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A capsule-based model for immature hard tick stages infestation on laboratory mice

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Editorial board of JoVE

Please consider the revised version of the manuscript entitled "**A capsule-based model for immature hard tick stages infestation on laboratory mice**", by Mateos-Hernandez et al., for publication in the Journal of Visualized Experiments (JoVE). Reviewers' constructive criticisms were greatly appreciated. In our revised manuscript we have improved the quality of the proposed manuscript dealing with the development of a simple, fast and very effective method for the feeding of immature hard ticks on the laboratory mice.

All our point by point answers to editorial and reviewers specific comments are in the Rebuttal document. At the end of this document is also "tracking changes version" of the manuscript.

Please let me know if you have any questions.

Sincerely,

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TITLE:

A Capsule-Based Model for Immature Hard Tick Stages Infestation on Laboratory Mice

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KEYWORDS:

ticks, infestation, feeding, nymphs, larvae, mice, capsule, glue, animal recovery

SUMMARY:

In this study, a feeding system for nymphal and larval stages of hard tick was developed using a capsule attached to laboratory mouse. The feeding capsule is made from flexible materials and remains firmly attached to the mouse for at least one week and allows comfortable monitoring of tick feeding.

ABSTRACT:

Ticks are obligatory blood feeding parasites at all stages of development (except eggs) and are recognized as vectors of various pathogens. The use of mouse models in tick research is critical for understanding their biology and tick-host-pathogen interactions. Here we demonstrate a non-laborious technique for the feeding of immature stages of hard ticks on laboratory mice. The benefit of the method is its simplicity, short duration, and the ability to monitor or collect ticks at different time points of an experiment. In addition, the technique allows attachment of two individual capsules on the same mouse, which is beneficial for a variety of experiments where two different groups of ticks are required to feed on the same animal. The non-irritating and flexible capsule is made from easily accessible materials and minimizes the discomfort of the experimental animals. Furthermore, euthanasia is not necessary, mice recover completely after the experiment and are available for re-use.

INTRODUCTION:

Ticks are important vectors of several pathogens and represent a serious risk to animal and human health[1]. Setting up an effective feeding system is crucial when studying their biology, tick-host-pathogen interactions, or establishing effective control measures. Currently, several artificial feeding systems which avoid the use of live animals are available for ticks[2-4] and these should be utilized whenever experimental conditions allow. However, in various experimental settings these systems fail to appropriately mimic the specific physiologic features and the use of live animals is necessary to achieve relevant results.

Laboratory mice are commonly used for the study of many biological systems and are routinely utilized as hosts for feeding ticks[5-9]. The two most common methods of feeding immature ticks on mice include free infestations and the use of confinement chambers attached to the mouse. Free infestations are primarily used for larval stages and engorged ticks can drop to an area where they can be recovered. Confinement chambers are usually composed of acrylic or polypropylene caps which are glued to the mouse's back. The first technique is an effective natural system for tick feeding but does not allow close monitoring during the experiment because the individual ticks are dispersed on different parts of the host body. Additionally, engorged ticks that drop to a recovery area can become contaminated with feces and urine[10-14] that may severely affect the tick fitness or they can be damaged or eaten by the mouse if there is no separation between the animal and the recovery area[15]. Chamber-based systems allow the confinement of ticks to a defined area, however, the gluing process is laborious and the caps are often weakly adherent to the glue and thus they often detach during the experiment[16-19]. The caps are also stiff, uncomfortable, and lead to skin reactions which prevent the re-use of the mice and necessitates their euthanasia after the experiment.

In our previous study, we successfully developed an effective system using chambers made of ethylene-vinyl acetate (EVA) foam for feeding ticks on laboratory rabbits[20]. Herein, we adapted this system to a mouse model and propose a simple and clean method to feed immature hard tick stages in closed capsules made from EVA-foam. Specifically, our system uses elastic EVA-foam capsules glued to the shaved mice back with fast drying (3 min), non-irritating latex glue. This technique allows firm and long-lasting attachment of capsules to the experimental mouse, as well as effective tick infestation/collection during the entire course of the experiment. The flat capsule is made from flexible materials and does not impede manipulation of the mouse for blood collection or other purposes. The system is suitable mainly for the nymphal tick stages, but with slight modification it can be used for feeding larvae as well. The method can be completed by one single experienced person and extensive training is not required.

