Video Article

RNA Interference in Aquatic Beetles as a Powerful Tool for Manipulating Gene Expression at Specific Developmental Time Points

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Abstract

RNA interference (RNAi) remains a powerful technique that allows for the targeted reduction of gene expression through mRNA degradation. This technique is applicable to a wide variety of organisms and is highly efficient in the species-rich order Coleoptera (beetles). Here, we summarize the necessary steps for developing this technique in a novel organism and illustrate its application to the different developmental stages of the aquatic diving beetle *Thermonectus marmoratus*. Target gene sequences can be obtained cost-effectively through the assembly of transcriptomes against a close relative with known genomics or de novo. Candidate gene cloning utilizes a specific cloning vector (the pCR4-TOPO plasmid), which allows the synthesis of double-stranded RNA (dsRNA) for any gene with the use of a single common primer. The synthesized dsRNA can be injected into either embryos for early developmental processes or larvae for later developmental processes. We then illustrate how RNAi can be injected into aquatic larvae using immobilization in agarose. To demonstrate the technique, we provide several examples of RNAi experiments, generating specific knockdowns with predicted phenotypes. Specifically, RNAi for the tanning gene *laccase2* leads to cuticle lightening in both larvae and adults, and RNAi for the eye pigmentation gene *white* produces a lightening/lack of pigmentation in eye tubes. In addition, the knockdown of a key lens protein leads to larvae with optical deficiencies and a reduced ability to hunt prey. Combined, these results exemplify the power of RNAi as a tool for investigating both morphological patterning and behavioral traits in organisms with only transcriptomic databases.

Introduction

The question of how specific genes contribute to the evolution of diverse traits is an exciting topic in biology. Over the last few decades, much progress has been made in regard to dissecting the genetic underpinnings of developmental processes in a few model organisms, such as the nematode *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster*, and the house mouse *Mus musculus*[1]. More recently, the invention of powerful gene-editing techniques such as clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9[2] has provided the ability to change the genetic code of non-model organisms (for examples see[3,4]). As a result, there has been a surge in genetic studies on a variety of organisms that had not previously been approached through molecular techniques. Considering the enormous diversity of our animal kingdom, with many interesting traits or trait variances that are only represented in specific species, this progress has made it an exciting time for evolutionary-developmental biology ("evo-devo") related work. However, genome-editing techniques that are available to non-model organisms are relatively restricted in regard to the developmental time points to which they can be applied, making it challenging to discern the temporal properties related to the role that specific genes play in any trait. In addition, transgenic techniques are often limited to genes that are nonessential for survival (i.e., whose knockout does not result in lethality). Therefore, while gene-editing techniques have started to become popular, there remains a need for effective techniques that are applicable to a variety of different organisms at specific developmental time points and facilitate partial knockdowns (rather than complete loss-of-function). Here, we draw attention to RNA interference (RNAi), a somewhat dated yet powerful gene knockdown technique[5] that is particularly valuable as a synergistic approach to gene editing. Specifically, we developed procedures that allow for the application of RNAi to aquatic diving beetles as an example that illustrates the implementation of this technique, from the acquisition of the necessary molecular sequences to the successful injection of double-stranded RNA (dsRNA) into eggs and larvae.

RNAi-based gene knockdown leverages an innate defense mechanism of organisms, in which dsRNA molecules facilitate the silencing of invading nucleic acid sequences, such as viruses and transposons[6]. In brief, dsRNA is taken up into the cell, where it is cut into 20–25 nucleotide pieces by the Dicer enzyme. These pieces then activate the formation of the RNA-induced silencing complex (RISC), which inhibits the targeted mRNA by binding to it at specific sites using the guide strand. This process ultimately leads to mRNA degradation and hence interferes with the translation of mRNA into the respective protein[6]. The RNAi-based gene knockdown technique presented here therefore relies on the injection of dsRNA. For animal models, this technique was originally developed in *C. elegans*[7] and *D. melanogaster*[8] but has since emerged as a powerful functional genetic tool in non-model organisms[9,10]. Owing to its highly effective nature in some insects, RNAi can even be applied in pest management[11].

As a research tool, RNAi has been used to examine how key molecular-developmental pathways function in nontraditional insect models. For example, RNAi in the flour beetle *Tribolium castaneum* has been instrumental in determining how deeply conserved genes contribute to specific traits in that beetle, as exemplified for the development of specifically shaped wings[12,13,14] and eyes[15,16]. The techniques that underlie the manipulations in *T. castaneum* have been well described[17] and rely on the ability to immobilize relatively dry eggs and larvae on a sticky surface. Such immobilization however is not possible for the wet developmental forms of aquatic organisms such as the Sunburst Diving Beetle *Thermonectus marmoratus*. As is the case for many nontraditional model organisms, it lacks an annotated genome. To manipulate gene expression in any organism without a genome, a reasonable and cost-efficient first step is to generate transcriptomes and identify the putative nucleotide sequences of the expressed genes of interest based on sequence similarity with related but more established model organisms, in this case, primarily *Tribolium* (Coleoptera) and *Drosophila* genes.

Here, to demonstrate how RNAi can be used on an aquatic organism, we first discuss protocols and software for RNA extraction and transcriptome generation and assemblage, which allow for the identification of specific targeted gene sequences. We then summarize the necessary steps for synthesizing gene-specific dsRNA. Subsequently, we illustrate how eggs can be injected in an aquatic environment and demonstrate incubation protocols for culturing developing embryos. In addition, we show how agarose gel can be used to completely immobilize larvae during the injection process, a technique that is generally useful during various procedures and could be applied to a variety of arthropods. To demonstrate how RNAi can be applied to different developmental stages, we include an example in which we silenced the eye pigmentation gene *white* in embryos. In addition, we describe an example in which the tanning gene *laccase2(lac2)* was silenced during both the second larval instar (to affect larvae of the third larval instar) and the third larval instar (to affect adults). Finally, we demonstrate that the injection of a lower concentration of dsRNA leads to partial knockdown, which shows that this technique can also be applied to genes where loss-of-function is known to be lethal.

