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# Pupal and Adult Injections for RNAi and CRISPR Gene Editing in *Nasonia vitripennis*

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**Citation**

Benetta, E.D., Chaverra-Rodriguez, D., Rasgon, J.L., Akbari, O.S. Pupal and Adult Injections for RNAi and CRISPR Gene Editing in *Nasonia vitripennis*. *J. Vis. Exp.* (), e61892, doi:10.3791/61892 (2020).

**Date Published**

November 11, 2020

**DOI**

10.3791/61892

**URL**

jove.com/t/61892

## Abstract

The jewel wasp, *Nasonia vitripennis*, has become an efficient model system to study epigenetics of haplo-diploid sex determination, B-chromosome biology, host-symbiont interactions, speciation, and venom synthesis. Despite the availability of several molecular tools, including CRISPR/Cas9, functional genetic studies are still limited in this organism. The major limitation of applying CRISPR/Cas9 technology in *N. vitripennis* stems from the challenges of embryonic microinjections. Injections of embryos are particularly difficult in this organism and in general in many parasitoid wasps, due to small embryo size and the requirement of a host pupa for embryonic development. To address these challenges, Cas9 ribonucleoprotein complex delivery into female ovaries by adult injection, rather than embryonic microinjection, was optimized, resulting in both somatic and heritable germline edits. The injection procedures were optimized in pupae and female wasps using either ReMOT Control (Receptor-Mediated Ovary Transduction of Cargo) or BAPC (Branched Amphiphilic Peptide Capsules). These methods are shown to be effective alternatives to embryo injection, enabling site-specific and heritable germline mutations.

## Introduction

CRISPR/Cas9 gene editing is a powerful technology for functional genetic studies, especially in many rising model organisms such as the jewel wasp, *Nasonia vitripennis*. The ease of rearing and the availability of a complete genome makes the jewel wasp an important experimental system for elucidating the molecular mechanisms of different biological processes. For example, *N. vitripennis* has recently been used to unravel the epigenetic basis of the haplodiploid sex determination system[1,2], the biology of B-chromosomes[3,4,5,6], and the genetic basis for circadian and seasonal regulation[7,8]. Some of the features that make *N.vitripennis* amenable to work with include short generation time (~2 weeks at 25 °C), high reproduction rates, easy sex separation at the pupal stage, and the ability to diapause and store strains at 4 °C. The life cycle begins with female wasps parasitizing the pupae of the blowfly, *Sarcophaga bullata*.

Through their ovipositor, females lay up to 50 eggs in the pupal case of the blowfly. Eggs develop into larvae that feed on the *S. bullata* pupa, continue to develop over the next several days, and then pupate, followed by adult eclosion and emergence from the host puparium[9].

Molecular tools to perform functional genetic studies in *N. vitripennis*, such as RNA interference (RNAi)[10] and CRISPR/Cas9[11,12], are available, but are limited, primarily due to difficulties in performing embryonic microinjections[13]. As *N. vitripennis* eggs require a pupal host for development, egg manipulation is very challenging. Pre-blastoderm stage embryos must be collected from the host blowfly pupae, quickly microinjected, and immediately transferred back to the host for development[13]. These steps require precision and specialized training to avoid damaging the microinjected embryos or the pupal hosts[13]. Moreover, the eggs are very small and fragile, especially after microinjections, with a very viscous cytoplasm causing a continuous clogging of the injecting needle[13]. These features make embryonic microinjections exceptionally challenging, requiring highly trained operators and specialized equipment that is absent in most *N. vitripennis* laboratories.

The optimization of alternative injection methods for the delivery of CRISPR reagents would contribute to the consolidation of *N. vitripennis* as a model organism. The manipulation of pupae and adults is less challenging than manipulating embryos and can be accomplished with a basic injection setup. Here, two protocols are described for injection of pupae and adults: one involving specialized equipment for injections, and the other involving the use of an aspirator tube assembly fitted with a glass capillary needle. The use of an aspirator tube is particularly suited for laboratories that do not have access to specialized equipment for embryo microinjections. Efficient injections of different developmental stages of *N. vitripennis*, including white or black pupae and adult wasps, are demonstrated. Wasps at the white pupal stage are particularly suited for RNAi-mediated knockdown experiments. Although RNAi in *Nasonia* was first described by Lynch and Desplan in 2006[10], there is no visual procedure available for how RNAi injections are performed. RNAi was recently used to discover the *haploidizer* gene of the B-chromosome PSR (paternal Sex Ratio)[3] and to study the involvement of the clock gene, *period*, in *N. vitripennis* biological rhythms[7].

