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Research Article

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The use of cell and larval assays to identify target genes for RNA interference-meditated control of the Australian sheep blowfly (*Lucilia cuprina*)

Yunjia Yang,[a] Bing Zhang,[b] Jiaxi Yong,[a] Peter James,[a] Zhi Ping Xu,[c] Neena Mitter,[a] Timothy J Mahony[a] and Karishma T Mody[a*]

Abstract

BACKGROUND: Flystrike, primarily caused by *Lucilia cuprina*, is a major health and welfare issue for sheep wool industries. Current chemical-based controls can have limited effectiveness due to the emergence of resistance in the parasite. RNA interference (RNAi), which uses double-stranded RNA (dsRNA) as a trigger molecule, has been successfully investigated for the development of innovative pest control strategies. Although RNAi offers great potential, the efficient identification, selection of target genes and delivery of dsRNA represent challenges to be overcome for the successful application of RNAi for control of *L. cuprina*.

RESULTS: A primary *L. cuprina* (blowfly) embryo cell line (BFEC) was established and confirmed as being derived from *L. cuprina* eggs by PCR and amplicon sequencing. The BFECs were successfully transfected with plasmids and messenger RNA (mRNA) expressing fluorescent reporter proteins and dsRNA using lipid-based transfection reagents. The transfection of dsRNA into BEFC in this study suggested decreased mRNA levels of target gene expression, which suggested RNAi-mediated knockdown. Three of the dsRNAs identified in this study resulted in reductions of in target gene mRNA levels in BFEC and loss of biological fitness by *L. cuprina* larvae in a feeding bioassay.

CONCLUSION: This study confirms that the novel BFEC cell line can be used to improve the efficacy of dsRNA-mediated screening to accelerate the identification of potential target genes in the development of RNAi mediated control approaches for *L. cuprina*. The research models established in this study are encouraging with respect to the use of RNAi as a blowfly control method, however further improvement and validation are required for field applicationsnot prefect, and could be ongoing developing.
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Supporting information may be found in the online version of this article.

Keywords: RNA interference based bioinsecticide 1; pest control 2; *Lucilia cuprina* 3; insect primary cell line 4; larval bioassay 5; livestock agriculture 6

1 INTRODUCTION

Pests and diseases have reduced the efficacy of primary production since farming began.[1] According to the Food and Agriculture Organization, 80% of the world's livestock population is exposed to parasites.[2] The parasitic fly *Lucilia cuprina* causes a significant economic burden globally and is responsible for over 80% of sheep flystrike events.[3] Flystrike is one of the most economically important diseases affecting the Australian sheep wool industry, costing more than $356 million annually in lost productivity.[4] There is therefore a pressing need to develop alternative and sustainable approaches for the prevention and improved management of flystrike events associated with *L. cuprina*.

RNA interference (RNAi) has emerged as a potential solution to enable the replacement of existing insecticides with a technology that is highly specific with low environmental impacts.[5] Unlike conventional insecticides, RNAi-based control uses the molecular mechanisms of the pest to confer silencing of essential genes. The successful application of RNAi can drive messenger

* *Correspondence to: KT Mody, Queensland Alliance for Agriculture and Food Innovation), The University of Queensland, Brisbane, QLD, 4072, Australia, E-mail: k.mody@uq.edu.au*

a *Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, Brisbane, Australia*

b *Department of Agriculture and Fisheries, Ecosciences Precinct (ESP), Brisbane, Australia*

c *Australian Institute for Bioengineering and Nanotechnology (AIBN), The University of Queensland, Brisbane, Australia*

RNA (mRNA) degradation in a sequence-specific manner to block targeted protein production. Depending on the developmental stage of the pest and the role of the targeted gene, this can result in the inhibition of larval growth and/or pest death.

Currently, the efficient selection and delivery of dsRNA is a major barrier to successful use of RNAi in many systems. To develop more efficient dsRNA target screening and to minimize time and costs, cell-based screening assays appear to be the most efficient method prior to laboratory and field testing of dsRNAs in insect trials.[6] The S2 cell line, produced from *Drosophila melanogaster* embryos, was the initial cell line employed to demonstrate passive dsRNA uptake by incubating the cells with dsRNA added directly to the growth media.[7] This approach became the most frequently used strategy for inducing the RNAi responses in S2 cells.[8,9] This approach has been widely applied for RNAi research in many different insect cell lines.[10] However, different cell lines can have varying capacities for direct dsRNA uptake. Some cells could simply take up dsRNA when naked dsRNA is directly added into the cell culture medium.[10–12] Other cells showed higher uptake rates when the dsRNA was complexed with a transfection reagent or only showed dsRNA uptake with the use of a specific transfection reagent.[13] Hence, transfection method development and optimization are critical for efficient utilization of RNAi in new cell lines when screening dsRNA activities.

To date, there have been no published studies reporting the production of a cell line for *L. cuprina*. In addition, there is a lack of RNAi studies that show insecticidal activity of dsRNA-mediated RNAi in *L. cuprina*. This study is the first to demonstrate the potential of dsRNA for future *L. cuprina* insect management (Fig. 1). The transfection methods and techniques used in this study can serve as a reference for future studies, providing an important tool to develop more effective transfection strategies. The transfection protocol and cell-basic gene selection model developed in this research article will increase the efficiency of target gene selection for the development of RNAi-based bio-insecticides.

2 MATERIALS AND METHODS

2.1 Primary BFEC establishment

2.1.1 Culture medium preparation

Culture medium (1 L) was prepared by combining 340 mL of Schneider's Drosophila Medium (1×), 340 mL of Dulbecco's Modified Eagle Medium (DMEM) (1×), 200 mL of fetal bovine serum (FBS), 100 mL of sterilized 30% yeast extract, 10 mL of GlutaMAX (100×), and 10 mL of antibiotic-antimycotic (Anti-Anti) (100×). The medium was stored at 4°C and used in a UV sterilized Class II biosafety cabinet. Cell freezing medium was prepared by combining 10% (v/v) dimethyl sulfoxide (DMSO) with the blowfly embryo cell line (BFEC) culture medium and mixing thoroughly.

2.1.2 Primary BFEC establishment and passaging

To establish a *L. cuprina* primary BFEC, freshly hatched blowfly eggs were collected and subjected to a series of washing steps. Initially, eggs (approximately $n = 300$) were washed with warm tap water and then with 2.5% (v/v) sodium hypochlorite for 20 min. The washed eggs were then moved to a sterilized Class II biosafety cabinet and washed for a third time with sterile phosphate-buffered saline (PBS). After centrifugation at 80 × *g* at 8°C for 10 min, the egg pellet was collected and gently rewashed with sterile PBS with a transfer pipette and recentrifuged. The egg pellet was then ground using a sterile pestle to disrupt the eggs and disassociate the embryonic cells. The

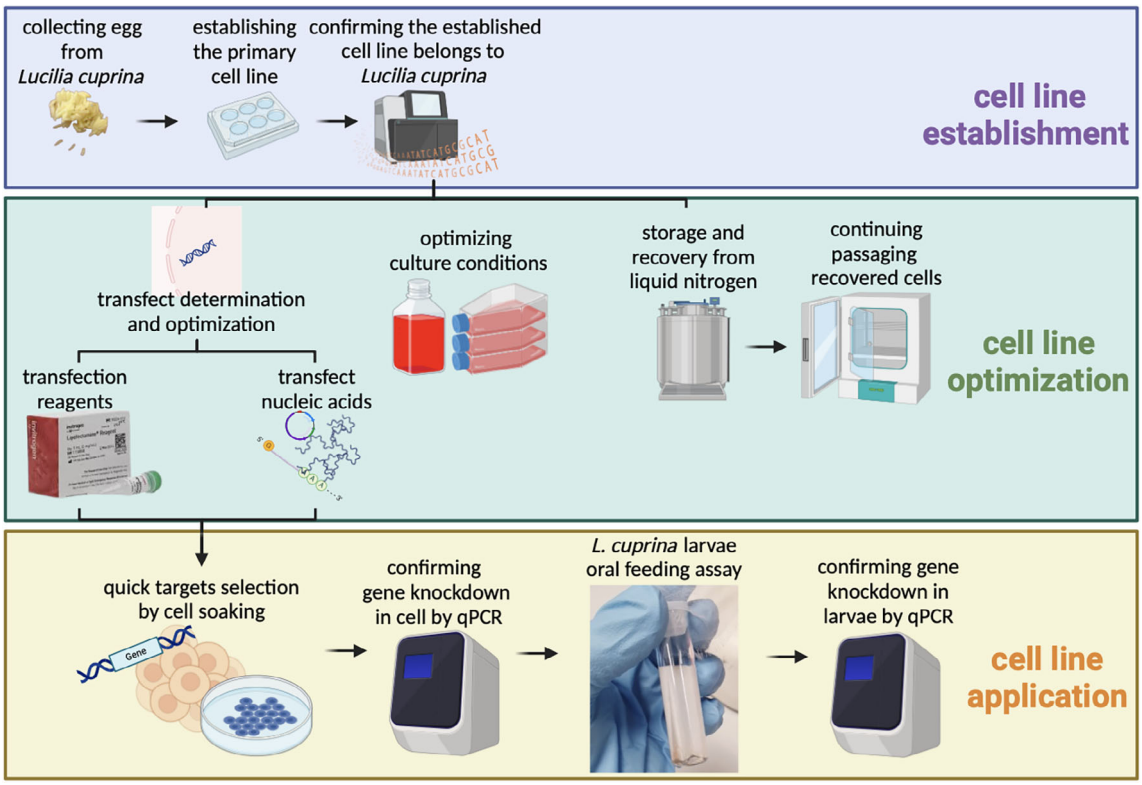

Figure 1. Schematic diagram showing *L. cuprina* (blowfly) embryo cell line (BFEC) establishment, maintenance, optimization, transfection and the following cell-basic gene selection model. The schematics were created with Biorender.com.