Protocol

1. RNA isolation and de novo transcriptome assembly

1. Perform total RNA isolation from late stage third instar diving beetle larval eye tubes and adult beetles using an RNA isolation kit (see **Table of Materials**) that is designed for lipid-rich tissue. Isolate total RNA from *T. marmoratus* and sequence it following previously described methods[18].
2. De novo transcriptome assembly
   NOTE: To assemble the transcriptome de novo, various bioinformatics platforms can be used (see **Table of Materials**). These platforms are commercially available and, in some cases, exist as Unix-based command line scripts. Since the assembly is de novo, it is recommended to assemble the transcriptomes independently on two platforms and compare the results to exclude false positives or false negatives.
   1. To start assembling the transcriptomes, upload the raw reads on the respective bioinformatics platform.
      NOTE: These files are usually compressed and in the format FASTQSANGER.GZ. There is no need to decompress the files as this format is accepted in the next step.
   2. Using the Trimmomatic command line, trim the raw reads to remove adapter sequences or short reads that fall below the default threshold. Decompress the trimmed raw reads; the files will now be in the FASTQ format. Concatenate the FASTQ raw reads for each sample to obtain files that are ready for de novo assembly.
   3. Assemble the concatenated raw reads de novo using any command line script that can assemble transcriptomes de novo. Save the assembled transcriptome, which will now have a list of contigs with their respective sequences, as a FASTA file. To increase the coverage of the assembly, combine and assemble multiple transcriptomes for the same species.
      NOTE: This process increases the chances of generating more accurate contigs and is especially important if low copy number genes are of interest.
   4. Once assembled, assess the transcriptome for completeness by calculating coverage scores (coverage assessment software is freely available, see **Table of Materials**).
      NOTE: A transcriptome with a coverage score >75% is considered good. However, the relevance of this score depends on the reason for transcriptome generation. For example, if the transcriptome is being used to identify low copy number genes, then a score >85% is better, as it signifies greater coverage.
   5. Annotate the de novo assembled transcriptome using a basic local alignment search tool (BLAST; see **Table of Materials**).
      NOTE: For this experiment, the transcriptome was annotated primarily against a database of known *Drosophila* proteins and a database of known beetle proteins. The list of annotations can be downloaded as a spreadsheet file and contig/contigs that indicate sequence similarity to the proteins of other organisms can be downloaded as a FASTA file.
   6. Identify the contig number/numbers of the protein of interest and extract the nucleotide sequences. Use BLAST to identify if protein-specific conserved regions (such as homeobox domains) are present in the annotated nucleotide sequence of interest. Check other contigs with the same annotation for nucleotide sequence overlaps to generate the sequence of a gene-specific transcript.
      NOTE: Identifying contigs with sequence overlap is not usually possible for all the genes, especially for low copy number genes.
   7. If the full-length sequence of the gene is needed, start with the contig that has the highest sequence similarity as an initiation point for identifying the nucleotide sequence of the whole mature transcript using techniques such as 3′ and 5′ rapid amplification of cDNA ends (RACE[19]).
   8. Annotate the assembly obtained from the second platform following steps 1.2.4–1.2.7.
      NOTE: Ideally, the results should be similar for the proteins of interest; however, coverage can differ between platforms to some extent.
   9. Use the assembled transcriptome to synthesize species-specific dsRNA as outlined in section 2.

2. Cloning and gene-specific dsRNA synthesis

NOTE: Gene-specific cloning and dsRNA synthesis has been described in detail for *T. castaneum*[20,21,22]. The following steps are a brief overview.

1. Cloning, bacterial transformation, and plasmid sequencing
    1. Isolate total RNA from the organism (as described in step 1.1) and create complementary DNA (cDNA) using a reverse transcription kit (see **Table of Materials**). Identify one or two 100–1000 bp nucleotide sequences from contigs that are specific for the genes of interest.
        1. Design primer pairs spanning the entire identified stretch of nucleotides and amplify the sequence through a polymerase chain reaction (PCR). Add an extra 30 min step at the end to create 3′ adenine overhangs in the amplified DNA product. Purify this product using a PCR purification kit (see **Table of Materials**).
    2. Clone the purified product into a pCR4-TOPO plasmid vector (see **Table of Materials**) following the vendor's protocol provided with the cloning kit. Using heat-shock treatment transform the cloned plasmids into competent bacterial cells (strain: *E.coli* DH10B) following the vendor's protocol for competent cells (see **Table of Materials**), and screen for successfully transformed cells.
    NOTE: Plates with colonies can be wrapped in paraffin film to retain moisture and stored at 4 °C for up to one month.
    3. Pick colonies of transformed cells using a 10 µL pipette tip and culture in lysogeny broth (LB) containing ampicillin (50 µg/mL) in a shaker-incubator at 37 °C and 200 rpm for 24 h.
    NOTE: For long-term storage, cultured cells can be diluted 1:1 with 100% glycerol and frozen at -80 °C as glycerol stocks.
    4. Isolate plasmids containing the gene of interest from this culture using a miniprep isolation kit (see **Table of Materials**). Store the isolated plasmids (minipreps) at -20 °C after assessing the yield spectrophotometrically. Specifically, measure the sample absorbance at 260 nm, 280 nm and 230 nm. Calculate the ratios A_{260}/A_{280} for quantifying DNA, and A_{260}/A_{230} for quantifying DNA purity.
    NOTE: A 260/280 ratio of 1.8 is generally accepted as sufficiently pure DNA, ratios lower than 1.5 indicate the presence of residual extraction reagents such as phenol. The 260/230 ratio should be greater than or equal to the 260/280 ratio. Lower ratios indicate residual reagent contamination. The yield typically ranges from 100 to 500 ng/µL.
    5. Sequence the isolated minipreps to confirm that the gene-specific fragment has been successfully integrated, being flanked between 5′ T7 and 3′ T3 promoter regions in the plasmid; this can be done using universal T7 and T3 sequencing primers[22]. Use the minipreps with the gene-specific sequence of interest for dsRNA preparation.