Black pupae and adult wasps can be used to induce CRISPR/Cas9 germline gene editing using ReMOT Control (Receptor-Mediated Ovary Transduction of Cargo) and BAPC (Branched Amphiphilic Peptide Capsules) protocols. These two ovary delivery methods have been recently described to be effective in *Nasonia* for generating germline mutations in the target gene, *cinnabar*[7,12]. Here, a simplified protocol is provided for injections including a visual procedure of a step-by-step methodology for both pupal and adult injections that can be utilized to generate functional genetics studies in *Nasonia* and likely in other parasitoid wasps, without requiring specialized equipment and bypassing embryonic microinjections.

## Protocol

### 1. Nasonia rearing

1. Set up ~20 mated females singularly in small glass test tubes plugged with cotton.
   NOTE: To reduce rearing space, 10 x 85 cm, 4 mL tubes are optimal. Females are easily distinguished from males because of the larger wings and the presence of the

ovipositor (**Figure 1A**). Detailed protocols for rearing *N. vitripennis* can also be found in other literature[13,14,15].

1. Add two *S. bullata hosts* per tube, and maintain the wasps at 25 ± 1 °C and 30% relative humidity, with a 12:12 light-dark cycle for 2-3 days.

   NOTE: Wasps can be maintained at lower temperature or room temperature; however, development will be slowed down.

2. After 2-3 days, with the help of a fine-tip paint brush, gently remove the females to avoid continuous oviposition and asynchrony in offspring development.

   NOTE: Removed females can optionally be re-hosted or stored at 5 °C for up to a month.

1. Maintain the parasitized host at 25 °C and 30% relative humidity, with a 12:12 light-dark cycle for 7 days if the desired injection stage requires white pupae; for 13 days if the required developmental stage is black pupae; or 14 days for young, newly emerged adults.

   NOTE: Yellow and black pupae can also be stored at 5 °C for up to one week. Longer storage will increase the frequency of diapause in larvae for the next generation, making the post-injection screening and establishment of mutant lines more difficult.

2. Crack open the host puparium with a dissecting needle to recover *N. vitripennis* pupae and adults at the desired stage (**Figure 1A**).

   NOTE: For injections at a particular adult age or for collecting virgin females, it is recommended to remove the dark pupae on day 13 from the host, separate by sex, and collect adults after emergence.

## 2. Alignment of white and black pupae

1. Prepare a glass slide by applying a line of school glue at the center. Spread the glue with a dissecting needle to obtain a thick layer (**Figure 1B**), which will be used to align the pupae. Let the glue dry for ~2 min before transferring the pupae.

   NOTE: Do not overdry the glue, otherwise the pupae will not be properly attached to the slide and will slip over during the injections.

2. Under a dissecting microscope, using the dissecting needle, apply glue on the back of the head of the pupa.

3. Attach the pupa to the glue layer on the slide with its abdomen facing up. Align 20-30 pupae side by side on the slide (**Figure 1C**).

   NOTE: Avoid touching the abdomen with the glue, and avoid submerging the pupae into the glue; otherwise, the adults will not be able to emerge.

4. Place the slide with the pupae in a Petri dish to let the glue dry for ~10 min. Before starting the injections, test the adherence of the pupae to the glue by pushing them gently with the dissecting needle. If most of the pupae are loosened, prepare a new slide. Alternatively, remove all the pupae that are loose, and proceed to inject those that are properly attached to the slide.

## 3. Needle preparation

1. Load one capillary glass tube into a needle puller, and pull needles following the manufacturer's instructions.

   NOTE: A P-1000 platinum filament needle puller and aluminum silicate glass capillaries are used in this demonstration. The operation manual for this instrument (see **Table of Materials**) explains how to properly load

capillary glass tubes and set up programs on this instrument. Use the following parameters (**Heat: 536; Pull: 80; Vel: 100; Delay: 70**) on aluminum silicate glass capillaries to inject yellow pupae, black pupae, and adults. In addition, the P-2000 needle puller was used to prepare quartz needles following the parameters (**Heat: 805; Pull: 145; Vel: 50; Delay: 145**). The manual provides instructions for loading capillary quartz tubes and setup of programs in this instrument. In the absence of a puller, customizable capillary glass is available.

2. Load the needle with 5 µL of the injection mixture (prepared in advance following previous protocols for RNAi[10] or CRISPR/Cas9 ribonucleoprotein[12] (RNP) injections in *N. vitripennis*) usingmicroloader pipette tips.
   NOTE: During the demonstration, the needle is loaded with double-stranded RNA (dsRNA) combined with red dye (see **Table of Materials**) for injection of white stage pupae and with BAPC-Cas9-sgRNA for injection of black pupae wasps and adults. These reagents will target the *cinnabar* gene. Wasps that are successfully knocked down/out will show red eyes instead of the wild type brown eyes.
3. Open the needle, sliding the tip on a surface made from two overlapping slides (**Figure 1D**). Alternatively, open the tip of the needle with a pair of fine forceps to create a sharp edge or by slowly piercing the thorax or abdomen of the wasp.

