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# Egg microinjection and efficient mating for genome editing in the firebrat Thermobia domestica

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**TITLE:**

# Egg Microinjection and Efficient Mating for Genome Editing in the Firebrat *Thermobia domestica*

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**KEYWORDS:**

genome editing, insect, arthropod, Evo-Devo, non-traditional model, microinjection

**SUMMARY:**

We provide a detailed protocol for rearing, microinjection of eggs and for efficient mating of the firebrat *Thermobia domestica* to generate and maintain mutant strains after genome editing.

**ABSTRACT:**

The firebrat *Thermobia domestica* is an ametabolous, wingless species that is suitable for studying the developmental mechanisms of insects that led to their successful evolutionary radiation on the earth. The application of genetic tools such as genome editing is the key to understanding genetic changes that are responsible for evolutionary transitions in an Evo-Devo approach. In this article, we describe our current protocol for generating and maintaining mutant strains of *T. domestica*. We report a dry injection method, as an alternative to the reported wet injection method, that allows us to obtain stably high survival rates in injected embryos. We also report an optimized environment setting to mate adults and obtain subsequent generations with high efficiency. Our method underlines the importance of taking each species' unique biology into account for the successful application of genome editing methods to non-traditional model organisms. We predict that these genome editing protocols will help in implementing *T. domestica* as a laboratory model and to further accelerate the development and application of useful genetic tools in this species.

**INTRODUCTION:**

*Thermobia domestica* belongs to one of the most basal insect orders, Zygentoma, which retains an ancestral ametabolous and wingless life cycle. Such basal phylogenetic position and ancestral characteristics set this species as an attractive model for studying the mechanisms underlying

the success of insects on Earth, which cover over 70% of the described animal species[1]. *T. domestica* has long been used mainly to study ancestral characteristics of insect physiology because of its suitable features as a laboratory model, such as a relatively short lifecycle (2.5–3.0 months from embryo to reproductive adult; **Figure 1A**) and an easy breeding. In the past three decades, its use has been expanded to investigate ancestral characteristics of various traits such as body plan, neural differentiation, and circadian rhythms[2–4].

The application of advanced genetic tools in *T. domestica* could further accelerate such contributions in a wide research area. Successful RNA interference (RNAi)-mediated gene knockdown in embryos, nymphs, and adults has been reported in *T. domestica*[4–6]. The efficiency of systemic RNAi is still highly species-dependent—for example, it is generally high in coleoptera whereas it is low in the lepidoptera order[7]. The efficiency and duration of the RNAi knockdown in *T. domestica* is yet to be assessed. In addition to RNAi, we have previously reported a successful CRISPR/Cas9-mediated gene knockout in *T. domestica*[8]. The CRISPR/Cas system has been widely applied for genome editing in insects particularly for targeted gene knockout. Its use could be expanded for other applications such as gene reporter assay, cell lineage tracking, and manipulation of transcriptional activity by knocking-in exogenous constructs after the establishment of a protocol for delivering components of the CRISPR/Cas system into nuclei[9]. Combined with the published genome assembly[10], the wide use and further development of the CRISPR/Cas-based genome editing in *T. domestica* would facilitate studies focusing on the evolutionary mechanisms behind the outstanding adaptive success of insects. Here, we describe a detailed protocol for embryo microinjection and for mating adult *T. domestica* to generate a mutant strain using CRISPR/Cas9. Considering this novel method, we discuss the importance of considering the unique biology of non-traditional model species for successful applications of these techniques.

**PROTOCOL:**

## 1. Maintenance of laboratory colonies

1.1. For the maintenance of wildtype and mutant populations, use a large plastic container (460 mm x 360 mm x 170 mm) with regular artificial fish food, water in plastic cups with a ventilation hole on the top, a folded paper for hiding the insects, and layered cotton for laying eggs (**Figure 2A**). Keep all *T. domestica* cultures inside 37 °C incubators and set the relative humidity (RH) inside each container to 60%–80%.

NOTE: Because *T. domestica* absorbs water vapor from the atmosphere[11], a direct water supply is not needed. The appropriate RH is maintained due to the presence of water vapor from plastic cups containing a ventilation hole on the top or from lidless cups inside each container. There is no need to humidify inside an entire incubator which contains cultures. The approximate duration of each developmental stage under the condition described in this protocol is shown in **Figure 1**. The developmental speed could be adjusted by changing the temperature and/or RH[12].

1.2. Add food periodically. Add water before it dries up.

1.3. Transfer the populations to a new clean container at least every 3 months given that adults stop laying eggs in a dirty environment and/or dense population (see **Discussion**).

## 2. Egg collection and microinjection

2.1. Design a guide RNA (gRNA)

2.1.1. Design a gRNA sequence for each required target and BLAST the gRNA sequences against the genome assembly to check possible off-target recognition sites.

2.1.2. Synthesize and purify the gRNAs according to the manufacturer's instructions[8].

NOTE: As an example, in the case of targeting the ATP-binding cassette transporter *white* gene, a 20 bp target sequence was designed and two synthesized DNA oligonucleotides 5´-TAATACGACTCACTATAGTAAGTGTTGTGGGAC-3´ and 5´-TTCTAGCTCTAAAACATCGGTCCCACAACACTTA-3´ were ordered. The DNA template was prepared by annealing these two oligonucleotides followed by PCR amplification and the gRNA is then in vitro transcribed from the template with a T7 RNA polymerase.

2.2. Prepare egg collection colonies

2.2.1. Transfer about 20 male and 20 female adults to a middle-sized container (200 mm x 150 mm x 90 mm) with food, water supply, a folded paper, and a small piece of layered cotton for egg laying (**Figure 2B**).

2.2.2. Set up several colonies to obtain large number of staged embryos in a short time period to be used for genome editing.

NOTE: About 20–40 eggs are expected to be collected from a colony after 8 h at 37 °C. It usually takes a few days for transferred adults to start laying eggs, possibly due to adaptation to a new environment.

2.3. On the day of injection, replace the cotton inside the containers with new ones.

2.4. Place a 76 mm x 5 mm double-sided tape on a regular 76 mm x 26 mm glass slide.

2.5. Eight hours later, collect the eggs from the layered cotton by separating the layers using forceps.

2.6. Align the eggs on the double-sided tape using a wet paint brush and keep a 2 mm distance between the eggs. All eggs should be oriented so that the longitudinal axis of an egg faces the injection side (**Figure 3A**). Gently press down the eggs with a paint brush for firm holding during the injection.

NOTE: In this protocol, fungus grows fast in damaged injected eggs under wet and warm condition. It is important to keep the distance between the eggs to prevent cross contamination and expansion of fungus.

2.7. Mix the gRNA and Cas9 protein to a final concentration of 100 ng/µL and 500 ng/µL, respectively. Use distilled water for dilution. Incubate the mix for 10 min at room temperature to promote ribonucleoprotein complex formation and then keep the mix on ice.