2. PCR amplification and in vitro dsRNA synthesis
    1. PCR amplification and product purification
        1. Design plasmid-specific or gene-specific primers that have the T7 promoter sequence on their 5' sides (see reference[22] for details) to amplify a linear fragment of the inserted gene. Optimize the yield by setting up at least 10 reactions of 20 µL each.
        NOTE: The resulting product is flanked by two 20 bp T7 polymerase binding sites.
        2. Purify the PCR product using a PCR product purification kit (see **Table of Materials**), following the vendor's column-based elution protocol. To increase the yield, repeat the final elution step up to 3 times with the same eluent. Assess the yield spectrophotometrically.
        NOTE: The yield usually ranges from 100 to 700 ng/µL. This purified product can be stored at -20 °C until further use.
    2. In vitro dsRNA synthesis and purification
        1. Use the gene-specific PCR purified product to synthesize dsRNA in vitro according to the protocol of a dsRNA synthesis kit of choice. If necessary, extend the incubation time for increased dsRNA production.
        2. Assess the progression of the reaction based on the opacity of the reaction mixture (the greater the amount of the dsRNA product, the higher the turbidity). At the end of the incubation, stop the reaction using a DNase treatment. To do so add 1 µL from a stock of 2 U/ µL, to the reaction mixture. Extend this treatment to 1–2 h to ensure the optimum degradation of the template DNA.
        3. Purify the dsRNA with a purification kit or via the following steps.
            1. Dilute the resulting product with 115 µL of nuclease-free water and add 15 µL of 3 M sodium acetate. Add 2 volumes of ice-cold 100% ethanol to this reaction mixture and precipitate the dsRNA overnight at -20 °C.
            NOTE: At this point, samples can be stored safely without affecting the final yield.
            2. Centrifuge the precipitate at 0 °C for 20 min at 9000 x *g* and discard the supernatant. Wash the product once with ice-cold 70% ethanol and centrifuge for 15 min at 13000 x *g*.
            3. Remove the supernatant and air dry the pellet for 5–15 min. Resuspend the pellet in up to 40 µL of nuclease-free water (this volume can be adjusted based on the isolated pellet size) and assess the yield and purity spectrophotometrically, as described in 2.1.4.
            4. Store the purified dsRNA at -20 °C until further use. Use the purified dsRNA for injection at the desired developmental stage.

3. Collection and preparation of early stage *T. marmoratus* embryos for dsRNA injections

1. Prepare an agarose plate for the incubation of *T. marmoratus* embryos.
    1. First, dissolve 20 mg of low-melting agarose powder in 100 mL of autoclaved distilled water (2% agarose) and then boil this mixture in a microwave until a clear solution is obtained. Take care with the hot solution, as air bubbles can cause it to overflow.
    2. Dispense this solution into a small Petri dish. To make grooves (which will hold the embryos) on the surface of the agarose plate, place three 1–2 mm wide plastic tubes (such as the tips of plastic transfer pipettes) on the surface of the liquid agarose before it solidifies.

3. Once the agarose solidifies, use a pair of blunt forceps to remove these tubes. Add 500 µL of autoclaved distilled water using a P1000 micropipette to cover these indentations in the agarose.

2. Using a fine natural hair paintbrush, collect *T. marmoratus* embryos that are 5–8 h old from the beetle nesting sites in a glass cavity dish filled with autoclaved distilled water. Monitor the nesting sites frequently to assure that the obtained eggs are of the proper age.
3. Dechorionate the embryos under a stereomicroscope using fine dissection forceps. To do so, visualize the chorion under the microscope (at the desired magnification) by positioning the light source at an appropriate angle, then grab the chorion from two sides with sharp forceps, rip it open, and gently slide the embryo out. As there are two layers, ensure that both are removed.
4. Using a fine natural hair paintbrush, carefully transfer the embryos from the glass cavity dish to the agarose plate and arrange them in the grooves under a stereomicroscope. Take great care during this process, as dechorionated embryos are very fragile. Use the prepared embryos for dsRNA injections.
NOTE: The slightly thicker end is the side of the embryo in which the head will develop.

4. dsRNA microinjections in early stage *T. marmoratus* embryos

1. To inject early stage embryos with gene-specific dsRNA, prepare microinjection needles using a microinjection needle puller (see **Table of Materials**). While visualizing the needle tip under a stereomicroscope, use a pair of fine forceps to break the needle tip, creating a sharp edge. Ideally, break the needle tip diagonally so that a sharper edge remains on one side, which will allow it to enter the embryo while causing minimal injury.
2. Thaw the purified stock dsRNA solution on ice, add 1 µL of the 10x injection buffer and top it off with double distilled water, to make 10 µL of injection solution. For injections, a dsRNA concentration of 1 µg/µL usually is suitable but can be adjusted according to the phenotypic severity of the resulting animals).
NOTE: Prepare 1 mL of 10x injection buffer[22] by adding 10 µL of 0.1 M sodium phosphate buffer (made by mixing 8.5 mL of 1 M Na2HPO4 and 1.5 mL of 1 M NaH2PO4 adjusted to a pH 7.6 at 25 °C with 100 µL of 0.5 M KCl), 100 µL of food dye and 790 µL of double-distilled water.
    1. Using a P10 pipette, backfill the microinjection needle with the working dsRNA solution. Once the solution has filled the tip of the needle, attach it to a microneedle holder connected to a microinjection system that utilizes pressure ejection technology (see **Table of Materials**).

3. Turn on the microinjection system and the pressurized air supply. Using the microvalve on the microinjection system, adjust the injection pressure to 15 psi. Adjust the injection duration using the time adjustment knob (set it to “Seconds”).
    1. As the injection duration depends on the required injection volume, if necessary, adjust this parameter on the microinjection system before each injection. Use an injection volume of 1–2 nL for early stage *T. marmoratus* embryos.
    NOTE: Higher injection volumes tend to interfere with the embryo survival rate.