PROTOCOL:

Please note that this protocol can be only applied when all welfare and safety measures are met in the laboratory. This protocol received permission to use mice for tick feeding by the Ethics Committee for Animal ExperimentsComEth Anses/ENVA/UPEC, Permit Numbers E 94 046 08. For the endpoint, the animals were exposed to CO_2 for 9 min in two phases of 4 and 5 min each one.

1. Preparation of the capsule

1.1 Stick 2 mm thick ethylene vinyl acetate (EVA)-foam and the adhesive double sticky foam together (**Figure 1A**).

1.2 Using a 20 mm diameter leather hole punch, cut a circle from the sticked foam pieces. Then, using a 12 mm diameter hole punch, cut the interior to create the double foam circle (**Figure 1B**).

NOTE: The frame thickness of the capsule should be greater than 3 mm in size to guarantee sufficient surface for the gluing process to the host skin (see below).

1.3 Peel the protective paper strip from the adhesive double sticky foam (**Figure 1C**) and attach a transparent circular plastic of 20 mm diameter (**Figure 1D**).

NOTE: If feeding larvae, do not remove the protective paper strip from the adhesive foam and directly move to the step 2 in the protocol. Glue the double foam ring, including protective paper strip to the mouse.

1.4 Make a ~ 1 cm slit in the transparent plastic (**Figure 1E**).

1.5 Create at least 10 small holes with an entomological pin (**Figure 1F**) to allow excessive moisture evaporation during the experiment.

NOTE: The capsule (**Figure 1G**) has a total height of 4 mm (2 mm EVA-foam together with 2 mm adhesive foam) and can be used to feed nymphs and larvae of all the hard tick species. The capsule size (**Figure 1H**) of 20 mm outer diameter is suitable for most of the mouse strains but can be modified if necessary.

2. Preparation of the mice before tick infestation

NOTE: In this study, 10 - 12 weeks old female experimental mice (strain C57BL/6 and BALB/cByJ) were maintained in standard cages with food and water offered *ad libitum* (Green line ventilated racks at -20 Pa) at the French Agency for Food, Environmental and Occupational Health & Safety (ANSES) accredited animal facilities in Maisons-Alfort, France. Animals were monitored twice daily by experienced technicians for any abnormal skin reactions, health problems or complications.

2.1 Anesthetize mouse with isoflurane in the induction chamber. Once anesthetized, place mouse to the manipulation pad and attach to a nose cone for the continuous isoflurane supply (**Figure 2A**). Monitor the breathing rate and reduce isoflurane level to ensure it is less than 80 breaths per minute.

NOTE: Prior to the manipulation, label the individual mouse by tattooing or radio-frequency identification chip if necessary. It is recommended to keep the individual mice in separate cages to avoid capsule damage by biting.

2.2 Shave the anterior part of the mouse from behind the shoulder blades up to the area just behind the ears (**Figure 2A**).

NOTE: The shaved area should be greater than the capsule surface.

2.3 Apply non-irritating latex glue to the entire EVA-foam site of the prepared capsule and wait for 1 min (**Figure 2B**).

2.4 Glue the capsule to the mouse back by slight 3 min constant pressure with the finger(s) (**Figure 2C**), especially on the left and right side of the capsule. Slightly lift the capsule to visually check its attachment to the skin. If non-attached regions are found, apply more glue using a spatula and press for another 3 minutes.

3. Tick Infestation

3.1 For nymph infestation, introduce the individual nymphs into the capsule via the cut made in step 1.4) (**Figure 2D**).

NOTE: For *Ixodes* tick species a maximum of 20 nymphs is recommended per one capsule.