## 4. Pupal injection with femtojet

1. Place the slide with the aligned pupae under a dissecting microscope. Insert the needle into the femtojet's injection tube and tighten the grip head.
2. Looking at the needle under the microscope, turn on the femtojet and set up Pc and Pi injection parameters by rotating the rotary knobs.
   NOTE: A Pc value of 600 hPa is recommended for a continuous flow of the liquid. The Pc value depends on the opening of the needle. For needles prepared using the indicated parameters in the P-1000 needle puller, Pc values between 500 hPa and 700 hPa produce a constant flow. If lower values of Pc are required, this indicates that the opening may be too large and could damage the insects, whereas higher values indicate that the needle is still closed, or that the opening is too small.
3. Carefully insert the needle between the 2 and 3 visible abdominal segments with a vertical angle of about 30° (**Figure 1E**). Inject with a continuous flow until the whole abdomen turns pink in the case of white-stage pupae, or until the abdomen increases in size in the case of black pupae. Stop injecting when it is clear that the abdomen cannot take any more liquid, or when the liquid starts flowing out of the body. Move carefully to the next pupa and repeat these steps.
   NOTE: Avoid touching and damaging the ovipositor with the needle during injection.
4. Transfer the slide with the injected pupae to a Petri dish containing a paper towel soaked with deionized water to maintain humidity. Cover the dish with its lid, and place it at 25 °C until wasp emergence (**Figure 1F**).

## 5. Pupal injection with aspirator tube

1. Calculate the amount of injection mix using the factor: 4 pupae injected/1 µL solution.

NOTE: A typical injection of 20-30 pupae requires ~ 5-8 µL of ribonucleoprotein (RNP) complex-saponin mix or RNP with BAPC[12].

2. Align the pupae, as suggested in section 2, and place one slide with the aligned pupae under a dissecting microscope (**Figure 1E**).

3. Take one of the capillary needles and break the tip between two glass slides, as indicated in step 3.4 (**Figure 1D**). Ensure that the needle tip is open enough to allow the injection solution to go out by blowing air with the mouth, but not too open to avoid losing liquid and damaging the pupae (**Figure 1E**).
   NOTE: This step is critical because the user will be using air from the mouth to push the injection mixture into the insect hemolymph. It is better to practice so as to ascertain what type of needle aperture is optimal for the viscosity of a particular solution mix. Five sets of injections of 20 pupae per set may be enough to get used to the mouth injection system.

4. Load one needle with the ribonucleoprotein solution[12] using a microloading tip, and insert the needle into the connector pack of the aspirator tube assembly.
   NOTE: During the demonstration, the needle is loaded with BAPC-Cas9-sgRNA targeting the *Nasoniacinnabar* gene[12].

5. Carefully insert the needle between the 2 and 3 visible abdominal segments with a vertical angle of about 30° (**Figure 1E**). Inject with a continuous flow until the whole abdomen turns pink in the case of white-stage pupae or until the abdomen increases in size in the case of black pupae. Stop injecting when it is clear that the abdomen cannot take any more liquid or when the liquid starts flowing out of the body. Move carefully to the next pupa and repeat these steps
   NOTE: Avoid touching and damaging the ovipositor with the needle during injection.

6. Transfer the slide with the injected pupae to a Petri dish containing a paper towel soaked with deionized water to maintain humidity. Cover the dish with its lid and place it at 25 °C until wasp emergence (**Figure 1F**).

## 6. Adult injection with aspirator tube

1. For adult preparation, separate groups of 20 virgin females in a clean small test tube with a fine paintbrush. Place the tube on ice for 5 min until the females are anesthetized. Alternatively, females can be anesthetized and injected using $CO_2$.
   NOTE: Ice is preferable as an anesthetic as adult wasps are cold-tolerant and recover easily after the injections. Females can take up more injected liquid than the pupae: 1 µL of solution can be used for three females instead of for four pupae. Prepare volumes accordingly.

2. Prepare an ice bucket, placing one glass slide on top of the ice. Align the females side by side on the cold slide with abdomens facing up (**Figure 1E**) under a dissecting scope.

3. Load one needle with the ribonucleoprotein solution[12] using a microloading tip, and insert the needle into the connector pack of the aspirator tube assembly. Support the females from the opposite side with blunted dissecting needles while injecting slowly into the abdomen from the other side. Avoid touching the ovipositor, this will severely damage this critical reproductive structure (**Figure 1E**).
   NOTE: Either orientation is acceptable, depending on operator preference.

