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## Drosophila melanogaster Larva Injection Protocol

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**TITLE:**

*Drosophila melanogaster* Larva Injection Protocol

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**KEYWORDS:**

*Drosophila melanogaster*, innate immunity, infection, micro-injection, host-pathogen interactions, animal model

**SUMMARY:**

*Drosophila melanogaster* adult flies have been extensively utilized as model organisms to investigate the molecular mechanisms underlying host antimicrobial innate immune responses and microbial infection strategies. To promote the *D. melanogaster* larva stage as an additional or alternative model system, a larval injection technique is described.

**ABSTRACT:**

The use of unconventional models to study innate immunity and pathogen virulence provides a valuable alternative to mammalian models, which can be costly and raise ethical issues. Unconventional models are notoriously cheap, easy to handle and culture, and do not take much space. They are genetically amenable and possess complete genome sequences, and their use presents no ethical considerations. The fruit fly *Drosophila melanogaster*, for instance, has provided great insights into a variety of behavior, development, metabolism, and immunity research. More specifically, *D. melanogaster* adult flies and larvae possess several innate defense reactions that are shared with vertebrate animals. The mechanisms regulating immune responses have been mostly revealed through genetic and molecular studies in the *D. melanogaster* model. Here a novel larval injection technique is provided, which will further promote investigations of innate immune processes in *D. melanogaster* larvae and explore the pathogenesis of a wide range of microbial infections.

**INTRODUCTION:**

*Drosophila melanogaster* has been immensely utilized in biological and biomedical research for several decades, as the sophisticated array of genetic and molecular tools have steadily evolved

for analysis of a wide range of studies[1-4]. The evolutionarily conserved aspects of development, homeostasis and innate immunity in *D. melanogaster* have made it a valuable model organism for studying various human and insect diseases[5,6]. Notably, the fundamental role of the *D. melanogaster* model for studying immunity has been largely exemplified in adult flies studies. However, *D. melanogaster* larvae studies have also contributed to the current knowledge and mainly explored cellular immune responses, specifically for wasp and nematode infections that occur through the insect cuticle[7–10]. *Drosophila melanogaster* larvae possess three different types of blood cells, collectively called hemocytes: plasmatocytes, crystal cells, and lamellocytes[11–13]. These cells can mount an array of immune responses when *D. melanogaster* larvae are infected with pathogens such as bacteria, fungi, viruses, and parasites[14–16]. Cellular immune responses include direct engulfment (phagocytosis) of small molecules or bacteria, melanization, encapsulation of larger pathogens such as parasitoid eggs, and production of reactive oxygen species (ROS) and Nitric oxide synthases (NOS)[17–19].

In contrast, fewer studies have been published on the use of the *D. melanogaster* larval model to analyze humoral immune responses. This is mainly due to the application of feeding assays for oral infection of *D. melanogaster* larvae and several challenges associated with microinjecting larvae including the precise handling of larvae and proper use of the microneedle, especially during penetration[20,21]. Thus, the limited knowledge of larval infection and technical difficulties (i.e., high mortality) have frequently made the *D. melanogaster* larval model difficult to use. A larval model will have the potential to identify novel molecular mechanisms that will provide further insights into host-pathogen interactions and the induction of specific host innate immune responses against pathogenic infections.

Here a simple and efficient protocol that can be used to inject *D. melanogaster* larvae with various pathogens, such as bacteria, is described in detail. In particular, *D. melanogaster* larvae are used for injections with the human pathogen *Photorhabdus asymbiotica* and the non-pathogenic bacteria *Escherichia coli*. This method can be used for the manipulation and analysis of *D. melanogaster's* immune responses to various microbial infections.

**PROTOCOL:**

## 1. Fly rearing

NOTE: The *D. melanogaster* life cycle is divided into four stages: embryo, larva, pupa, and adult. The generation time with optimal rearing conditions in the laboratory (~25 °C, 60% humidity, and sufficient food) is approximately 10 days from fertilized egg to eclosed adult. Females lay ~100 embryos per day, and embryogenesis lasts about 24 h[22]. The larvae undergo three developmental stages (instars; L1–L3) in ~4 days (L1 and L2: 24 h, and L3: 48 h). The first instar larvae begin to feed immediately on the surface of the medium. Second instar larvae burrow into the medium, whereas third instar larvae leave the medium and wander up the vial walls, looking for a place to pupariate for 24–48 h. The *D. melanogaster* line used for this protocol is *Oregon R* (FBsn0000276).