4. To measure the volume of injection fluid that is dispensed by the microinjection system, calibrate the injection needle by assessing the volume of the fluid bubble that is formed at the tip of the needle when injecting into air. For *T. marmoratus* embryos, use an optimal bubble size of 100–200 µm. The injection needle is of good quality if this can be achieved at 15 psi with an injection duration of ~3 s.
5. Align the needle and the agarose plate containing the embryos under a stereomicroscope, such that the needle approaches the embryos at an angle between 45° and 60°.
6. Using the micromanipulator, move the microneedle slowly while monitoring the progress through the stereomicroscope. Once the tip of the needle is touching the surface of an embryo, carefully move the needle forward until it pierces the surface of the embryo. Do not perforate the embryo deeply with the needle tip since this can affect the survival of the embryo. To reduce variability, deliver the injection in the middle of the embryo.
7. Once the needle is inside the embryo, carefully press the injection button of the micro-injector to deliver the dose of dsRNA to the embryo. After injecting 1–2 nL of dsRNA, slowly retract the needle from the embryo, such that it does not cause the embryo to rupture.
    1. Inject the other embryos in a similar fashion. If the microinjection needle becomes blocked during the procedure, unblock it by increasing the injection pressure and duration. Visually identify successfully injected embryos by the presence of a colored spot at the site of the injection (since the injection buffer contains food coloring).

8. Carefully place the dish with injected embryos in a humidity chamber.
    1. To construct such a chamber, take any plastic box with a lid, sterilize it with 70% ethanol, allow it to dry, and place tissue soaked in autoclaved water at the bottom to provide a humid environment. Place this humidity chamber in an incubator with an appropriate temperature (in this case, 25 °C) and light cycle of 14 h light and 10 h dark.

9. Allow injected embryos to develop into first instar larvae, which requires 4–5 days. Monitor the progress of embryo development daily using a stereomicroscope to identify morphologically visible knockdown phenotypes as depicted in **Figure 2**.
10. Document this progress by taking digital images using a high-resolution camera. Remove dead embryos and replenish the water on the agarose plate daily to avoid desiccation and the possible spread of microbial contamination to other surviving embryos during the incubation period.
11. Perform appropriate controls for dsRNA injections, including buffer injections, injections of dsRNA synthesized against the sense strand of the gene of interest, and injections of dsRNA synthesized against sequences that do not exist within the target organism. Confirm RNAi knockdown using additional molecular methods[22].

5. Preparation of *T. marmoratus* larvae for dsRNA injections

NOTE: Unlike early stage embryos, *T. marmoratus* larvae are relatively sturdy and can be injected with larger volumes. For example, second instars can be injected with up to 2 µL of dsRNA working solution and third instars with at least 3 µL without noticeable negative effects on the survival rate. To inject dsRNA, it is helpful to immobilize larvae by embedding them in agarose.

1. Before collecting the larvae, melt 2% agarose and keep it in a water bath at 60–70 °C to maintain it in liquid form. Prepare an immobilization dish by pouring melted 2% agarose into a small Petri dish and then cooling until solidified. Use blunt forceps to make a shallow groove in the agarose plate (roughly the size of the arthropod to be embedded) for each animal that will be injected.
2. Collect young larvae at the appropriate developmental stage (one stage prior to the stage desired for analysis). To do this, carefully drain the water from the culture cups containing the larvae and follow the steps mentioned below.
3. Anaesthetize on ice (carefully pick the larva out from its water cup and place it gently on ice) until the larva shows no notable movements. Use blunt, soft-ended forceps to carefully lift the larva from the ice and place it on the immobilization stage with its neck and body in the groove but its tail located above the agarose surface so that it will not be covered, which allows the larva to continue breathing through its tail spiracles.
4. Cover the larva with a thick layer of agarose that is still liquid but not dangerously hot. If it was accidentally covered, use the forceps to free the tail and the two segments below it from the agarose. Once the agarose solidifies, use the larva for dsRNA injections.

6. dsRNA microinjections in *T. marmoratus* larvae

1. Prepare and backfill a microinjection needle with the desired amount of gene-specific dsRNA working solution (as described in steps 4.1–4.2). Attach the needle to a needle holder connected to a manually controlled microinjection syringe. Before attaching the needle, pull the syringe plunger all the way out to avoid running out of injection pressure mid-procedure.
2. Position the immobilized larva such that the injection site, which is located dorsally between the third and fourth body plate segments, is aligned with the trajectory of the microinjection needle at a relatively flat angle (nearly parallel to the stage).
   NOTE: Angling the needle and the larva in this position is important, as it provides the path of least resistance for the needle tip to perforate the larvae with minimum damage.
3. Once the needle and the larva have been positioned, carefully move the needle tip into the larva using the micromanipulator while monitoring the progress through a stereomicroscope.
4. After the tip perforates the tissue, slowly and carefully apply pressure to the syringe. Adjust the injection pressure by observing the movement of the colored injection fluid in the needle under the microscope.
5. Carefully retract the needle after the injection. Use soft forceps to peel away and hence free the larva from the agarose. Transfer the larva back into a container with water (at room temperature) and allow it to develop further in an incubator or culture room at 25–28 °C and a light cycle of 14 h light and 10 h dark.
6. Employ appropriate control injections and knockdown quantifications, as outlined in step 4.10.

