Journal of Visualized Experiments

Electroporation-mediated RNA interference method in Odonata

--Manuscript Draft--

Article Type:	Invited Methods Collection - Author Produced Video
Manuscript Number:	JoVE61952R2
Full Title:	Electroporation-mediated RNA interference method in Odonata
Corresponding Author:	Ryo Futahashi Kokuritsu Kenkyu Kaihatsu Hojin Sangyo Gijutsu Sogo Kenkyujo Tsukuba, Ibaraki JAPAN
Corresponding Author's Institution:	Kokuritsu Kenkyu Kaihatsu Hojin Sangyo Gijutsu Sogo Kenkyujo
Corresponding Author E-Mail:	ryo-futahashi@aist.go.jp
Order of Authors:	Genta Okude
	Takema Fukatsu
	Ryo Futahashi
Additional Information:	
Question	**Response**
Please specify the section of the submitted manuscript.	Developmental Biology
Please indicate whether this article will be Standard Access or Open Access.	Open Access (US$3000)
Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below:	I agree to the Author License Agreement
Please provide any comments to the journal here.	Due to the timing of the sample and COVID19's accessibility restrictions, we prefer a video produced by authors via dropbox.

TITLE

Electroporation-mediated RNA interference method in Odonata

AUTHORS AND AFFILIATIONS

Genta Okude[1,2], Takema Fukatsu[1,2,3], Ryo Futahashi[2]

[1]Department of Biological Sciences, Graduate School of Science, The University of Tokyo, Bunkyo-ku, Tokyo, Japan

[2]Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Central 6, Tsukuba, Ibaraki, Japan

[3]Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki, Japan

Corresponding Author:
Genta Okude
gentaokude@gmail.com

Corresponding Author:
Ryo Futahashi
ryo-futahashi@aist.go.jp

KEYWORDS

RNA interference, RNAi, *in vivo* electroporation, Gene knockdown, Dragonfly, Damselfly, Odonata, *Ischnura senegalensis, Pseudothemis zonata*

SUMMARY

We provide a detailed protocol for electroporation-mediated RNA interference in insects of the order Odonata (dragonflies and damselflies) using the blue-tailed damselfly (*Ischnura senegalensis*: Coenagironidae: Zygoptera) and the pied skimmer dragonfly (*Pseudothemis zonata*: Libellulidae: Anisoptera).

ABSTRACT

Dragonflies and damselflies (order Odonata) represent one of the most ancestral insects with metamorphosis, in which they change their habitat, morphology, and behavior drastically from aquatic larvae to terrestrial/aerial adults without pupal stage. Odonata adults have a well-developed color vision and show a remarkable diversity in body colors and patterns across sexes, stages, and species. While many ecological and behavioral studies on Odonata have been conducted, molecular genetic studies have been scarce mainly due to the difficulty in applying gene functional analysis to Odonata. For instance, RNA interference (RNAi) is less effective in the Odonata, as reported in the Lepidoptera. To overcome this problem, we successfully established an RNAi method combined with in vivo electroporation. Here we provide a detailed protocol including a video of the electroporation-mediated RNAi method as follows: preparation of larvae, species identification, preparation of dsRNA/siRNA solution and injection needles, ice-cold anesthesia of larvae, dsRNA/siRNA injection, in vivo electroporation, and individual rearing until

adult emergence. The electroporation-mediated RNAi method is applicable to both damselflies (suborder Zygoptera) and dragonflies (suborder Anisoptera). In this protocol, we present the methods for the blue-tailed damselfly *Ischnura senegalensis* (Coenagrionidae) as an example of damselfly species and the pied skimmer dragonfly *Pseudothemis zonata* (Libellulidae) as another example of dragonfly species. As representative examples, we show the results of RNAi targeting the melanin synthesis gene *multicopper oxidase 2*. This RNAi method will facilitate understanding of various gene functions involved in metamorphosis, morphogenesis, color pattern formation, and other biological features of Odonata. Moreover, this protocol may be generally applicable to non-model organisms in which RNAi is less effective in gene suppression due to the inefficiency and low penetrance.

INTRODUCTION

Dragonflies and damselflies (the order Odonata) are among the most ancestral groups of insects that exhibit "metamorphosis"[1,2]. By metamorphosis, they change their habitat, morphology, and behavior drastically from aquatic larvae to terrestrial/aerial adults[3]. Odonata adults have a well-developed color vision and represent a remarkable diversity in body colors and patterns across sexes, stages, and species[3-5]. While many ecological and behavioral studies on Odonata have been conducted[6,7], molecular genetic studies have been hindered mainly by the difficulty in applying gene functional analysis to Odonata.

The conventional RNA interference (RNAi) method, in which double-stranded RNA (dsRNA) is injected to suppress the function of the gene of interest[8], turned out to be ineffective in Odonata insects[9], as reported in Lepidopteran insects[10]. On the other hand, previous reports have suggested that electroporation-mediated RNAi is effective in Lepidopteran species, especially in epidermal tissues[11-13]. We recently found that the electroporation-mediated RNAi works effectively in the tiny dragonfly *Nannophya pygmaea* (Libellulidae: Anisoptera)[9], but *N. pygmaea* is a relatively rare species and therefore not suitable for molecular genetic studies.

Most Odonata species are classified into either of the two suborders, Zygoptera (damselflies) or Anisoptera (true dragonflies)[3]. Here we focused on the blue-tailed damselfly *Ischnura senegalensis* (Coenagrionidae; **Figure 1A**) as a representative Zygopteran species and the pied skimmer dragonfly *Pseudothemis zonata* (Libellulidae; **Figure 1B**) as a representative Anisopteran species. The two species are among the most common Odonata species in natural and urban ponds in Japan, including those in Tsukuba City, and we can collect many larvae of the two species in the field. Recently we established a laboratory rearing system for individual larvae of *I. senegarensis*, which enabled continuous monitoring of development and morphogenesis of the Odonata larvae in detail[14].

In this report, we provide a refined method and a video protocol for the electroporation-mediated RNAi in *I. senegalensis* and *P. zonata*. In Japan, *I. senegalensis* and *Ischnura asiatica*, which are genetically close, are often found sympatrically[15], and they are difficult to distinguish in larvae[16]. We also describe how two *Ischnura* species can be distinguished by restriction fragment length polymorphism (RFLP).

For evaluating the effectiveness of the electroporation-mediated RNAi, we select *multicopper oxidase 2* gene (*MCO2*; also known as *laccase2*) as a representative target gene, on account of the visible phenotype of paler cuticle color upon knockdown of the gene expression[9]. *MCO2* is known to be essential for darkening of the epidermis in a variety of insect species[17,18].

PROTOCOL

NOTE: The overall scheme of the protocols is shown in **Figure 1**.

1. Preparation of larvae of dragonflies or damselflies.

1.1. Collect larvae in the field using a hand net.

NOTE: *I. senegalensis* larvae often cling onto water plants floating on the water surface, while *P. zonata* larvae often stay among leaf litter at the bottom. In Tsukuba City, the final instar larvae of *I. senegalensis* are found mainly from March to June, and those of *P. zonata* from May to June. *I. senegalensis* larvae can be reared from eggs in the laboratory[14], but the larvae collected in the field have the clearly higher success rate of adult emergence.

1.2. Species identification by restriction fragment length polymorphism (RFLP) of PCR-amplified products.

NOTE: This step is only necessary if the species is difficult to identify from the appearance of the larvae, as in *I. senegalensis*.

1.2.1. Hold one of the caudal gills of a larva using forceps. Then, the larva falls off its caudal gill itself (causing autotomy).

NOTE: Larvae of Zygopteran damselflies usually have three caudal gills (**Figure.1A**). When they are attacked by a predator, they can take off their own caudal gills to escape. If the caudal gill is not available, a portion of the leg is dissected.

1.2.2. Put one caudal gill into 100 µL of PBS solution [0.8% NaCl, 0.02% KCl, 0.115% Na_2HPO_4, and 0.02% KH_2PO_4 (w/v)] and homogenize with a hand mixer using a deposit pestle.

1.2.3. Spin down the mixture at 5,000 x g for 10 seconds, and subject 0.5 µL of the supernatant to PCR-amplification using DNA polymerase and primers to amplify the internal transcribed spacer 1 (ITS1) region of the nuclear DNA.

