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# Applications of RNA Interference in American Cockroach

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**TITLE:**

# Applications of RNA Interference in American Cockroach

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**KEYWORDS:**

dsRNA; RNAi; injection; cockroaches

**SUMMARY:**

The present protocol describes step-by-step guidelines for the RNAi operation techniques in *P. americana.*

**ABSTRACT:**

Cockroaches, a sanitary pest, are essential species in insect developmental and metamorphic studies due to their easy feeding and hemimetabolous characteristics. Altogether with well-annotated genome sequences, these advantages have made American cockroaches, *Periplaneta americana,* an important hemimetabolous insect model. Limited by the shortage of knockout strategy, effective RNA interference (RNAi)-based gene knockdown becomes an indispensable technique in functional gene research of *P. americana*. The present protocol describes the RNAi operation techniques in *P. americana*. The protocol includes (1) selection of the *P. americana* at

proper developmental stages, (2) preparation for the injection setting, (3) dsRNA injection, and (4) gene knockdown efficiency detection. RNAi is a powerful reverse genetic tool in *P. americana*. The majority of *P. americana* tissues are sensitive to extracellular dsRNA. Its simplicity allows researchers to quickly obtain dysfunctional phenotypes under one or multiple targeting dsRNA injections, enabling researchers to better use the *P. americana* for developmental and metamorphic studies.

**INTRODUCTION:**

RNA interference (RNAi), an evolutionarily conserved mechanism, gradually becomes an essential reverse-genetic tool to inhibit gene expression in many organisms[1], since Andrew Fire and Craig Mello[2] developed the double-stranded RNA (dsRNA) mediated gene silence strategy. dsRNA is cleaved into fragments of 21-23 nucleotides, small interfering RNAs (siRNAs), by the enzyme Dicer in cells to activate the RNAi pathway. Then siRNAs are incorporated into the RNA-induced silencing complex (RISC), which couples to the target mRNA, causes mRNA cleavage, and finally results in the loss of gene function[3-5]. Among the insect species, many systemic RNAi experiments have so far been reported in lots of insect orders, such as Orthoptera, Isoptera, Hemiptera, Coleoptera, Neuroptera, Diptera, Hymenoptera, Lepidoptera, and Blattodea[5-8].

Cockroaches (Blattaria) are an essential insect family in developmental and metamorphic studies with their rapid growth cycles, strong adaptability to the environment, and high developmental plasticity[9]. Before discovering that RNAi was compatible with cockroaches, previous research only focused on cockroach prevention and control due to a scarcity of genetic manipulation techniques in cockroaches. The cockroach ootheca's unique structure made it challenging to perform embryo injection-based gene knockout with the CRISPR-Cas9 system. Besides, most tissues in cockroaches (such as *P. americana*) show robust systemic RNAi response, allowing for the rapid generation of dysfunctional phenotypes by injecting one or more targeting dsRNAs[9-11]. These features made RNAi an indispensable technique in gene functional research in *P. americana*.

Even though the use of RNAi in functional gene research in *P. americana* has been reported, no detailed or step-by-step description was available. This report provides one step-by-step operational guideline for RNAi in *P. americana*, useful for gene function study in other cockroaches. Furthermore, this guide is not limited to Blattodea and can be applied to many other insects with minor modifications.

**PROTOCOL:**

The line of *P. americana* was initially provided by Dr. Huiling Hao. This species has been maintained with inbreeding for 30 years[9].

## 1. Hatching and feeding of *P. americana*

1.1. Collect fresh oothecae (immediately post egg-laying) of *P. americana* and incubate in the dark incubator at 25 °C and 60% humidity for ~25 days. Then increase the temperature to 30 °C and 75% humidity for 3 days before hatching.

1.2. Use a sieve with 4 mm aperture to separate the hatched nymphs from oothecae.

1.3. Keep the nymphs in cylindrical containers (12 cm in diameter and 10 cm in height) in the dark at 28 °C, 70% humidity. Brush the inside edge of the containers with vaseline to prevent cockroaches from escaping. Provide rat food, water, and shelter (egg trays). Pay attention to the activity of the nymphs and regularly clean up the feces and debris.

## 2. Selection of the nymphs in proper instar

2.1. Use a glass tube to pick out the freshly molted *P. americana* (white in body color). Keep them in new containers and wait for the correct stage for treatment.

NOTE: After ~19 days under the above feeding conditions, the nymphs in the 3$^{rd}$ instars will be available for injection. The color of freshly molted cockroaches is white, and the instars are clarified by molting times.

## 3. Preparation of the target fragment with T7 promoters

3.1. Using the cDNA of *P. americana* as a template, design paired primers to perform PCR to obtain 300-800 bp DNA fragment of the target gene[9]. Then clone the PCR fragment into a pTOPO vector (see **Table of Materials**) for sequencing.

3.2. Using the target fragment DNA as a template, synthesize one new pair of primers with T7 promoter sequence (5'-GGATCCTAATACGACTCACTATAGG-3') at the 5' terminals and perform another round of PCR to obtain the target fragment with T7 promoters on each side[9].

## 4. Transcription and synthesis of dsRNA *in vitro*

4.1. Add 10 µL of T7 2X Buffer, 2 µL of the Enzyme Mix, T7 Express (see **Table of Materials**), and 2 µg of PCR product (obtained in step 3.2) in the reaction system and add up the total volume to 20 µL with dd$H_2O$. Gently mix upside down manually and incubate at 37 °C for 30 min and 70 °C for 10 min, and then slowly cool to room temperature.

4.2. Dilute RNase A solution (4 µg/µL) with $ddH_2O$ at a ratio of 1:200. Then add 1 µL of RQ1 RNase-Free DNase (1 U/µL) and 1 µL of diluted RNase A solution to the system (see **Table of Materials**).

NOTE: Now, the volume of a single system is 22 µL.

4.3. Incubate at 37 °C for 30 min. Then add 10% of the total volume (when performing N reactions simultaneously, the total volume is N x 22 µL) of Sodium acetate and three volumes of the total volume of isopropanol.

4.4. Gently mix upside down manually, place on ice for 5 min, and centrifuge at 13,000 x *g* for 10 min at 4 °C.

4.4.1. Remove the supernatant with a pipette, wash the residue with 75% ethanol (with 25% diethyl pyrocarbonate (DEPC) treated water), and then centrifuge at 13,000 x *g* for 5 min at 4 °C.

4.5. Remove supernatant again. Air-dry the pellet for 15 min at room temperature. Use ~100 µL of DEPC water to dissolve dsRNA, then dilute the dsRNA to the final 2 µg/µL.

NOTE: The dsRNA could be stored at -80 °C for 6 months.

## 5. Loading dsRNA solution into the syringe

5.1. Set up the program in the micro-injection pump (see **Table of Materials**) in advance to ensure that the volume of each injection is consistent. Before using the syringe, clean the syringe by filling it with DEPC water 8-10 times.