Representative Results

Using the protocol described above, we knocked down three different genes, namely, *white*, *laccase2* (*lac2*), and *Lens3* (**Table 1**), at a variety of different developmental stages of the Sunburst Diving Beetle *T. marmoratus*. We performed RNAi in *T. marmoratus* by injecting dsRNA at a very early stage during embryogenesis (**Figure 1A**). As some of the embryos do not survive the process and turn necrotic (**Figure 1B**), they need to be removed to keep the remaining embryos healthy. Exemplified here are the injections of dsRNA against the *white* gene. This gene is well known in *Drosophila* as one of three ATP-binding cassette (ABC) transporters involved in the uptake and storage of the precursors of eye pigment[23]. Accordingly, its loss-of-function results in an unpigmented, white-eye phenotype. Our results show that the injections of dsRNA targeting the orthologous *white* gene in *T. marmoratus* embryos leads to the loss of eye pigmentation in newly emerging larvae. In this case, wild type larvae are characterized by heavily pigmented eyes, and the RNAi knockdown of *white* leads to various levels of reduction and even complete elimination of eye pigment. Overall, we observed at least some reduction in eye coloration in 34% of surviving embryos (n = 35). **Figure 2A** compares a control individual and an individual with slightly lighter eye color. **Figure 2B** illustrates a more severe knockdown in an individual, in which the more ventral eyes (Eyes 2–5) of the cluster are completely unpigmented, whereas the dorsal eyes still show some pigmentation. These differences highlight how the efficiency of the knockdown can vary regionally, which is possibly related to differences in the efficacy of dsRNA penetration in dense tissue. Another individual shows essentially complete pigment loss in all eyes (**Figure 2C**).

To investigate how well RNAi works at the larval stage of *T. marmoratus*, we injected dsRNA targeting the tannic gene *lac2* into second instar larvae a few days before they were due to molt into third instars (**Figure 3**) and evaluated the effect on the cuticular coloration of third instar larvae. Lac2 is a type of phenoloxidase that oxidatively conjugates proteins to make them insoluble, harder, and darker. Knockdown in the flour beetle *T. castaneum* has been shown to lead to lighter colored individuals in low doses but is considered lethal in high doses[24]. **Figure 4** illustrates that this treatment also leads to lighter colored Sunburst Diving Beetle larvae. Specifically, in this experiment, 75% of the surviving injected larvae (n = 12) had reduced pigmentation (compared to 0% in the control group). **Figure 4A** shows an individual with relatively mild depigmentation, whereas **Figure 4C** illustrates the head of a *T. marmoratus* larva in which the dark coloration of the cuticle is nearly absent. Depigmentation was particularly evident for the central dark patterning that is typical for these larvae, whereas this pattern remained clearly visible in a control-injected individual (**Figure 4B**). In addition, a lightening of the tail trachea was observed, as depicted in **Figure 4D**. In the case where *lac2* dsRNA was injected into third instar larvae, lighter adult individuals were obtained (**Figure 5**). Note that the wings of the knockdown beetle are somewhat deformed, likely due to its unusual softness.

In addition to altering morphological traits, it is possible to use RNAi to target genes that affect behavior. To demonstrate this, we performed RNAi against a key lens protein coding gene, *Lens3*[18], and injected it into second instar larvae to affect the optical properties of third instar larval eyes. Any effects on the lens observed here are likely because the eyes of *T. marmoratus* larvae undergo major eye growth at this transition, which also involves major optical changes of the lens[25]. RNAi knockdown in this experiment was highly efficient. Verification through qPCR showed

a 100% success rate; out of 13 tested individuals, 12 were knocked down to less than 10% of the expression level of control individuals, with the remaining individual having an expression level of 17% of the control level (unpublished observation). At the phenotypic level, only some individuals were severely handicapped or incapable of prey capture, as is illustrated in **Figure 6** for an individual that repeatedly approached its prey from a very close distance but consistently overshot it.

Table 1: Primer sequences and amplicons for white, lac2, and Lens3 proteins. Please click here to download this table.

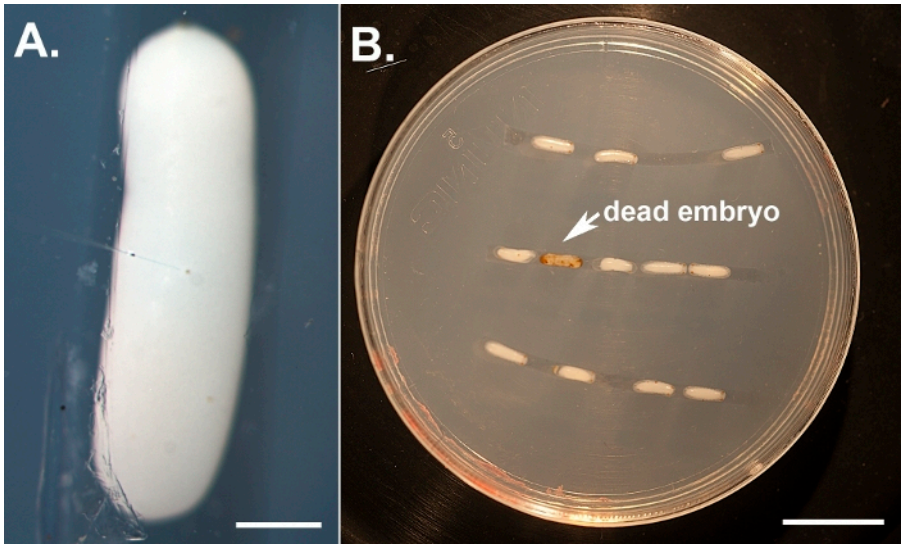

Figure 1: Illustration of embryo injections. (**A**) Dechorionated embryos lined up on an agarose plate and injected near their center using a microinjection needle filled with dsRNA and food-dye-containing injection buffer. The scale bar represents 500 µm. (**B**) An individual with a more severe knockdown. Injected embryos are kept in a humidity chamber and monitored daily to score phenotypes and remove dead individuals (which turn brown). The scale bar represents 5 mm. Please click here to view a larger version of this figure.