NOTE: Perform the PCR according to the manufacturer's protocol. The combination of ITS-F0 (5'-GGA AAG ATG GCC AAA CTT GA -3') and 5.8S-AS1 (5'- GCC GGC CCT CAG CCA G -3') primers can amplify ITS1 region in almost all Odonata species[19].

1.2.4. Incubate the mixture of 1 µL of PCR-amplified product, 0.3 µL of 10x M Buffer, 0.1 µL of

DraI restriction enzyme, and 1.6 µL of water at 37 °C for 1 h.

NOTE: Select the best restriction enzymes, depending on the combination of species. If there are no restriction enzymes suitable for species identification, confirm by DNA sequencing.

1.2.5. Load 2 µL of the products with loading dye on 2% agarose gel and run electrophoresis.

NOTE: It is difficult to identify species when using 1% or 1.5% agarose gel.

1.2.6. Check the electrophoresis pattern to identify the species (**Figure 2**).

NOTE: RFLP patterns are species- and population-dependent. In Tsukuba population, *I. asiatica* has a major band of 400–500 bp, whereas *I. senegalensis* has a major band of approximately 200 bp and an additional band of less than 100 bp (an arrowhead in **Figure 2**). The ITS1 region exists in multiple copies in the genome, and in *Ischnura* species, microsatellite polymorphism within the ITS1 region is often present in the same individual, affecting the pattern of RFLPs.

1.3. Rear the collected larvae in the laboratory until use for RNAi.

1.3.1. Place each larva separately into each well of a 12-well plate with approximately 3 mL of water.

NOTE: *I. senegalensis* larvae must be kept individually because they frequently cannibalize each other, while *P. zonata* larvae can be kept in a group because they rarely cannibalize.

1.3.2. Feed *I. senegalensis* larvae with *Artemia* brine shrimp every day and *P. zonata* larvae with bloodworms and/or *Tubifex* worms at least twice a week until they grow to the suitable developmental stage for RNAi.

NOTE: Frequent feeding is critical to increase the success rate of adult emergence.

1.3.3. Change the water as soon as it becomes dirty with feces or leftovers.

NOTE: Frequent water change is also important to increase the success rate of adult emergence.

1.3.4. Judge the appropriate developmental stage for RNAi.

NOTE: The final instar larvae of *I. senegalensis* can be classified into five developmental stages[14,20]. The first stage (stage A, before the wings begin to expand) or the second stage (stage B) of the final instar larvae is suitable for RNAi experiments, when considering the clear phenotype of RNAi after adult emergence (see Discussion). In *P. zonata*, the final instar larvae before wing expansion (corresponding to stage A of *I. senegalensis*) were used in this study.

2. Preparation of dsRNA/siRNA solution and injection needles for RNAi.

NOTE: Select either small interfering RNA (siRNA) (step 2.1) or double-stranded RNA (dsRNA) (step 2.2) as the solution for RNAi.

2.1. Preparation of the siRNA solution

2.1.1. Design siRNA using siDirect program version 2.0 (http://sidirect2.rnai.jp/)[21], following the guidelines previously reported in Lepidopteran insects[22].

2.1.2. Obtain commercially synthesized siRNA.

NOTE: The sequences of siRNA targeting *MCO2* gene of *I. senegalensis* used in this study were as follows: 5'- GCA CUU UCC GUU AUC AAU AUA -3' for sense strand and 5'- UAU UGA UAA CGG AAA GUG CUC -3' for antisense strand. As a negative control, the sequences of siRNA targeting *enhanced green fluorescent protein* (*EGFP*) gene were as follows: 5'- GCA UCA AGG UGA ACU UCA AGA -3' for sense strand and 5'- UUG AAG UUC ACC UUG AUG CCG -3' for antisense strand[11].

2.1.3. Dilute the siRNA to 100 µM with injection buffer [100 mM CH_3COOK, 2 mM $Mg(CH_3COO)_2$, 30 mM HEPES-KOH, pH 7.4][22].

2.1.4. Store at -80 °C until use.

2.2 Preparation of dsRNA solution.

2.2.1 Select a 300-400 bp region for dsRNA using primer3 program version 4.1.0 (http://bioinfo.ut.ee/primer3/)[23] and design the primer sets.

2.2.2. Obtain commercially synthesized primer sets.

NOTE: To produce templates for dsRNA synthesis, the primer sets used in this study were as follows: (5'- GCC TGT CAG CTT TGT CTT CC -3' for forward primer and 5'- GGT GTC TGG CGG ACA ACT AT -3' for reverse primer) for *MCO2* genes of *I. senegalensis* (*IsMCO2*, accession no. LC589180) and (5'- CCG CAC AGC TCA CTA TTC AA -3' for forward primer and 5'- GGA GGA TTC CTT CAT CGA CA -3' for reverse primer) for *MCO2* genes of *P. zonata* (*PzMCO2*, accession no. LC589179). As a negative control, the primer set for dsRNA targeting *β-lactamase* (*bla*) gene on the cloning vector were (5'- CTA TGT GGC GCG GTA TTA T -3' for forward primer and 5'- CAG AAG TGG TCC TGC AAC T -3' for reverse primer).

2.2.3. Extract RNA from Odonata larvae using a commercially available RNA extraction kit and perform cDNA synthesis using reverse transcriptase according to the manufacturer's protocol.

NOTE: cDNA library prepared for RNA sequencing analysis can be also used.

2.2.4. Amplify the target sequences using the synthesized cDNA and the designed primer set and

clone them into the cloning vector using a commercial ligase according to the manufacturer's protocol.

2.2.5. Transform the plasmid into *E. coli* competent cells and pick up a single colony after overnight incubation.

2.2.6. PCR-amplify the insert region using primers on the vector.

2.2.7. Confirm the cloned sequence by Sanger sequencing.

2.2.8. PCR-amplify the insert using vector primers containing the T7 polymerase promoter sequence[24, 25].

NOTE: In this study, the following primers were used: T7-F (5' - TAA TAC GAC TCA CTA TAG GGA GAC TAG TCA TAT GGA T - 3') and T7-R (5'- TAA TAC GAC TCA CTA TAG GGA GAC CCG GGG ATC CGA T - 3') [25].

2.2.9. Purify the PCR product using the PCR purification kit according to the manufacturer's protocol, elute the DNA with 50 µL of distilled water, and concentrate the eluted DNA solution to approximately 10 µL using a centrifugal evaporator.

2.2.10. Synthesize dsRNA by in vitro transcription according to the manufacturer's protocol. Use a total of 1000 ng template DNA and elute synthesized dsRNA with 100 µL of elution buffer.

2.2.11. Measure the concentration of dsRNA using a spectrophotometer and confirm the quality of dsRNA by electrophoresis on a 1.5% agarose gel.

NOTE: A single band can be seen when dsRNA synthesis is successful.

2.2.12. Dilute dsRNA to 1000 ng/µL with elution buffer and store at -20 °C until use.

2.3 Preparation of injection needles

2.3.1. Pull a glass capillary by using a glass needle puller.

2.3.2. Place the tip of the pulled capillary onto a double-sided adhesive tape and break the tip of the capillary with forceps.

2.3.3. Set the capillary to an injector.

NOTE: It is easier to handle the capillary if you wear nitrile gloves.

2.3.4. Load siRNA/dsRNA solution to the prepared capillary.

NOTE: Do not use the capillary repeatedly since the tip of the injected capillary may become clogged with dirt because the larvae collected in the field lived in the mud.

3. siRNA/dsRNA injection

NOTE: The procedure is slightly different for damselflies (step 3.1) and dragonflies (step 3.2).

3.1. Injection to a Zygopteran (damselfly) larva (e.g., *I. senegalensis*).

3.1.1. Anesthetize a larva covered with a wet paper on crushed ice for 50-70 seconds (**Figure 3A-C**).

NOTE: Duration of ice-cold anesthesia depends on the condition of the larva; if the larva begins to move after 70 seconds of ice-cold anesthesia, an additional 70 seconds of ice-cold anesthesia is applied. For larvae that rarely move (e.g., *P. zonata*), this procedure is not essential.

3.1.2. Attach two pins on both sides of the prothorax and fix the larva to a fixed stand (e.g., a piece of Styrofoam) (**Figure 3D**).

NOTE: Adjust the position and number of pins, depending on the place of injection.