NOTE: Neutral red at a 1% final concentration could be added to the injection solution to monitor the injected amount.

2.8. Load 2 µL of the gRNA/Cas9 solution in a glass injection capillary with a microloader. Make sure there are no air bubbles in the solution before the injection. If necessary, tap the needle to remove the bubbles.

NOTE: In this experiment, a ready-made needle is used to obtain a fine needle tip (**Figure 3B**). A homemade needle with a similar shape could be used instead.

2.9. Fix the glass injection capillary, previously loaded with the gRNA/Cas9 solution, to a holder equipped with a manipulator and connect the holder to an electronic microinjector.

2.10. Optimize the shape of the needle tip by breaking it slightly with forceps to prevent clogging and getting better durability throughout a series of injections (an example of an appropriate needle is shown in **Figure 3C**).

NOTE: It is recommended to change a needle when it is clogged. It is possible to continue injecting with the same needle if it is broken again with forceps, but a wider tip leads to more egg damage and lowers their survival rate. As *T. domestica* eggs are soft and fragile, keeping a fine needle tip is key for having a high survival rate after injection.

2.11. Insert the needle at the midpoint of the longitudinal axis of an egg and inject a slight amount of the solution (see **Figure 3D–H** for reference on the amount of injection). Adjust the configuration of the electronic microinjector during injection, depending on the shape of the needle tip.

NOTE: It is recommended to apply a constant pressure during injection, otherwise sticky egg contents can easily flow into the glass needle and may clog it. The solution may be to either inject with a short pressure pulse or with a constant pressure, depending on the amount of liquid injected. Keep in mind that when a needle tip has a wide opening, too much solution is injected into an egg, which causes lethality (**Figure 3F–H**). In that case, maintain a constant liquid flow by constant pressure, then insert the needle into an egg and pull it out immediately. If it causes too much overflow or the egg bursts, change the needle.

2.12. Keep the injected eggs in a container with the appropriate size for the number of injected eggs (<20 eggs: small dish; >20 eggs: middle-sized container) with 60%–80% RH and 37 °C.

**3. Mating**

3.1. Check the injected eggs periodically and discard damaged eggs with forceps to avoid fungal growth (**Figure 3I,J**). In case too much fungus is growing on an egg, clean up the surface of the egg with 70% EtOH.

3.2. Before hatching (approximately 10 days at 37 °C after egg laying), dip the glass slide with the injected eggs into talcum powder to coat the surface of the double-sided tape. This will avoid the stacking of hatched nymphs. Transfer the powder-coated glass slides to a middle-sized container with food, water, and a folded paper for hiding of the insects.

3.3. Remove the glass slides after the nymphs have hatched. Periodically supply food until they reach adulthood.

NOTE: It takes about 2.0–2.5 months for individuals to reach adulthood after they hatch (**Figure 1A**). The adult stage is judged based on a well-developed ovipositor in females (**Figure 1B**).

3.4. To mate the individuals, transfer as many as needed wildtype female adults from a laboratory colony to the medium-sized container and incubate them for at least 14 days at 37 °C to make sure they are virgin.

NOTE: It is not necessary to collect virgin females from a laboratory colony because adult *T. domestica* have a repeated cycle of molting and mating called "reproductive and molting cycle", during which females throw away sperm with each molt and mate again in the next fertilization cycle[13].

3.5. Transfer either a male or a female G0 adult that developed from an injected egg and wildtype adult(s) to a small plastic dish (⌀ 100 mm x 40 mm) with food, a folded paper, and a small piece of cotton for laying G1 eggs (**Figure 2C'**; mating dish). Keep the mating dishes in a larger container with 60%–80% RH (**Figure 2C**).

NOTE: Multiple wildtype adults can be included in a dish to increase the chance of successful mating, although high success rates have been achieved with one-to-one pairing.

**4. Genotyping**

4.1. Design PCR primer pairs for each gRNA to amplify a 100–200 bp product that includes the site targeted by the gRNA. BLAST each primer sequence against the genome assembly to check its specificity (an example is shown for the targeting of the *white* gene in **Figure 4A**).

4.2. Check the germline transformation of G0 adults.

4.2.1. Five days after the eggs are laid, collect individual G1 eggs from each G0 adult pair into 0.2 mL tubes (one egg per tube; store collected samples at -20 °C for a long-term storage). Separate the cotton layers to collect the eggs.

NOTE: It is recommended to genotype at least 12 G1 nymphs from each mating pair to evaluate the success of the germline transmission (see **Representative Results**).

4.2.2. Add 15 µL of a 0.25 mg/mL Proteinase K solution (dissolved in Tris-EDTA buffer) to each tube, briefly homogenize samples with toothpicks, and incubate at 55 °C for 3 to 16 h.

4.2.3. Inactivate the Proteinase K by placing the samples at 95 °C for 10 min.

4.2.4. Add 90 µL of distilled water to each tube and mix well. Use 2 µL of supernatant in a 10 µL PCR reaction mix containing the primers designed in step 4.1.

NOTE: The use of a DNA polymerase optimized for crude templates is recommended to reach enough PCR amplification.

4.2.5. To analyze the PCR products, perform an heteroduplex mobility assay (HMA) with a microchip electrophoresis system (**Figure 3B**; see Ohde et al., 2018)[8].

NOTE: Mutations could be assessed with two alternative methods: (1) HMA with standard gel polymers, such as 8% polyacrylamide[14]; (2) digestion of PCR products with T7 endonuclease followed by agarose gel electrophoresis[15].

4.2.6. Keep only the G1 nymphs resulting from G0 adults that contain mutations in their germline and discard the others.

4.3. Individual genotyping of G1 nymphs/adults

4.3.1. Isolate G1 nymphs into 24-well plates with an aspirator or a paint brush. Place the 24-well plates in a larger container (e.g., the medium-sized container used in this protocol) with water supply as described above to keep a RH of 60%–80% (**Figure 1D**). Maintain the supply of artificial regular fish food (**Figure 1D'**).

NOTE: Although this step can be performed at any point of nymphal and adult stages, it is recommended to perform it after reaching adulthood and just before pairing (>2.5 months after injection; **Figure 1B**) because it is easier to maintain firebrats in a large container. Individual rearing is required to track the genotype of each G1 nymph on the following steps. G1 nymphs from the same G0 adult can have different mutations.

4.3.2. Pinch and pull cerci and the caudal filament from a nymph/adult using forceps and collect them into a 0.2 mL tube containing 50 µL EtOH (store the collected samples at -20 °C for a long-

term storage).

NOTE: Tissue samples are collected in EtOH because it prevents the loss of these small samples due to static electricity. If one needs to stop the motion of insects, anesthetize nymph/adult on ice when taking tissue samples. Because *T. domestica* cannot survive after long-term cooling on ice, do not anesthetize them for more than a minute and immediately move them back to room temperature. Ablation of cerci and the caudal filament causes no increase of mortality.