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disassociated egg cells were again washed five times with culture medium as described above to minimize the risk of bacterial contamination and remove any residual impurities. The cultures were maintained in an incubator at 26°C under atmospheric conditions.

2.1.3 Storage cell line in liquid nitrogen and recovery

For long-term storage, the putative BFECs were dislodged using gentle pipetting with a sterile transfer pipette, washed with sterile PBS, and then centrifuged at 120 × *g* at 10°C for 10 min. The cell pellet (~6 × 10^7 cells) was gently resuspended in 1 mL of freezing medium. The cell suspension was immediately transferred into 1.5-mL cryovials, placed in a precooled (4°C) cryo-freezing container, and kept at −80°C. After 16 h, the tubes were transferred (on dry ice) to a liquid nitrogen container for long-term storage.

To recover the putative BFECs from liquid nitrogen, the frozen cells were transported on dry ice and subsequently rapidly thawed in a 37°C water bath for 1 min. Once thawed, each 1 mL of frozen BFECs was washed with 9 mL of culture medium to remove any residual freezing medium and centrifuged at 140 × *g* at 10°C for 10 min.

2.1.4 Established cell line sequence confirmation

To confirm that the putative BFEC cell line was derived from *L. cuprina* eggs, a specific primer pair for the amplification of the gene endoplasmic reticulum resident protein 44 (ER) (Table S1) was selected for PCR amplification and direct sequencing to verify that the putative BEFCs after culture establishment and after recovery from low temperature storage (Passage 2, stored for 86 weeks) were derived from *L. cuprina*. See detailed the method in Data S1.

2.2 Transfection of BFEC and SF9

For all transfection experiments in this study, only cells in wells that reached more than 65% confluency were selected for transfection. Commercial transfection reagents (Lipofectamine™, GeneJuice®, Cellfectin® II, and Lipofectamine™ MessengerMAX™) were investigated to analyse the transfection efficiency of BFEC cells and Sf9 cells (positive control) using a fluorescence-activated cell sorting method, and the transfection efficiency of the cells was evaluated by imaging them with a Leica TCS SP8 laser scanning confocal microscope.

All transfection experiments utilized 24-well plates. Wells were seeded with 1 × 10^5 Sf9 cells or 1 × 10^6 BFECs. For all following transfection experiments, confocal images were taken at 24 h, 48 h, and 72 h.

2.2.1 Transfection of BFEC and Sf9 by plasmids

Three DNA-specific transfection reagents (Lipofectamine™, GeneJuice®, and Cellfectin® II) and two specific insect-reported plasmids (pLC-exCas-GFP and pLC-exCas-dsRED) were tested in this section.

2.2.1.1. Cloned insect-specific plasmids preparation. The insect-specific green fluorescent protein (GFP) reporter plasmid was designed and synthesized using a DNA sequence fragment encoding the insect promoter, ie + hr, and an open reading frame encoding GFP (corresponding bases 6618 to 8069, GenBank accession number EU594544.1) with an insect-specific transcription terminator, hsp70T (bases 5105 to 5348 GenBank accession number EU594544.1). The complete fragment was commercially synthesized (Integrated DNA Technologies, Singapore).[14] A second reporter fragment with the insect promoter 3 × P3-hsp70, dsRed open reading frame and the SV40 transcriptional terminator (corresponding to bases 5619 to 6565 of GenBank accession number EU594544.1) was also synthesized (Integrated DNA Technologies). Each gene block was amplified by PCR using specific primer pairs (Table S1). The resulting PCR products were purified and cloned and inserted into the pGEM-T Easy cloning system (Promega, Madison, USA, A1360). Plasmids containing the GFP and red fluorescent protein (RFP) expression cassettes verified by DNA sequencing were deemed pLC-exCas-GFP and pLC-exCas-dsRED, respectively.

2.2.1.2. Transfection of BFEC and Sf9 with insect-specific reporter plasmids by Lipofectamine™ or GeneJuice®. The commercial protein-free medium ESF 921 Insect Cell Culture Medium (Fisher Scientific International L.L.C., Waltham, USA) was applied for Sf9 cell maintenance. The capacity of BFEC to express the reporter genes was evaluated using the insect cell-specific transfection reagent Lipofectamine™ (Thermo Fisher Scientific, Waltham, USA, 11 668 019) or GeneJuice® (Sigma-Aldrich, 70 967) according to the manufacturer's instructions. Two different amounts (200 or 600 ng) of plasmids (pLC-exCas-GFP and pLC-exCas-dsRED) were used in conjunction with Lipofectamine™ (Waltham, USA 02451) or GeneJuice® (Melbourne, Australia) in each well of a 24-well plate to estimate the transfection efficiency.

2.2.1.3. Transfection of BFEC and Sf9 with cloned insect-specific plasmids by Cellfectin® II. The commercial transfection reagent Cellfectin® II (Thermo Fisher Scientific, 10 362 100) was also tested for transfection of BFEC cells.

To further optimize transfection of the BFECs, the original medium mentioned in Section 2.1.1 and protein-free commercial ESF 921 insect cell culture medium with 20% (v/v) FBS were tested separately. Commercial protein-free ESF 921 insect cell culture medium and protein-free medium with 10% (v/v) FBS were provided for Sf9 cells before overnight incubation. The experimental design for this Cellfectin® II reagent was two types of cells (BFEC and Sf9) × 2 culture medium for each cell, two plasmids (pLC-exCas-GFP and pLC-exCas-dsRED), two quantities of plasmid (200 or 600 ng), and with or without Plus Reagent).

2.2.2 Transfection of BFEC and Sf9 by fluorescently labelled dsRNA and eGFP mRNA

Two transfection reagents (Cellfectin® II and Lipofectamine™ MessengerMAX™) and two RNA molecules (eGFP mRNA and *L. cuprina*-specific fluorescein-labelled dsRNA frizzled-like protein [FL]) were tested.

2.2.2.1. Synthesis of fluorescein-labelled L. cuprina*-specific dsRNA.* As described in Section 2.1.4 for ER amplification, primers for *L. cuprina*-specific dsRNA and FL were designed to amplify selected genes by PCR from blowfly larval cDNA (Brisbane, Australia) (the same cDNA preparation was used in the larval feeding assay). For dsRNA synthesis, one reaction included 1200 ng of purified PCR product, 8 μL of NTP from commercial Thermo Scientific™ (London, UK) NTP Set, 2 μL of T7 RNA polymerase mix, 2 μL of T7 RNA buffer mix, and 2 μL of fluorescein RNA labelling mix (Roche, REF 11685619910), adjusted to a volume of 20 μL and incubated in a 37°C water bath overnight. The fluorescein-labelled dsRNA product was then purified using a RNeasy Plus Kit (Qiagen, 74 034).

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2.2.2.2. Transfection of BFEC and Sf9 with eGFP mRNA and fluorescent dsRNA. Two transfection reagents, Lipofectamine™ MessengerMAX™ (Thermo Fisher Scientific, LMRNA001) and Cellfectin® II, were evaluated. Briefly, 500 ng of fluorescent mRNA or dsRNA was applied to each well of a 24-well plate with the transfection reagents. Based on the results from BFECs were only used in the original culture medium and Sf9 was only applied in commercial protein-free ESF 921 insect cell culture media supplemented with 10% heat-inactivated FBS (56°C, 30 min).

2.3 BFECs transfected with dsRNAs specific for *L. cuprina* genes

Three *L. cuprina* gene-specific dsRNAs, trifunctional purine biosynthetic protein adenosine-3 (GART), segmentation protein cap'n'-collar (SPCNC), and FL (Table S1), were selected for BFEC transfection experiments. Lipofectamine™ MessengerMAX™ was used to evaluate dsRNA uptake. After 24 h, the culture medium was removed and the BFEC cells were washed with room temperature PBS. The recovered cells were centrifuged at 140 × *g* for 10 min, and the pelleted cells were investigated using RT-qPCR.

2.4 *L. cuprina* larvae dsRNA feeding assay

Freshly hatched *L. cuprina* larvae ($n = 20$) with similar body sizes were placed on 12 × 3 cm filter paper and then folded into a soda glass vial (19 × 50 mm) (Labco, Batch No. 425671), representing one biological replicate. Five biological replicates were included for all feeding experiments. Freshly hatched larvae were fed 100% heat-inactivated sheep serum (56°C, 30 min) during the selection process. dsRNA treatments were administered on days 1 and 2 of the experiment. Naked dsRNA (1 mL, 500 µg mL^{-1}) mixed with 100% heat-inactivated sheep serum (1 mL) was provided on days 1 and 2. From day 3 through day 10, 100 µL of heat-inactivated sheep serum was provided daily as a food source. The larvae were maintained in the same artificial environment throughout the duration of the trial at a constant temperature of 27°C.