3.1.3. Stretch the inter-segmental membrane between the 7th and 8th abdominal segment for RNAi in the abdomen or between the prothorax and synthorax (fused mesothorax and metathorax) for RNAi in the thorax using hands and forceps.

3.1.4. Keep the inter-segmental membrane stretched by hand.

3.1.5. Insert the tip of the prepared capillary into the stretched inter-segmental membrane (**Figure 3D, 3F**).

3.1.6. Inject 1 µL of siRNA/dsRNA solution.

3.2. Injection to an Anisopteran (dragonfly) larva (e.g., *P. zonata*).

3.2.1. Wipe the water off the larval surface with a paper towel.

3.2.2. If needed, attach two pins on both sides of the prothorax and fix the larva to a fixed stand (e.g., a piece of Styrofoam) (**Figure 3H**).

NOTE: Adjust the position and number of pins, depending on the place of injection. In the case of *P. zonata*, it is not essential to fix the larvae with pins at this step.

3.2.3. Stretch the inter-segmental membrane between the 4th and 5th abdominal segment by hand.

3.2.4. Make a small hole with a fine needle in the inter-segmental membrane between the 4th and 5th abdominal segment.

NOTE: This procedure is necessary for many Anisopteran (dragonfly) species because the inter-segmental membrane is too hard to insert a glass capillary directly.

3.2.5. Insert the tip of the prepared capillary into the prepared hole (**Figure 3I**).

NOTE: As shown in **Figure 3H**, part of the dorsal side of the larvae corresponds to the ventral side of the adult, so the phenotype appears ventrally when treated as shown in **Figure 3H-J**.

3.2.6. Inject 1 µL of siRNA/dsRNA solution.

4. in vivo electroporation

4.1. If needed, add more pins to fix the larva to a fixed stand (e.g., a piece of Styrofoam).

4.2. Following the injection of the siRNA/dsRNA solution, apply two droplets of ultrasound gel on the larval surface using forceps.

4.3. Place electrodes on the ultrasound gel, with the positive electrode on the side injected with the siRNA/dsRNA solution and a negative electrode on the opposite side (**Figure 3E, 3G, 3J**).

NOTE: Do not directly touch the epidermis of the larva to avoid the burning effect of electroporation.

4.4. Generate 10-times electroporation pulses (280 ms/s each) using an electroporator.

NOTE: Adjust the voltage of electroporation, depending on the species, stages and tissues. In this study, 25 V was applied to *I. senegalensis* and 45 V to *P. zonata*.

4.5. Wipe off the remaining gel on the surface with a paper towel.

4.6. Keep the treated larvae resting on a wet paper towel for approximately one day for recovery and transfer them to a rearing case on the following day.

5. Site-specific phenotypic analysis

5.1. Keep *I. senegalensis* individually in a Petri dish (5 cm in diameter) containing approximately 10 mL of water and a piece of paper towel, and keep *P. zonata* in a plastic cage with a disposable non-woven mesh (eclosion cage[14]).

5.2. For *I. senegalensis*, after the larvae stop eating, move them individually into a plastic cage

with a disposable non-woven mesh.

NOTE: Do not put two or more larvae in the same cage. Otherwise, they will cannibalize each other immediately after they become adults.

5.3. After the emergence of the adult, observe and photograph the phenotype around the region where the positive electrode was placed for electroporation.

NOTE: The phenotype appears only in patches. Phenotypes are often difficult to recognize immediately after emergence due to incomplete pigmentation.

5.4. In order to examine the efficiency of RNAi (e.g., quantitative RT-PCR), if the phenotype is visible, dissect the region and compare it with the region without the phenotype.

NOTE: The levels of RNAi phenotype, namely size and location of the cuticle decolorization, often exhibit considerable variation between individuals (see **Figure 4**).

5.5. Keep the emerged adults in 100% EtOH for future analyses.

NOTE: Body color of Odonata species quickly fades after death, so it is important to store them in ethanol before they die. Ethanol sometimes discolors the insects, in such cases that the insects are frozen before they die.

REPRESENTATIVE RESULTS

We applied the above protocol to electroporation-mediated RNAi targeting *MCO2* gene and negative control genes (*EGFP* for siRNA and *bla* for dsRNA) (i) in the abdomen of *I. senegalensis* (**Figure 4**), (ii) in the thorax of *I. senegalensis* (**Figure 5**), and (iii) in the abdomen of *P. zonata* (**Figure 6**). The results of the RNAi experiments are summarized in **Table 1**. Because negatively charged siRNA/dsRNA is incorporated only into positively charged cells, RNAi phenotypes were observed around the region where the positive electrode was placed for electroporation.

In both *I. senegalensis* and *P. zonata*, inhibition of melanin pigmentation (i.e., black, brown, and reddish brown) appeared in patches around the region where the positive electrode was placed (white arrowheads and dotted lines in **Figures 4-6**) when *MCO2* RNAi was performed in combination with electroporation (**Table 1**), as previously reported in *N. pygmaea*[9]. By contrast, no phenotypic effects were observed around the electroporation site when the control gene was injected (*EGFP* siRNA or *bla* dsRNA) (**Figures 4-6, Table 1**). In addition, injecting the *MCO2* gene without electroporation had no effect on adult pigmentation (**Figure 4, Table 1**), indicating that electroporation is essential for RNAi in Odonata. It should be noted that the blue, green, and yellow colorations are not affected by the RNAi of *MCO2* gene that is involved in melanin synthesis in the cuticle, which plausibly reflect the fact that these body colors are attributed to pigment granules present in the epidermal cells that are visible through the transparent cuticle[26]. As shown in **Figure 4**, no remarkable phenotypic differences were recognized between the individuals subjected to siRNA treatment and dsRNA treatment, whereas considerable variation

in size and location of the RNAi phenotype was observed among different individuals subjected to the same RNAi treatment (ex. compare two examples of *IsMCO2* dsRNA in **Figure 4**).

To determine the developmental stage most suitable for the RNAi treatment, we compared the phenotypic consequences of the RNAi treatment at five morphological stages (stage A-E) in the final larval instar of *I. senegalensis* (**Figure 7A**). Inhibition of melanin pigmentation caused by *MCO2* RNAi was observed in all emerged adults when injected at the stages A and B (**Figure 7C**). When injected at the stages C and D, suppression of melanin pigmentation was certainly observed in some emerged adults, but other adults exhibited abnormal coloration caused by wounds (**Figure 7B**).

FIGURE AND TABLE LEGENDS

Figure 1: Electroporation-mediated RNAi methods in Odonata. A. *Ischnura senegalensis* (Coenagirionidae) as a representative damselfly species. **B.** *Pseudothemis zonata* (Libellulidae) as a representative dragonfly species. **C.** The overall scheme of the protocols. Blue and orange boxes indicate the protocols for *I. senegarensis* and *P. zonata*, respectively. The purple boxes indicate the common protocols applied to both species.

Figure 2: A representative result of restriction fragment length polymorphism (RFLP)-based species identification for *Ischnura* species. Arrowhead indicates the *I. senegalensis*-specific band. 1, 2, 4: *I. asiatica*, 3: *I. senegalensis*, M: 100-base pair ladder marker.

Figure 3: Electroporation-mediated RNAi method in Odonata. A-C. Ice-cold anesthesia of *I. senegalensis*. Arrowheads indicate a larva. **A.** Putting a larva on crushed ice with a wet paper. **B.** Magnified view of a larva on ice. **C.** A larva covered with a wet paper on ice. **D-G.** RNAi method for *I. senegalensis*. **D.** Injection into the thorax. **E.** Electroporation on the thorax. **F.** Injection into the abdomen. **G.** Electroporation on the abdomen. **H-J.** RNAi method for *P. zonata*. **H.** Making a small hole on the abdomen. **I.** Injection into the abdomen. **J.** Electroporation on the abdomen. Arrows indicate the point of making a hole or injection. +, -: Positive/negative side electrodes. Numbers indicate the abdominal segment.

Figure 4: Dorsal views of RNAi phenotypes on the abdomen of *I. senegalensis*. White arrowheads indicate the regions of suppressed melanization.

Figure 5: Lateral and dorsal views of RNAi phenotypes on the thorax of *I. senegalensis*. White arrowheads and dotted lines indicate the regions of suppressed pigmentation.

Figure 6: Ventral views of RNAi phenotypes in the abdomen of *P. zonata*. White arrowheads and dotted lines indicate the regions of suppressed melanization.