1.1. Place a mixture of at least 20 newly emerged male and female adult flies in a narrow polystyrene vial (25 mm x 95 mm) that contains a cornmeal-soy-based diet.

1.2. Add dry diet to the vial to about 1/5 to 2/5 volume. Then add 9 mL of water and allow the vial to sit for 1 min until the diet is completely hydrated.

1.3. Add about 10 granules of dry baker's yeast to the vial and incubate at 25 °C on a 12:12-h light: dark photoperiodic cycle.

1.4. To ensure life cycle progression, check the fly vials daily and record the initial development stages.

## 2. Larvae selection for infection

2.1. Select larvae with a fine paintbrush (camel's hair is best) once they reach the wandering third instar stage on the day the infection will be carried out (**Figure 1**).

2.2. Wash the selected larvae with Ringer's solution (100 mM NaCl, 1.8 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES pH 6.9) in a small Petri dish (60 mm x 15 mm) upon removal from their original vial[23].

2.3. Place the larvae on filter paper (150 mm diameter) that is slightly moistened with around 5 mL of Ringer's solution in a Petri dish (100 mm x 15 mm) (**Figure 2A**).

2.4. Add ~1 mL of Ringer's solution daily to the filter paper as needed to avoid dryness and larval desiccation.

## 3. Bacterial preparation

3.1. Culture *P. asymbiotica* bacteria on Luria Bertani (LB) agar media at 28 °C for 48 h.

3.2. Use a biosafety level 2 cabinet to transfer a single colony of *P. asymbiotica* to inoculate a liquid culture in 10 mL of LB media.

3.3. Incubate the *P. asymbiotica* liquid culture overnight at 28 °C in a rotary shaker set to 220 rpm.

3.4. Culture *E. coli* bacteria in a similar fashion, but carry out the initial growth on agar media and liquid culture incubation at 37 °C overnight.

3.5. Centrifuge each overnight bacterial culture for 3 min at 13,000 x *g* and 4 ˚C.

3.6. Discard the supernatant and wash the resulting bacterial pellets three times with 10 mL of sterile PBS.

3.7. Centrifuge the pellets again for 3 min at 13,000 x *g* and 4 ˚C.

3.8. Dilute each pellet with sterile PBS to adjust bacterial concentrations to the optical density (600 nm) of 0.25 for *P. asymbiotica* and 0.015 for *E. coli*, which correspond to 100–300 colony forming units per larva of each bacterial preparation, using a spectrophotometer.

## 4. Injector preparation

4.1. Prepare capillaries using 3.5" glass capillary tubes and a micropipette puller. Set the instrument as follows: heat: 580; pull: 143; velocity: 25; delay: 1; pressure: 500.

4.2. Use straight serrated medium point forceps to open the glass capillary to the degree that will allow the delivery of the experimental treatments while causing minimal damage to the larvae (**Figure 2B**). To optimize this procedure while practicing injections, use Trypan Blue solution (dilute the stock 0.4% with PBS to 0.2%) to easily track leakage while injecting and the survival of the blue larvae.

4.3. Fill the capillary with mineral oil using a plastic, disposable 20 mL syringe with a hypodermic needle (22 G, 25 mm length).

4.4. Set up the nanoliter injector and eject the oil out of the capillary (**Figure 2C**).

4.5. Fill the capillary under a stereoscope with the desired bacterial preparation for injection. Take the solution from a drop (~20 µL) that is placed on parafilm. In this protocol, 50.2 nL of two bacterial stocks were injected.

## 5. Larvae injection

5.1. Anesthetize *D. melanogaster* larvae using carbon dioxide for ~2 min prior to the procedure.

5.2. Gently transfer the anesthetized larvae to a filter paper moistened in Ringer's solution in preparation for injection under a stereomicroscope. The larvae will be lethargic or passive at this point.