For gene expression analysis, four larvae from each biological replicate were taken on days 3 and 5, and this number was reduced to 2 on day 7 due to larger-sized larvae. The assay was concluded on day 10, and all the remaining live larvae were collected for RT-qPCR analysis.

Since live larvae were collected at different time points for further gene expression analysis (Fig. 2), the number of mortalities, pupation and growth performance score were shown as a percentage for different dates for comparative purposes.

2.4.1 *Mortality*

The number of dead larvae was recorded on days 2, 3, 5, 7 and 10. Binomial percentage mortality data were arcsine transformed in Microsoft Excel using the formula (total remaining larvae on the examined date − alive larvae number)/total remaining larvae on the examined date × 100%. Based on the experimental setup (Fig. 2), total remaining larvae numbers were 20, 20, 16, 12, and 10 on days 2, 3, 5, 7, and 10, respectively.

2.4.2 *Pupation rate*

The number of larvae turning to pupae for each treatment was recorded from day 7 to day 10. Binomial percentage pupation data were arcsine transformed in Microsoft Excel using the formula pupa number/total remaining larvae on the examined date

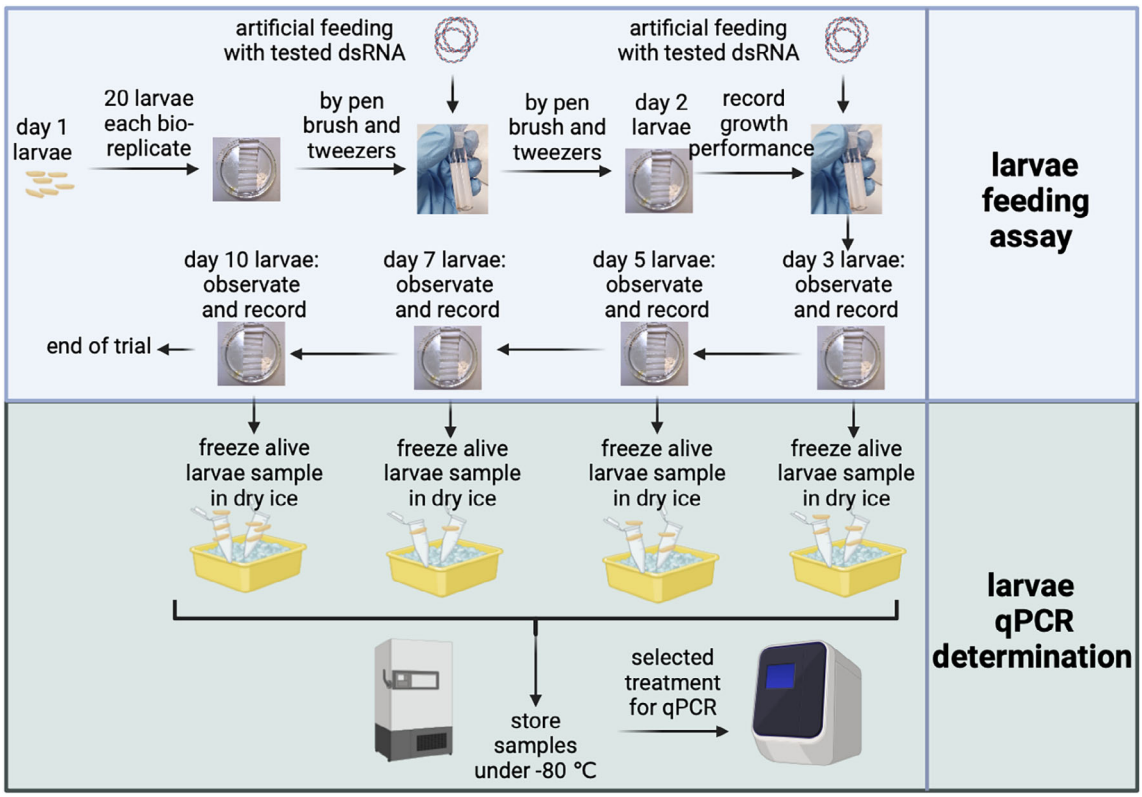

Figure 2. Schematic diagram demonstrating the dsRNA target feeding bioassay setup and gene knockdown analysis using RT-qPCR. The schematics were created with Biorender.com.

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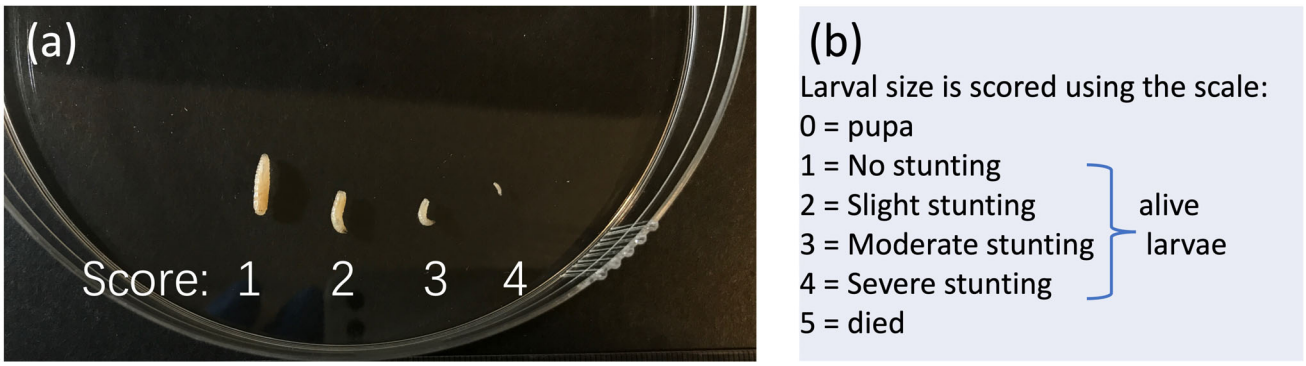

Figure 3. Different sizes of live larvae from the same vial on day 3 (A) and growth performance score assisted value (B). Clear body size variation of live moving larvae from the same biological replicate, hence this growth performance score is vital to reflect biological performance.

Table 1. Name and abbreviations of the tested dsRNAs in the *L. cuprina* larval oral feeding assay

Gene name	Abbreviation	Accession number	Function
Trifunctional purine biosynthetic protein adenosine-3	GART	XM_023449722.1	Purine metabolism
Segmentation protein cap'n'collar	SPCNC	XM_023446792.1	DNA binding
Frizzled-like	FL	XM_023449379.1	Transmembrane signaling receptor activity
Akirin	AKI	XM_023449147.1	Transcription coregulator activity
Alanine aminotransferase 1	AT1	XM_023440000.1	Alanine-glyoxylate transaminase activity
Alcohol dehydrogenase class-3	ADH3	XM_023441817.1	*S*-(hydroxymethyl)glutathione dehydrogenase activity
Abnormal spindle-like microcepha associated protein homolog	ASPM	XM_023451111.1	Calmodulin-binding
V-type proton ATPase subunit E	V-ATPase	XM_023447483.1	Proton-transporting ATPase activity, rotational mechanism
Discs overgrown protein kinase	DCO	XM_023443405.1	ATP binding
Division abnormally delayed protein	DALLY	XM_023445883.1	Act as a coreceptor for growth factors
Protein tumorous imaginal discs, mitochondrial	TID	XM_023441699.1	Act as a tumor suppressor in larval imaginal disks
Ecdysone receptor	ER	XM_023438347.1	Core promoter sequence-specific DNA binding

× 100%. Based on the experimental design, 12 and 10 pupae were expected to be present on days 7 and 10, respectively.

2.4.3 *Growth performance score*

The larval size was estimated on days 2, 3, 5, 7 and 10 and was assigned an arbitrary value from 1 to 4, where 1 represents no stunting, 2 represents slight stunting, 3 represents moderate stunting, and 4 represents severe stunting compared to the control larvae (Fig. 3). Any dead larvae were assigned a value of 5, and pupae on days 7 and 10 were assigned a value of 0. Binomial percentage growth performance scores were arcsine transformed in Microsoft Excel using the formula total value of one vial/(total remaining larvae on the examined date × 5) × 100%.

L. cuprina-specific dsRNAs ($n = 12$) were selected according to their gene function that provided from National Center for Biotechnology Information (NCBI) database (Table 1). They were tested individually, including the three genes (trifunctional purine biosynthetic protein adenosine-3 [GART], segmentation protein cap'n'collar [SPCNC], and frizzled-like [FL]), tested in the above BFEC transfection experiments. The primer details of the tested dsRNAs are shown in Table S1. Based on the observed effects of target dsRNA feeding on larval biological performance, six dsRNA treatment groups (GART, SPCNC, FL, AKI, ASPM, and V-ATPase) were selected for RT–qPCR analysis. Three individual replicate trials were conducted using the hatched larvae from three different hatching events.