Figure 7: Stage dependent *IsMCO2* RNAi effects during the final larval instar of *I. senegalensis*. (A) Morphological changes in the compound eyes at five morphological stages (stage A–E) and the number of days to adult emergence in this study. Numbers in parentheses are from previous report[14]. (B) Abnormal pigmentation due to wounds. Arrowhead indicates electroporation site.

(C) The effect of RNAi at five morphological stages on adult pigmentation in *I. senegalensis*. The number on the bar indicates the number of individuals.

Table 1. The effect of RNAi on adult pigmentation in *I. senegalensis* and *P. zonata*. Results at stage A are shown in *I. senegalensis*. *IsMCO2*: *multicopper oxidase 2* gene of *I. senegalensis*, *EGFP*: *Enhanced green fluorescent protein* gene, *bla*: *beta lactamase* gene from pGEM-T Easy Vector, *PzMCO2*: *multicopper oxidase 2* gene of *P. zonata*. The results for the control genes represent the total number of experiments the authors have conducted to date.

DISCUSSION

Lethality of RNAi treatment

We found that the lethality of the RNAi treatment depends strongly on the rearing history and condition of the Odonata larvae. The larvae soon after collection in the field are generally healthy and exhibit low mortality rates after the electroporation-mediated RNAi treatment. By contrast, the larvae reared in the laboratory for a long period of time (e.g., one month) tend to suffer low success rates of adult emergence. In *I. senegalensis*, instead of the larvae collected in the field, the larvae reared in the laboratory from eggs can be used[14], but the success rates of RNAi using the laboratory-reared larvae tend to be considerably lower (many individuals died during metamorphosis) than those using the field-collected larvae. In addition, frequent larval feeding and clean water rearing are important for increasing the success rates of adult emergence and reducing the lethality of the RNAi treatment.

Efficiency of RNAi treatment

As described above, the levels of RNAi phenotype, namely size and location of the cuticle decolorization, often exhibited considerable variation between individuals subjected to the same RNAi treatment (e.g., **Figure 4**), but the levels of the phenotypic penetrance seem to be remarkably different between the Odonata species. The observed phenotypic regions were larger and more prominent in *I. senegalensis* (**Figures 4-5**) than in *P. zonata* (**Figure 6**) and *N. pygmaea*[9]. This difference may be due to the thickness of the cuticle on the larval surface, considering that the cuticle of *I. senegalensis* is thinner than the cuticle of *P. zonata* and *N. pygmaea*). As far as we examined, no clear difference was recognized between the effects of siRNA and dsRNA (**Figure 4, Table 1**).

Appropriate developmental stage for RNAi

It should be noted that proper larval staging is important for performing RNAi efficiently. Inhibition of adult pigmentation was caused by *MCO2* RNAi before the stage D (approximately 3 days before adult emergence), which is consistent with the previous report on *N. pygmaea*[9]. The RNAi phenotypes observed when injected at the stages C and D were less conspicuous than those treated at the stages A and B, which indicate that the stages C and D may be too late to sufficiently suppress the gene expression. The appropriate timing for RNAi treatment depends on the timing of gene expression, and *MCO2* gene exhibits transiently high expression during adult emergence[9], as in other insects[17,18]. In the stinkbug *Plautia stali*, RNAi knockdown of *MCO2* gene was observed from day 4 onwards after injection[27], which is consistent with the present results.

Our previous study on *I. senegalensis* showed that, after the stage B, days to adult emergence exhibit relatively small variation among the majority of final instar larvae, suggesting that the stage B may correspond to the onset of the process toward adult emergence, after which the developmental processes for metamorphosis proceed in a prefixed and coordinated manner[14]. Morphological abnormalities caused by wounds were often observed when the larvae were RNAi-treated at the stages C and D (**Figure 7B, 7C**). This is likely to be associated with a dramatic progression of metamorphosis during these stages, suggesting that RNAi treatment should be avoided from the stage C and on. In summary, we recommend that final instar larvae at the stage A or B (or at the stage before the larval wings expand significantly) should be used for RNAi experiments.

Usefulness and superiority of electroporation-mediated RNAi method

The conventional RNAi is a simple and powerful experimental method, but some insect lineages like butterflies[10], aphids[28] and dragonflies[9] exhibit low RNAi efficiency, for which establishment of gene function analysis is a major challenge. In this study, we found that electroporation-mediated RNAi can induce local gene suppression in dragonflies with almost 100% efficiency, at least in epidermis, if treated at appropriate developmental stages (**Table 1**). Recently, CRISPR/Cas9-based gene knockouts have been successfully applied to a variety of insects, providing a powerful molecular genetic tool for non-model organisms[29]. Here, however, we point out that CRISPR/Cas9 is certainly great but the electroporation-mediated RNAi method may be superior to CRISPR/Cas9 in some respects.

Firstly, in the electroporation-mediated RNAi method, the body region where RNAi phenotypes appear can be easily controlled experimentally by the position of the positive electrode upon electroporation. In addition, since the region where the gene expression is suppressed is limited around the region where the positive electrode was placed, the RNAi phenotypes can be easily compared with the control phenotypes side by side in the same individual. Second, compared to CRISPR/Cas9 method in which injected eggs have to be reared to adulthood to observe the knockout phenotypes, the electroporation-mediated RNAi is superior in that the gene knockdown phenotypes can usually be observed in much shorter time. For example, it takes three to four months for *I. senegalensis* and one to two years for *P. zonata* from eggs to adults[14,30]. However, in order to observe RNAi phenotypes within the adult epidermis, it takes less than one month from dsRNA injection into final instar larvae at the stage B to adult emergence for both *I. senegalensis* and *P. zonata* (**Figure 7**). Thirdly, the electroporation-mediated RNAi method entails dsRNA injection into large larvae, which is easier than microinjection into tiny eggs required for CRISPR/Cas9 method. In addition, the electroporation-mediated RNAi is applicable to insect species whose newly laid eggs are difficult to collect. For example, females of *P. zonata* lay eggs onto floating plants on water surface during flight, and thus it is difficult to collect their eggs both in the field and in the lab. Hence, we expect that this protocol may be generally applicable to non-model organisms in which the conventional RNAi method does not work efficiently.

ACKNOWLEDGMENTS

We thank Minoru Moriyama for technical advice and supports, Bin Hirota and Ryutaro Suzuki for collecting Odonata larvae, and Misa Shinya for helpful comments on the manuscript. This work

was supported by JSPS KAKENHI Grant Numbers JP18J21561 to GO and JP18H02491, JP18H04893, JP19H03287, and JP20H04936 to RF.

DISCLOSURES

The authors have nothing to disclose.

REFERENCES

1. Misof, B. et al. Phylogenomics resolves the timing and pattern of insect evolution. *Science*. **346** (6210), 763–767 (2014).

2. Wipfler, B. et al. Evolutionary history of Polyneoptera and its implications for our understanding of early winged insects. *Proceedings of the National Academy of Sciences of the United States of America*. **116** (8), 3024-3029 (2019).

3. Corbet, P. S. *Dragonflies, Behavior and Ecology of Odonata*. Cornell University Press, Ithaca, NY (1999).

4. Futahashi, R. Color vision and color formation in dragonflies. *Current Opinion in Insect Science*. **17**, 32–39 (2016).

5. Futahashi, R. Diversity of UV reflection patterns in Odonata. *Frontiers in Ecology and Evolution*, **8**, 201 (2020).

6. Córdoba-Aguilar, A. Dragonflies and damselflies. Model organisms for ecological and evolutionary research. Oxford University Press, Oxford (2008).

7. Bybee. S, et al. Odonata (dragonflies and damselflies) as a bridge between ecology and evolutionary genomics. *Frontiers in Zoology*. **13**, 46 (2016).

8. Linz, D. M., Clark-Hachtel, C. M., Borràs-Castelles, Tomoyasu, Y. Larval RNA Interference in the Red Flour Beetle, *Tribolium castaneum*. *Journal of Visualized Experiments*. **92**, 52059 (2014).

9. Okude, G. et al. Electroporation-mediated RNA interference reveals a role of multicopper oxidase 2 gene in dragonfly's cuticular pigmentation. *Applied Entomology and Zoology*. **53** (3), 379–387 (2017a).