5.2. Install the 10 µL Syringe on the micro-injection pump, start the pump, and fill the syringe with 10 µL of dsRNA solution (prepared at step 4.5). Inject 1 µL of the dsRNA into a 3$^{rd}$ instar nymph (see step 2) and ensure that it does not leak out to the body.

## 6. Injecting dsRNA to *P. americana*

6.1. Anesthetize the cockroaches with an increased concentration of $CO_2$ in a container until the cockroaches do not move anymore, and then proceed immediately with the injection.

6.2. Gently pick up the cockroach with tweezers, and deliver the cockroach toward the needle with hand. Next, insert the needle *via* the gap between two abdominal somites horizontally against the epidermis; insert depth the shallower, the better.

6.3. Then, inject the dsRNA solution into the cockroach. Finally, pull out the needle tip. Ensure that the needle tip is as close as possible to the epidermis to avoid damaging internal organs.

NOTE: The injection should be made under a dissection microscope.

6.4. Put the injected cockroaches into clean bioassay containers. Wait for about 10-20 min to let them recover from the $CO_2$ effects. Label the containers with the date of injection, type and dose of dsRNA, and age of the *P. americana*.

6.5. Place the injected cockroaches in a dark environment at 28 °C, 70% humidity, provide water, feed, and shelter, and observe possible changes in the phenotype of the cockroaches regularly.

**7. Knockdown confirmation and phenotypic analysis**

7.1. Evaluate the efficiency of RNAi using any available molecular biology techniques such as quantitative Real-Time PCR (qRT-PCR) and Western blotting. For detailed qRT-PCR and Western blotting procedures, see References[1,12].

7.2. To observe and analyze RNAi-related phenotypes, use the microscope suitable for living animals.

NOTE: RNAi may affect the morphology, behavior, molting, limb regeneration, and other physiological activities of cockroaches. The specific frequency of phenotype is calculated according to specific conditions.

**REPRESENTATIVE RESULTS:**

**Figure 1** shows a successful injection. The microinjection syringe with a micro diameter needle should be horizontally placed on the booster (**Figure 1A**). The needle is inserted *via* the gap between two abdominal somites horizontally against the epidermis (**Figure 1B**). Ensure that the liquid goes into the *P. americana* abdomen. The too steep angle of the needle will damage the internal organs (**Figure 1C**), and improper injection leads to leakage of the dsRNA solution out of the abdomen (**Figure 1D**). If any dsRNA leaks out, the animal needs to be discarded, and another injection is performed. The *P. americana* needs to be completely anesthetized during the injection.

Like other biological experiments, control groups are needed when RNAi treatment is performed. The treatment differences need to be confirmed caused by targeted RNAi instead of due to the off-target effects of dsRNA, the damage during the injection, or $CO_2$ anesthesia. Here *Ddc* (*Dopa decarboxylase*) and *Dpp* (*decapentaplegic*) genes were selected as two examples to observe the

phenotype of the molted cockroaches after dsRNA injection, and a negative control ds*Mock*[11] which has no target in the animals, was performed as a negative control, the RNAi efficiency was detected by qRT-PCR (**Figure 2**). Taking the injection of ds*Ddc* as an example (**Figure 3A,B**), the *P. americana* with knocked down *Ddc* gene would have the white epidermis for a long time since the melanization is blocked (**Figure 3B**). In the experiment of ds*Dpp* injections, which are necessary for limb regeneration, the RNAi disrupted the limb regeneration (**Figure 4**), which was reported previously[9].

**FIGURES LEGENDS:**

**Figure 1: Instrument and correct injection operation**. (**A**) The syringe on the microinjection instrument. (**B**) Correct position of the injection needle; no liquid comes out. (**C**) The too steep angle of the injection needle may damage the internal organs. (**D**) Failed injection results in the dsRNA solution or hemolymph flowing out.

**Figure 2: RNAi efficiency detected by qRT-PCR**. The knockdown efficiency of *Ddc* and *Dpp* genes were detected by qRT-PCR, and the ds*Mock* was used as a negative control. Three replicates were used for each treatment, and the error bars indicate standard deviation of the three replicates. The significance of differences was analyzed by Student's t-test. *: $P < 0.05$, **: $P < 0.01$.

**Figure 3: Examples of successful RNAi for disruption of epidermis melanization in *P. americana***. (**A**) Normal cockroach after injection of ds*Mock* with normal melanization. (**B**) *Ddc* RNAi mediated albinistic *P. americana* after molting.

**Figure 4: Examples of successful RNAi for disruption of limb regeneration in *P. americana***. (**A**) A *P. americana* with an amputated hind leg. (**B-C**) *P. americana* after RNAi treatments and molting. The hindleg was regenerated in ds*Mock* control group (**B**) but not when the *Dpp* (**C**) gene was silenced.

**DISCUSSION:**

This report described a methodological step-by-step RNAi strategy in *P. americana*; of note, it also can be applied to other cockroaches (*Blattella germanica,* for example) and many other insects with minor changes. However, the gene silencing efficiency of RNAi is not always high enough, with an obvious disadvantage compared with the gene knockout strategy[13]. The following residual effect of gene-level may interfere with the real phenotypes. To ensure the RNAi treatment is successful, several essential conditions need to be considered, including the physical condition of animals, selection of control group, the length and concentration of the dsRNA molecules, the avoiding of the possibility of Off-target effects (OTE), and different sensitivity of tissues to dsRNA.

**Physical condition of *P. americana***

Healthy animals are the premise for the success of RNAi and different gene functional studies in *P. americana*. Besides, to keep experiments consistent, only cockroaches that have been raised in the same conditions are chosen. And only nymphs that are older than the 3rd instar (about 19 days post-hatching) can be used because younger nymphs are too small for injection in body size.

**Selection of control group**

ds*Mock*[11] and ds*EGFP* and the same dose of cockroach saline[6] have been used as negative controls. The target sequences of negative controls should be exogenous and have no homology with the endogenous genetic sequence[11]. ds*Mock* was preferred because using ds*EGFP* would delay the growth and development of *P. americana* to a certain degree.

**dsRNA length**

The efficiency of RNAi is affected by the length of the dsRNA molecules. Longer dsRNA fragments are more effective at mRNA silencing, possibly because it produces more siRNAs, or shorter dsRNA fragments are taken up by cells less efficiently [1]. In *P. americana* and *B. germanica*, dsRNA between 300 bp and 800 bp appears ideal for RNAi experiments[6,9,10,14]. A short dsRNA may be insufficient to induce a systemic RNAi response, whereas a long dsRNA may increase OTE risk[1].

**dsRNA concentration**

The concentration of dsRNA can also influence the efficiency of RNAi[1,15]. For the 3rd instar nymphs of *P. americana*, 1 μg of dsRNA appears to be a reasonable amount, and 4-6 μg is suitable for adult[6,9,14,16]. The precise dsRNA amount used for injection can be adjusted based on the genes, tissues, and body size of the *P. americana*.