2.5 Gene expression analysis

The BEFC pellets or larvae were retained for total RNA extraction using TRIsure (Bioline, BIO-38032, London, UK). To remove any contaminating genomic DNA (gDNA), total RNA preparations were treated with a DNase I (RNase-free) kit (BioLabs, M0303L, Cambridge, UK). The purified RNA was then used as the template for RT-qPCR, which was performed in a 384-well format with four technical replicates.

RT-qPCR was carried out using the SensiFAST SYBR No-ROX one-step kit (Bioline, BIO-72005). The 10-μL reaction was set up with 1× SensiFAST SYBR No-ROX one-step mix, 200 nM RT–qPCR primers (Table S1), reverse transcriptase, RiboSafe RNase Inhibitor, and 10 ng of total RNA with the following conditions: reverse transcription at 45°C for 10 min, polymerase activation at 95°C for 2 min, then 40 cycles of denaturation at 95°C for 5 s, primer annealing at 60°C for 10 s, and extension at 72°C for 5 s. For each RT-qPCR experiment, using beta-actin as a transcript normalizer, the relative expression of a target gene was calculated using the

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ΔΔCT technique.[15] The raw data were produced using Rotor-Gene Q series software version 2.3.1.

2.6 Statistics

Statistical test parameters are reported in the figure legends and source data. All statistical analyses were performed using GraphPad Prism v.9.1.0. Experimental data were analyzed with one-way analysis of variance (ANOVA) with Tukey's test. Experiments on the larval oral dsRNA feeding assay were performed three times and the imaging experiments were repeated twice with three independent replicates.

3 RESULTS

3.1 Primary BFEC establishment

Cell-like structures were observed attached to the plate surfaces by light microscopy 1 week after the homogenized material from the blowfly eggs was added to the culture plates (Fig. 4(A)). The cells were uniformly round and were estimated to range in size from 3 to 5 μm with an average diameter of 4 μm.

Cells were stored in liquid nitrogen for 59 days and then recovered into culture, maintaining growth and morphological features that were indistinguishable from the parental cells (Fig. 4(B)). The original cells were passaged at weekly intervals for 52 consecutive passages, with no changes in overall morphology or size observed (Fig. 4(A),(C)).

3.2 Established cell line sequencing confirmation

To confirm that the BFEC were derived from *L. cuprina*, a PCR amplicon targeting the transcript from the ER gene was amplified. The ER reverse transcriptase PCR (RT-PCR) yielded an amplicon of 254 bp, which was consistent with the expected size (Fig. S1). The nucleotide sequence of the ER transcript amplicon was 100% identical to the corresponding transcript of the *L. cuprina* ER transcript templates from both fresh and recovery BFEC (data not shown).

3.3 Other observations within the BFEC line

Two other characteristics were identified in the BFEC cultures. First, comparatively large crystalline structures were observed in the cultures using light microscopy (Fig. S2). These structures changed under different composed culture medium (Table S2) and were not observed when using commercial ESF 921 insect cell culture medium (data not shown). In comparison, in the original culture medium, these crystalline structures were not observed on the day the cells were passaged. The pH of the medium used increased over time, and these crystalline structures were first observed from day 2 after passaging. It was hypothesized that these crystalline structures are byproducts from culture medium components due to changes of the pH over time. However, buffering with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid to maintain pH had minimal impact on the formation of these structures (Table S2). The presence of these factors did not appear to affect the growth.

The second observation was the presence of motile low-density bacterial cells in the cultures by light microscopy. Conventional PCR analysis of total DNA extracts from BFECs with a universal bacterial 16S rRNA gene yielded an amplicon of the expected size of 1550 bp (Table S1). Subsequent direct sequencing and BLAST analysis of the amplicon demonstrated 100% identity with multiple sequences from bacteria assigned to the genus *Bosea*. Based on the antimicrobial susceptibilities reported for bacteria from this genus in published studies, several antimicrobials and combinations thereof were tested in attempts to remove the bacteria (Table S2).[16] However, none of the antimicrobials or combinations of antimicrobials tested were able to prevent the persistence of *Bosea* spp. in the BFEC cultures. While the bacterial cells persisted in the BFEC cultures, they remained at low levels and did not appear to influence the growth characteristics of the BFECs.

3.4 Transfection studies of BFECs

3.4.1 Transfection of BFEC and Sf9 with reporter gene plasmids

No fluorescence was observed in the BFECs or Sf9 cells transfected with GeneJuice™ or Lipofectamine™ Plus Reagent with both pLC-exCas-GFP and pLC-exCas-dsRED at any time point (24, 48, and 72 h).

Red fluorescence was observed in Sf9 cells transfected with pLC-exCas-dsRED (200 ng) using Cellfectin® II with Plus Reagent recovered in ESF 921 containing 10% (v/v) FBS medium at 72 h (Fig. 5(A)). Sf9 cells transfected with pLC-exCas-GFP (200 ng) as shown in Fig. 5(B) confirmed transfection. Complete fluorescence confocal microscope images of the BFECs and Sf9 cells are given in Figs S3 and S4.

When BFECs and Sf9 cells were transfected with pLC-exCas-GFP combined with Cellfectin® II, both cell lines showed evidence of

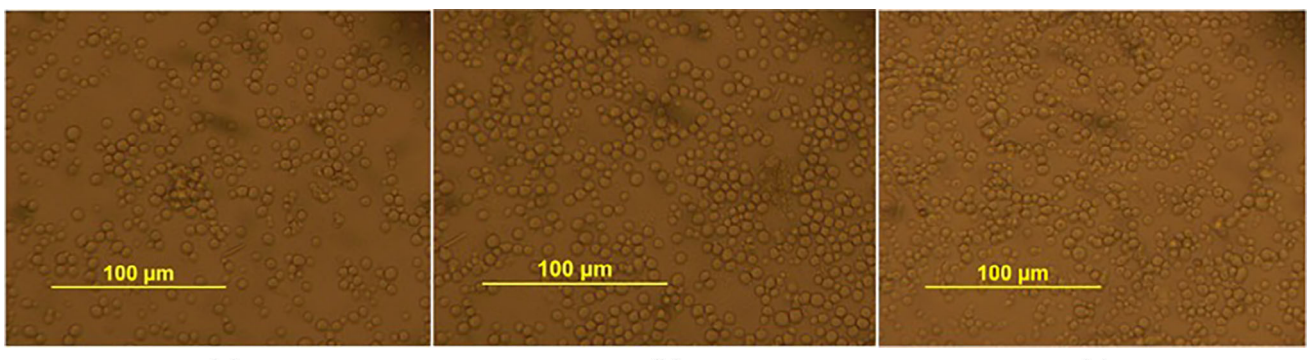

Figure 4. Light microscopy of primary blowfly embryo cell line (BFEC) established in this study. (A) BFEC on day 7 post-established cells. (B) BFEC after recovery from liquid nitrogen storage, stored at passage 8, recovered after 59 days. (C) BFEC after 52 consecutive passages. Microscope observation confirmed recovery and long-passaging (52 consecutive passages equal to 1 year) cells showed similar morphology to the newly established cells (7 days), which proved that this primary cell line was established successfully. The background color in the photographs is due to the yeast extract in the culture medium.

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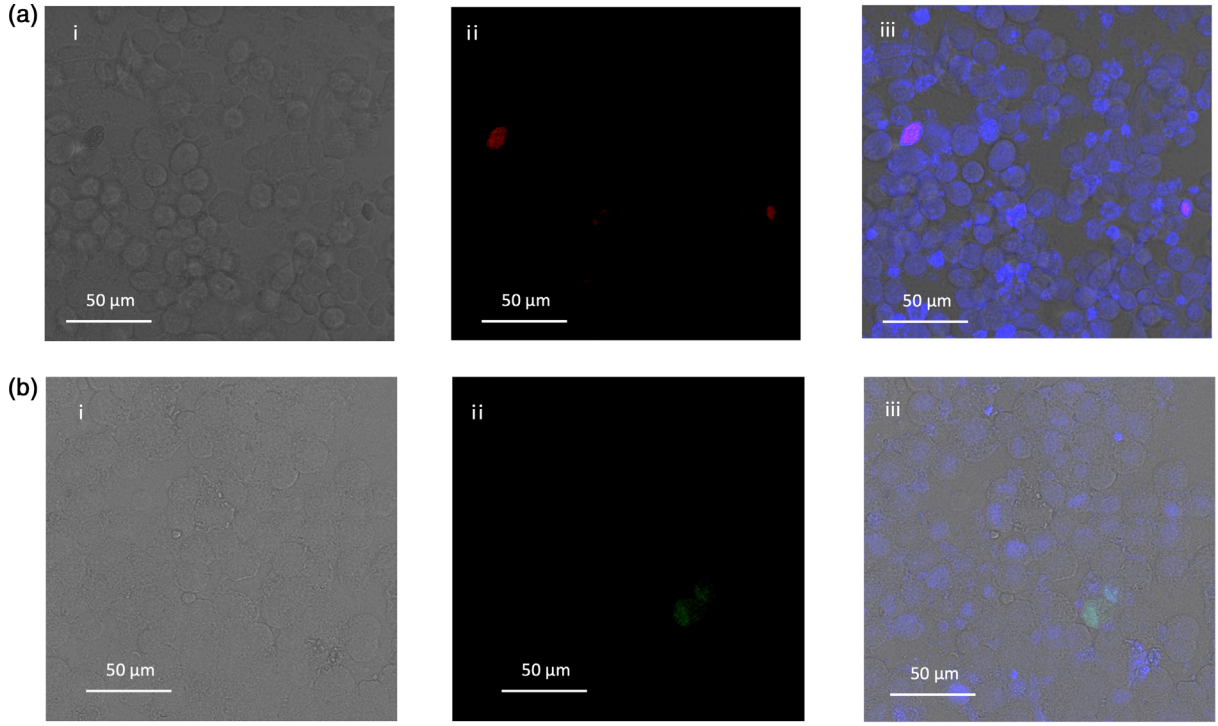