5.3. To inject larvae, apply firm pressure on the dorsal side of the posterior end (the tracheal spiracles are apparent at the posterior end versus black mouthparts at the anterior end) using forceps (**Figure 2D**).

5.4. Insert the needle horizontally towards the posterior end of the larvae, near the cuticle. A successful injection will not result in a leakage of the injected solution from the larvae (**Figure 3**).

5.5. Remove the forceps that were used to apply pressure on the larvae's tail before withdrawing the needle. If not removed first, the forceps can force hemolymph and/or the intestine out of the larvae from the wound site.

5.6. Infect the appropriate number of larvae for the study being performed. In this particular protocol, 20 larvae for each experimental condition were injected.

5.7. Using forceps, gently transfer the injected larvae to a separate moist filter paper in a Petri dish (10 larvae per dish) or a food vial (depending on the purpose of the experiment) to allow for recovery.

5.8. Place the Petri dishes or food vials in an incubator at 25 °C. If larvae are kept in a Petri dish, add Ringer's solution as necessary to prevent desiccation.

## 6. Recording survival/mortality

6.1. Record the number of dead and live *D. melanogaster* larvae according to the set experimental time points. Live larvae will continue to develop into pupae and adults.

6.2. Use statistical programs, such as Prism, to enter the raw survival/mortality data, analyze them with the log-rank (Mantel-Cox) test, and represent the results in figures.

## REPRESENTATIVE RESULTS:

When performed correctly, injections of *D. melanogaster* larvae show a bacterium-specific effect. The survival data were collected at several time points following infections of *P. asymbiotica* (strain ATCC43943), *E. coli* (strain K12), and PBS (**Figure 4**). Whereas *D. melanogaster* larvae are susceptible to *P. asymbiotica*, which compromises survival rapidly, larvae injected with *E. coli* or PBS controls exhibit prolonged survivals[24–26]. Particularly, in comparison to larvae infected with *P. asymbiotica*, which exhibit a 57% survival rate 24 h following injection, larvae injected with *E. coli* show an 85% survival rate at the same time point.

## FIGURE AND TABLE LEGENDS:

**Figure 1: Selection of *Drosophila melanogaster* larvae for injection.** The life cycle of *Drosophila melanogaster*, from egg fertilization to adult life, takes approximately 10 days. During larval growth, larvae feed until ready to pupate and change to adults. For the purpose of injection experiments, third instar larvae, which leave the culture medium and wander up the walls of the vial, are selected.

**Figure 2: Depiction of *Drosophila melanogaster* larval injection procedure.** (**A**) Wandering third instar larvae are selected, washed with Ringer's solution, and placed on filter paper in a Petri dish in preparation for injection. (**B**) Using forceps, the glass capillary is broken to allow the delivery of the experimental treatments. (**C**) The programmable nanoliter injector is set up in preparation for injection under a stereomicroscope. (**D**) To inject larvae, pressure is applied on the dorsal side of the larvae's tail using forceps.

**Figure 3: Illustration of delivering bacterial cells into *Drosophila melanogaster* larvae through microinjection.** The dorsal side of the posterior end is stabilized using forceps. Then, the capillary is inserted horizontally towards the posterior end of the larvae, near the cuticle. Following the injection, the forceps are removed before carefully withdrawing the capillary to prevent hemolymph leakage from the wound site.

**Figure 4: Survival of *Drosophila melanogaster* larvae following injection of pathogenic and non-pathogenic bacteria.** *Oregon R* larvae of *D. melanogaster* were injected with 50.2 nL of *Photorhabdus asymbiotica* (ATCC43943), *Escherichia coli* (K12), or PBS. Whereas PBS and *E. coli* controls showed no significance in survival ratios, *P. asymbiotica* injections compromised fly survival rapidly. Each survival curve is comprised of measurements from three independent trials, each including 20 larvae (*** $p < 0.001$).