**Off-target effects**

The OTE of RNAi is hard to avoid altogether[17,18]. Two non-overlapping regions of the dsRNA can be designed to reduce the effect of OTE on phenotypes[5]. If dsRNAs targeting two distinct regions produce the same effect, the possibility of OTE-induced false positive phenomenon could be excluded.

**Method for RNAi efficiency detection**

The phenotypic analysis is the most intuitive approach for evaluating RNAi, and qRT-PCR and Western blotting analysis are the two golden standards for measuring knockdown efficiency. qRT-PCR is a straightforward method for determining interference efficiency at mRNA level[6,9,10]. The primers should be designed out of the dsRNA targeting region. The Western blotting analysis is another method for analyzing protein-level interference but requires specific antibodies. However, no particular phenotypic effects are sometimes observed, even with high silencing

efficiency (>90%). One possible explanation for this residual effect is massive gene family expansion in cockroaches; the redundancies of genes control many particular functions and phenotypes. Another possible explanation is that the minimum gene expression level required for functional enough is extremely low. These lead to failure in obtaining a particular phenotype even with high enough RNAi efficiency.

**Tissue-specificity for the RNAi sensitivity**

The interference efficiency in some cockroach tissues (such as the ovary and accessory glands) is not high enough, which could be the penetrating barrier for dsRNA[5,19]; the different RNAi efficacy in different insect species is also observed, which depends on the enzymatic degradation of dsRNA in hemolymph[15,20]. A few ideas are reasonable for improving RNAi efficiency in *P. americana* tissues, such as digesting dsRNA into siRNAs *in vitro*[21], increasing the dsRNA dose and injection times, and using nanomaterials as a carrier for delivery.

In conclusion, the high efficiency of RNAi together with well-annotated genome sequences[9] makes *P. americana* a good model for investigating gene function in a wide range of developmental, physiological, and behavioral studies. Similarly, this report can serve as a valuable reference for other insects sensitive to dsRNA and hard to make knockout mutations.

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**DISCLOSURES:**

The authors declare that they have no conflicts of interest.

**REFERENCES:**

1. Miller, S. C., Miyata, K., Brown, S. J., Tomoyasu, Y. Dissecting systemic RNA interference in the red flour beetle *Tribolium castaneum*: Parameters affecting the efficiency of RNAi. *PloS One*. **7** (10), e47431 (2012).
2. Fire, A. et al. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature*. **391** (6669), 806–811 (1998).
3. Ambesajir, A., Kaushik, A., Kaushik, J. J., Petros, S. T. RNA interference: A futuristic tool and its therapeutic applications. *Saudi Journal of Biological Sciences*. **19** (4), 395–403 (2012).

4. Younis, A., Siddique, M. I., Kim, C. K., Lim, K. B. RNA interference (RNAi) induced gene silencing: A promising approach of hi-tech plant breeding. *International Journal of Biological Sciences*. **10** (10), 1150–1158 (2014).
5. Bellés X. Beyond Drosophila: RNAi in vivo and functional genomics in insects. *Annual Review of Entomology*. **55**, 111–128 (2010).
6. French, A. S., Meisner, S., Liu, H., Weckström, M., Torkkeli, P. H. Transcriptome analysis and RNA interference of cockroach phototransduction indicate three opsins and suggest a major role for TRPL channels. *Frontiers in Physiology*. **6**, 207 (2015).
7. Hennenfent, A., Liu, H., Torkkeli, P. H., French, A. S. RNA interference supports a role for Nanchung-Inactive in mechanotransduction by the cockroach, *Periplaneta americana*, tactile spine. *Invertebrate Neuroscience: IN*. **20** (1), 1 (2020).
8. Immonen, E. V. et al. EAG channels expressed in microvillar photoreceptors are unsuited to diurnal vision. *The Journal of Physiology*. **595** (16), 5465–5479 (2017).
9. Li, S. et al. The genomic and functional landscapes of developmental plasticity in the American cockroach. *Nature Communications*. **9** (1), 1008 (2018).
10. Zhao, Z. et al. Grainy head signaling regulates epithelium development and ecdysis in *Blattella germanica*. *Insect Science*. **28** (2), 485–494 (2021).
11. Lozano, J., Belles, X. Conserved repressive function of Krüppel homolog 1 on insect metamorphosis in hemimetabolous and holometabolous species. *Scientific Reports*. **1**, 163 (2011).
12. Philip, B. N., Tomoyasu, Y. Gene knockdown analysis by double-stranded RNA injection. *Methods in Molecular Biology (Clifton, N. J.)*. **772**, 471–497 (2011).
13. Zheng, Y. et al. CRISPR interference-based specific and efficient gene inactivation in the brain. *Nature Neuroscience*. **21** (3), 447–454 (2018).
14. Garbutt, J. S., Bellés, X., Richards, E. H., Reynolds, S. E. Persistence of double-stranded RNA in insect hemolymph as a potential determiner of RNA interference success: Evidence from Manduca sexta and *Blattella germanica*. *Journal of Insect Physiology*. **59** (2), 171–178 (2013).
15. Parrish, S., Fleenor, J., Xu, S., Mello, C., Fire, A. Functional anatomy of a dsRNA trigger: differential requirement for the two trigger strands in RNA interference. *Molecular Cell*. **6** (5), 1077–1087 (2000).
16. Lemonds, T. R., Liu, J., Popadić, A. The contribution of the melanin pathway to overall body pigmentation during ontogenesis of *Periplaneta americana*. *Insect Science*. **23** (4), 513–519 (2016).
17. Jackson, A. L., Linsley, P. S. Noise amidst the silence: Off-target effects of siRNAs? *Trends in Genetics: TIG*. **20** (11), 521–524 (2004).
18. Patel, M., Peter, M. E. Identification of DISE-inducing shRNAs by monitoring cellular responses. *Cell Cycle (Georgetown, Tex.)*. **17** (4), 506–514 (2018).
19. Ventós-Alfonso, A., Ylla, G., Montañes, J. C., Belles, X. DNMT1 promotes genome methylation and early embryo development in cockroaches. *iScience*. **23** (12), 101778 (2020).

352 20. Wang, K. et al. Variation in RNAi efficacy among insect species is attributable to dsRNA
353 degradation *in vivo*. *Insect Biochemistry and Molecular Biology*. **77**, 1–9 (2016).
354 21. Bi, F., Liu, N., Fan, D. Small interfering RNA: A new tool for gene therapy. *Current Gene Therapy*.
355 **3** (5), 411–417 (2003).