4. Carefully insert the needle between the 2 and 3 visible abdominal segments with a vertical angle of about 30° (**Figure 1E**). Inject the solution into the female abdomen, stopping when no more liquid can enter, or when leaking of surplus solution is observed. Move carefully to the next pupa and repeat these steps

   NOTE: Inject slowly, leaving the needle inside the abdomen for about 3 s before removing it very slowly. This will help to adjust the internal liquid pressure and avoid solution leaking from the injection site following needle removal.

5. When finished, gently transfer single injected females to a new tube with one host using a paintbrush. Leave to recover at room temperature for approximately 1 h, confirming survival, and then return the tubes to the rearing incubator.

   NOTE: During the demonstration, the needle is loaded with BAPC-Cas9-sgRNA targeting the *Nasoniacinnabar* gene[12].

## 7. Post-injection care and mutant screening

1. After adult eclosion from the injected pupae, place single wasps into a glass tube plugged with cotton and insert one *S. bullata* host (**Figure 1G**). In contrast, place injected females in individual tubes with hosts immediately after injection.

2. For CRISPR/Cas9 knockout experiments, allow females to parasitize the hosts for one day, and replace the host each day for three consecutive days.

   NOTE: Owing to the haplodiploid sex determination system in *N. vitripennis*[16], virgin females will produce haploid male broods, which facilitates the detection of mutations. For knockdown via RNAi of male-specific genes, the injected individuals can be singularly mated with wild type individuals and allowed to parasitize hosts. Use one host per day, and replace the hosts based on the experimental setup.

3. Place the parasitized hosts at 25 °C until emergence of the G0 male offspring (for ~13-14 days).

4. Under a dissecting microscope, screen G0 males for the mutated phenotypes. The *cinnabar* gene is responsible for eye pigmentation[12]: wasps with brown eyes are wild type, and wasps with bright red eyes or variations between red and brown eyes are mutants (**Figure 1H**).

## 8. Post-injection crosses and rearing

1. Place all the mutant G0 males with red eyes (the phenotypically disrupted *cinnabar*$^{12}$ gene) with wild type virgin females for 1-2 days (**Figure 2A**).

   NOTE: If the disrupted gene does not confer a visible phenotype, polymerase chain reaction (PCR) followed by sequencing of the target gene is required to identify the mutant animals. Before obtaining the DNA from G0 males for PCR, it would be ideal to mate them with wild type virgin females. Alternatively, extract DNA from a leg of a G0 male to identify mutants, and mate only those with verified mutations.

2. Add two *S. bullata* hosts per female, and allow to oviposit at 25 °C and 30% relative humidity with a 12:12 light-dark cycle. Replace the hosts every 2-3 days.

   1. Store parasitized hosts at 25 °C and 30% relative humidity, with a 12:12 light-dark cycle for ~10 days.

   2. After ~10 days, crack open the parasitized host with a dissecting needle, and remove each *N. vitripennis* pupa from the host.

3. With the help of a fine-tip paintbrush, select female pupae (**Figure 1A**).

   NOTE: Females are diploid and heterozygous, and thus the eye color will be wild type.

3. Place 15-20 female pupae in glass tubes at 25 °C until emergence. After emergence, add ~20 *S. bullata* hosts and let the virgin G1 females parasitize the hosts for 2-3 days.

   NOTE: These females will produce only G2 mutant males.

   1. Store parasitized hosts at 25 °C and 30% relative humidity, with a 12:12 light-dark cycle until G2 male adult emergence.

   2. Place the remaining G1 pupae at 5 °C for 8-10 days . After this period, remove them and place them at 25 °C and 30% relative humidity, with a 12:12 light-dark cycle until emergence (**Figure 2A**).

4. After emergence of the G2 males in step 8.3.1, screen for the presence of red-eyed mutant phenotype. With the help of a fine-tip paintbrush, separate red-eyed males from wild type males.

   NOTE: The presence of red-eyed wasps in this generation (G2, **Figure 2A**) indicates that gene editing occurred in the germline, and that the mutation is hereditary.

5. Place red-eyed males with the G1 females newly emerged in step 8.3.2 (**Figure 2A**), and let them mate for 1-2 days. Add 2 *S. bullata* hosts per female, and place them at 25 °C and 30% relative humidity with a 12:12 light-dark cycle for 10-11 days.

1. After 10-11 days, crack open the parasitized host with a dissecting needle, and remove each *N. vitripennis* pupa from the host (**Figure 2A**).

2. With the help of a fine-tip paintbrush, separate red-eyed wasps (males and females) from wild type (**Figure 1H**). Continue rearing of red-eyed wasps together in glass tubes, and do not mix with the wild type wasps.