4.3.3. Place sample tubes with the lids open on a thermal block for 15 min at 70 °C to evaporate the EtOH.

4.3.4. Repeat steps 4.2.2–4.2.5 for genotyping.

4.3.5. Submit the PCR products in which a mutant band pattern is observed to a standard Sanger sequencing service.

4.3.6. Keep the G1 nymphs/adults with the desired mutations and discard the others (see **Figure 4C** for a representative sequencing result).

4.4. Cross the adults containing the desired mutations in a mating dish and obtain the next generations to establish a homozygous mutant strain.

**REPRESENTATIVE RESULTS:**

In our hands, about 100 eggs can be well injected with a single injection capillary when it has the adequate tip (**Figure 3C**). Injection of gRNA/Cas9 ribonucleoprotein complex in embryos within the first 8 h after egg laying results in indels at the gRNA targeted site. This causes biallelic mutations in some cells of the injected generation (G0) and thus mutant mosaic phenotypes are usually obtained in G0. For example, when this protocol was used to inject a gRNA that is designed to target the *white* gene, 32.6% of G0 nymphs display partial loss of pigmentation in their compound eyes and dorsal regions (**Figure 5**)[8].

Using the presently described dry injection method, when 80–120 eggs are injected the survival rate of the injected embryos is as high as 40%–60%. This is in contrast with the previous wet injection method, in which eggs are injected and maintained on an agarose plate, occasionally resulting in a survival rate of less than 10%.

Assessment of the germline transformation of G0 adults and mutated G1 individuals was done by genomic PCR followed by HMA. In HMA, wildtype and mutant alleles anneal in each possible combination, which typically results in four distinct bands on a gel electrophoresis (two homoduplexes and two heteroduplexes)[14]. In G1 samples, differential band patterns between wildtype and mutated samples are clearly distinguishable (**Figure 4B**). Germline transformation was found in 39.1% of the G0 adults when we targeted the *white* gene[8]. In our experience, the percentage of mutated individuals in G1 nymphs from a single G0 pair varies from 25% to 100%.

To evaluate the effect of our mating environment on the mating success, we crossed wildtype adults in a mating dish and obtained a success rate of 95.8% (23/24 pairs).

**FIGURE AND TABLE LEGENDS:**

**Figure 1: The lifecycle of *T. domestica*.** (**A**) Approximate duration of each developmental stage in *T. domestica*. (**B**) Dorsal view of an adult female. Arrowhead indicates a well-developed ovipositor.

**Figure 2: Artificial environments used in this protocol.** (**A**) A large container for laboratory colony, (**B**) a medium-size container for egg collection, (**C**) mating dishes with water supplies in a large container, (**C'**) a mating dish, (**D**) a 24-well plate with water supply in the medium-size container. Boxed region is magnified in (**D'**) to show a *T. domestica* individual.

**Figure 3: Dry injection of *T. domestica* eggs.** (**A**) Eggs aligned on a glass slide. Black arrow indicates the point where a needle is inserted. (**B**) Shape of a glass needle tip used for injection. (**C**) The same glass needle before (top) and after (bottom) breaking the tip. Needles were filled with 1% neutral red. Arrowhead indicates the tip of the needle. Scale bar is 1 mm. (**D**) Uninjected egg. (**E**) A good example of an injection. Arrow indicates the point of injection. (**F–H**) The solution is overflowing from the injected site (**F**) or from the opposite side (**G**) of the injected egg; too much volume of injection caused a burst (**H**). Arrowhead indicates overflowed egg content. (**I**) Normally developed late embryo. Arrowhead indicates the colored compound eye. (**J**) Shrunken damaged egg 3 days after injection.

**Figure 4: Genotyping of G1 individuals.** (**A**) PCR primers are designed to amplify a 120 bp genomic region that includes the gRNA target site. (**B**) Multiple bands of homoduplex and heteroduplex DNAs are detected in mutated samples while single bands appear in unmutated samples. L: DNA ladder, U: unmutated samples, M: mutated samples. (**C**) Representative result of direct sequencing of PCR products from wildtype and heterozygous mutant samples. Sequences of wildtype and mutant (⊿4) allele are shown on top. The forward primer shown in (A) was used as a sequencing primer. The sequence of a heterozygous mutant (bottom) is indicated by two overlapping sequences from the predicted cleavage site (arrow).

**Figure 5: Mosaic loss of pigmentations in compound eyes and in the dorsal region after targeting the *white* gene with gRNA/Cas9 protein injection.** (**A**) Wildtype and (**B**) *white* gRNA/Cas9 protein-injected first instar nymphs. Partial loss of black and pink pigmentations in eyes (arrowhead) and in the dorsal region (arrow) are indicated. Scale bar is 200 μm.

**DISCUSSION:**

For the successful generation of the desired *T. domestica* mutant with CRISPR/Cas9, it is first important to collect a sufficient number of staged embryos for injection. For a constant collection of a sufficient number of *T. domestica* eggs, the key is to select an appropriate size of the container to have a lower population density because it would help the successful completion of a series of complex mating behaviors, which is repeated after every adult molt[13]. A male *T.*

*domestica* adult transfers its sperm indirectly to a female via a spermatophore. Sweetman (1938) reported that it takes male adults 20 to 35 min from the initiation of the mating behavior to the placement of a spermatophore[12]. It is likely that disturbance of the interaction between a mating pair by other individuals prevents successful fertilization, which could happen more frequently in a dense environment.

Although eggs are collected 8 h after replacing the cotton inside a container to collect enough eggs in our protocol, the higher efficiency of genome editing may be achieved by collecting eggs and injecting them within a shorter time (e.g., 4 h after egg laying) when injected materials have more chance to be delivered to the large proportion of nuclei. Injection to a sufficient number of eggs within a shorter time could be done by (1) increasing the number or the size of containers if space allows, or (2) repeating the same procedure to obtain a sufficient number of injected eggs.

The site of injection is generally considered to be important for successful germline transformation in insects. *T. domestica* eggs are usually ellipsoidal in shape and contain a germ band at one pole of their longitudinal axis. It is recommended to inject the gRNA/Cas9 mixture at the midpoint of the egg longitudinal axis because it is hard to identify the pole where a germ band is formed in early embryos due to the variable shape of *T. domestica* egg. Although the gRNA/Cas9 solution is not directly injected to the site of the germ band formation, we have achieved germline transformation in as high as 39.1% of G0 adults[8].

During the microinjection of eggs, it is important to use a fine needle tip to obtain a high survival rate, as it is the case for other animal models. We obtained a higher survival rate after injection with ready-made needles than when using homemade glass needles in *T. domestica* eggs. However, a similarly high survival rate could be achieved by replicating the fine needle shape with homemade needles (as shown in **Figure 3B,C**). According to our experience, the dry injection method results in a higher survival rate than the previously reported wet injection method[8]. We found that egg incubation in a dry environment is key for this improvement, taking advantage of the fact that *T. domestica* eggs and early nymphs are resistant to desiccation. This is in accordance with previous reports suggesting that this species prefers a dry environment particularly during early developmental stages and that a wet environment can even be harmful[16]. Instead of a plastic plate, an agarose gel can be used for a quick alignment of the eggs; however, transferring the injected eggs to a dry surface leads to a higher survival rate.