Figure 1

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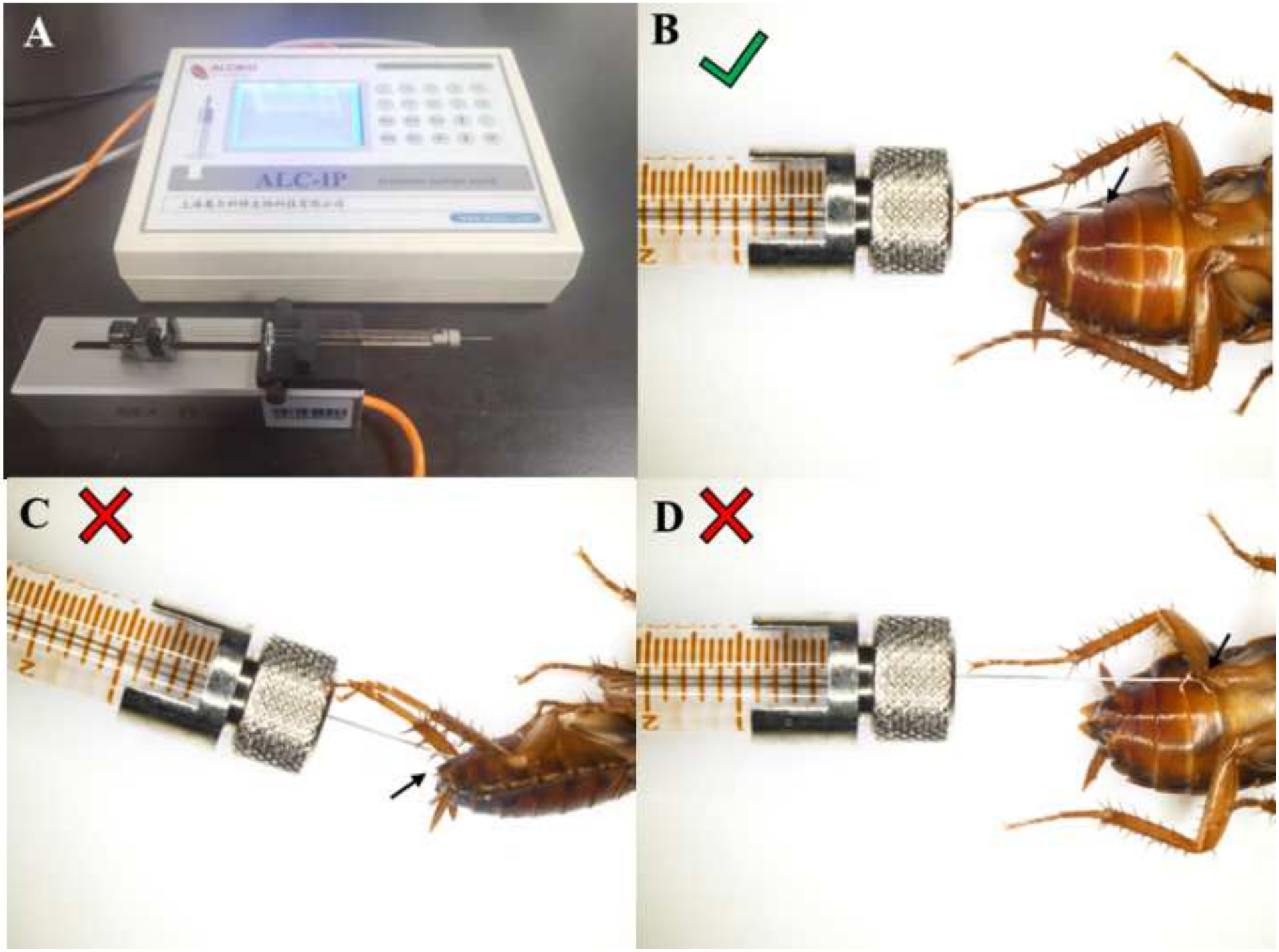

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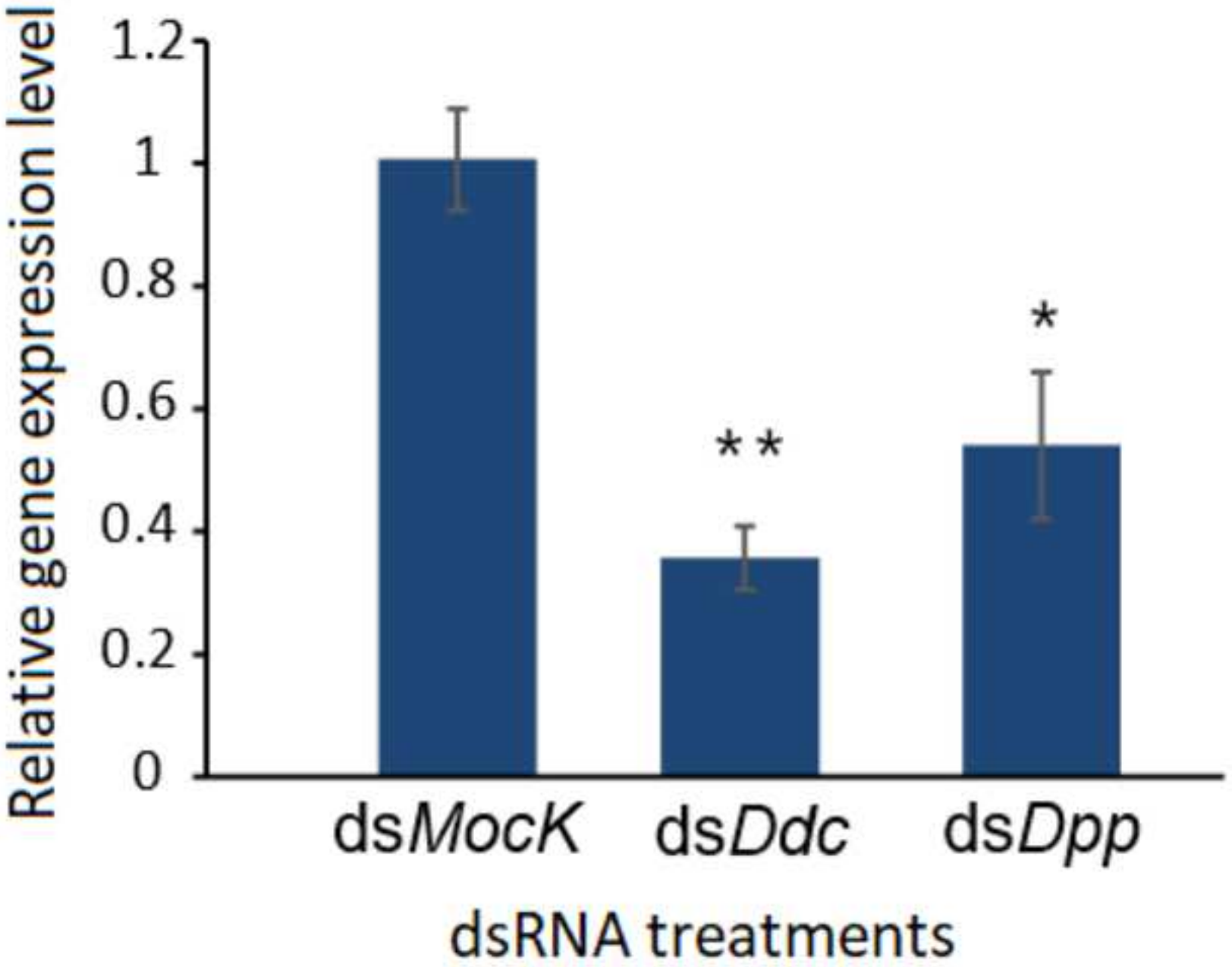