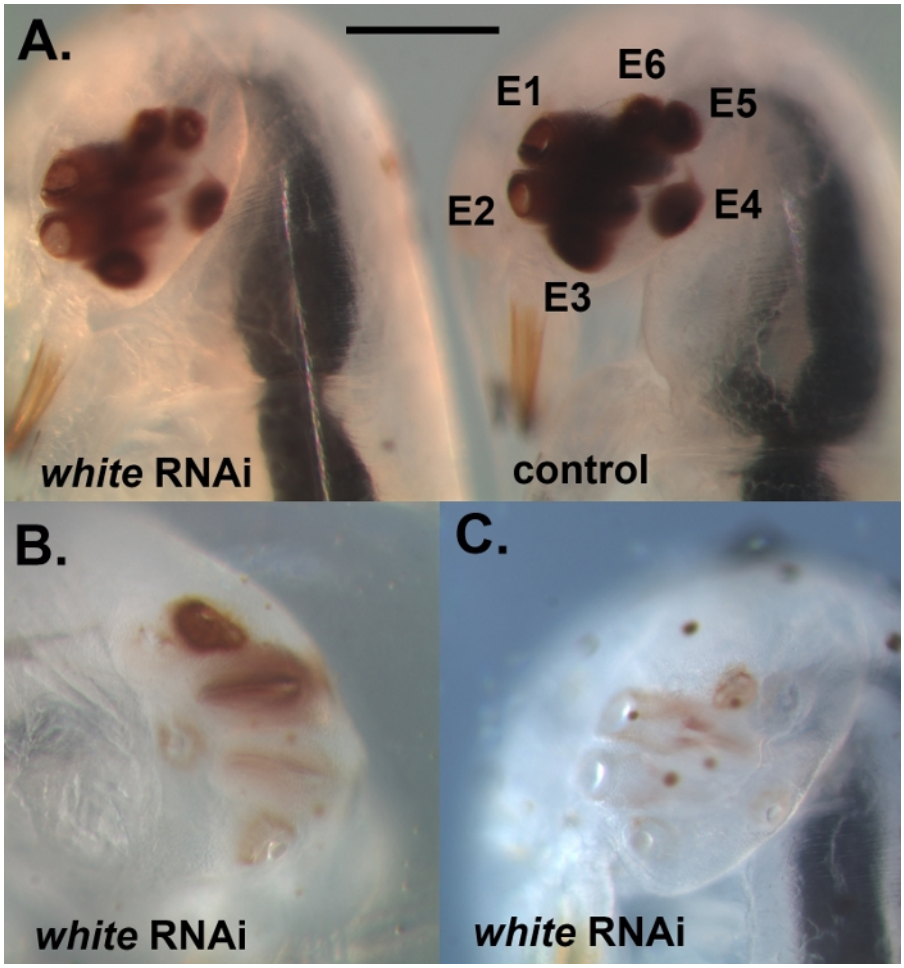

Figure 2: Example of fully developed embryos that were injected with dsRNA against *white*. (**A**) Comparison of an individual with a reduction in eye pigmentation (left) and a control-injected individual (right). E1–E6 refer to Eyes 1–6. (**B**) An individual with a more severe knockdown phenotype, which illustrates that, at times, some of the eyes within the cluster are more severely affected by the knockdown than others. (**C**) Individual with a nearly complete loss of eye pigmentation. The scale bar represents 200 µm; Panels B and C are represented at the same scale. Please click here to view a larger version of this figure.

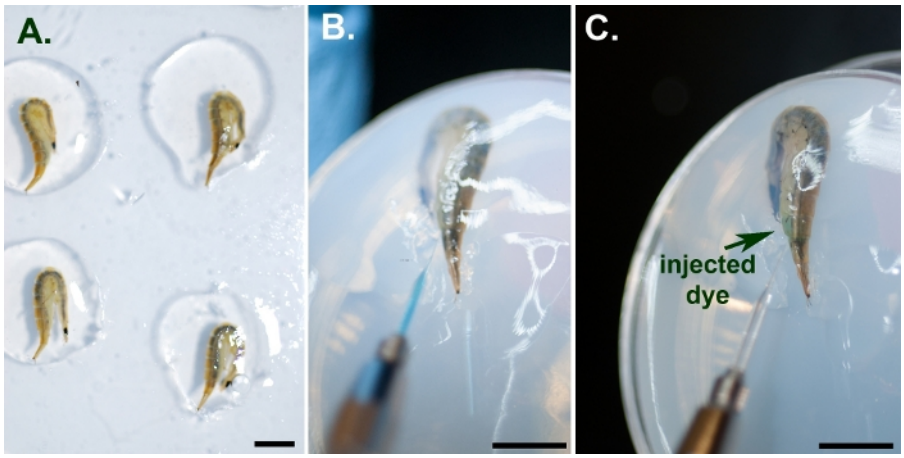

Figure 3: Illustration of larval injections. (**A**) Several larvae immobilized by embedding in 2% agarose with their tail spiracles left clear of any agarose. (**B**) Microelectrode containing the injection solution placed so that its tip can penetrate the fine membrane between two segments. (**C**) Blue injection dye visible at the injection site after the injection. Scale bars represent 1 cm. Please click here to view a larger version of this figure.