Figure 5. Confocal microscopy images of Sf9 cells transfected by using Cellfectin® II with Plus Reagent with 200 ng of insect-specific expression plasmid for red fluorescent protein (RFP) at 72 h (A) and 200 ng of insect-specific expression plasmid for green fluorescent protein (GFP) at 24 h (B). (i) Bright view, Sf9 cell had blue autofluorescence and could confirm the cell location, RFP and GFP view (ii) was used to detect the cell uptake of the red fluorescent plasmid, and the brightfield + RFP/GFP view + autofluorescence (iii) was used to image the whole cells with nucleus and cellular uptake of plasmid expressing RFP/GFP. Images were analyzed using a Leica TCS SP8 confocal fluorescence microscope.

fluorescence on recovery. BFECs recovered in original medium started to show evidence of reported gene expression following transfection with Cellfectin® II with (Fig. 6(D)–(F)) and without (Fig. 6(A)–(C)) Plus Reagent, both formulated with 200 ng pLC-exCas-GFP, at 24 h after treatment. Images about Sf9 were in Fig. S4a–e. Confocal images showed that the transfection efficiency was higher with Plus Reagent (Fig. 6(D)–(F)).

At the 24-h time point, similar observations were made for Sf9 cells, as only limited fluorescence was observed for the treatment of 200 ng pLC-exCas-GFP by Cellfectin® II with Plus Reagent in ESF 921 insect cell culture medium with 10% (v/v) FBS medium (Fig. 6 (F)). No fluorescence was observed in either BFECs or Sf9 cells at 24 h under any of the conditions used with 600 ng pLC-exCas-GFP (Table 2).

At the 48-h time point, BFECs only showed fluorescence with Plus Reagent in the original medium, with both the 200g and 600 ng amounts of the pLC-exCas-GFP reporter plasmid. The cells treated with 200 ng of plasmids showed a higher transfection rate than the 600-ng groups (Fig. S3).

At the 72-h time point, only Sf9 with an extra 10% FBS treatment showed fluorescence (Fig. S4). Similar to the results observed at 24 and 48 h, a higher transfection rate was observed in BFECs by adding Plus Reagent during transfection to both 200 and 600 ng pLC-exCas-GFP (Table 2). Interestingly, in the treatment without Plus Reagent, the plasmid amount first showed a positive correlation with the transfection rate and efficiency.

3.4.2 Transfection of BFEC and Sf9 with a GFP encoding mRNA

Following the BEFC transfection results using the fluorescent reporter plasmids with Cellfectin® II, an mRNA encoding GFP and a FITC-labelled dsRNA were used to investigate RNA transfection. The DNA-specific transfection reagent Cellfectin® II had a higher transfection rate in BFECs with both mRNA encoding GFP and fluorescently labelled dsRNA (Table 3). Compared to *L. cuprina*-specific fluorescently labelled dsRNA the Sf9 cells showed a lower transfection rate (Table 3).

Confocal microscopy imaging indicated that the BFECs were successfully transfected with mRNA and dsRNA (Fig. 7).

3.5 Transfection of BFECs with *L. cuprina*-specific dsRNAs

The three *L. cuprina*-specific dsRNAs (GART, SPCNC, and FL) showed evidence of gene knockdown. The gene knockdown observed for each of the target genes was 23%, 5%, and 25% for GART, SPCNC, FL, respectively, compared to the control treatment (Fig. 8).

3.6 *L. cuprina* larvae oral feeding assay

The parameters mortality, pupa rate, and growth performance were recorded.

Twelve dsRNAs were investigated in the larval bioassay, including the GART, SPCNC and FL targets evaluated in the BEFCs experiments. The initial assessment of the 12 dsRNAs (Table 1) in the screening experiment were first evaluated based on the larval pupation rate. Treatments that showed lower pupation rates, two negative control dsRNAs (LAC, *Escherichia coli* lac operator; TSWV, tomato spotted wilt virus) and no treatment control were also used for RT-qPCR. The same selection methods were considered for both mortality and growth performance scores, and detailed results can be found in Figs S7–S9.

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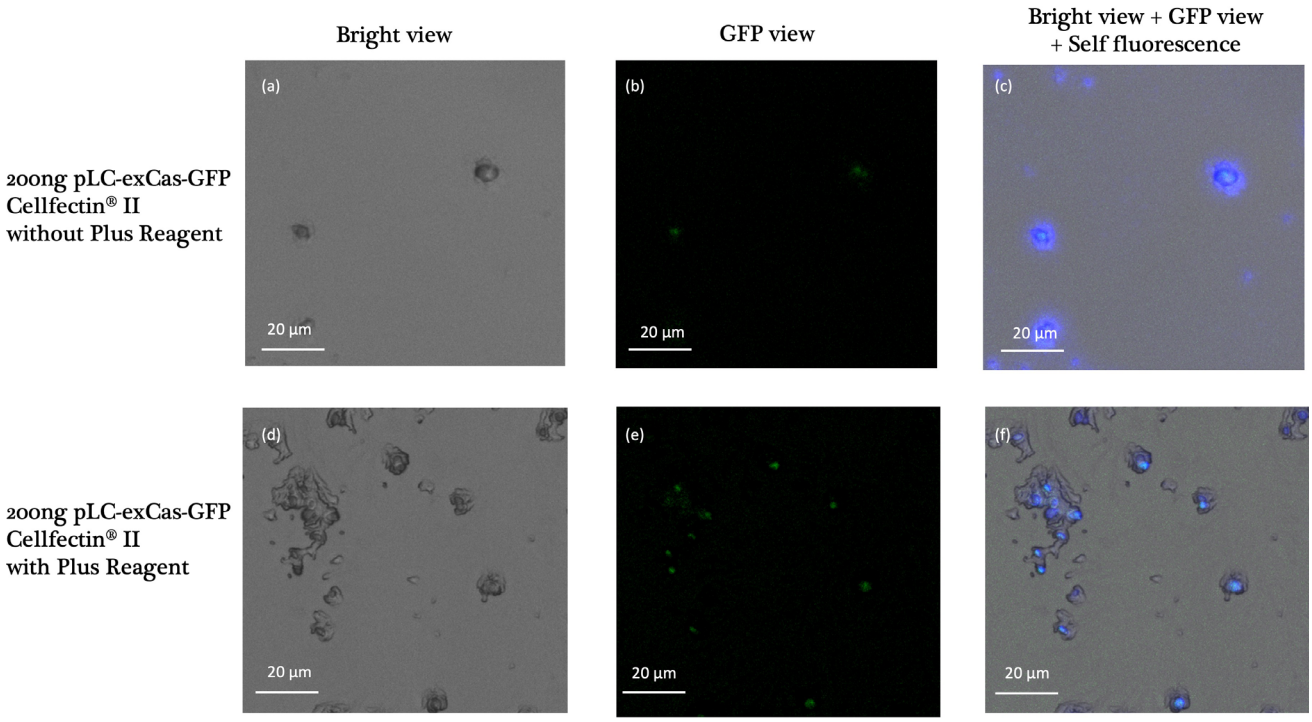

Figure 6. Confocal microscope images of the blowfly embryo cell line (BFEC) transfected with 200 ng of the insect-specific expression plasmid encoding green fluorescent protein (GFP) using Cellfectin® II only (A–C) or Cellfectin® II with Plus Reagent (D–F) at 24 h (A and D), Bright field view, BFECs have blue autofluorescence, and this was used to confirm the cell location. (B and E) GFP fluorescence was used to detect the cellular uptake of the reporter plasmid. (C and F) Overlay of brightfield + GFP fluorescence and autofluorescence was used to image whole cells with nuclei and cellular uptake of plasmids expressing GFP. Images were analyzed using a Leica TCS SP8 confocal fluorescence microscope.

Table 2. Summary of the transfection efficiencies of Cellfectin® II with insect-specific reporter plasmids in BEFCs and Sf9 cells over time

		BFECs			Sf9		
		Original medium			ESF 921 + 10%FBS		
		24 h	48 h	72 h	24 h	48 h	72 h
pLC-exCas-GFP	200 ng without Plus Reagent	++					
	200 ng with Plus Reagent	+++	++		+		++
	600 ng without Plus Reagent						+
	600 ng with Plus Reagent		+				++
pLC-exCas-dsRED	200 ng without Plus Reagent						
	200 ng with Plus Reagent						+
	600 ng without Plus Reagent						
	600 ng with Plus Reagent						

Empty cells equate to no fluorescence observed. The highest observed transfection rate was 10%; hence, +++ represents a transfection rate between 10% and 5%, ++ represents a transfection rate between 5% and 1%, and + represents a transfection rate between 1% and 0. No fluorescence was observed for BFECs with ESF 921 containing 20% FBS or Sf9 cells with ESF 921 without FBS.