   NOTE: At this stage (G3, **Figure 2A**), haploid males and diploid females carry and show phenotypes for the *cinnabar* mutations. If the affected gene encodes for an invisible phenotype, cross G0 male singularly with one wild type female for ~1 day. Then, remove the male for molecular characterization of the mutation by PCR and sequencing, and let the female parasitize a host. Continue the crossing scheme only with the offspring of a confirmed mutant male (**Figure 2B**). If the goal is the knockdown by RNAi of a candidate gene, perform the desired functional assay (such as lethality, sterility, or diapause induction assay[3,7]) directly using individuals injected with dsRNA. As RNAi is transient, it is not possible to generate a colony with the desired phenotype (**Figure 2C**)[3,7].

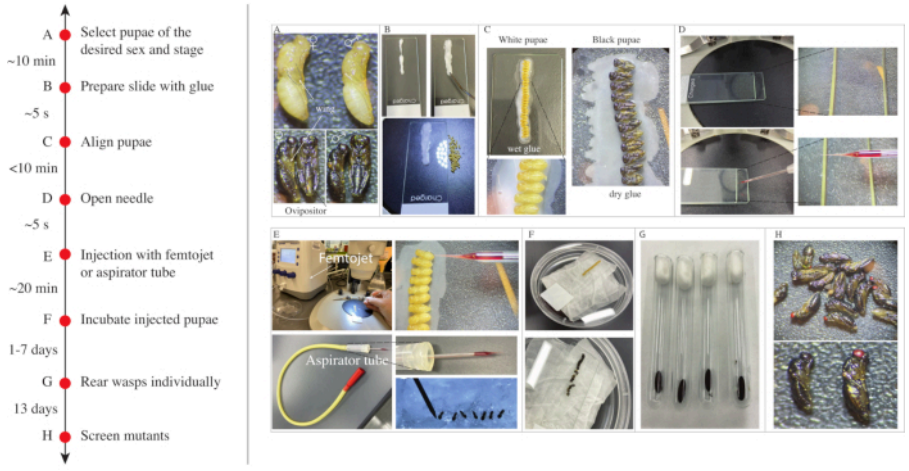


**Figure 1: Timeline for pupal and adult *Nasonia vitripennis* microinjection.** (**A**) Male and female white (top) and black (bottom) pupae are collected, (**B-C**) aligned and glued to a glass slide for injection. (**D**) Capillary needle is prepared and opened, sliding it on two overlapping slides. (**E**) Needle is attached either to the Femtojet (top) or to an aspirator tube (bottom) for injections.**(F**) The injected pupae on the slide are transferred to a Petri dish with a wet tissue on the bottom to keep humidity. Upon emergence, (**G**) females are placed singularly in small glass tubes and allowed to oviposit on *Sarcophaga* pupa. (**H**) Screening of the offspring to detect mutants. Please click here to view a larger version of this figure.