10. Terenius, O. et al. RNA interference in Lepidoptera: An overview of successful and unsuccessful studies and implications for experimental design. *Journal of Insect Physiology*. **57**, 231–245 (2011).

11. Ando, T., Fujiwara, H. Electroporation-mediated somatic transgenesis for rapid functional analysis in insects. *Development*. **140**, 454–458 (2013).

12. Nishikawa, H. et al. A genetic mechanism for female-limited Batesian mimicry in *Papilio*

butterfly. *Nature Genetics*. **47** (4), 405-409 (2015).

13. Osanai-Futahashi, M. et al. Positional cloning of a *Bombyx pink-eyed white egg* locus reveals the major role of *cardinal* in ommochrome synthesis. *Heredity*. **116** (2), 135-145 (2016).

14. Okude, G., Futahashi, R., Tanahashi, M., Fukatsu, T. Laboratory rearing system for *Ischnura senegalensis* (Insecta: Odonata) enables detailed description of dragonfly's larval development and morphogenesis. *Zoological Science*. **34** (5), 386–397 (2017b).

15. Ozono, A., Kawashima, I., Futahashi, R. *Dragonflies of Japan* (3rd edition). Bunichi-Sogo Syuppan, Co Ltd, Tokyo (2017).

16. Ozono, A., Kawashima, I., Futahashi, R. *The Handbook of Japanese Aquatic Insects. Volume 3: Dragonfly larvae*. Bunichi-Sogo Syuppan, Co Ltd, Tokyo (2019).

17. Arakane, Y., Noh, M. Y., Asano, T., Kramer, K. J. Tyrosine metabolism for insect cuticle pigmentation and sclerotization. in *Extracellular Composite Matrices in Arthropods* (eds Cohen, E. & Moussian, B.) 165-220, Springer (2016).

18. Asano, T. et al. Mini-review an insect-specific system for terrestrialization: Laccase-mediated cuticle formation. *Insect Biochemistry and Molecular Biology*. **108**, 61–70 (2019).

19. Futahashi R., Okude G., Sugimura M., Ugai S. Interspecific hybrids in Japanese Odonata. *Tombo*. **60**, 1–49 (2018).

20. Okude, G., Fukatsu, T., Futahashi, R. Interspecific crossing between blue-tailed damselflies *Ischnura elegans* and *I. senegalensis* in the laboratory. *Entomological Science*. **23** (2), 165–172 (2020).

21. Naito, Y., Yoshimura, J., Morishita, S., Ui-Tei, K. siDirect 2.0: updated software for designing functional siRNA with reduced seed-dependent off-target effect. *BMC Bioinformatics*. **10**, 392 (2009).

22. Yamaguchi, J., Mizoguchi, T., Fujiwara, H. siRNAs induce efficient RNAi response in *Bombyx mori* embryos. *PLoS ONE*. **6** (9), e25469 (2011).

23. Untergasser, A. et al. Primer3--new capabilities and interfaces. Nucleic Acids Research. **40** (15), e115 (2012).

24. Futahashi, R. Whole-mount *in situ* hybridization of sectioned tissues of species hybrids to detect *cis*-regulatory changes in gene expression pattern. *Methods in Molecular Biology*. **772**, 319–328 (2011).

25. Matsuura, Y., Kikuchi, Y., Miura, T., Fukatsu, T. Ultrabithorax is essential for bacteriocyte

development. *Proceedings of the National Academy of Sciences of the United States of America*. **112** (30), 9376–9381 (2015).

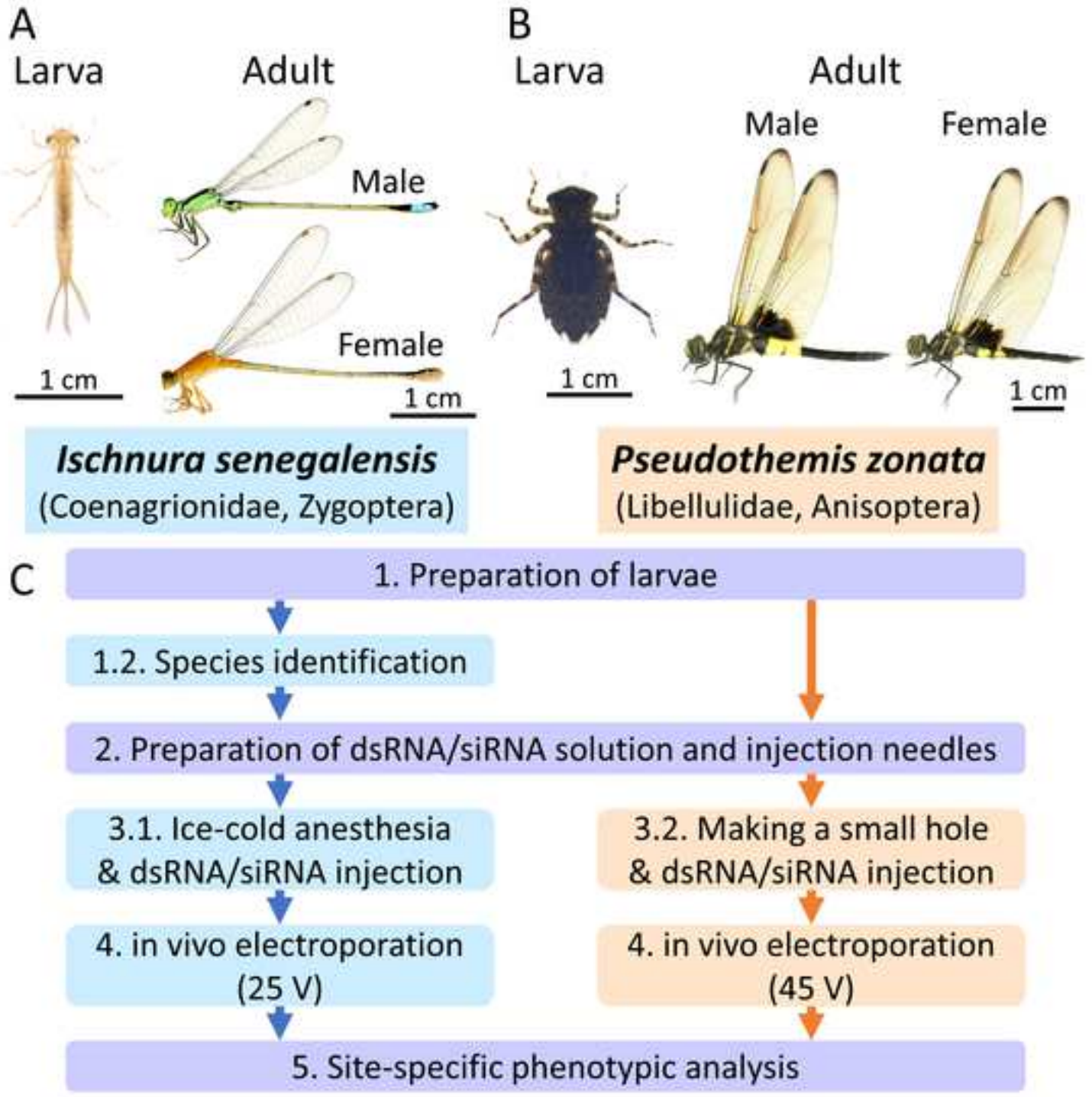
26. Henze, M. J., Lind, O., Wilts, B. D., Kelber, A. Pterin-pigmented nanospheres create the colours of the polymorphiic damselfly *Ischnura elegans*. *Journal of The Royal Society Interface*. **16**, 20180785 (2019).

27. Nishide, Y. et al. Diversity and function of multicopper oxidase genes in the stinkbug *Plautia stali*. *Scientific Reports*. **10** (1), 3464 (2020).

28. Christiaens, O., Swevers, L., Smagghe, G. DsRNA degradation in the pea aphid *(Acyrthosiphon pisum)* associated with lack of response in RNAi feeding and injection assay. *Peptides*. **53**, 307–314 (2014).

29. Sun, D., Guo, Z., Liu, Y., Zhang, Y. Progress and prospects of CRISPR/Cas systems in insects and other arthropods. *Frontiers in Physiology*. **8**, 608 (2017).

30. Miyakawa, K. A study of the life-history of *Pseudothemis zonata* (Burm.) (Odonata, Libellulidae) II. Immature stage. *Kontyû*. **37** (4), 409–422 (1969).