In conclusion, our method underlines the importance of taking the unique biology of *T. domestica* into account for a successful genome editing: keeping a sparse environment for collecting a sufficient number of eggs and a dry environment for having a higher survival rate of injected embryos. The basic protocols for injection, mating, and culture maintenance reported here are used not only for generating mutant strains with genome editing, but also for applications of other genetic tools such as RNAi and transgenesis and will help to understand the mechanisms underlying the early evolution of insects.

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**DISCLOSURES:**

The authors have nothing to disclose.

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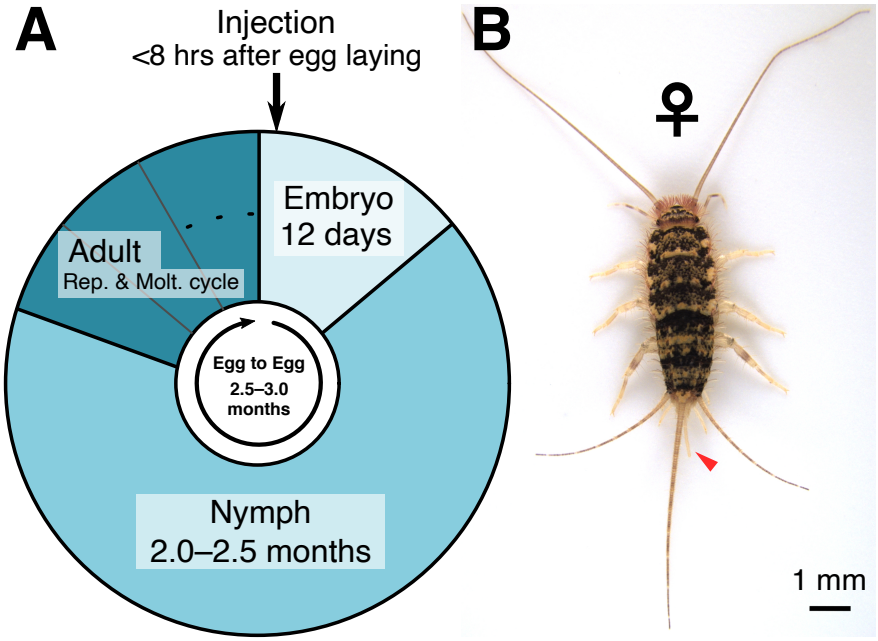

Figure2

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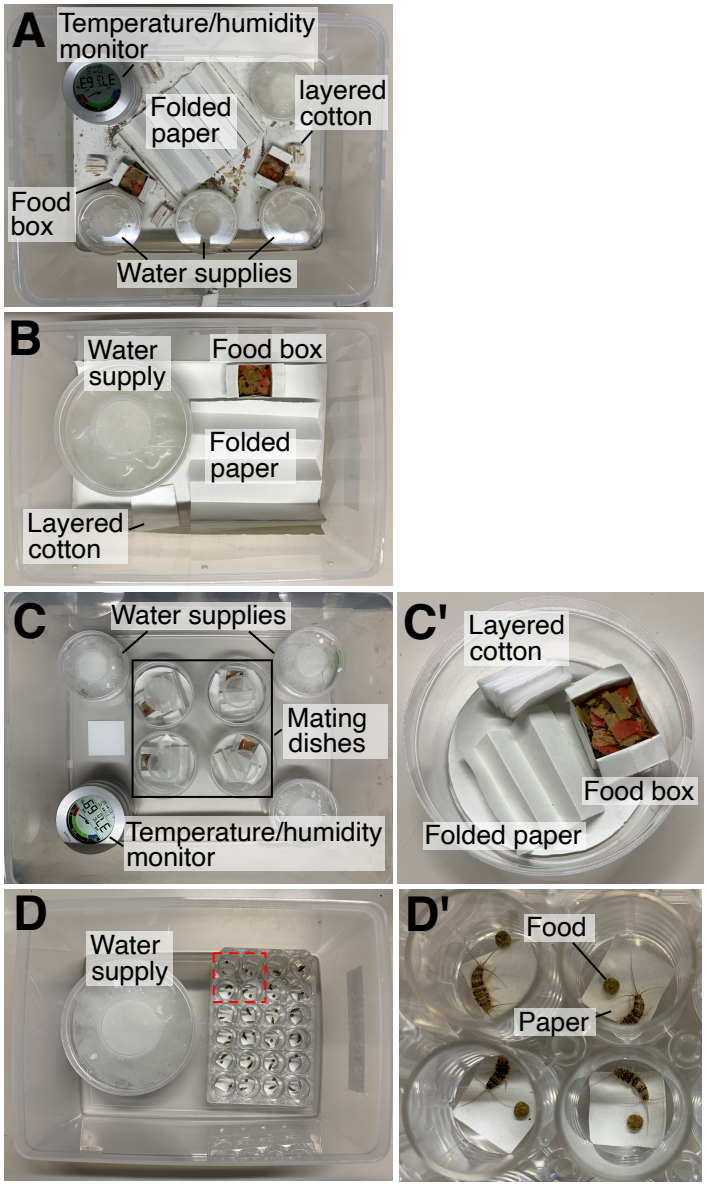