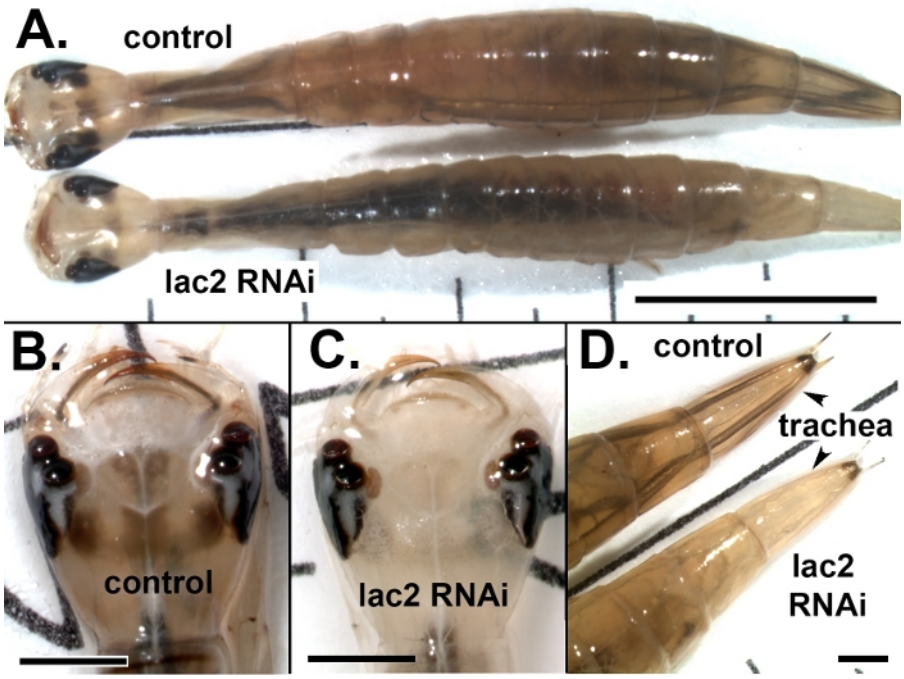

Figure 4: RNAi for *laccase2* applied to second instar larvae resulting in reduced cuticle coloration in third instar larvae. (**A**) Relatively mild loss of coloration in a *lac2* RNAi individual (bottom) when compared with a control-injected individual (top). The scale bar represents 5 mm. (**B**) Head of a control individual showing the characteristic dark colored pattern at the center of the head. (**C**) Relatively severe knockdown of *lac2* leading to a nearly complete loss of central head coloration. (**D**) Loss of coloration in the major tail cuticle of a *lac2* RNAi individual (bottom) when compared with a control-injected individual (top). Scale bars in B–D represent 1 mm. Please click here to view a larger version of this figure.

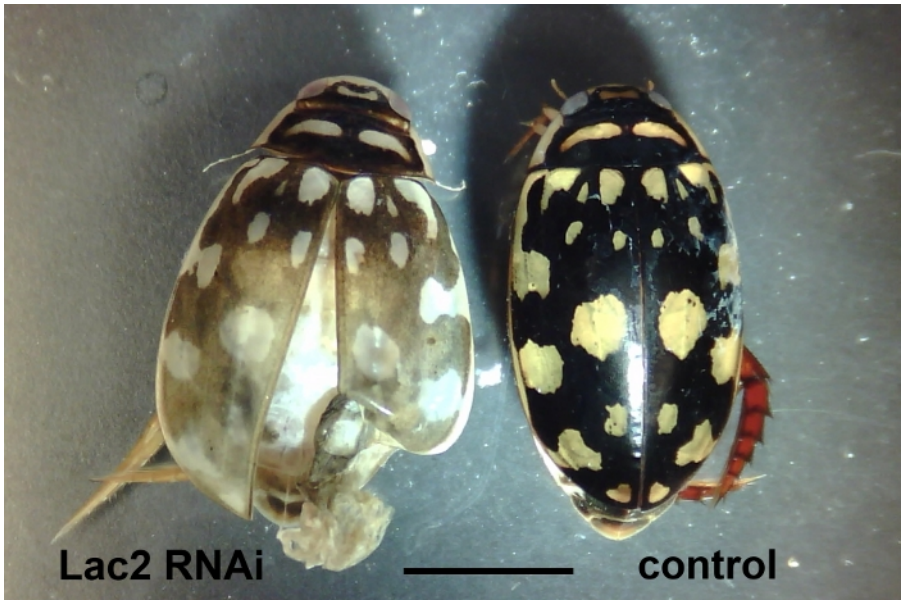

Figure 5: RNAi for *laccase2* applied to a third instar larva resulting in reduced cuticle coloration in an adult. The knockdown individual (left) is also characterized by very soft elytra when compared with the control (right). The scale bar represents 5 mm. Please click here to view a larger version of this figure.

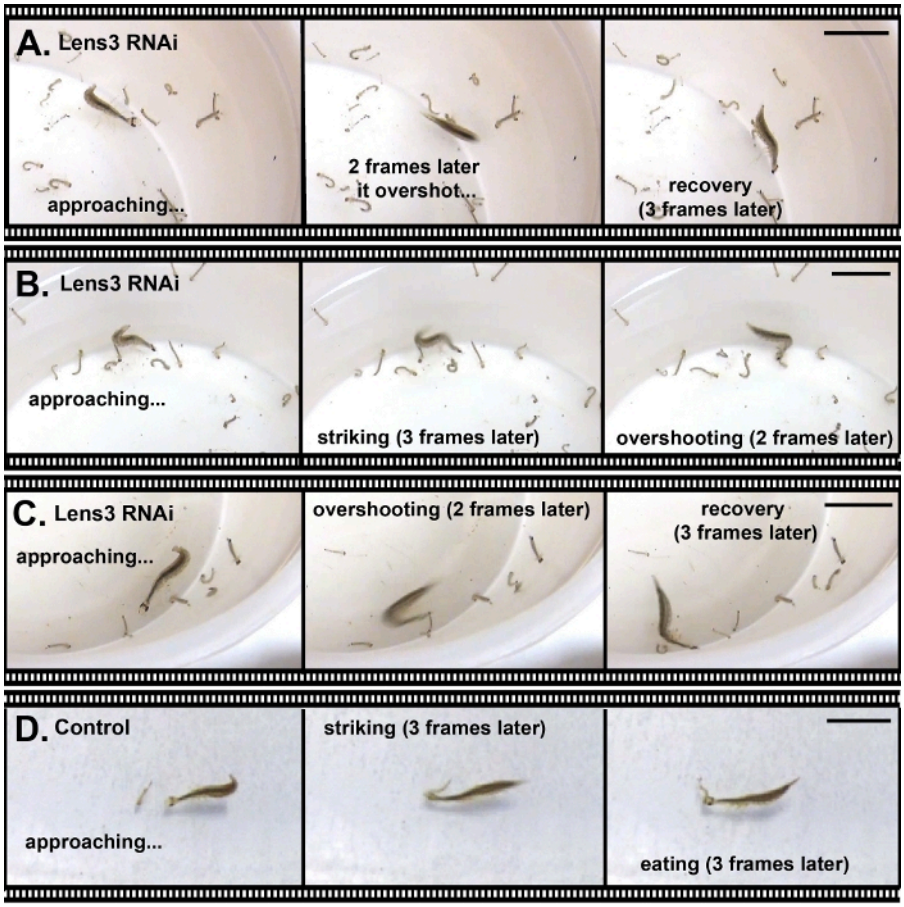

Figure 6: Knockdown of a major lens protein leading to deficiencies in prey capture. (**A–C**). Three examples wherein a Sunburst Diving Beetle larva, in which optical deficits were induced through RNAi, was unable to capture prey (mosquito larvae). Representative still images were selected from a video recording of characteristic prey captures. (**D**) Control larva catching prey. All scale bars represent 2 cm. Please click here to view a larger version of this figure.