3.2 Slightly squeeze the capsule from two sides to allow the transparent plastic to bend for easier introduction of individual nymphs using fine dissection forceps (**Figure 2D**). Push the individual nymphs via the cut inside the capsule. Once inside, turn the forceps in 90˚ and pull out the forceps to deposit ticks inside the capsule.

3.3 For larvae infestation remove the paper slip from the attached capsule (**Figure 2E**). Place the syringe, containing larvae (**Figure 2F**), directly inside the capsule and deposit ticks by pushing the syringe plunger. Gently turn the plunger towards the skin to remove the remaining larvae attached.

NOTE: Place larvae into a 1 mL syringe with cut end plugged by piece of cotton prior to the experiment.

3.4 Once the larvae are deposited onto the skin, close the capsule by attaching the transparent plastic (**Figure 2G**).

3.5 Apply the protective plastic band around the capsule (**Figure 2H**).

NOTE: The protective plastic band greatly improved the durability of the capsule for the entire duration of the experiment (**Figure 2I,J**). It is possible to attach two capsules to one individual mouse (**Figure 2K**). In this case, a minimum of 3 mm space between the capsules is required and the shaved area should be increased appropriately.

3.6 Return the mice to the cage.

4. Collection of Ticks

4.1. Anesthetize the mouse as in step 2.1 above.

4.2 Make a cross shaped cut (**Figure 3A**) to the plastic with a scalpel.

NOTE: This cross shaped cut enables easy collection of engorged ticks or detachment of the feeding ticks if necessary.

4.3. If needed, reclose the capsule by sticking an adhesive plastic patch to the transparent plastic (20 mm diameter, **Figure 3B**).

NOTE: If collection of ticks at multiple time points is desired, the same sticky plastic patch can be used. If the protocol requires, one may also euthanize the mouse, remove the capsule, and collect/detach the ticks (**Figure 3C**).

5. Recovery of the mice

5.1 Keep the mice in cage for one additional week.

5.2 Let the capsule detach naturally.

NOTE: In this case, it takes about 8-9 days for capsules to fall off. When the capsule is removed, it is important to check for abnormal reactions on the skin of the mice. In case of irritation apply an emollient lotion, although normally no treatment is required. If the ethical protocol allows, the recovered mice (**Figure 3D**) can be reused for another tick infestation or different experiment(s).

REPRESENTATIVE RESULTS:

We propose the detailed step-by step method for feeding immature hard tick stages in EVA-foam capsules applied to a mouse's back (**Figure 2**). This non-laborious protocol is suitable for various types of experiments when precise tick monitoring and collection is required. The main advantages of this method are its simplicity, easily accessible cost-effective materials, and short duration. In addition, we succeeded in attaching two capsules to one mouse individual (**Figure 2K**) allowing to us to feed two different groups of ticks on the same animal. The use of the highly effective, fast-drying, and non-irritating latex glue ensures that the capsule is firmly attached within 3 min. Also, the capsule remained attached for at least one week (**Figure 2J**) which was enough time for engorgement of most of the immature hard tick species[21-24]. Due to the capsule elasticity, further manipulation of the mouse for blood collection or other purposes was very convenient. This procedure also allows complete recovery of the mice after the experiments (**Figure 3D**) giving the opportunity to reuse the animals and avoid euthanasia. Our system has been successfully used to feed *Ixodes ricinus* nymphs **(Figure 4)**. A moderate to high engorgement success rate was achieved in C57BL/6 and BALB/cByJ mouse strains, respectively. In both cases

all nymphs finished the feeding within 4 – 5 days, while the majority (~75%) dropped off on the fourth day.

FIGURE AND TABLE LEGENDS:

Figure 1: **EVA-foam capsule preparation.** (**A**) Attachment of EVA-foam (black) and adhesive double sticky foam (white). (**B**) Cutting 20 mm diameter outer and 12 mm inner circle using leather hole punches. (**C**) Removal of the paper protection tape from the adhesive double sticky foam. (**D**) Attachment of the transparent plastic to the capsule. (**E**) Cutting the slit in the transparent plastic with a scalpel. (**F**) Creation of holes using an entomological pin in the plastic. (**G-H**) Schematic drawing of the different parts of the capsule and dimensions.