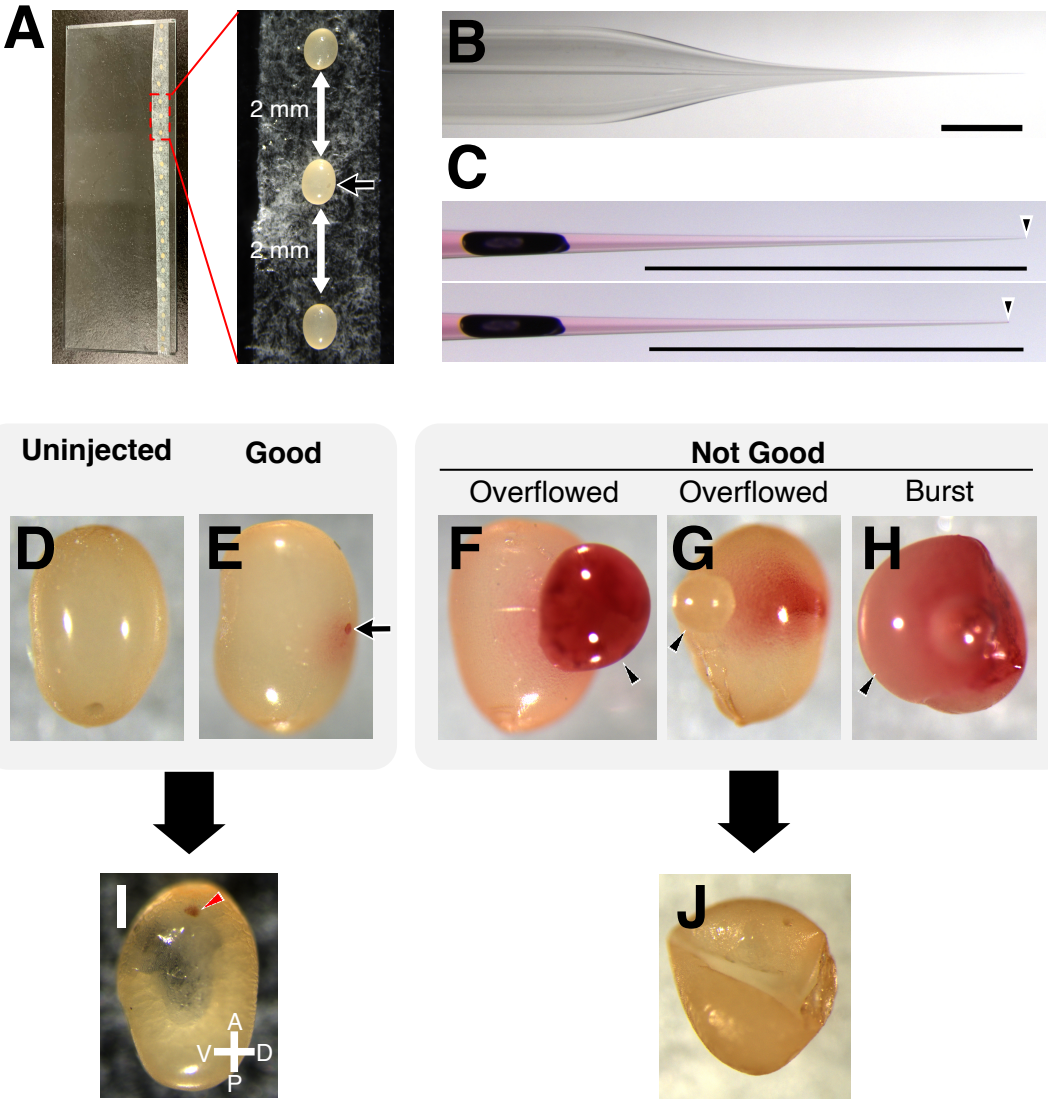

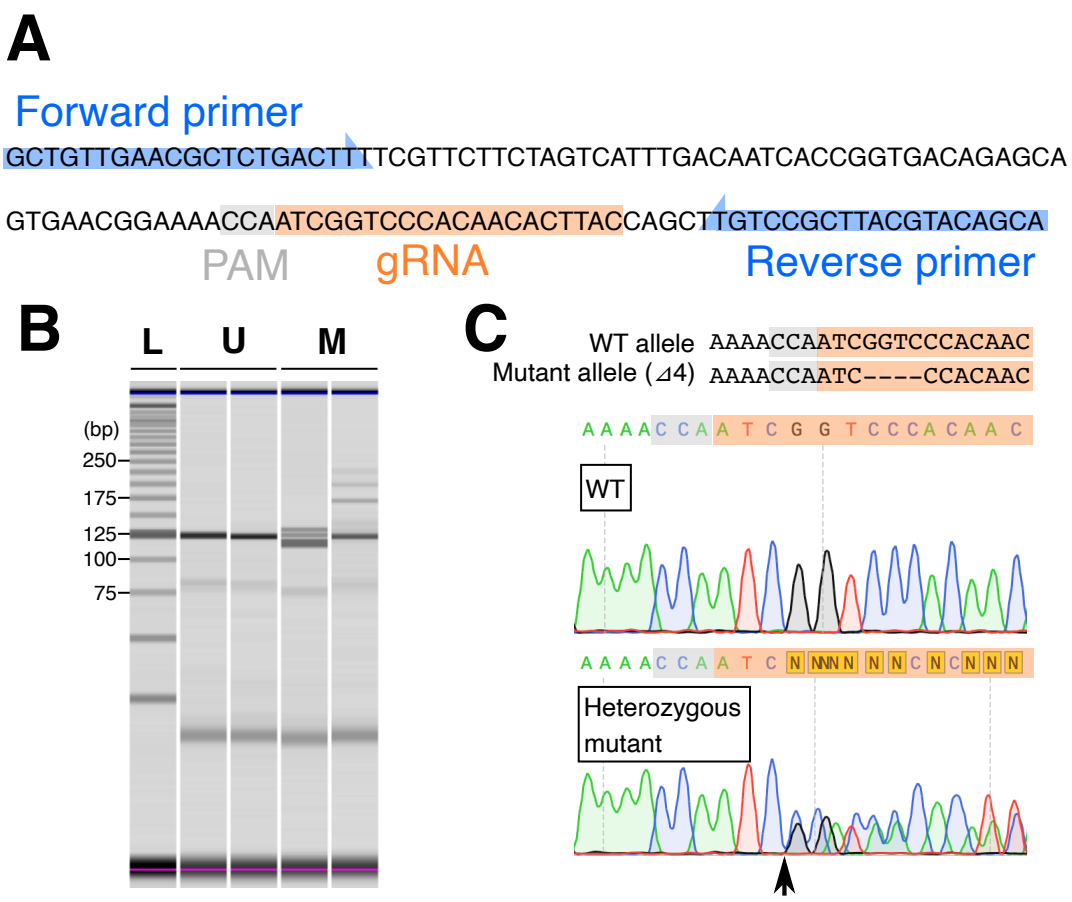

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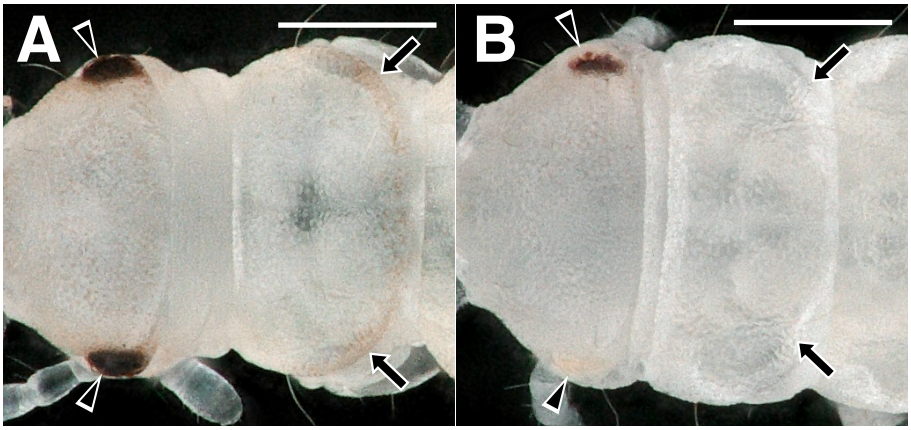