Table 3. Transfection efficiency and rate of GFP-labelled mRNA and dsRNA by transfection reagents in BFECs and Sf9 cells

		BFECs			Sf9		
		Original medium			ESF 921 + 10%FBS		
		24 h	48 h	72 h	24 h	48 h	72 h
mRNA	Cellfectin® II	+++	+	+	+		
	Lipofectamine™ MessengerMAX™	+++	++	+			
dsRNA	Cellfectin® II	+	+		+		
	Lipofectamine™ MessengerMAX™	+			+		

Full fluorescence confocal microscope images of BFECs transfected with Cellfectin® II are shown in Fig. S5, and images of BFECs transfected with Lipofectamine™ MessengerMAX™ are shown in Fig. S6. Empty means no fluorescence was observed. The highest observed transfection rate was 10%; hence, +++ represents a transfection rate between 10% and 5%, ++ represents a transfection rate between 5% and 1%, and + represents a transfection rate between 1% and 0.

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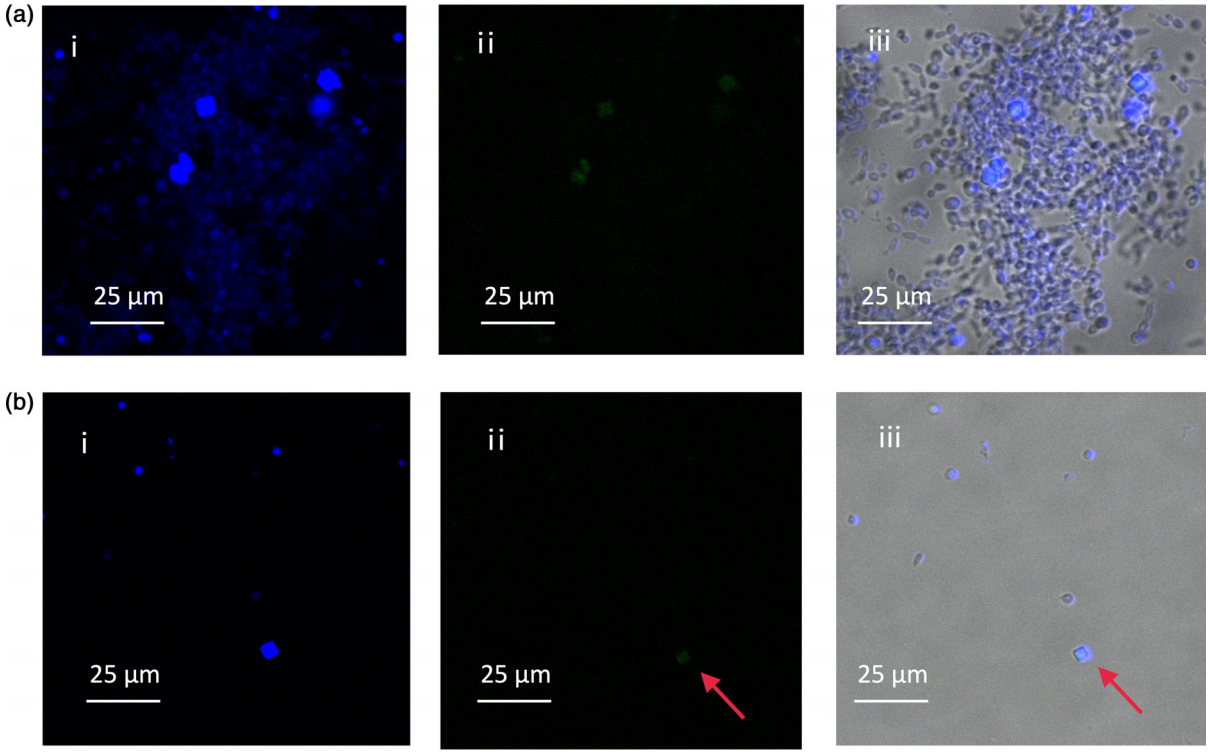

Figure 7. Confocal microscope images of BFECs transfected with Lipofectamine™ MessengerMAX™ with (A) 500 ng of eGFP mRNA or (B) 500 ng of *L. cuprina*-specific fluorescent dsRNA (FL) at 24 h. (i) BFECs have auto-blue fluorescence and could confirm the cell location, (ii) GFP view was used to detect the cell uptake of the green fluorescent plasmid, and (iii) brightfield + GFP view + auto fluorescence was used to image whole cells with nucleus and cellular uptake of plasmid expressing GFP. Images were analyzed using a Leica TCS SP8 confocal fluorescence microscope.

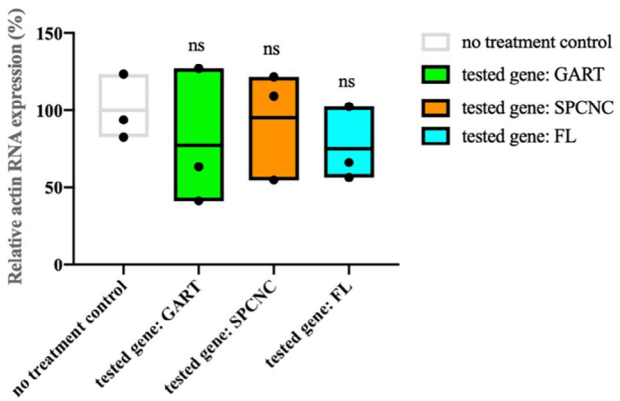

Figure 8. Relative target gene expression in blowfly embryo cell soaking trials following three dsRNA treatments, with 23% 5% and 25% knockdown in the GART, SPCNC, and FL treatments, respectively. The mean knockdown ± standard error of the mean (SEM) in one independent bioassay ($n = 3$ cell wells of a 24-well plate) are shown. Using GraphPad Prism v.9.1.0, values are mean expression relative to the no treatment controls ±SEM, with $n = 3$ biological replicates.

From the selected six dsRNA experimental groups, three candidate dsRNAs (GART, SPCNC, and FL) showed consistent knockdown in all three replicates of independent bioassays, and knockdowns were observed at multiple timepoints (Fig. 9). GART dsRNA resulted in gene knockdown percentages of 86%, 48%, 78%, and 66% on days 3, 5, 7 and 10, respectively. The gene knockdown observed in the larvae treated with SPCNC was 83%, 37%, 87%, and 49% on days 3, 5, 7 and 10, respectively, and FL was 85%, 50%, 72%, and 56% on days 3, 5, 7 and 10, respectively (Fig. 9). These three candidate dsRNAs also showed grow retardation. FL dsRNA showed a 27% increase in mortality and GART dsRNA showed a 34% decrease in pupation rate (Fig. 10). Meanwhile, GART dsRNA showed up to 87% gene knockdown relative to no treatment control on day 5 (Fig. 11). Furthermore, these are the three candidate dsRNAs (GART, SPCNC, and FL) that were screened in the cell-model.

4 DISCUSSION

Target gene validation and stable dsRNA delivery remain two major challenges for the successful application of RNAi as a bio-insecticide. Cell-based assays offer an effective method for target gene validation.[17] This study reports the use of two experimental models with the capacity to improve target gene selection for *L. cuprina*. The BEFC primary cell line and the *L. cuprina* larval feeding assay proved informative for screening dsRNA targets for RNAi efficacy. These BFEC cells were successfully transfected using Cellfectin® II and Lipofectamine™ MessengerMAX™ (Tables 2 and 3). Sf9 cells, which have a proven transfection capability,[18] were included in this research for comparative purposes. Interestingly, most of the transfection methods evaluated worked successfully in this study, and the primary cell line BFECs had higher transfection efficiency than the commercial Sf9 cells (Tables 2 and 3). Moreover, cell-based RNAi target screening has the potential to increase the number of genes evaluated as they are amendable to high-throughput systems.[19]