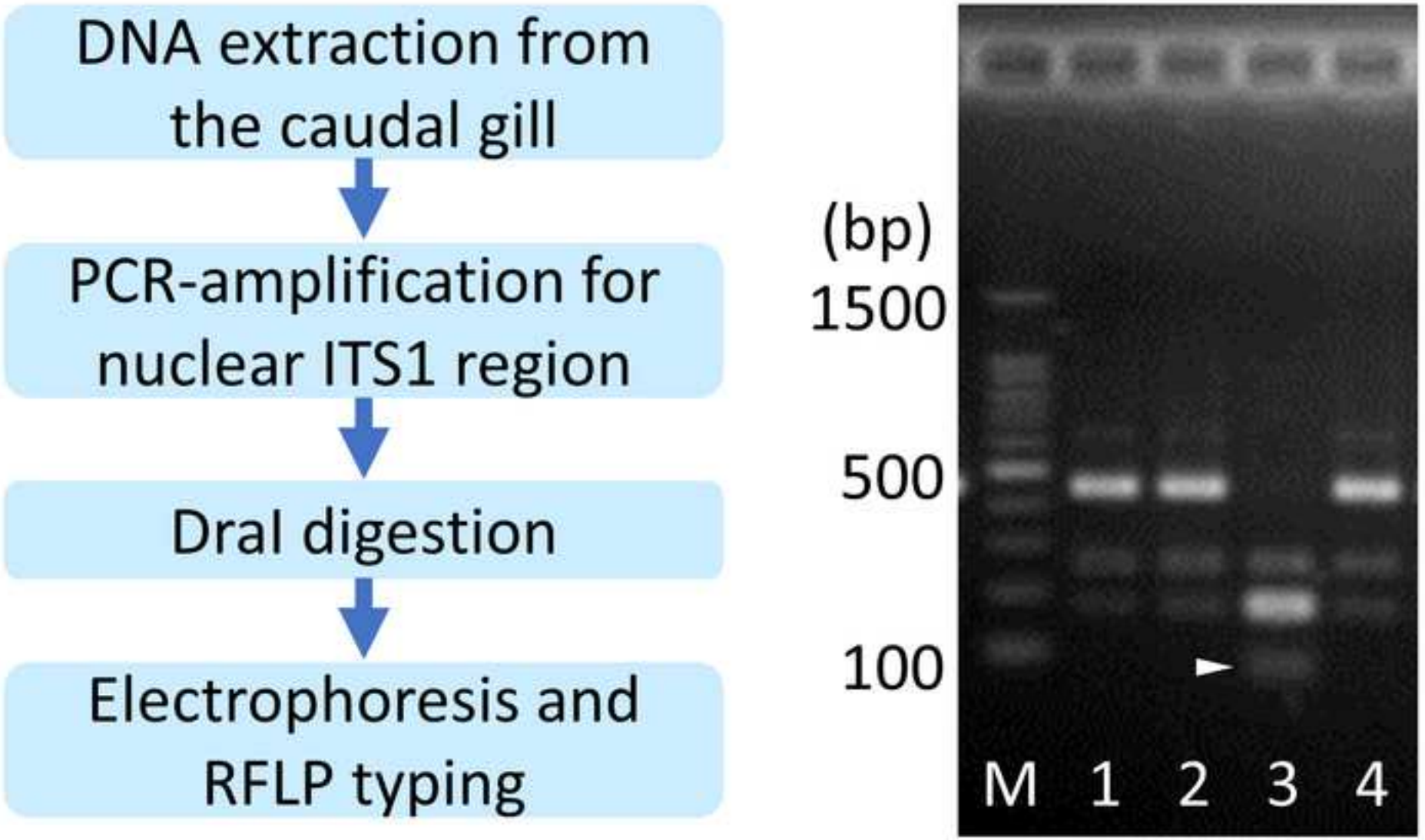


Figure3

Click here to access/download;Figure;Fig3.TIF

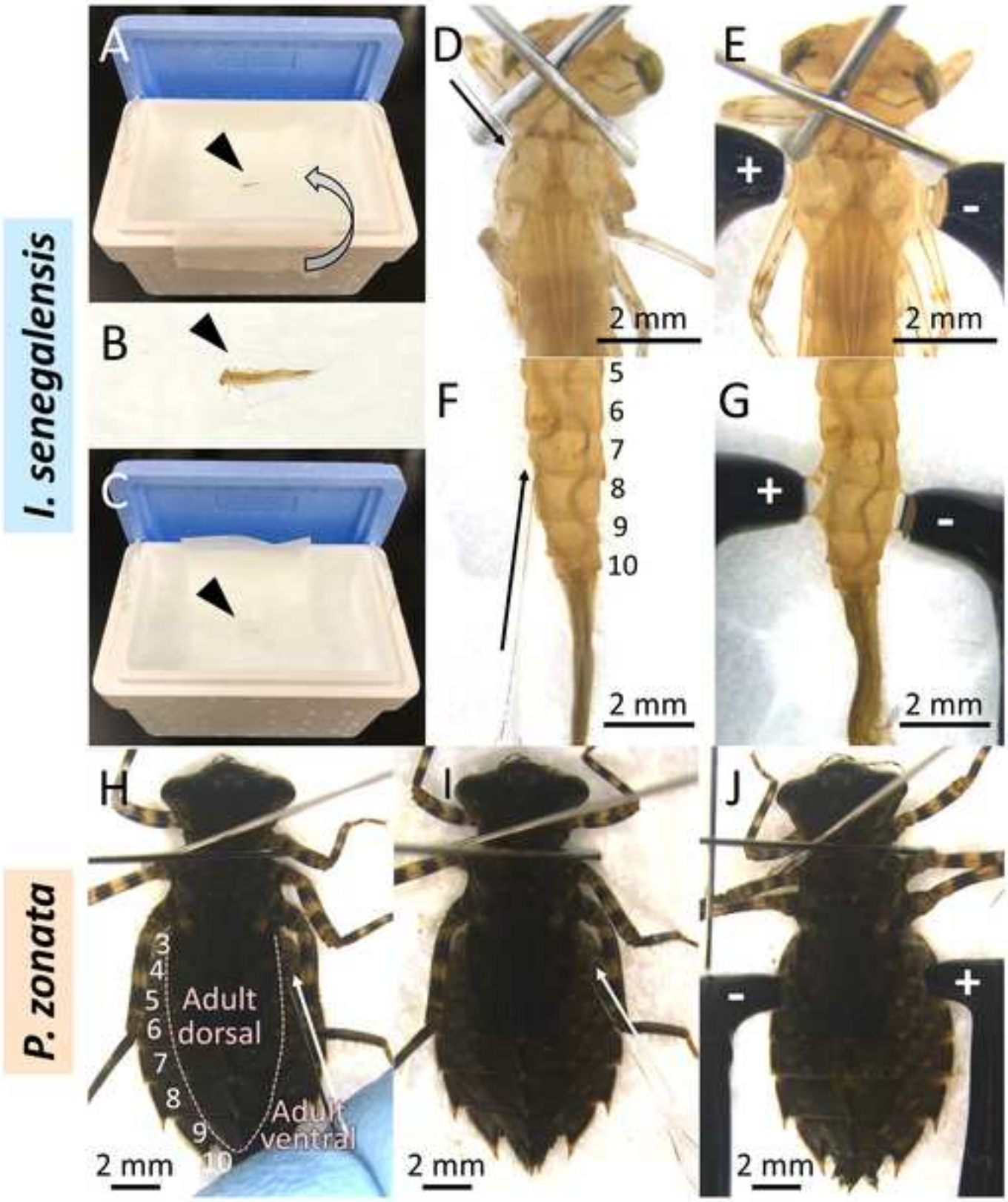
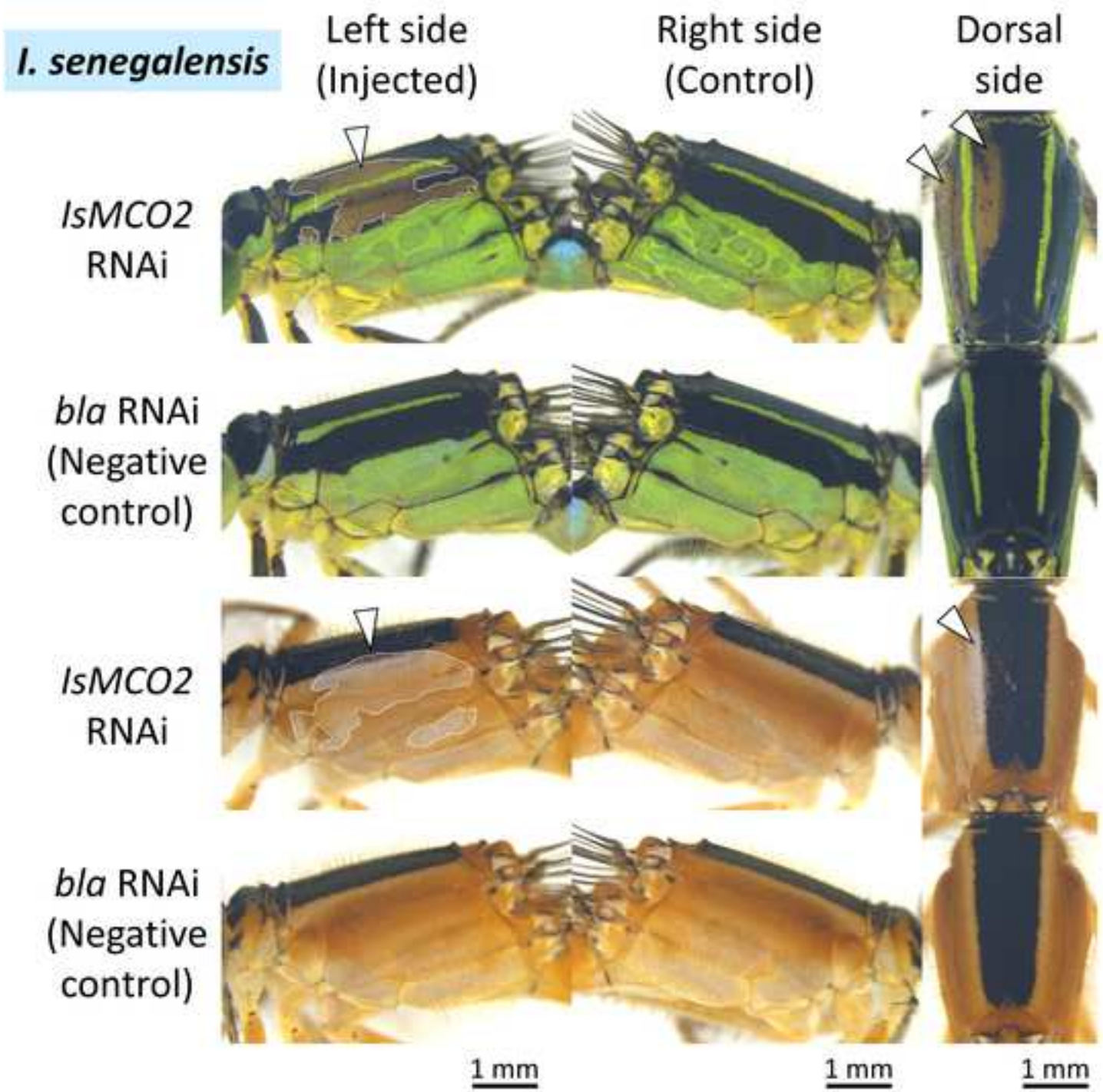