Figure 3



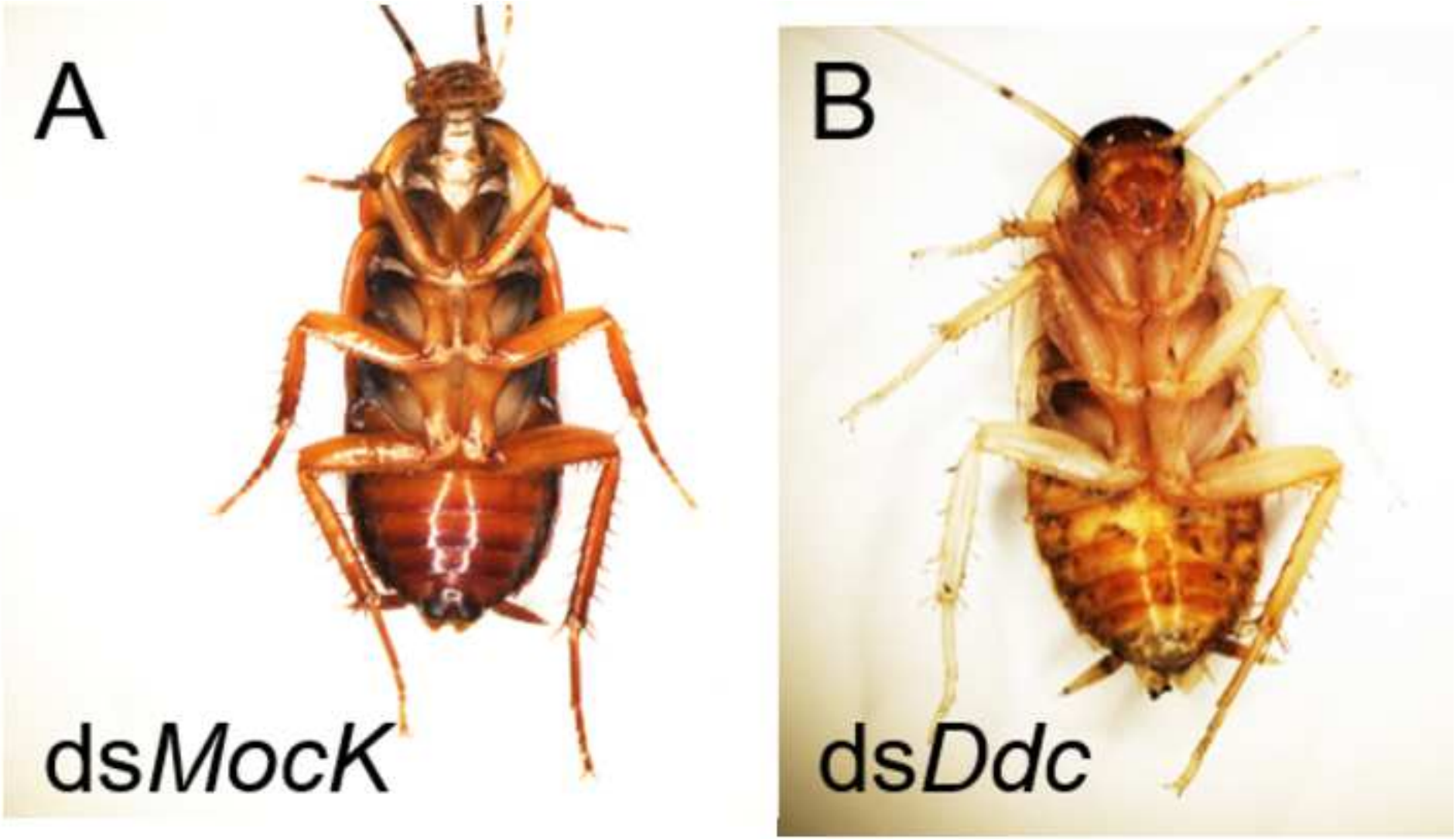
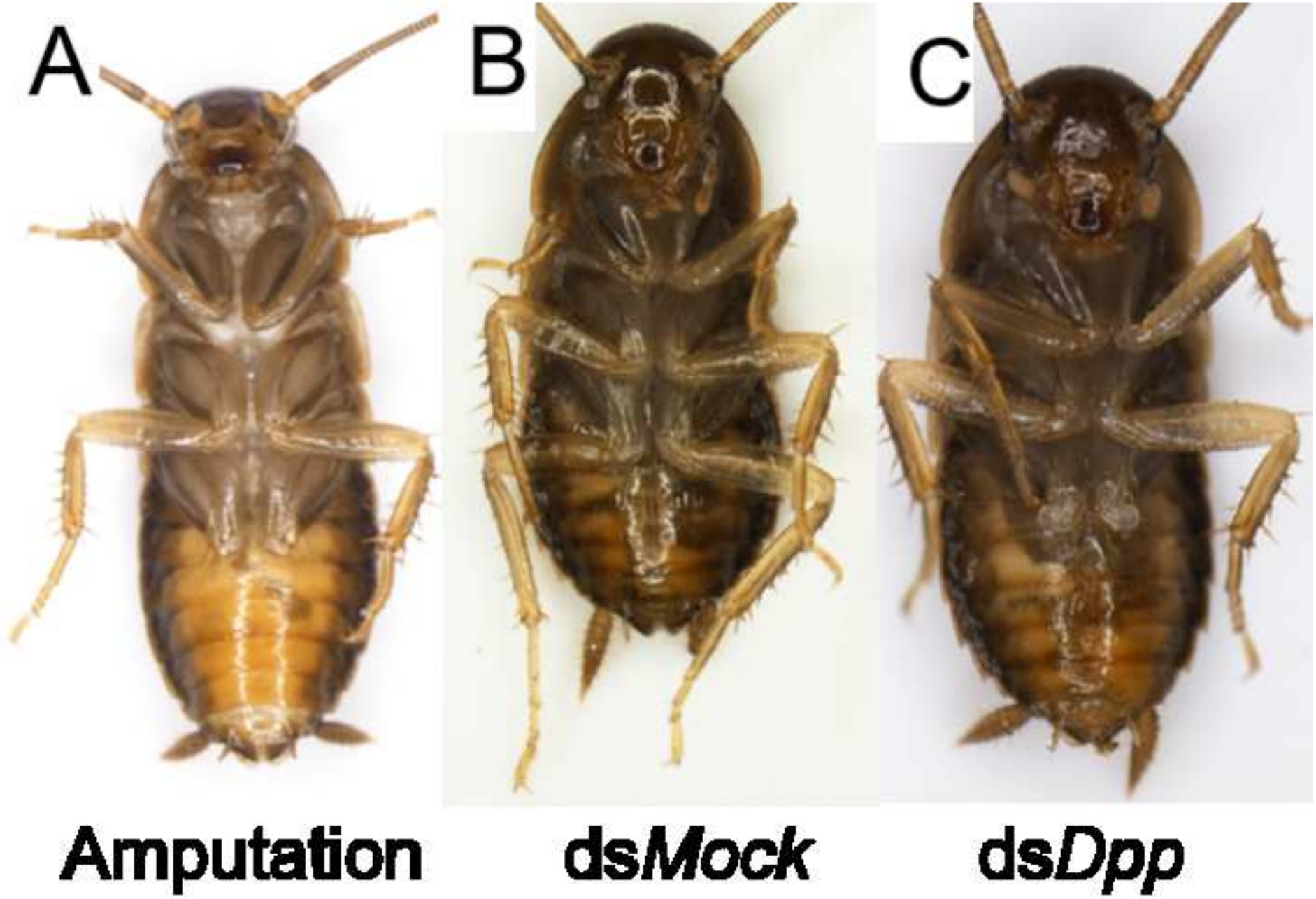