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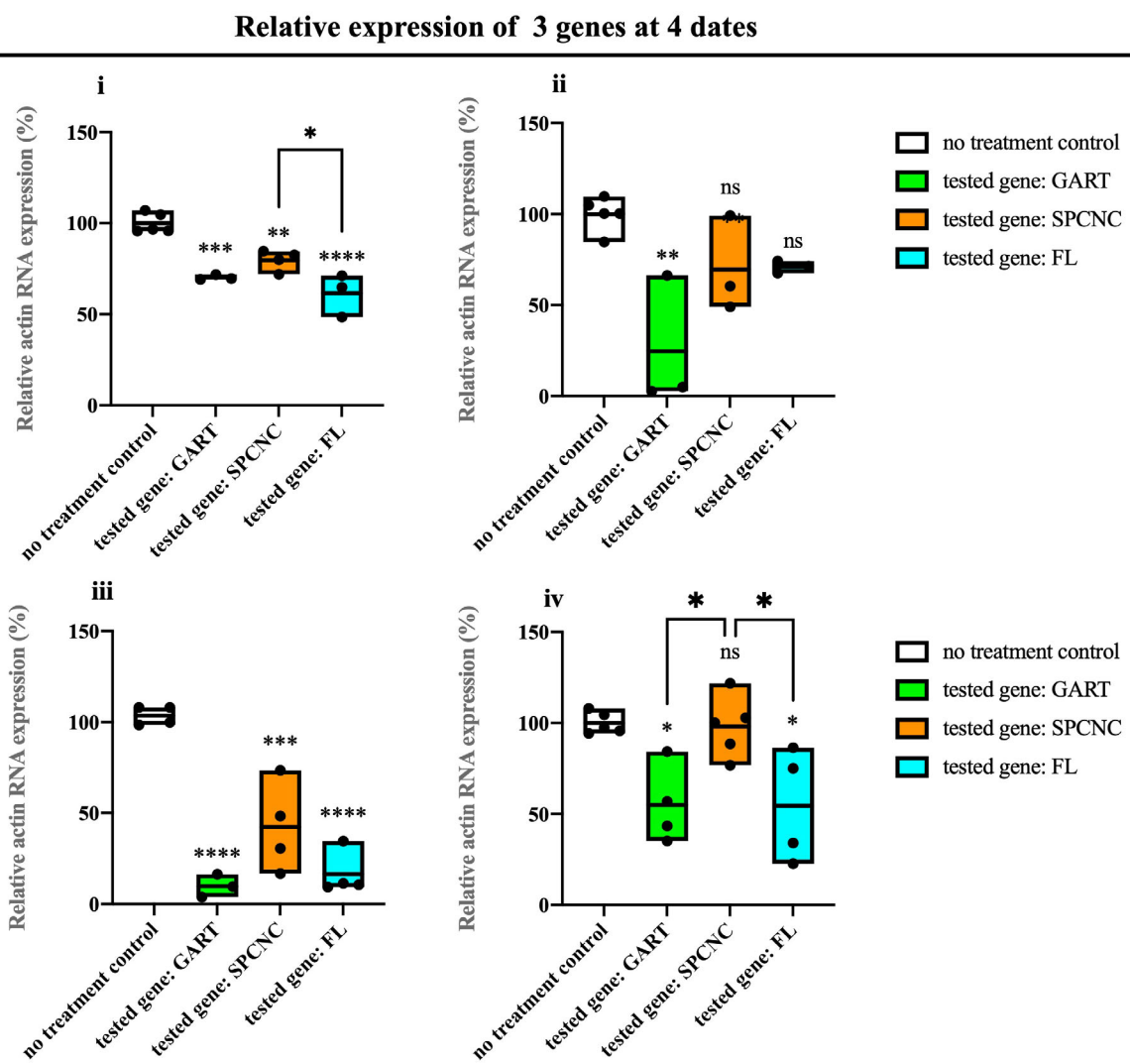

Figure 9. Relative expression of three candidate genes at four dates (day 3, 5, 7 and 10). Relative expression of target genes in *L. cuprina* fed naked dsRNA showing gene knockdown on days 3 c(i), 5 c(ii), 7 c(iii), and 10 c(iv). In (A), (B) and (C) indicate biological performance or knockdown ± SEM in three independent bioassays ($n = 5$ soda glass tubes with 20 larvae each) are shown. All statistical comparisons shown are relative to the no treatment control. Asterisks show statistical significance: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$.

It has been demonstrated that oral delivery of dsRNA can elicit gene silencing and insecticidal activity in insect larvae,[20] and this was in line with the findings of this study. Growth retardation with higher mortality was found in dsRNA feeding groups. Six of 12 candidate dsRNAs showed larval growth retardation in feeding trials, and three dsRNAs (GART, SPCNC, and FL) showed consistent gene knockdown from three independent replicate groups. Similarly, transfection assays in BEFC cells also showed successful RNAi silencing, but at much lower levels. This is probably due to the same genes being expressed at different levels in growth stages. While the mechanisms by which dsRNA enters and distributes itself in *L. cuprina* have not been fully elucidated, the fact that ingested dsRNA can induce RNAi in larvae offers some intriguing possibilities for future applications. The low transfection efficacy of the BEFCs is likely to have impacted on the capacity to detect significant reductions in the levels of the target mRNAs as most cells remained unaffected. Future studies could test if non-lipid-based systems such as electroporation improve the introduction of exogenous RNA into the BEFCs.

Despite the successful establishment of a *L. cuprina* cell line, the low transfection rate was one of the major factors that requires future improvement to enable this cell line to be used as an efficient screening tool for candidate dsRNAs. Transfection efficiency can be affected by cell growth stage, confluency, culture medium reagents, and transfection operation and can be more challenging in primary cell lines.[21] It has been reported that the most suitable cell stages for transfection are those that have reached the exponential growth stage after several passes.[21] At this point it is anticipated that the cells are growing vigorously and are therefore easier to transfect. If cell cultures are passaged in the laboratory for a prolonged time, their genetic makeup may change through the accumulation of mutations, total chromosome recombination/translocations, and/or changes in gene regulation and expression. These alterations can result in changes in cell behavior associated with transfection.[21]

For further diet-based assessment of dsRNA activity in the *L. cuprina* larvae feeding assay, a more objective method for

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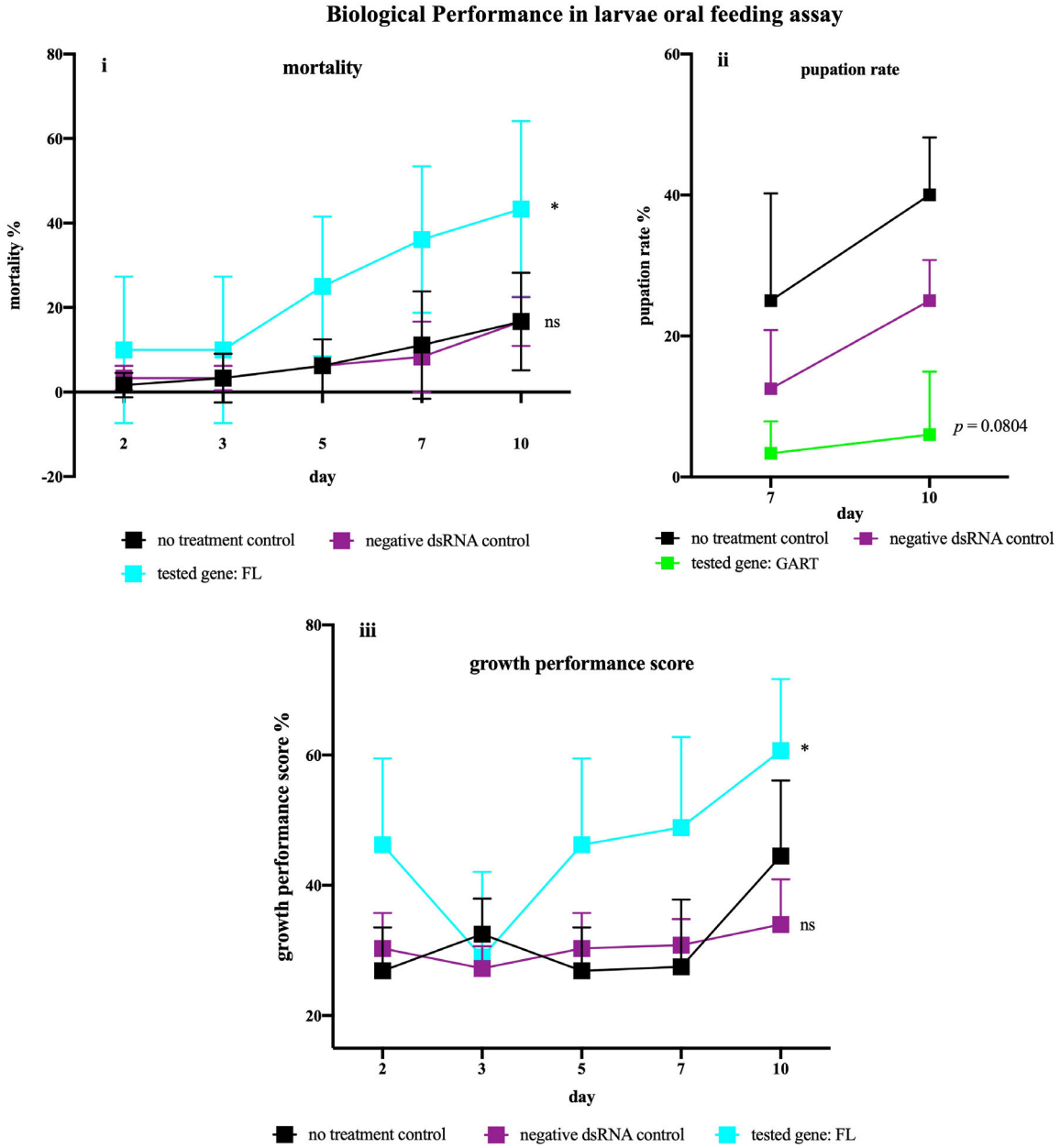

Figure 10. Biological performance in *L. cuprina* larvae oral feeding assay. Elevated corrected mortality rates (%), pupation rates (%), and growth performance score (%) in *L. cuprina* after feeding on 12 target dsRNAs. The best candidate dsRNA (FL) showed 27% increased corrected mortality a(i) and 34% decreased pupation rate a(ii). Asterisks show statistical significance: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$.

the growth performance observation system with a double-blind procedure would be ideal. In our assays, we tried to incorporate larval body weight as a more objective measure. However, this required drying of the larval body surface to minimize error, and it was difficult to apply this approach while avoiding mechanical damage to larvae. When using this approach, it was found that larvae for which body weight had been measured had higher mortality than the control group. As a result, the recording of body weight measurements was deemed to be impracticable and it was discontinued. It may be possible to objectively and quantitatively measure the impacts of dsRNA on larval development using a smart laboratory approach. For example, a software-based system such as Wormlab could be a good alternative.[22] The design of Wormlab enables auto-calculation of the moving larvae body surface and larvae moving speed using a short video with minimal intervention, although the current Wormlab system has been designed specifically for *Caenorhabditis elegans* and as such it may require modification for use with another species such as *L. cuprina*. Moreover, for further RNAi-based bio-insecticides, combining two or more target dsRNA together could result in cumulatively more pronounced effects in terms of larval mortality and pupation rates that are easier to quantify. Indeed, any future RNAi-based bio-insecticide for *L. cuprina* would likely contain dsRNAs targeting multiple genes to maximize efficiency and minimize the development of resistance.