| Name of Material/ Equipment | Company | Catalog Number |
|---|---|---|
| 24-well plate | Corning | 83-3738 |
| Alt-R S.p. HiFi Cas9 Nuclease V3, 100 µg | Integrated DNA Technologies | 1081060 |
| Anti-static cleaner | Hozan | Z-292 |
| Barrier Box 20.7L | AS ONE | 4-5606-01 |
| FemtoJet 4i | Eppendorf | 5252000013 |
| Femtotip II, injection capillary | Eppendorf | 5242957000 |
| High Pack 2440mL | AS ONE | 5-068-25 |
| Incubator | Panasonic | MIR-554-PJ |
| KOD Fx Neo | Toyobo | KFX-101 |
| Magnetic stand | Narishige | GJ-8 |
| Microloader | Eppendorf | 5242956003 |
| Micromanipulator | Narishige | MM-3 |
| Microscope | Olympus | SZX12 |
| MultiNA | Shimadzu | MCE-202 |
| NiceTac | Nichiban | NW-5 |
| Paint brush (horse hair) | Pentel | ZBS1-0 |
| Plant culture dish | SPL Life Sciences | 310100 |
| Proteinase K, recombinant, PCR Grade Lyophilizate from Pichia pastoris | Roche | 3115836001 |
| SZX 12 microscope | Olympus | SZX 12 |
| Talcum powder | Maruishi | 877113 |
| Tetra Goldfish Gold Growth | Spectrum Brands | |

| | Comments/Description |
|---|---|
| | for removing static electricity from a 24-well plate |
| | Large container |
| | Electronic microinjector |
| | Glass injection capillary |
| | Middle-sized container |
| | for 37 ℃ incubation. No need to humidify inside the incubator. |
| | PCR enzyme for genotyping. Optimized for an amplification from crude templates. |
| | for holding the micromanipulator |
| | for microinjection. More than 35X magnification is sufficient for the microinjection |
| | Microchip electrophoresis system |
| | Double-sided tape to place eggs on a glass slide |
| | Mating dish and water supplies for a large and middle-sized containers |
| | More than 35X magnification is sufficient for the microinjection |
| | Artificial regular fish food |

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September, 29th 2020

We submit a revised version of our paper entitled "**Egg microinjection and efficient mating for genome editing in the firebrat *Thermobia domestica***" to be considered for publication as an article in *Journal of Visualized Experiments*.
We were grateful to have positive responses from reviewers. We addressed each issue raised by reviewers, and their constructive comments improved our manuscript.

We hope that you will consider our revised paper suitable for the publication, and look forward to hearing from you.

Yours sincerely,
Takahiro Ohde

**Editorial comments:**

We appreciate the editor for helpful comments on our manuscript. Below we address each issue raised.

**Changes to be made by the Author(s):**

1. **Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.**

We submitted the manuscript to a language proof-reading service.

**2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points**

We used the provided format to make the revised version of the manuscript and confirmed that it is formatted accordingly.

**3. Please provide an email address for each author.**

We provided email addresses for all authors.

**4. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.**
**For example: FemtoTip II, Femtojet 4i, MultiNA DNA-500 kit, etc.**

We replaced all commercial languages with generic terms in the manuscript.

**5. 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.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."**

We changed the tense of sentences in the protocol section.

**6. Please ensure you answer the "how" question, i.e., how is the step performed?**

We answered all questions from reviewers.

**7. 2.11: Please include the injection volume.**

Although we cannot tell the exact volume of injection, we added pictures that show eggs after injecting a solution containing red dye to give an idea of how much the appropriate volume is.

**8. There is a 10-page limit for the Protocol, but there is a 3-page limit for filmable content. Please highlight 3 pages or less 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.**

We highlighted steps that should be included in the video.

**9. Please obtain explicit copyright permission to reuse any figures from a previous 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]."**

All figures are created for this manuscript.

**10. As we are a methods journal, please ensure that the Discussion 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 revised the Discussion accordingly.

**11. Please remove the link to youtube videos from the discussion section and include publications instead.**

We removed the link. The videos may be included in this publication.

**12. Please do not abbreviate the journal titles in the references section.**

Full journal titles are provided.

We appreciate each reviewer's careful analysis on our manuscript. Below we address each issue raised.

**Reviewer #1:**

**Manuscript Summary:**

**This is a very nice manuscript that will be useful for the scientific community. The protocols appear to be quite successful and will likely be used by a number of Evo-Devo labs.**

Thank you for finding the usefulness of this manuscript and for giving constructive comments.

**Major Concerns:**

**I ask that the authors add a section, or possibly a table with a timeline to clearly indicate how long each life stage lasts for this species, under the conditions described.**

Although we don't know the precise schedule of each stage, we added a schematic illustration that shows how long the embryo and nymph approximately last in Figure 1A.

**Minor Concerns:**

**Throughout, there are multiple small grammar mistakes that should be corrected. Additional requested changes and additions are listed below.**

Thank you for correcting the language mistakes. We used a language proof-reading service for making the revised version of the manuscript.

**Introduction**

*** The following sentence is unclear: "The situation that multiple hypotheses have been under tested is similar in the discussion…"**

According to the suggestion, we removed an unnecessary paragraph and improved the introduction.

*** It's unclear why the following point is being made: "The firebrat Thermobia domestica shows no drastic disproportional changes in sizes of body parts except for genital organs during post-embryonic development"**

According to the suggestion, we removed an unnecessary paragraph and improved the introduction.

**2. What microscope is used for the microinjection procedure? What magnification is needed?**

We added the information in the material list.

**2.1.1. What kind of plastic folder is used? Perhaps other materials would work, and it would be helpful to explain what about this product makes it useful so people can find suitable alternatives.**

We regularly use the plastic plate for injection of this species but found that a regular glass slide can be used instead to achieve the goal of this protocol. The protocol is revised accordingly.

**2.1.2. What is the plastic plate? What double-sided tape is used? It seems like different tapes stick to some species' chorions better than others, so it would be helpful to include this product in the product list at the end.**

We revised the comment for the double-sided tape in the material list for more clarity.

**2.7. Explain how microinjection needle is pulled and loaded, including exact needles name, brand and dimensions. It looks like there is very little information about this product on the Eppendorf website, so any details you can add would be helpful. Are they pre-opened? In 2.10, you suggest breaking it to improve its durability, so this is not clear.**

We use the ready-made injection capillary for injection, but a homemade glass needle could be used if readers replicate needle shapes of the ready-made needle. We added a panel that shows the tip of the ready-made needle to provide an idea of how the shape should be in Figure 3B, C.

**2.10. Please add some measurement of the size of the needle opening when it's considered "good".**

We added a new figure (Figure 3B, C) that shows an example of a good needle.