Figure 4





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Sincerely yours,

Chonghua Ren

Dear editor,

We greatly appreciate your evaluation on the novelty, impact, and general interest of our work. We agree with your and reviewers' constructive comments and we have revised the whole manuscript according to these insightful suggestions. We believe that we have addressed all the concerns point to point and the manuscript has been significantly improved.

Sincerely yours,

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**Editorial comments:**

1.Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Thanks for your suggestion. We have carefully scrutinized the manuscript, and made corresponding revisions including some typos, grammatical errors and long sentences, etc.

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We have asked a native speaker to proofread our manuscript, and hope it is good enough now.

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4. Please provide a Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"

We have added this sentence "Here, we present a protocol to help researchers quickly master cockroach RNAi operation techniques." in abstract.

5. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Thanks for your comments. We've revised the text according to your suggestion.

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Thanks for your comments, we have corrected all the details in revised manuscript.

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a) A clear statement of the overall goal of this method
b) The rationale behind the development and/or use of this technique
c) The advantages over alternative techniques with applicable references to previous studies
d) A description of the context of the technique in the wider body of literature
e) Information to help readers to determine whether the method is appropriate for their application
Thanks a lot for your insightful and constructive comments. We have rewritten Introduction according to your suggestions.
a) "This report provides one step by step operational guideline for RNAi in P. americana, which will be useful for gene function study in cockroaches."
b) "To activate the RNAi pathway, dsRNA is cleaved into fragments of 21-23 nucleotides, small interfering RNAs (siRNAs), by the enzyme Dicer, and then siRNAs are incorporated into the RNA-induced silencing complex (RISC), which couples to the target mRNA, causes mRNA cleavage and finally results in the loss of function".
c) "Even though the use of RNAi in gene functional research in cockroaches has been reported, no detailed or step by step description was available. This report provides one step by step operational guideline for RNAi in P. americana, which will be useful for gene function study in cockroaches."
d) "Furthermore, this guide is not limited to Blattodea and can be applied to many other insects with minor modifications."
e) "This report provides one step by step operational guideline for RNAi in *P. americana*, which will be useful for gene function study in cockroaches. Furthermore, this guide is not limited to Blattodea and can be applied to many other insects with minor modifications".

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b. Line 73: please specify the number of days cockroaches were fed here.
c. Step 3.1: please include the details of primer and target gene sequences. Please also include the details of cloning and the vector used. Alternatively, provide references to published material.
d. Line 87: what is the dilution solvent for RNase A solution.

Thanks for your comments.

a) "After 19 days under above feeding conditions, the nymphs in the 3rd or higher instars will be available for injection to ensure that the body sizes are large enough."

b) "After 19 days under above feeding conditions, the nymphs in the 3rd or higher instars will be available for injection to ensure that the body sizes are large enough."
c) We have added these information in revised manuscript.
d) The dilution solvent for RNase A solution is dd$H_2O$. We've revised the manuscript.

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12. Step 6: Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.).
Done

13. Please give a single-line space between steps and substeps of the protocol. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.
Done

14. Place all the figure legends after Representative Results.
Done

15. Figure 1 legend: Please include a one-liner title in the legend.
Done

16. Figures: Please remove the text 'Figure X' within the respective figures and upload individual figures to your Editorial Manager account.
Done

17. Figure 2: labels are missing
Done

18. Figure 3 and wherever applicable: Please obtain explicit copyright permission to reuse any figures from publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."
Thanks for your recommendation, to avoid the conflict with published data, we change the pictures with our new treatments.

19. Line 175: Please briefly discuss in which way the use of EGFP affected the growth and development of insects.
We are extremely grateful to you for pointing out this problem. We've changed "because using EGFP as the control group had some effects on insect growth and development in our experiment (both in *P. americana* and *B. germanica*)." to "Mock was chosen because using EGFP would delay the growth and development of cockroaches".

20. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
We sincerely thank you for thoroughly examining our manuscript and providing very helpful comments to guide our revision. Critical steps within the protocol are listed as subtitles in discussion, the limitation of the technique is added in the beginning of discussion as "However, the RNAi efficiency is not always high enough, and residual effect of gene transcriptional level may interfere with the

real phenotypes".

**Reviewers' comments:**

**Reviewer #1:**

We sincerely thank you for thoroughly examining our manuscript and providing very helpful comments to guide our revision. We also thanks for the references, which are now included in the revised manuscript. Specific references are listed as follows: 6,7,8. We thoroughly read the two papers you recommended, as well as the other papers in this group. And we have tried our best to revise the manuscript according to your kind and constructive comments and suggestions.

Line 32: "found" should be "developed"
Done

Line 37: remove "phenomenons"
Done

Line 39: "Blattaria" is based on old taxonomy, the current term is "Balttodea" (cockroaches and termites).
Done

Reference 5 in line 39 is from 2010 and there is a lot of work done after that using RNAi in insects including cockroaches.
Based on your suggestions, we've added several examples of successful RNAi work.

Line 40: change "species in" to "family of"
Done

Lines 42-44: See above: multiple papers have used RNAi on cockroaches successfully.
Please accept our apologies for not being clear. "However, due to the shortage of genetic manipulation techniques in cockroaches, the researches mainly focused on prevention and control of cockroaches in the past, and it's difficult for the gene functional and developmental study" has been replaced by "Prior to the discovery that RNAi was compatible with cockroaches, previous research focused primarily on cockroach prevention and control due to a scarcity of genetic manipulation techniques in cockroaches."

Line 53: "circumstantial" should be "detailed"
Done

Line 53: remove "methodological"
Done

Line 57: "Blattaria: should be "blattodea", but this could be removed, since the method can be used for any insects.
Done, “Furthermore, this method is not only suitable for insects of Blattidae, but also can be applicable to other insect families of Blattaria with minor modifications.” has been replaced by “Furthermore, this guide is not limited to Blattodea and can be applied to many other insects with minor modifications.”