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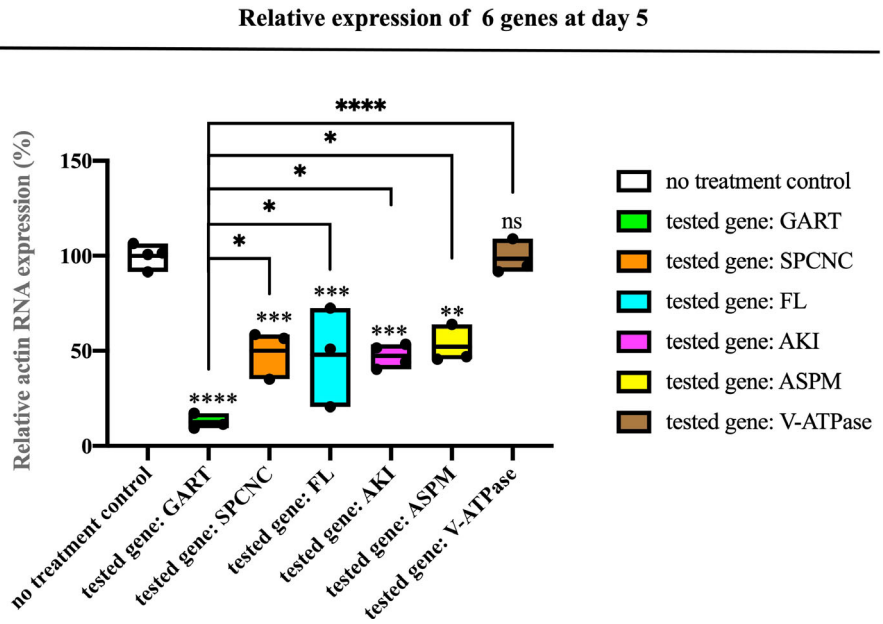

Figure 11. Relative expression of six candidate genes at day 5. Relative expression of target genes in *L. cuprina* fed on naked dsRNA FL showed up to 87% gene knockdown relative to no treatment control on day 5. Depending on the biological performance of larval oral feeding trials, six of 12 candidate dsRNA treatment groups were selected for qPCR determination. The same trial was repeated three times from different hatched larvae to minimize individual differences. Asterisks show statistical significance: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$.

5 CONCLUSION

In conclusion, a primary cell line derived from *L. cuprina* eggs, BEFC, was successfully generated and transfected with plasmids encoding fluorescent reporter genes, eGFP-mRNA, and *L. cuprina*-specific dsRNA. Additionally, for the first time, we have identified three novel *L. cuprina*-specific dsRNA targets, GART, SPCNC, and FL, that induced gene silencing and growth retardation, leading to higher mortality, reduced pupa rate and impaired growth performance in a larval bioassay. The fact that dsRNA induced RNAi effects in both the BFEC cells and the blowfly larvae holds a significant value and resource for studies focusing on understanding host–parasite interactions. Established research models in this study are encouraging, and further improvement and validation could be perfected, such as using multiple stable reference genes. Overall, the BFECs, transfection methods, gene screening, and larvae oral feeding techniques investigated and developed in this study offer valuable insights for future development of RNAi mediated by dsRNA as a biocontrol approach for animal health targets.

AUTHOR CONTRIBUTIONS

All authors contributed to the study conception and design. Conceptualization: Yunjia Yang, Timothy J. Mahony, Bing Zhang, Zhi Ping Xu, Neena Mitter, and Karishma T. Mody. Methodology: Yunjia Yang, Timothy J. Mahony, Bing Zhang, Zhi Ping Xu, and Karishma T. Mody. Result interpretation: Yunjia Yang, Timothy J. Mahony, Bing Zhang, Jiaxi Yong, and Karishma T. Mody. The first draft of the manuscript was written by Yunjia Yang and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

REFERENCES

1 FAO, *The State of Food and Agriculture 2001*. Food & Agriculture Org, Rome, Italy (2001).
2 Narladkar B, Projected economic losses due to vector and vector-borne parasitic diseases in livestock of India and its significance in implementing the concept of integrated practices for vector management. *Vet World* **11**:151–160 (2018).
3 Stevens J and Wall R, The evolution of ectoparasitism in the genus Lucilia (Diptera: Calliphoridae). *Int J Parasitol* **27**:51–59 (1997).
4 Smith J, Managing flystrike in sheep. https://www.agric.wa.gov.au/livestock-parasites/managing-flystrike-sheep.

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5 Fletcher SJ, Reeves PT, Hoang BT and Mitter N, A perspective on RNAi-based biopesticides. *Front Plant Sci* **11**:51 (2020).

6 Yu N, Christiaens O, Liu J, Niu J, Cappelle K, Caccia S *et al*., Delivery of dsRNA for RNAi in insects: an overview and future directions: delivery of dsRNA for RNAi in insects. *Insect Sci* **20**:4–14 (2013).

7 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* **97**:6499–6503 (2000).

8 Zhou R, Mohr S, Hannon GJ and Perrimon N, Inducing RNAi in Drosophila cells by soaking with dsRNA, in *Cold Spring Harbor Protocols*, Cold Spring Harbor Protocols, New york, USA (2014).

9 Li C and Zamore PD, RNAi in Drosophila S2 cells by dsRNA soaking. *Cold Spring Harb Protoc* **3**:pdb-prot097477 (2019).

10 March JC and Bentley WE, RNAi-based tuning of cell cycling in drosophila S2 cells—effects on recombinant protein yield. *Appl Microbiol Biotechnol* **73**:1128–1135 (2007).

11 Shah C and Förstemann K, Monitoring miRNA-mediated silencing in Drosophila melanogaster S2-cells. *Biochim Biophys Acta, Gene Regul Mech* **11**:766–772 (2008).

12 Caplen NJ, Fleenor J, Fire A and Morgan RA, dsRNA-mediated gene silencing in cultured Drosophila cells: a tissue culture model for the analysis of RNA interference. *Gene* **252**:95–105 (2000).

13 Valdes VJ, Sampieri A, Sepulveda J and Vaca L, Using double-stranded RNA to prevent in vitro and in vivo viral infections by recombinant baculovirus. *J Biol Chem* **278**:19317–19324 (2003).

14 Lukacsovich T, Hamada N, Miyazaki S, Kimpara A and Yamamoto D, A new versatile gene-trap vector for insect transgenics. *Archiv Insect Biochem Physiol* **69**:168–175 (2008).

15 Pfaffl MW, A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* **29**:e45 (2001).

16 La Scola B, Bryskier A and Raoult D, Comparison of the in vitro efficacy of telithromycin (HMR 3647) and levofloxacin with 22 antibiotic compounds against Bosea and Afipia species. *J Antimicrob Chemother* **53**:683–685 (2004).

17 Christiaens O, Petek M, Smagghe G and Taning CNT, The use of nanocarriers to improve the efficiency of RNAi-based pesticides in agriculture. *Nanopesticides*:49–68 (2020).

18 Kiely A, Authier A, Kralicek AV, Warr CG and Newcomb RD, Functional analysis of a *Drosophila melanogaster* olfactory receptor expressed in Sf9 cells. *J Neurosci Methods* **159**:189–194 (2007).

19 Echeverri CJ and Perrimon N, High-throughput RNAi screening in cultured cells: a user's guide. *Nat Rev Genet* **7**:373–384 (2006).

20 Taning CNT, Christiaens O, Berkvens N, Casteels H, Maes M and Smagghe G, Oral RNAi to control *Drosophila suzukii*: laboratory testing against larval and adult stages. *J Pest Sci* **89**:803–814 (2016).

21 Thiel C and Nix M, Efficient transfection of primary cells relevant for cardiovascular research by nucleofection®. *Cardiovasc Dis* **2**:255–266 (2007).

22 Roussel N, Sprenger J, Tappan SJ and Glaser JR, Robust tracking and quantification of *C. elegans* body shape and locomotion through coiling, entanglement, and omega bends, in *Worm*. Taylor & Francis, **3**:e982437 (2014).

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