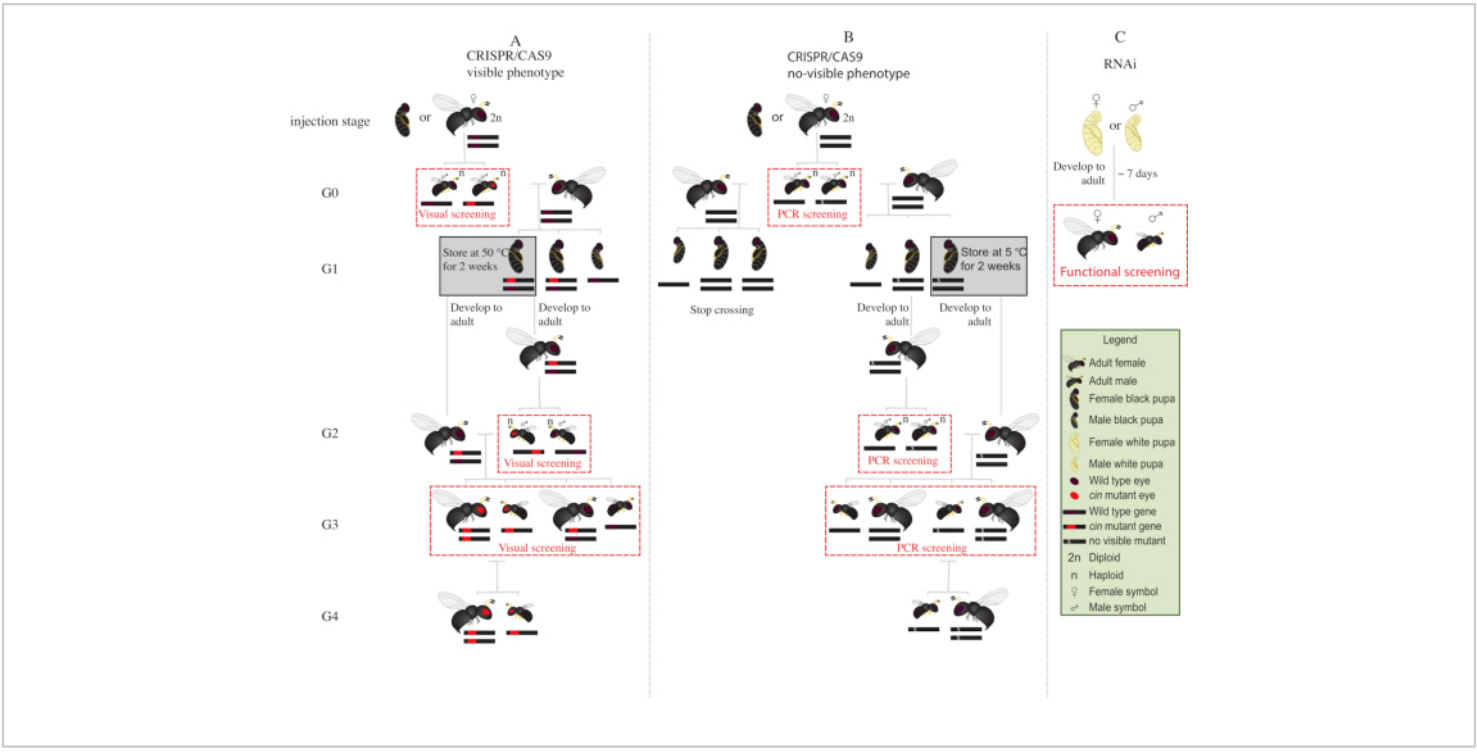


**Figure 2: Crossing scheme after injection.** Schematic representation of crossing scheme procedure in the case of CRISPR/Cas9-mediated gene editing of genes **(A)** that induce a visible phenotype and **(B)** that do not confer visible phenotype. **(C)** Schematic representation of RNAi screening procedure. Abbreviations: *cin* = *cinnabar*; PCR = polymerase chain reaction. Please click here to view a larger version of this figure.

## Representative Results

This paper presents two easy methods for pupal and adult microinjection, either using a femtojet or an aspirator tube. The first method allows a more precise injection of liquid, which is important for RNAi consistency, whereas the second one allows the injection of larger amounts of liquid into *Nasonia* pupae or adults.

Representative results presented in **Table 1** show good survival rates (from 20% to 89%) and efficiency of getting the desired phenotype (either knockdown via RNAi or gene editing via CRISPR) using the injection methods described here without requiring laborious egg injections.

| Stage | Reagent | Concentration ng/µL (Protein/gRNA or dsRNA) | N Injected | N Survival† (%) | Injected individuals laying offspring | | Injected individuals producing offspring with phenotypes | | | Offspring showing *knockdown/ knockout* phenotypes | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | N | % | N | % out of the total injected† | % out of individuals | N | Mutants/ injected |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | producing offspring | | pupae or adult |
| **White pupae** | dsRNA* | 3000-4000 | 80 | 69 (86%) | 69 | 100% | 45 | 56% | 65% | 1263 | NA |
| **Black pupae** | Cas9 | 2903/2741 | 39 | 34 (87%) | 19 | 49% | 2 | 5% | 11% | 2 | 0.05 |
| | P2C-CAS9-EGFP** | 454/227 | 40 | 6 (15%) | 2 | 5% | 1 | 3% | 50% | 3 | 0.08 |
| | P2C-Cas9 | 725/750 | 28 | 28 (100%) | 16 | 57% | 0 | 0% | 0% | 0 | 0.00 |
| | BAPC | 500/200 | 20 | 11 (55%) | 9 | 45% | 3 | 15% | 33% | 5 | 0.25 |
| | BAPC | 400/600 | 20 | 17 (85%) | 10 | 50% | 0 | 0% | 0% | 0 | 0.00 |
| | BAPC | 360/360 | 20 | 16 (80%) | 16 | 80% | 2 | 10% | 13% | 3 | 0.15 |
| **24 h-48-h-old adult** | Cas9 | 2769/3290 | 11 | 10 (90%) | 1 | 9% | 0 | 0% | 0% | 0 | 0.00 |
| | P2C-CAS9-EGFP | 454/227 | 34 | 30 (89%) | 12 | 35% | 3 | 9% | 25% | 3 | 0.06 |
| | P2C-Cas9 | 2903/3076 | 36 | 36 (100%) | 17 | 47% | 4 | 11% | 24% | 4 | 0.05 |

*RNAi experiments were performed on males that were then mated to non-injected wildtype females. Therefore the numbers reperesent the number of crosses that produced offspring and those which produced phenotypes.