Figure4

Click here to access/download;Figure;Fig4.TIF

Figure5

Click here to access/download;Figure;Fig5.TIF



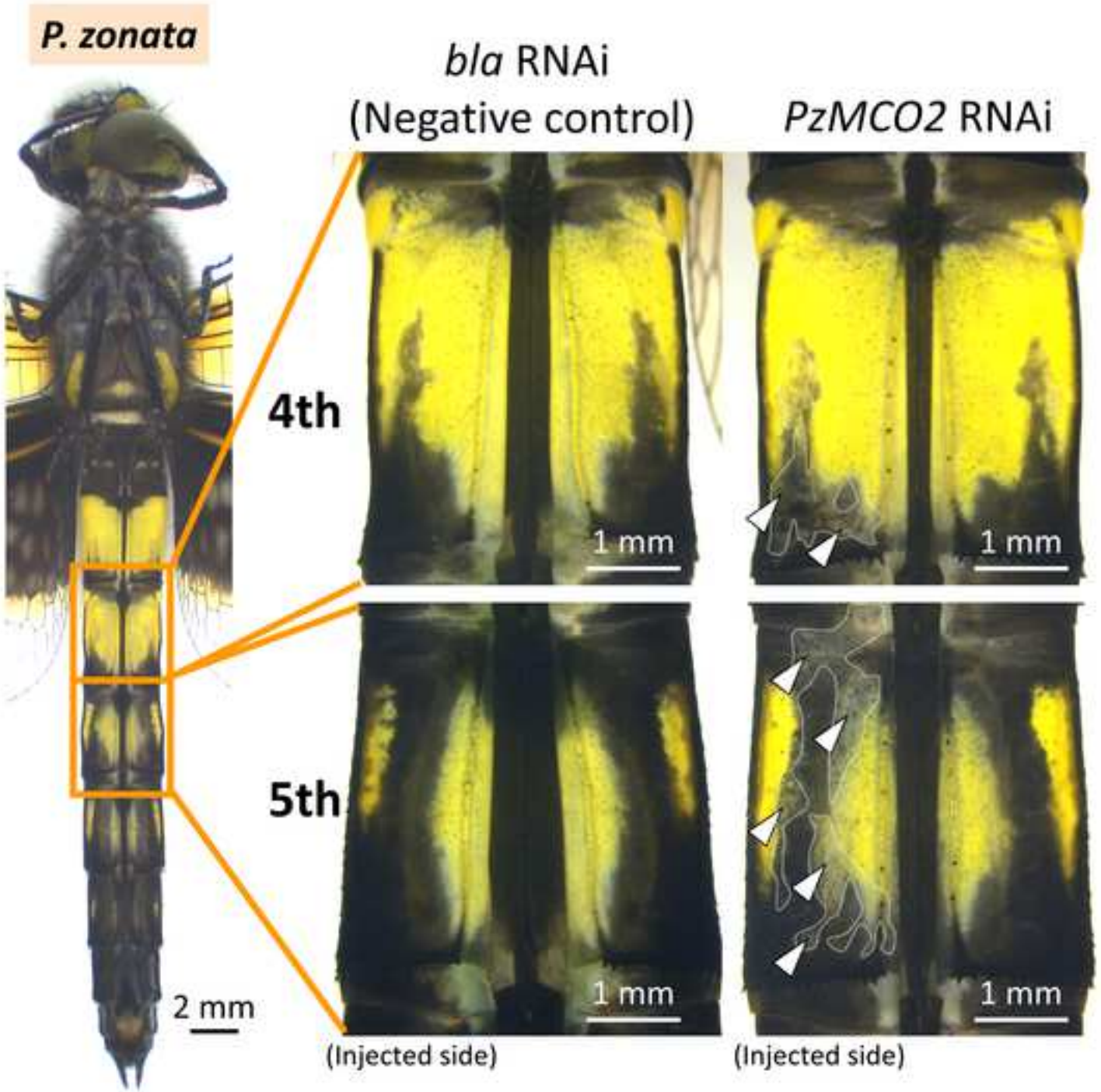


Figure7

Click here to access/download;Figure;Fig7.TIF

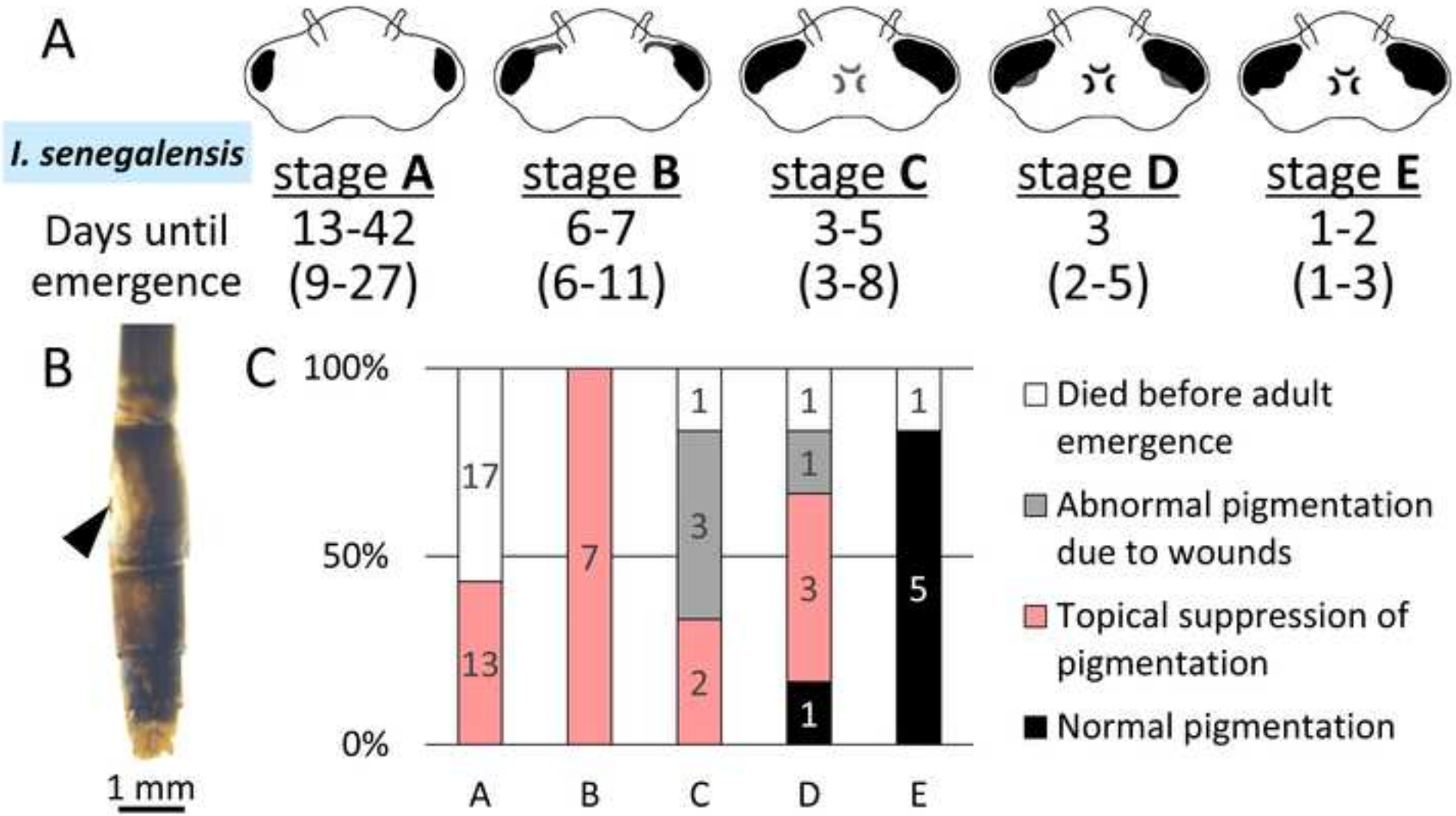


Table1

Species	*I.senegalensis*							*P.zonata*		
Injected region	Abdomen					Thorax		Abdomen		
siRNA/dsRNA	siRNA		dsRNA			dsRNA		dsRNA		
Target gene	*IsMCO2*	*EGFP*	*IsMCO2*	*IsMCO2*	*bla*	*IsMCO2*	*bla*	*PzMCO2*	*PzMCO2*	*bla*
Electroporation	+	+	+	-	+	+	+	+	-	+
Injected larvae	22	25	30	6	53	12	20	17	5	9
Emerged adults	7	6	13	4	40	11	14	11	2	5
Adults with less pigmented regions (ratio)	7 (100%)	0 (0%)	13 (100%)	0 (0%)	0 (0%)	10 (91%)	0 (0%)	11 (100%)	0 (0%)	0 (0%)

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
12-well plate	Violamo	VTC-P12	For rearing larvae before injection
Brine shrimp eggs	JAPAN PET DESIGN	4975677012396	
Calibrated micropipette (1-5 µL)	Drummond	2-000-001-90	
Deposit pestle 1.5 mL	Thermo Fisher Scientific	K749520-0090	
Digital high defenition microscope camer	Leica Microsystems	MC170HD	
Disposable non-woven mesh	HAKUGEN EARTH	DSC-105A	
DNA Ligation Kit Ver.2.1	Takara Bio	6022	
DraI	Takara Bio	1037A	
Electrode (1mmϕ)	NEPAGENE	CUY650P1	
Electroporator	CellProduce	Cure-gene	
Forceps	KOWA Forceps Industry	K-10 No1	
Glass needle puller	NARISHIGE	PN-3	
Hand mixer	AS ONE	1-229-02	
Hand net	HOGA	IS33-3B	
HEPES	FUJIFILM Wako Pure Chemical	346-01373	For injection buffer
Insect pin capitate No. 3	Shiga Konchu Fukyusha	N230	For fixing larvae
KCl	FUJIFILM Wako Pure Chemical	163-03545	For PBS
KH_2PO_4	FUJIFILM Wako Pure Chemical	169-04245	For PBS
KOAc	FUJIFILM Wako Pure Chemical	160-03175	For injection buffer
KOH	FUJIFILM Wako Pure Chemical	168-21815	For injection buffer
Laboratory Jack (150x150)	AS ONE	1-4642-11	For adjusting the position of the manipulator
LOGIQLEAN Gel for Ultrasound Hard type	GE Healthcare	2369385	
Manipulator	Muromachi Kikai Co., Ltd.	SJ-1	For adjusting the position of the injector and the capillary
MEGAscript RNAi kit	Thermo Fisher Scientific	AM1626	
$Mg(OAc)_2$	FUJIFILM Wako Pure Chemical	130-00095	For injection buffer
Na_2HPO_4	FUJIFILM Wako Pure Chemical	197-02865	For PBS
NaCl	FUJIFILM Wako Pure Chemical	191-01665	For PBS
Petri dish (5 cm diameter)	Iwaki	1010-060	For rearing injected larvae