**DISCUSSION:**

*Drosophila melanogaster* is among the most valuable, experimentally manipulated models used for investigations of innate immunity and pathogenesis of various microbial infections. This is due to its simple and fast life cycle, simple upkeep in a laboratory, well-established evolutionary genetics, and diverse genetic toolbox. Previous methods of *D. melanogaster* larvae injections, such as using a hybrid microfluidic device or a Narishige micromanipulator, require highly specialized equipment and can be costly[21,27]. In the current protocol, to expand the use of *D. melanogaster*, a simple injection technique that represents an efficient and rapid method to deliver bacteria into the hemocoel of *D. melanogaster* larvae is outlined. The essential part of the technique described here is the actual injection of the desired pathogen or other liquid substances using a microinjector. In addition to describing this essential operation, we also describe accessory methods such as growing and culturing bacteria and handling materials.

The principal advantage of this method is that the needle is held approximately parallel to the larva, and the holding is done with very little pressure on the larva, thus reducing the possibility of hemolymph leakage, fatal injury, or inadequate delivery of the desired substance. The needle is then inserted toward the posterior end of the larva to complete the injection. The precise way of holding the larva, the manner of inserting the needle, the speed at which the desired substance is injected, and the direction of withdrawal of the needle are all matters that can best be improved by practice. The most challenging step to overcome is withdrawing the needle out of the larva without any resulting leakage of essential organs. However, with preciseness and experience, the difficulty encountered during this step becomes less.

This technique is also an excellent tool in multiple applications for introducing a variety of substances in a uniform manner that can be repeated several times, thus allowing for consistent results. The rapid mortality observed in *P. asymbiotica*-infected larvae reflects the high virulence of this bacterial strain to insects[24–26]. *P. asymbiotica* is well documented for expressing virulence genes during infection that increase bacterial survival and pathogenicity against insect hosts by inhibiting hemocyte migration and phagocytosis[24,25]. Also expectedly, larvae are resistant to injections with the non-pathogenic bacterial strains of *E. coli* as well as injections with PBS, thus

confirming previous results in this research field[26]. Hence, microinjection is achieved without any adverse effect on animal viability, allowing its use in molecular and immunological studies to explore the pathogenesis of a wide range of microbial infections.

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**DISCLOSURES:**

The authors declare no competing interests.

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Figure 1

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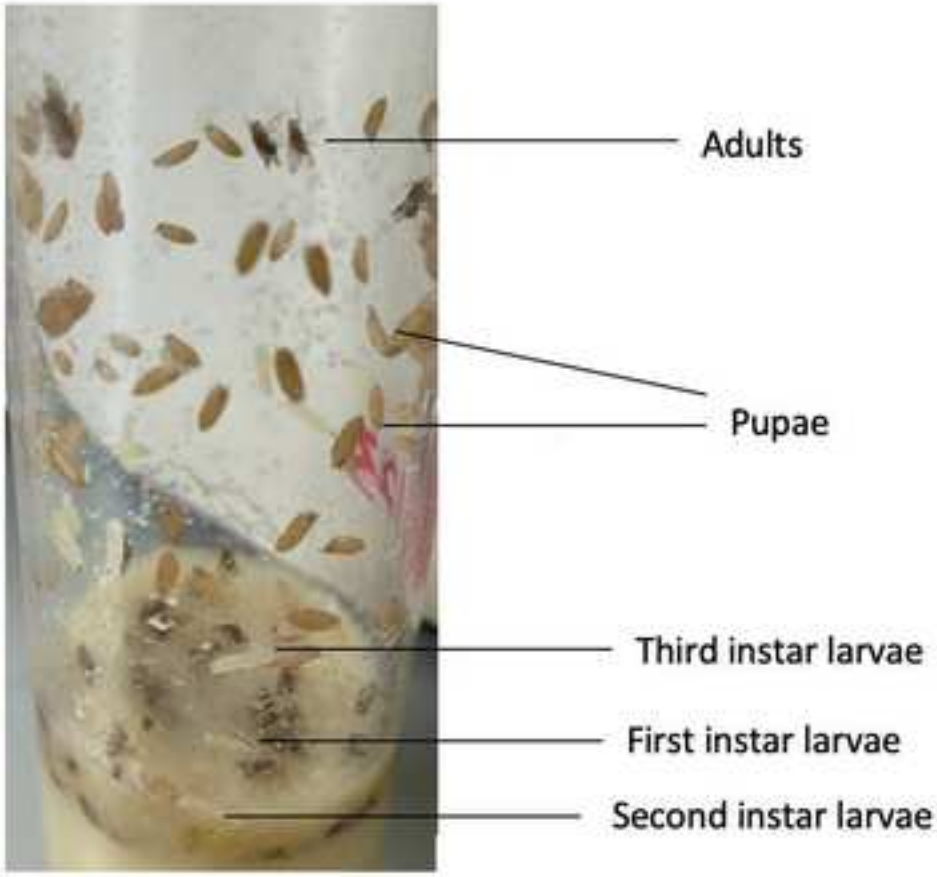