** Very First injection on pupae by the authors. Abbreviations: dsRNA = double-stranded RNA; EGFP = enhanced green fluorescent protein; BAPC = branched amphiphilic peptide capsules.

**Table 1: Survival and efficiency rate of RNAi and CRISPR/Cas9 via pupal and adult injections.** Efficiency in RNAi experiments represents efficiency in knockdown of the gene of interest, conferring a measurable effect such as sex ratio distortion[3]. For CRISPR/Cas9, efficiency represents the number of injected females producing mutant offspring. Abbreviations: dsRNA = double-stranded RNA; gRNA = guide RNA; EGFP = enhanced green fluorescent protein; BAPC = branched amphiphilic peptide capsules.

## Discussion

With the recent increased use of *Nasonia vitripennis* as a model organism for various biological questions[2,3,7,17], there is a need to develop and optimize injection methods to enable a simplified and efficient protocol for the functional analysis of *N. vitripennis* genes. The current methods involving embryonic microinjection of gene editing reagents are challenging[13], requiring specialized equipment and highly trained personnel. Thus, to make gene editing technology more accessible to non-specialized laboratories, it is critical to develop alternative delivery approaches of CRISPR reagents. Therefore, demonstrated here is a detailed visual technique that describes two alternative injection procedures to allow the delivery of editing reagents (CRISPR/Cas9 or dsRNA) into insect hemolymph and ovaries.

Injection of dsRNA into the insect hemocoel is a common approach for delivering dsRNA in various species of arthropods[18,19,20,21,22]. It is also efficient in *N. vitripennis* when used in white pupae, as reported previously[3,7]. CRISPR/Cas9 methods for germline insect mutagenesis rely almost exclusively on embryonic microinjections. Few successful experiments delivering CRISPR reagents into nymphs or adults have been reported[12,23,24,25,26,27]. Overall, the methods described here offer a number of significant benefits with respect to existing methods[13]. First, they enable the injection of several developmental stages of *N. vitripennis* wasps: white, black pupae, and adults. Additionally, injection into the hemolymph allows either knockdown via RNAi or germline gene editing via CRISPR, bypassing embryonic microinjections.

The first step in this protocol is to determine and screen *Nasonia* wasps for the right developmental stage based on the functional test of interest (e.g., genome editing, RNAi). It is important to get used to identifying both sexes in white and dark pupae and adults. *N. vitripennis* females lay up to fifty eggs in the pupal case of the blowfly, *S. bullata*, and wasp pupae can be easily collected and screened in large numbers, starting from the white stage. Additionally, the possibility of storing pupae at different stages to slow down the development rate for weeks at 5 °C makes the system very flexible in terms of time and organization[9].

The survival of pupae and adults after injection is an aspect that must be optimized by users. For instance, in this protocol, the first injection of CRISPR/Cas9 reagents in pupae affected survival dramatically (15%) compared to the survival of injected adults (90%). However, in the second session, pupae survival increased up to 60%. After several injection sessions, it was possible to maintain a similar survival (80%-100%) after injecting pupae or adults. Indeed, survival after injection can be improved if parameters related to aligning the wasps, selecting the right aperture for the needle, and avoiding piercing or stabbing the wasps with the needle are optimized.

Aligning and preparing pupae and females for injections, based on the protocol presented here, can reduce mortality. Furthermore, the method presented to align the immobile pupae by gluing them to the slide can ease the injection process, because it prevents them from moving during injection. Thus, pupae can then be injected very rapidly, overcoming the time-consuming nature of the steps involved in embryonic microinjections. In addition, as injections can be done with a mouth aspirator, no specialized equipment is required to accomplish gene editing events. However, this technique requires the wasps to be placed in a timely manner on the glue; otherwise, they will not be properly attached and will slip off from the slide during the needle insertion. This can be corrected after several trials of alignment.

Adults cannot be aligned using this method because they can move their abdomen and legs; hence, they need to be anesthetized. The preferred method of choice in this protocol involves placing the tube containing the wasps in ice for ~5 min until the wasps are knocked down, and then transferring them in groups to a pre-chilled slide on ice. This method allows alignment and preparation of the wasps for injection in groups of 10-15 wasps facing upward, with a new Petri dish containing ice for each new injection group. Preventing the melting of the ice during the injection will help keep the slide stable and decrease the risk of breaking the needles or killing the wasps by accident. This step can be improved by using a chilling table, if accessible.