Discussion

Our goal is that this compilation of methods will make RNAi widely available, especially as this tool remains a powerful synergistic technique to CRISPR/Cas9-based gene editing, with the advantage that it can be applied to the desired developmental stages of studied organisms. To exemplify this strength, we injected dsRNA into embryos and into different larval stages. Injections into eggs affected the development of embryos (**Figure 2**), injections into the second larval stage had apparent effects on the third larval stage (**Figure 4** and **Figure 6**), and injections into the third larval stage showed effects in the adults (**Figure 5**). While the exact timing has to be established experimentally, generally, injections take effect within a few days. The success of this process can be affected by the length of the dsRNA sequence. Here, we presented examples using a little over 200 bp to more than 800 bp. As a general rule, sequences between 100 and 600 bp are preferred to limit off-target effects, but sequences up to 1000 bp yield good results[22]. One question in regard to RNAi is the duration of knockdown that can be achieved through this technique. Since the phenotypes were terminal at each stage, we cannot comment on this issue based on our presented results. However, it has previously been noted that RNAi effects are generally relatively long lived, and that higher concentrations lead to longer lasting knockdowns[20].

One limitation of this technique is that it works better for some organisms than for others, and there seems to be no direct way of predicting how well it will work a priori. Nevertheless, it has been found to work well for a large range of different organisms. Within arthropods, this includes arachnids[26], crustaceans[27], and a variety of insects, with particularly high success rates in beetles[28]. A further complication is that differences in phenotype severity often occur between individuals despite the application of the same amount of dsRNA. As illustrated in **Figure 2B**, variation can even occur within an individual. In our RNAi studies targeting different genes involved in *T. marmoratus* larval eye development, we have frequently found that some eyes are affected more severely than others. This phenomenon may be related to the relatively dense tissue of the eye cluster, with the dsRNA better able to reach some of the units.

For the successful execution of RNAi experiments, it is critical that several parameters are optimized for the target gene. For example, the concentration of the dsRNA and the length of the targeted gene can strongly influence the outcome[20]. Another critical parameter is how the injections are executed, as this process can greatly influence the survival rate. For embryos, we achieved the best results by targeting the center of the embryo. A well-laid-out plate allows for the injection of 100 or more embryos in a single session. For larvae, it is critical to insert the injection needle between the segments. These injections require more dsRNA, and based on larvae availability, our injection sets here typically only consisted of a few animals at a time. For all injections, it is critical to prevent air from entering the organism.

In some cases, the feedback loops of a gene regulatory network and genetic redundancy can influence the penetrance of RNAi phenotypes, despite consistent knockdowns. This seems to be the case for our behavioral observations of larvae with highly successful knockdowns of a prominent lens protein, Lens3[18]. Although we verified the high efficiency of these knockdowns through qPCR, considerable variation was observed in the associated phenotypes. This result highlights the necessity of properly quantifying RNAi knockdowns (for details on options see[22]). If there is no clear a priori expectation in regard to the resulting phenotypes, a good way of controlling for the off-target effects of RNAi is to target the same gene with two non-overlapping sequences of dsRNA and to evaluate the results for common phenotypes.

In contrast to gene-editing techniques, RNAi is also a powerful tool for studying lethal genes, and there are two ways to do so. For example, if one is interested in the functional contribution of a gene where loss-of-function early in development is known to be lethal, a functional investigation of such a gene can be achieved by simply allowing the animal to develop normally and then knocking down the gene via RNAi later in development (i.e., in the adult). Alternatively, a gene where complete loss-of-function is known to be lethal can be investigated through a partial knockdown, which can be achieved by injecting a range of dsRNA concentrations. Some of our results show knockdowns of *lac2*, which are known to be lethal if the cuticle in insects becomes overly soft[24]. Even the *lac2* RNAi beetle depicted in **Figure 5** would be unlikely to survive outside laboratory conditions. Another lethal gene is *cut*, which codes for a transcription factor that is fundamental for cell-fate specification in various organ systems in arthropods and has been linked to glia development in the *Drosophila* visual system[29]. Based on our experience with *cut* RNAi in *T. marmoratus* embryos, we can evoke informative eye phenotypes in embryos that are able to complete their embryonic eye development (unpublished observations). Here, higher dosages appear to lead to higher lethality rates, while lower doses result in observable and informative phenotypes.

Our protocol not only lists the necessary steps for a researcher to pursue RNAi experiments on *T. marmoratus*, as illustrated, but also is generally applicable to other organisms, especially aquatic organisms. Among aquatic organisms, there are already several examples within crustaceans such as the water fleas *Daphnia*[30] and shrimp (for a recent review, see reference[31]). There are ample opportunities among aquatic insects, as they have been estimated to comprise about 6% of all insect diversity, with likely more than 200,000 species[32]. Furthermore, RNAi has already been performed on water striders that tend to inhabit the surface of aquatic environments[33]. If no genome is present, then a transcriptome can be assembled de novo. As long as this process reveals contigs of a few hundred nucleotides, dsRNA against specific genes can be designed. Our protocol for immobilizing insects in agarose will likely also be useful for other procedures, especially for soft, malleable, and aquatic organisms. Taken together, RNAi remains a powerful technique for manipulating gene expression in a diverse group of organisms, even when no other molecular and genetic tools are available.

Disclosures

The authors have nothing to disclose.

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