Figure 2



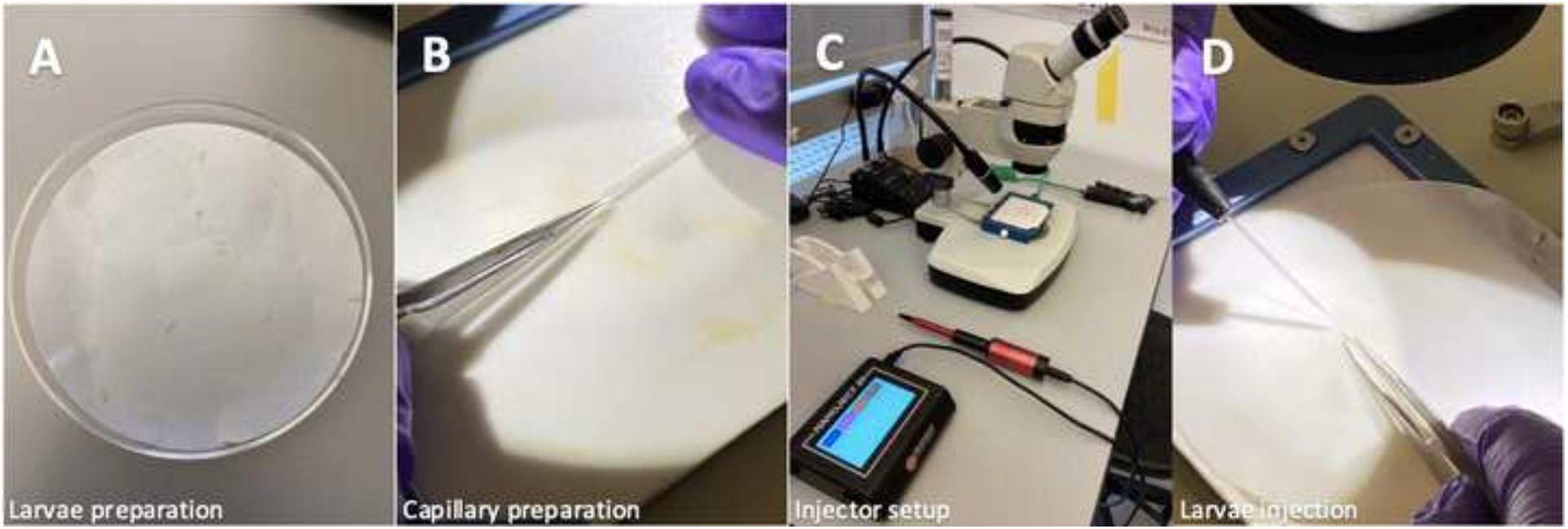


Figure 3



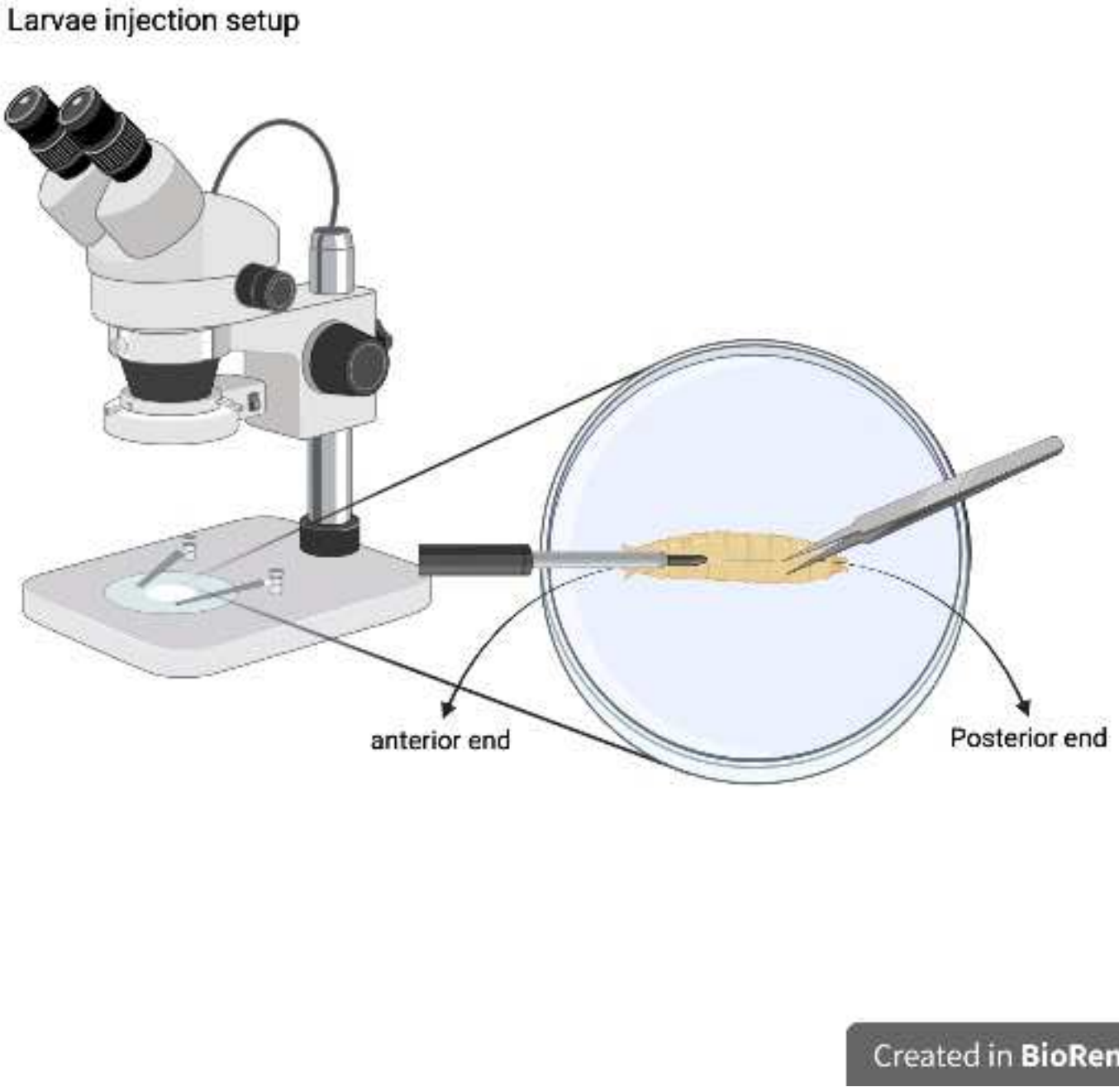
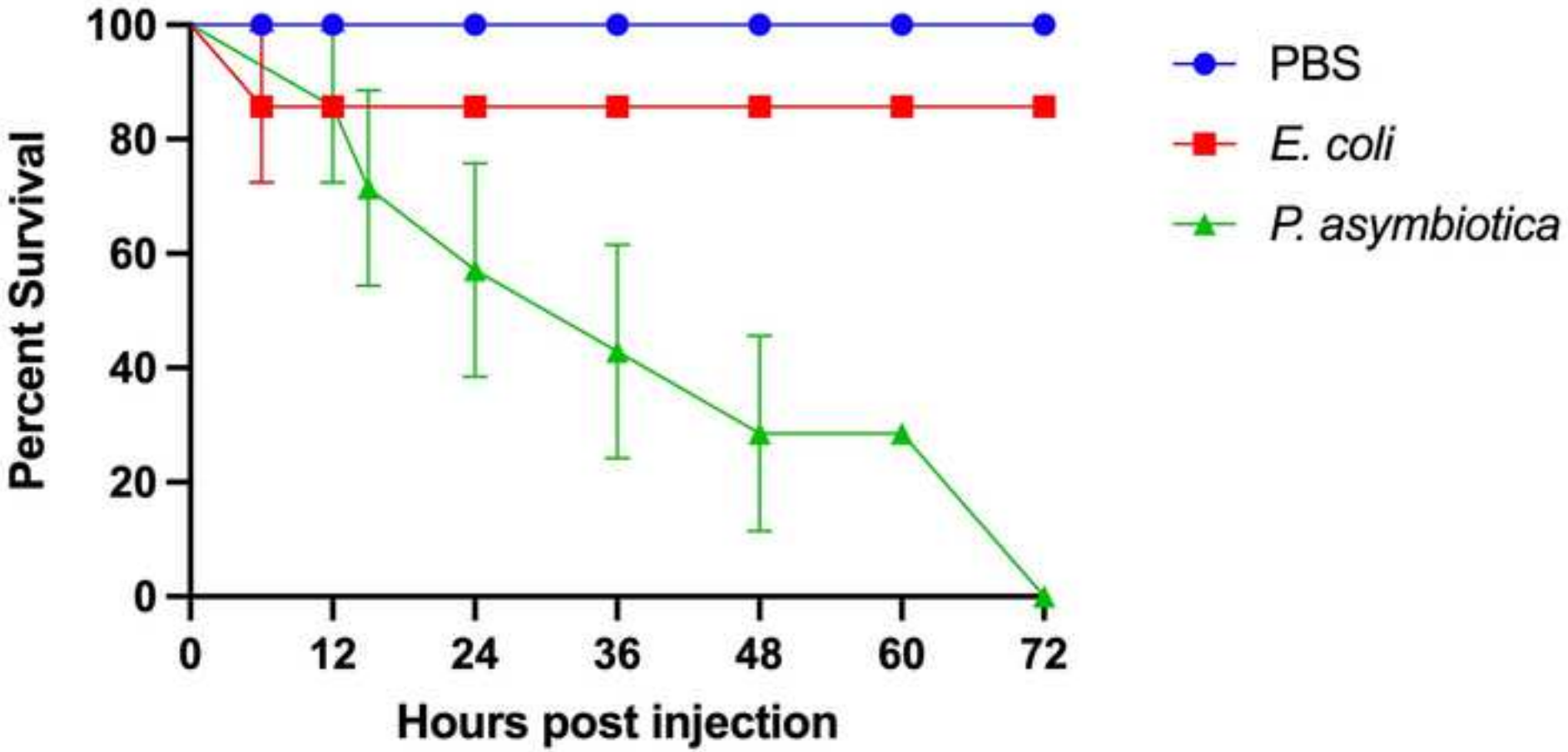


Figure 4





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**Editorial comments:**

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please note that the manuscript has been formatted to fit the journal standard. Comments to be addressed are included in the manuscript. Please review and revise accordingly.

**Response: We have checked the manuscript throughout for correctness. Thank you for formatting the manuscript according to the journal guidelines.**

2. Please revise the lines to avoid the issue of plagiarism: 36-39, 80-83,85-87,137-139,236-238. Please refer to the iThenticate report attached.

**Response: We have modified the text in the indicated lines. We are afraid we cannot change the wording more. The current text is original.**

3. Figure 2/3: Please label the figure to make it more informative.

**Response: We have labeled Figures 2 and 3, as suggested.**

4. Please consider highlighting section 2 (Larvae selection for infection) also to benefit novice researchers.

**Response: We have highlighted section 2, as indicated.**

**Other points:**

**Section 4.1:** These are the settings on the instrument, there is no reference to cite and no specific units. We would like not to change the text.

**Section 4.4:** There are no specific setting here. By “set up”, we mean plugging all the cords in and position the injector on the bench. We would like not to change the text.

**Acknowledgements and Disclosures:** We have completed these sections.