6.50004-5

60. Marmaras VJ, Lampropoulou M. Regulators and signalling in insect haemocyte immunity. Cell Signal. 2009; 21: 186–195. https://doi.org/10.1016/j.cellsig.2008.08.014 PMID: 18790716

61. Negri P, Maggi M, Ramirez L, Szawarski N, De Feudis L, Lamattina L, et al. Cellular immunity in Apis mellifera: studying hemocytes brings light about bees skills to confront threats. Apidologie. 2016; 47: 379–388. https://doi.org/10.1007/s13592-015-0418-2

62. Hystad EM, Salmela H, Amdam GV, Münch D. Hemocyte-mediated phagocytosis differs between honey bee (Apis mellifera) worker castes. PLoS ONE. 2017; 12: e0184108. https://doi.org/10.1371/journal.pone.0184108 PMID: 28877227

63. Sorescu I, Tanss R, Gheorghe L, Mardare A, Chioveanu G. Attempts to in vitro cultivate honey bee (Apis mellifera L) haemocytes. Stud Res Vet Med. 2003; 9: 123–131. https://scholar.google.com/scholar?cluster=14637902619676646393&hl=en&oi=scholarr

64. Liang T, Ji H, Du J, Ou J, Li W, Wu T, et al. Primary culture of hemocytes from Eriocheir sinensis and their immune effects to the novel crustacean pathogen Spiroplasma eriocheiris. Mol Biol Rep. 2012; 39: 9747–9754. https://doi.org/10.1007/s11033-012-1840-4 PMID: 22561121

65. Defferrari MS, da Silva R, Orchard I, Carlini CR. Jack bean (Canavalia ensiformis) urease induces eicosanoid-modulated hemocyte aggregation in the Chagas’ disease vector Rhodnius prolixus. Toxicon. 2014; 82: 18–25. https://doi.org/10.1016/j.toxicon.2014.02.006 PMID: 24561121

66. Duressa TF, Huybrechts R. Development of primary cell cultures using hemocytes and phagocytic tissue cells of Locusta migratoria: an application for locust immunity studies. In Vitro Cell Dev Biol Animal. 2016; 52: 100–106. https://doi.org/10.1007/s11626-015-9952-5 PMID: 26427710

67. Fruttero LL, Moyetta NR, Uberti AF, Grahl MVC, Lopes FC, Broll V, et al. Humoral and cellular immune responses induced by the urease-derived peptide Jaburetix in the model organism Rhodnius prolixus. Parasit Vectors. 2016; 9: 412. https://doi.org/10.1186/s13071-016-1710-3 PMID: 27455853

68. Hong M, Hwang D, Cho S. Hemocyte morphology and cellular immune response in termite (Reticulitermes speratus). J Insect Sci. 2018; 18: 46. https://doi.org/10.1093/jisesa/eyq039 PMID: 29718507