Pneumatic Injector	NARISHIGE	IM-12	
pT7Blue T-Vector	Novagen	69820	
QIAquick PCR Purification Kit	QIAGEN	28106	
RNAiso Plus	FUJIFILM Wako Pure Chemical	9109	
RNeasy Mini Kit	QIAGEN	74106	
Shiga micro insect pin with stainless heac	Shiga Konchu Fukyusha	N251	For making a small hole
Stereoscopic microscope	Leica Microsystems	S8APO	
SuperScript IV Reverse Transcriptase	Thermo Fisher Scientific	18090010	
TaKaRa Ex Taq	Takara Bio	RR001B	For PCR-amplification from plasmid
Tks Gflex DNA Polymerase	Takara Bio	R060B	For PCR-amplification from caudal gill

Dear Editor,

Here we submit a revised version of our manuscript. We carefully read and addressed all the comments made by the reviewers.

Editorial and production comments.

[1. Please the black bars on the side and use the full screen resolution. Please do not use pillar-boxing (https://en.wikipedia.org/wiki/Pillarbox).
2. Cut down some of the time between the steps of the protocol, such as the gaps between injection and applying ultrasound gel at 3:16 through 3:37 and 6:06 through 6:39, and the time between pinning and applying gel at 10:03 through 10:17]

We revised the video as you suggested.

Reviewer 1.

[Page 8 of 6 Line #367 may want to add NOTE: "Ethanol will fade other color pigments of insects, green, yellow, red, so freeze insects prior to death IF your study is targeting those pigment colors"]

We added the following sentences in Line 374-375.
“Ethanol sometimes discolors the insects, in such cases that the insects are frozen before they die.”

Reviewer 2.

[Please discuss why only the position of the positive electrode upon electroporation have the RNAi phenotypes!]

We added the following sentences in Line 382-384.
“Because negatively charged siRNA/dsRNA is incorporated only into positively charged cells, RNAi phenotypes were observed around the region where the positive electrode was placed for electroporation.”

[Figure 7 should move forward as Fig. 2.]
We deleted “Figure 7A” in Line 172.

We hope that the revised version of the manuscript will be considered for publication in *Journal of Visualized Experiments.*

Yours sincerely,

January 14, 2021

Ryo Futahashi
Genta Okude

National Institute of Advanced Industrial Science and Technology (AIST)
Tsukuba 305-8566, JAPAN
E-mail: ryo-futahashi@aist.go.jp Tel & Fax: 81-29-861-6812