**2.11 Is it possible to provide any more information about the amount of liquid injected? Is dye added to monitor the liquid? How easy is it to observe the liquid entering/inside the egg? Perhaps you could add a panel to Figure 2 of injected embryos, so readers can easily compare injected versus uninjected.**

Thank you for the constructive suggestion. We found that the addition of neutral red at 1% concentration helps to monitor the amount of injected solution without harm to embryos. We added a

figure that shows good and not good examples for the amount of injection.

**2.12 What is the moist chamber? Do you have problems with fungus, and if so, how to deal with this?**

Larger containers shown in Fig. 1 could be used. We revised the step for clarifying that point.
Dealing with fungus growth is an important point. We added some sentences to explained how to prevent and deal with fungus growth.

**3.1. The addition of talc powder is a very clever solution. However, it's not clear why such a wide time frame is given for when to add the powder. What needs to happen before you do this? Also, how can you tell which embryos are dead at this point?**

Thank you for finding this solution good. Talc powder could be added at any point before hatching, but the best timing should be before hatching after the removal of dead embryos. We removed the confusing time window and specified to about 10 days after egg laying.

**3.2. When do you expect hatching to occur at these rearing conditions? It seems like you need to move the embryo before it hatches, so this is an important time frame to include. Also, when you mention keeping the well plates in a moist chamber, does this mean you put it in another container with water?**

We added a figure that shows the approximate duration of the life stage of this species in Fig. 1. For the second question, we added a panel in Figure 2 to show how to keep humidity with a larger container.

**3.3 After removing the nymph, where is it placed? It sounds like you raise each insect individually in a well of the 24-well plate until adulthood. This seems particularly cumbersome so it would be helpful to explain why this is done.**

The review is right. Individual rearing is not necessary for G0 individuals. We revised the protocol accordingly.

**3.5 To clarify, matings are set up with one male and one female?**

We clarified that point.

**Fig. 1C: I don't see any water in the mating dish.**

We added a panel that shows how humidity is kept with a larger container.

**4.2.1 Is this one egg per tube? How many eggs are analyzed from each G0 cross? What is the expected rate of germline transmission? That is, how many G0s and how many eggs should be analyzed in order to have a good chance of finding a mutation?**

We added Note to explain the number of eggs needed to be analyzed based on the rate of germline transformation in our published data.

**4.2.5. Can you explain more about the MultiNA DNA-500 kit? It appears to be more like a specialized piece of equipment, rather than a kit, so are there alternatives for labs that don't have this?**

We added a brief explanation about heteroduplex shift assay in the Results and provided two alternative methods for this step.

**4.2.6. Once again, it's unclear why the insects are being reared in 24-well plates. I imagine it is for ease of tracking an individual's identity, but couldn't siblings be reared together? Could you explain why you prefer to do it this way, as well as any special challenges that may arise from this method of rearing? A Figure 1 panel showing individuals being reared this way should be shown, as it's hard to imagine having space for food, water, and the insect all within a single well.**

As the reviewer mentions it is necessary to rear G1 nymphs individually because genotypes of each individual must be tracked as it could be different even among each G1 nymph from the same G0 parents. We clarified this point by adding Note.
Also, we added a panel in Figure 2 to show how to keep firebrats in a 24-well plate under the controlled RH.

**4.3. Have you noticed any mortality after dissecting the cerci and caudal filament?**

No, it appears to have no effects on the survival rate. We added a Note to describe this point.

**4.3.1. How do you remove the cerci and caudal filament?**

We explained how to do it in 4.3.2.

**4.3.2. This seems like an unnecessary step; why not move directly to 4.2.2? It would be helpful to state more explicitly why this is done.**

It is for the ease in the sample collection. We explained the necessity of this step clearer in a Note.

**It would be helpful to add a section, or possibly a table with a timeline to clearly indicate how long each life stage lasts. This would be helpful for planning experiments.**

We agree with the usefulness of such information about developmental stages, but the precise timing of each developmental event is unknown yet for this species. We added a figure that shows the approximate duration each stage lasts under the condition in this protocol.

**The representative results show G0 mosaics but should also show information about germline transmission. Exact numbers of animals should be reported for each step of the protocol, rather than making general statements about possible success rates.**

We added the information about the germline transformation in the Results section.

**Discussion**

*** Especially since you state a preference for the premade Femtotips, it would be helpful to explain what makes these suitable for your needs (including needle dimensions, material, etc.)**

The point is the shape of the needle. To give an idea of the good needle shape, we added pictures in Figure 3.

**Reviewer #2:**

**Manuscript Summary:**

**The paper "Egg microinjection and efficient mating for genome editing in the firebrat Thermobia domestica" by Ohde et al. describes a method for genome editing in a basal insect Thermobia domestica (Zygentoma). This is a welcome contribution to the methods that can be used for studies on Thermobia and I congratulate the authors on achieving this. Thermobia has a convenient phylogenetic position to address many questions on insect evolution. The protocol described here will be useful to a broad spectrum of Evo-Devo and physiology researchers.**

Thank you for finding the usefulness of this manuscript, and giving many constructive comments.

**Major Concerns:**

**The introduction needs to be improved. It provides too much unnecessary details about the two authors' chosen topics. The species is not sufficiently introduced, its phylogenetic position is not explained clearly, we do not learn if it has been long used as a model, what other genetic tools are available for the species etc. The method is little introduced and explained.**

We appreciate the constructive suggestion. We made a major revision on the introduction: removed unnecessary paragraphs and added some background information about available techniques in this species.

**The protocol is a very specific description of how the authors do it in the lab. Alternatives are little discussed.**

Thank you for pointing out the limitation of the application of this protocol in other laboratories. To improve this point, we revised our manuscript for providing some alternatives in steps such as injection and genotyping.

**(Details of issues that I suggest for editing are below.)**

**Minor Concerns:**

**The text could be discussed with an English native speaker.**

We used a language proof-reading service to make the revised version of the manuscript.

**Specific comments - suggestions for editing:**

**Abstract:**

**first line: mention that it is a BASAL insect**

We revised the introduction as suggested.

**Introduction:**

**1. Explain clearly the phylogenetic position of Thermobia (one of the two basalmost insect orders). How long has it been used as a laboratory model (long before, just recently etc.) and why (generation time, easy breeding, numerous progeny etc.).**

We included the information in the introduction.

**2. Authors mention that Thermobia is useful for understanding the evolution of complete metamorphosis. But complete metamorphosis (holometaboly) evolved from hemimetaboly, not ametaboly. I think it would be better to say that ametabolous Thermobia will help understand origins of insect metamorphosis (metamorphosis in general).**

We revised the introduction accordingly.

**3. What genetic tools do we already have for Thermobia, such as: does RNAi work very well/poorly (embryonic, systemic), is transgenesis using transposable elements established, is TALEN-mediated genome editing established, are the genome and transcriptome sequenced?**

We included the information in the introduction as suggested.