Alternatively, $CO_2$ pads can be used to anesthetize the wasps. To do this, it would be necessary to optimize the

time of exposure to $CO_2$ during injection and to determine the effects of $CO_2$ exposure on survival and fertility, as reported previously for other species[18]. One additional hurdle when injecting adults relies on the method used to hold the anesthetized wasp for injection. A blunt-pointed object that can hold the wasp without the risk of piercing or stabbing it is most suitable. In general, dissection needles or blunt forceps work well.

After the alignment and proper holding of the wasps is mastered, the injection process may proceed very quickly. Pupae and adult injections can be injected with solution volumes in the range of hundreds of nanoliters, facilitating its preparation at different concentrations based on the user's preference. However, it is important to use proper needles to inject the correct amount of liquid without the risk of damaging and killing the wasp. In general, very long needles with very small apertures are better because they cause less physical damage to the abdomen of the pupae. Moreover, the flow of the liquid is slow, avoiding big changes in pressure and most importantly, preventing the leakage of the solution through the hole created by the needle.

The disadvantages of a small aperture are that the needles can break easily when touching the hard exoskeleton of the wasps and can also get clogged with the material from inside the abdomen. Therefore, small aperture needles work very well using a femtojet or a nanoject, and their use requires great care to avoid breaking them. These needles are not suited for a mouth aspirator because it is very difficult to inject liquid with the mouth through a small aperture. For this method, the ideal needle requires the minimal aperture that allows the solution to flow smoothly while preventing the damage that can be caused with a big aperture needle. Again, this aspect needs to be optimized by the users. In any case, changing the needle often will help to avoid clogging and damaging the tip that will decrease the survival and efficiency.

In *N. vitripennis,* any place in the abdomen can be used to deliver reagents into the hemolymph. However, extra care must be taken to avoid damage to the wasp's ovipositor as this is used for feeding and egg-laying. For both pupae and adults, immobilization techniques have been suggested in this protocol to allow injecting away from the ovipositor, mainly in the ventral area toward the anterior part of the abdomen. Additionally, in adults, this can also be achieved by injecting on the sides or in the dorsal area. The aligning method for pupae described herein may facilitate side injection, but not dorsal. In any case, efforts to prevent piercing other body parts or damage the abdomen are a priority.

Once the needle is inserted, the flow of the liquid should be slow and constant. The femtojet allows this easily, although the pressure may have to be adjusted for each needle. When using a mouth aspirator, injection of large amounts of liquid too fast should be avoided because this may cause it to leak through the pore of the injection. In fact, besides observing the abdomen swelling with liquid, leakage is a sign to stop an injection. After the injection finishes, the adult wasps must be left on the slide until they recover and then transferred to a tube with hosts.

The systems presented here can be easily adapted to other insect species that are particularly challenging for egg microinjections. For example, many parasitoid wasps lay eggs into other insect larvae. Eggs are therefore impossible to reach and inject in those species. The eggs of other pest species, such as the Asian citrus psyllid *Diaphorina citri,* are also very difficult to reach for injection because they are attached to the plant where they are laid. A recent report suggests that BAPC can be used for CRISPR gene editing

in the Asian citrus psyllid, *D. citri*[27]. Similarly, P2C-mediated delivery of the protein, mCherry, was accomplished in the ovaries of this species[28], suggesting that these injection techniques described here could facilitate the implementation of RNAi and CRISPR in a wide range of insect pest species and help in designing control strategies that help prevent the crop damage associated with them.

The implementation in other species requires optimization based on the life cycle of the species of interest. For example, it is important to establish the right stage of injection also based on the functional test of interest. Thus, for delivery of CRISPR/Cas9 into ovaries, it is important to know when vitellogenesis is active to allow the uptake of the gene editing reagents by the ovaries[24,25,26,27,28]. In contrast, for an efficient knockdown, it is important to know when the gene is expressed and functional. Future implementation of those injection techniques could involve delivery of different types of nucleic acids, such as plasmid DNA, into insect hemolymph and ovaries. This makes it possible to promote transient expression of genes present in the plasmid DNA. In addition, combining CRISPR/Cas reagent with a template DNA could help mediate transgenesis via homology-directed repair (HDR) or induce a transposon-mediated transgenesis, circumventing embryonic microinjection. In conclusion, this improved technique can be used to generate many kinds of functional analysis, such as knockdown via RNAi, induction of mutations, deletions, and possibly even transgene insertions to generate transgenic *N. vitripennis*, associating transposons with BAPC, which should greatly accelerate functional research in this organism.

## Disclosures

JLR and DCR have filed for provisional patent protection on ReMOT Control technology. O.S.A is a founder of Agragene, Inc., has an equity interest, and serves on the company's Scientific Advisory Board. All other authors declare no competing interests.

## Acknowledgments

This work was supported in part by UCSD startup funds directed to O.S.A. and NSF/BIO grant 1645331 to J.L.R.

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