Line 60: remove "incubator"
Done

Line 61: "at the beginning" should be replaced by the number of days under these conditions
Changed to about 25 days.

Line 63: "suitable aperture" Please, be more specific. What kind of sieve is used?
Done

Line 64: Cockroaches do not have larval stages, the small ones are called nymphs. "Place the nymphs in a culture tank in the dark at 28°C, 70% humidity. Provide feed, water and shelter"
We are extremely grateful to you for pointing out this problem. We have changed all the “larvae” to “nymphs”

Line 68-69 1.4 is unnecessary
Removed.

Line 71 "more than" should be "older than". "size is big enough" should be removed
Done

Line 73: "feed them" should be "keep them" Change "for several days according to experimental needs" to "until used for experiments"
Done

Line 80: what does "are designed" refer to?
We have added Reference 9 in this part.

Line 85 and 91: "mix gently" be more specific, how is the mixing done? Manually or some other way?
Changed to “Gently mix upside down manually”.

Line 87: Where are these reagents purchased from? What dilution buffer is used?

We’ve revised the manuscript.

Line 89: add "incubate at" at the beginning of the sentence. What is the total volume of sodium acetate?
Done. We've revised the manuscript.

Line 92: replace "the upper liquid" with "supernatant"
Done

Line 94: replace "place" with "incubate"
Done

Line 95: "appropriate amount" please be specific.
Done. We have added "100uL DEPC water" in revised manuscript.

Line 98: add the details of the instrument after it is first mentioned.
Done

Line 98-99: replace "The volume of the fourth-instar larvae injected in vivo is generally 1-2 μl, too much volume may flow out of the worm body and affect the normal growth." With "Inject 1-2 μL of the dsRNA into a fourth instar nymph making sure it does not leak out to the body"
Done

Line 101: replace "draw 8-10 times of DEPC water to clean the syringe." With "clean the syringe by filling it with DEPC treated water 8-10 times"
Done

Line 102: replace "draw the dsRNA that needs to be injected with "fill the syringe with 1-2 μL of dsRNA solution"
Done

Line 110- 13: It is not clear to me how the CO2 is applied; Normally CO2 is applied from the cylinder via tubing to single insect in a small container and it does not take 2 minutes to anesthetise even an adult cockroach. It should be impossible for the user to suffocate and obviously the user would not leave the room until the experiment is completed. So, this paragraph should read: "Place a cockroach that is to be injected into a small container and anesthetize with CO2. Once the cockroach does not move anymore, proceed immediately with the injection."
Done. Thanks for your comments

Line 115-116: replace " gently hold the cockroach with your hand, and send it to the tip of the needle…." With "gently push the cockroach toward the needle placing the needle between two somites on the cockroach abdomen avoiding excessive force that could damage the animal."
Done

Line 121-122: replace "with the help of a microscope" with "under a dissection microscope". This should be done what ever the age of the roach.
Done

Line 123: what is the waiting period for? Instead of "feed" use "provide water, feed and shelter" (animal food is called feed)
Done

Line 124: "appropriate markings…" should be "label the tank with the date of injection, type of dsRNA, dose and age of the cockroach"
Done

Line 127-128: "observe the RNAi phenotype" should be "observe possible changes in the phenotype". I do not understand why the Note is here? If staging is provided, it should be at the beginning of the methods section and include all stages and ages.
Thanks. We deleted the Note.

Line 140-142: "packaged" should be "placed" "Inserting the needle from the gap between the two abdominal somites horizontally and press it against the epidermis…." Should be " insert the needle via the gap between two abdominal somites horizontally against the epidermis (Fig 1B). Make sure that the liquid goes into the cockroach abdomen. Too steep angle of the needle will damage the internal organs (Fig. 1C) and too shallow angle leads to leakage of the dsRNA out of the abdomen (Figure 1D). Cockroach should be deeply anesthetised during the injection."
Thanks for your kind comments, we have replaced all the information according to your suggestions.

Line 152: Lozano 20118 probably should be 2018?
We've replaced it by "a negative control dsMock[11]".

Line 153-154: "which are in shortage of" should be "with knocked down Ddc gene" and "keep" should be "have"
Done

Line 156: remove "obviously"
Done

Line 160-161: add "and insects" after "cockroaches" this method is quite generic and can be used for most insect. Remove "depending on the context and organism"
Done

Line 167: "RNA silence" should be "RNAi".
Done

Line 168: replace "the consistency of growth" with "experiments consistent" and remove "we"
Done

Line 169-171 replace sentence starting "The size…." With " Use only cockroach nymphs that are older than 3 instar (about 30 days). Younger nymphs are too small for injection."
Done

Line 178: remove "interference".
Done

Line 181: In addition to your own work you should refer, French et al 2015 and Hennnenfent et al 2020
Done

Line 182: add "be" between "may" and Insufficient"
Done

Line 188: replace "phenotype" with "effect"
Done

Line 189: change "RNA" to "dsRNA" and remove "interference"
Done

Line 191: replace "to be avoided" with "to avoid"
Done

Line 192: replace "are usually designed" with "can be designed"
Done

Line 193: replace "phenotype" with "effect"
Done

Lines 203-207: RNAi has been very efficient in P americana nerve tissue, e.g. compound eye (French et al 2015 + multiple other papers from the same group) and also on mechanosensory neurons (Hennenfent et al 2020). These are nervous tissue.
We are grateful to you for pointing out this problem. We've removed "nerve" in revised manuscript.

Line 209: "made this technique can be used to investigate" should be " makes this method useful for investigating…"
Done

Line 210: change "responses" to "studies"
Done

Line 260-262 Change as : B. Correct position of the injection needle…. C. Too steep angle of the injection needle…D. Too shallow position of the injection needle. Remove the last sentence, it is unnecessary.
Done

Line 271-273: Change as: A) A cockroach with and amputated hindleg. (B-D) Cockroach after molting and various RNAi treatments. The cockroach hindleg was regenerated when the CK gene was silenced (B) but not when the Dpp (C) or Mad (D) genes were silenced.
Done

**Reviewer #2:**

Thank you for your recognition of our work and valuable feedback. We have tried our best to revise the manuscript according to your kind and constructive comments and suggestions.

Minor Concerns:
1. The initial part of the abstract talks about cockroaches in general from the family Blattidae. However, this is misleading since the paper is mostly about P. americana. Authors should specifically refer to American cockroaches throughout the abstract.
Thanks for your suggestion. We have meticulously revised the abstract.

2. INTRODUCTION: Again, authors begin by talking about cockroaches specific to the order Blattidae and then later in the section talk about German cockroaches which are from the order Blattellidae. So I would again recommend keeping the introduction specific to P. americana in the initial paragraphs and then referring to other species of cockroaches in the latter paragraphs of the intro section
Thanks for your constructive suggestion, which is highly appreciated. We have changed the information according to your suggestion.