**4. Explain the method presented here in more depth. Is it superficial to RNAi, is it already broadly used among insects? Is the protocol described here suitable for both knockout and knock-in, have you already achieved both?**

We revised the introduction accordingly.

**Protocol:**

**1. In introductory paragraph "relative humidity inside a container is kept around 60-80%": how do you achieve this humidity? E.g., do you also need to keep a dish with water inside the incubator (outside boxes with animals)?**

There is no need to keep humidity in an incubator. We clearly describe it in the revised manuscript. We explained that Thermobia can take up water from the atmosphere by citing a paper (Noble-Nesbitt, 1969).

**2. For all dimensions that you give (size of boxes) give rounded numbers.**

We corrected all dimensions of boxes in rounded numbers.

**3. "Fish food": is it a regular fish food? Then I suggest writing a "regular fish food". Or did you chose the particular one type, because it is enriched in some unique component, e.g. algae?**

Yes, it is regular food. We added “regular” to the word as suggested.

**4. I suggest using "folded paper" instead of "corrugated".**

The word is substituted as suggested.

**5. Step 2.6: briefly describe how you get sgRNA, what is it**

We added a brief description of how to prepare a gRNA and provided an example for *white* gene.

**6. Step 2.9: "glass needle": is it Femtotip II?**

Yes, it is. It becomes clearer after removing all commercial words from the protocol.

**7. Step 2.10: "vulnerable": I suggest changing e.g., to "delicate" , "fragile"**

We substitute the word as suggested.

**8. Step 3.1. "at this point" can be left out**

We removed the words as suggested.

**9. Step 3.3. "until adults": change to "until they reach adulthood"**

We corrected the expression as suggested.

**10. Step 3.4. "as much as" change to "as many as"**

We substitute the word as suggested.

**11. Step 4.1 "design specific PCR primer pairs for each gRNA": could you briefly expand, give a example (I think this may not be clear to a beginner)**

We added an example for targeting the *white* gene with a figure.

**12. Step 4.2.5. "MultiNA DNA-500 kit": is specifically this kit required?, why, can you give alternatives**

We provided alternative methods in a Note.

**13. Step 2.8. I suggest to add an image of the injection set up (micromanipulator, injector, needle) showing the correct angle of the needle for injection (this is important is my experience).**

In the case of the dry injection method described here, the angle could be flexible from 0° to 30°. An example will be included in the video.

**Representative results:**

**1. "Annealing procedure after PCR": what does this mean? Expand.**

We removed this part to avoid confusing readers.

**Figures:**

**1. Fig. 4: Explain more what you do here and how (briefly), where can we obtain the genomic information?**

We add a brief description of the heteroduplex shift assay in the Results section.

**Discussion:**

**1. The first sentence is not clear. I think that it is expected (not surprising) that for obtaining fertilized eggs both males and females are required.**

We removed the sentence.

**2. "…about 20-40 eggs/colony.." I suggest to refer to the information mentioned before about the size of the colony: "…about 20-40 eggs/colony (20 females plus 20 males, see 2.2)"**

For clarity, we moved this part to the Protocol section as a Note.

**3. "key to having" should be changed to "key to have"**

We changed as suggested.

**4. The needle is an important topic and it is great to read that the authors found a satisfactory solution. If one cannot use Femtotip II and has to use a home pulled needle from a glass capillary, what shape of the needle (long vs. short) is the best, what glass is most suitable? I think that if the authors have an experience, they should mention such an alternative.**

We added a picture that shows the needle shape we use in Figure 3.

**5. The content of the egg is gluey and sticky (personal experience) and when a needle is inserted into the egg it clogs easily. Could you comment on this.**

Constant pressure would help to avoid a needle clogged. We added a Note to state this.

**6. "at the middle point": change to "at the midpoint"**

We changed the word as suggested.

**Reviewer #3:**

**Manuscript Summary:**

**In this article, the authors are describing protocols for generating and maintaining mutant strains of T. domestica, with the main innovation being the development of dry injection to obtain stable high survival rates of injected embryos. As primitive wingless insects firebrats occupy a critical phylogenetic position, and development of genetic tools (such as genome editing) in this species will be critical for understanding major evolutionary innovations in insects, such as origins of wings. Hence, this study is a necessary first step and I recommend its publication following the revision. For more details, please see below.**

Thank you for finding the usefulness of this article and supporting the publication. We appreciate the constructive comments.

**Major Concerns:**

**The authors should keep in mind that the vast majority of evo-devo researchers would have absolutely no knowledge of how to establish and maintain a firebrat lab culture, let alone how to do microinjections in this species. So, the more details the authors can provide that are specific to firebrats, the greater the benefit to the entire field. For example, in step 2.10, pg. 2, the text emphasizes that it is important to keep a fine needle point - this is true for any kind of injections. What authors have to emphasize are specific modifications of the general insect injection protocol that they developed in firebrats.**

1) **The authors should include a new figure (or add a new panel to Fig. 2) that show the location of germ band in an embryo. This can be done with DAPI and phalloidin staining.**

Thank you for the suggestion. Unfortunately, we leave this analysis for future works due to time limitation, although we agree that the suggested information of the germ band position in this species would be helpful to improve the effectiveness of genome editing and other techniques in this species.

**2) More details should be provided for step 3.1, both in the text and when making videos.**

We added a little more detail in the step. We will include this step in the video.

**Minor Concerns:**

**Comments regarding figures**

**3) Fig. 1 -- First, please clarify if the water supply dish is used for the actual drinking by Thermobia? In my personal experience with raising firebrats, a water supply is used for**

**maintaining humidity in the dish, these insects do not "drink" water.**

We added a Note to emphasize that no direct water supply is needed because Thermobia can take up water from the atmosphere.

**Second, what is the difference between B and C? Isn't the mating dish also used for egg collection?**

The middle-sized container is needed to collect a sufficient number of eggs within a short time for injection because it is important to keep a sparse environment to have successful completion of the mating behavior as we stated in the Discussion.

**Third, it seems that C is lacking a water supply, is this correct? No water is provided during mating??**

We added a panel in Figure 2 that shows how humidity is kept with a larger container.

**4) Fig. 2 -- Include the larger image of an entire slide, then add the current (magnified) image. Also, include the image that shows the actual needle entering the egg.**

We added the larger image as suggested. Unfortunately, we are not able to take a picture that shows the needle entering because we don’t have a camera on the microscope for injection. It will be included in the video.

**5) Fig. 3 -- Be consistent in regard to figure labels. First, label left side as (A) and right side as (B). Second, when showing pinkish pigmentation around posterior T1 margin, use additional arrows to point to the middle and opposite side of this segment. Also, change color of arrowheads from red to black (so its consistent with the eye labeling). Third, when showing mutant phenotype, point to lack of eyes on both sides. Also, now use the different color for arrowheads. Similarly, point with multiple arrows (of different color) that the entire pinkish pigmentation is absent.**

We revised the figure labels as suggested.