3. Line 125/ section 5.3: While it is okay to refer to the rearing box as "culture tank", the use of this term is confusing when readers are being asked or instructed to transfer dsRNA injected insects to the "culture tank". Therefore, I recommend authors to refer to "culture tanks" as "bioassay containers" or "bioassay boxes" in all steps that follow the dsRNA injection step.
Thanks for your comments. We've revised the manuscript according to your suggestion.

4. Gene names are not spelled out at first mention in the "Representative Results"

section and in the figure captions. This needs to be included for clarity and understanding the biological function of the target and control dsRNAs.
Done.

5. Selection of control group: In the protocol and the discussion sections, no information is provided to describe the criteria or procedures used for selecting an appropriate "control dsRNAs". This information needs to be included.
To address the reviewer's concern, we have rewritten this sentence as follows: The target sequences of negative controls should be exogenous and have no homology with the endogenous genetic sequence.

6. DISCUSSION section: In my experience, knocking out a particular gene provides high silencing efficiency that can be verified by qPCR or western blots. However, even with >90% silencing efficiency no particular phenotypic effects are observed. One possible explanation for this is that there is massive gene family expansion in German and American cockroaches. Due to this gene family expansion, there are redundancies in genes that control any particular function or phenotype. This leads to failure in obtaining a particular phenotype even with >90% gene expression silencing. This important topic needs to be discussed in the "DISCUSSION" section.
Done. "However, sometimes even with high silencing efficiency (such as >90%), no particular phenotypic effects are observed. One possible explanation for this residual effect is that there is massive gene family expansion in cockroaches, the redundancies of genes control many particular function and phenotype. Another possible explanation is that the minimum level of gene expression required for functional enough is extremely low. These lead to failure in obtaining a particular phenotype even with high enough RNAi efficiency."

**Reviewer #3:**
Sincere thanks should be given to you for the constructive comments and suggestions. Our responses to the questions are shown below.

Major Concerns:
1. In different parts of the text, the authors describe aspects of the physiology or anatomy of Periplaneta americana as if they were general for all cockroaches, and this is not the case. The biological cycle is different, the regulation of oocyte growth is different, the hormonal profiles also are different. It is best to make explicit reference to the species under study and not generalize.
Sorry for our misleading at the previous version. We explicitly state in the revised manuscript that the American cockroach is the animal we focused on in this work, and we have revised the abstract, introduction and the rest sections.

2. The taxonomy of the Dictyoptera included in the text must be revised. There are important errors. Blattidae doesn't exist.
Order Blattodea

Suborder Blattaria (cockroaches)
Suborder Isoptera (termites)
Thank you for the advice. According to your suggestion, we've changed "Blattidae" to "Blattaria".

3. The description of the anatomical part of the cockroach where the injection will be done must be described accurately. Not saying that the syringe is introduced between somites in the abdomen. The correct is, in the membrane between abdominal sternites.
Done

4. The protocol must be described with more precision.
Thanks for your comments, we have revised the protocol section with more efforts.

Minor Concerns:

The keywords are included in the title.
We have changed the keywords according to your suggestion.

line 17: Between cockroaches, only Blattella germanica and Periplaneta americana have the genome sequenced
Thanks for your suggestion. We've changed "cockroaches" to "*Periplaneta americana*" in abstract as we made *Periplaneta americana* as the focused research specie in this manuscript.

Lines 56-57: the protocol can be (it is) useful to any insect species
Sorry for our misleading at the previous version. We've changed "Furthermore, this method not only suitable for insects of Blattidae, but also can be applicable to other insect families of Blattaria with minor modifications." to "Furthermore, this guide is not limited to Blattodea and can be applied to many other insects with minor modifications".

line 60: What means fresh ootheca. Clarify the time from oviposition
Done

Point 2. How is identified the correct stage?
Done

line 77: revise fragmented DNA.
Done

line 80: indicate the position of T7 promoter with respect to the primer.
Done

Line 87-88: Give the final concentrations of both enzymes.
Done

Line 89: replace 300% with three volumes.
Done

Line 124: replace resuscitate by recovering from CO2 effects or wake up.
Done

line 146: The authors say "If this happens, please do another injection instead". The correct is to discard the animal because is not possible to know the dose injected.
Done

Line 181: The references Garbutt, J. S., Belles, X., Richards, E.H., Reynolds, S.E. 2013. Persistence of double-stranded RNA in insect hemolymph as a potential determiner of RNA interference success: Evidence from Manduca sexta and Blattella germanica. Journal of Insect Physiology 59: 171-178, is missed.
Sorry for the missing about this information. We've added this reference and relative information.

Line 203: The bibliography based on Blattella germanica does not suggest any penetrating problem for RNAi. Conversely, it seems that RNAi works perfectly in all tissues studied in B. germanica.
It's a very nice comment and RNAi is effective in most Blattella germanica tissues. Actually, dsRNA mediated RNAi efficiency in ovary and male accessory gonads are ineffective according to our experiments. We've added these information in discussion section.

**Reviewer #4:**
Major Concerns:
1. The authors rear the cockroaches under the constant darkness condition which will allow the cockroaches to free-run on their endogenous circadian clock and make them to grow dis-synchronized. If the rearing condition changes to 12L:12D or 16L:8D, the cockroaches will develop in synchronization and the operation will not disrupt their circadian clocks.
Thank you very much for your suggestion. Actually, both *Blattella germanica* and *Periplaneta americana* prefer live in darkness, and we did rear them in darkness for many years. Moreover, many labs and papers also used the same rear condition. However, we will try the rearing condition 12L:12D or 16L:8D to see what will happen.

2. The preparation of RNAi injection requires immobilization of the cockroach. The authors use CO2 to anesthetize the cockroach, but it tends to kill the animal with over time exposure. Even the treated cockroaches survive the operation, the sublethal dosage

of CO2 might interfere the results. Therefore, I would suggest the authors to use crush ice to knock down the cockroach.
Thank you for the advice. We did compare with $CO_2$ and ice side by side, and found $CO_2$ is much friendly than ice to cockroaches. To avoid the side effects of $CO_2$, the anesthetized *P. americana* was proceeded immediately with the injection.

3. The dosage of dsRNA used in the experiment depends on the size and hemolymph volume of the target animal. If the authors want to set up a standard for the RNAi operation, they need to provide a formula for the calculation of the dosage of dsRNA.
This is a very good suggestion. We have added more information in "dsRNA concentration" section.

Minor Concerns:
The effectiveness of RNAi can be quantified by real time PCR. But, the knockdown effect of the RNAi is not complete sometimes and some of the residual effect might interfere with the phenotype expression. These are the points need to be considered.

Thank you for the advice. We have added more information in "**Method for RNAi efficiency detection**" section. "However, sometimes even with high silencing efficiency (such as >90%), no particular phenotypic effects are observed. One possible explanation for this residual effect is that there is massive gene family expansion in cockroaches, the redundancies of genes control any particular function or phenotype. Another possible explanation is the minimum level of gene expression required for functional is extremely low. These lead to failure in obtaining a particular phenotype even with high enough RNAi efficiency."