Figure 2: **Gluing the capsule to the mice and tick infestation.** (**A)** Shaving Mouse's back anterior part. (**B**) Application of the latex glue to the EVA-foam side of the capsule. (**C**) Attachment of capsule to the mouse. (**D**) Placing the nymph in the capsule via the cut in the transparent plastic. (**E**) Peeling the paper protection tape from the adhesive double sticky foam before larvae infestation. (**F**) Injections of larvae inside the capsule using a cut syringe. (**G**) Closing the capsule with the transparent plastic. (**H**) Placing a protective plastic band around the capsule. (**I**) Mouse with the attached capsule - 1st day. (**J**) Mouse with the attached capsule - 7th day. (**K**) Mouse with two capsules attached.

Figure 3. Tick collection and mouse recovery. (**A**) Cutting cross-shape opening for tick collection. (**B**) Resealing the capsule with adhesive plastic patch. (**C**) Capsule removal from a euthanized mouse. Arrows show the attached ticks. (**D**) Recovered mouse after dropped off capsule.

Figure 4. Engorgement success and feeding duration of *Ixodes ricinus* nymphs feeding on mice. (**A**) Total percentage of engorged nymphs in C57BL/6 and BALB/cByJ mice. (**B**) Duration of nymph engorgement in C57BL/6 and BALB/cByJ mice. The (n) numbers for infested nymphs are 130 and 25 for 15 individual C57BL/6 and 5 individual BALB/cByJ mice, respectively.

DISCUSSION:

The most critical step in the protocol is firm gluing of the capsule to the mouse skin. Therefore, the latex glue should be homogenously applied to the entire EVA-foam surface of the capsule and constant pressure for 3 minutes should be applied, especially to the left and right side of the capsule. We also recommend placement of the capsule as far forward on the back as possible to avoid its removal by the mouse using its rear paws. In our experiments, only the adhesion of the EVA-foam and latex glue to the mouse skin has been validated and we cannot guarantee the achievement of same results using different materials.

During our experiments, detachment of the capsule from the skin within first seven days was not observed. We strongly recommend protecting the outer surface of the capsule using the plastic band (**Figure 2H**). If the protective band is damaged over the course of tick feeding, it can be replaced with a new one. The diameter of the capsule can be modified for different mouse strain sizes. We suggest monitoring the feeding ticks at least twice daily and to collect engorged ticks

immediately after detachment to avoid their desiccation.

The number of infested ticks is limited by the capsule diameter, as well as the host size. In our experiments we used maximum of 20 nymphs or 100 larvae of *I. ricinus* for one mouse. For the larger size ticks such *Amblyomma* or *Hyalomma* sp., etc the number of infested ticks should be reduced to avoid harm to the host from blood loss[19,26,27]. Therefore, this technique is not suitable for the maintenance of tick rearing colonies, where large numbers of ticks are required to feed. For this purpose, larger hosts like rabbits or sheep are recommended[27] to reduce overall animal requirement.

Our technique is suitable for various types of experiments where a mouse model is required, and it is necessary to keep ticks in enclosed area for easy collection and/or monitoring of their biological parameters. Compared to other techniques[10-18], this simple protocol greatly reduces the overall anesthesia time (approximately 5 minutes) per mouse and the fast drying, non-irritating latex glue does not cause harm to the animal. The highly adhesive EVA-foam capsule protects the tick feeding area and minimizes the risk of lost, damaged, or eaten ticks as reported in free infestation systems[10-15]. The great advantage of the proposed technique is the flat-shape capsule and its firm long-lasting attachment to the skin allowing easy manipulation with the mouse if required. Special attention has been paid on usage of elastic and non-irritating materials to reduce the discomfort to the experimental animals allowing complete recovery of the mouse host after experiment (**Figure 3D**).

The method is expected to be used for a variety of the experiments when studying tick-host-pathogen interactions, tick manipulation of host immune systems, evaluating different tick control measures or tick biology.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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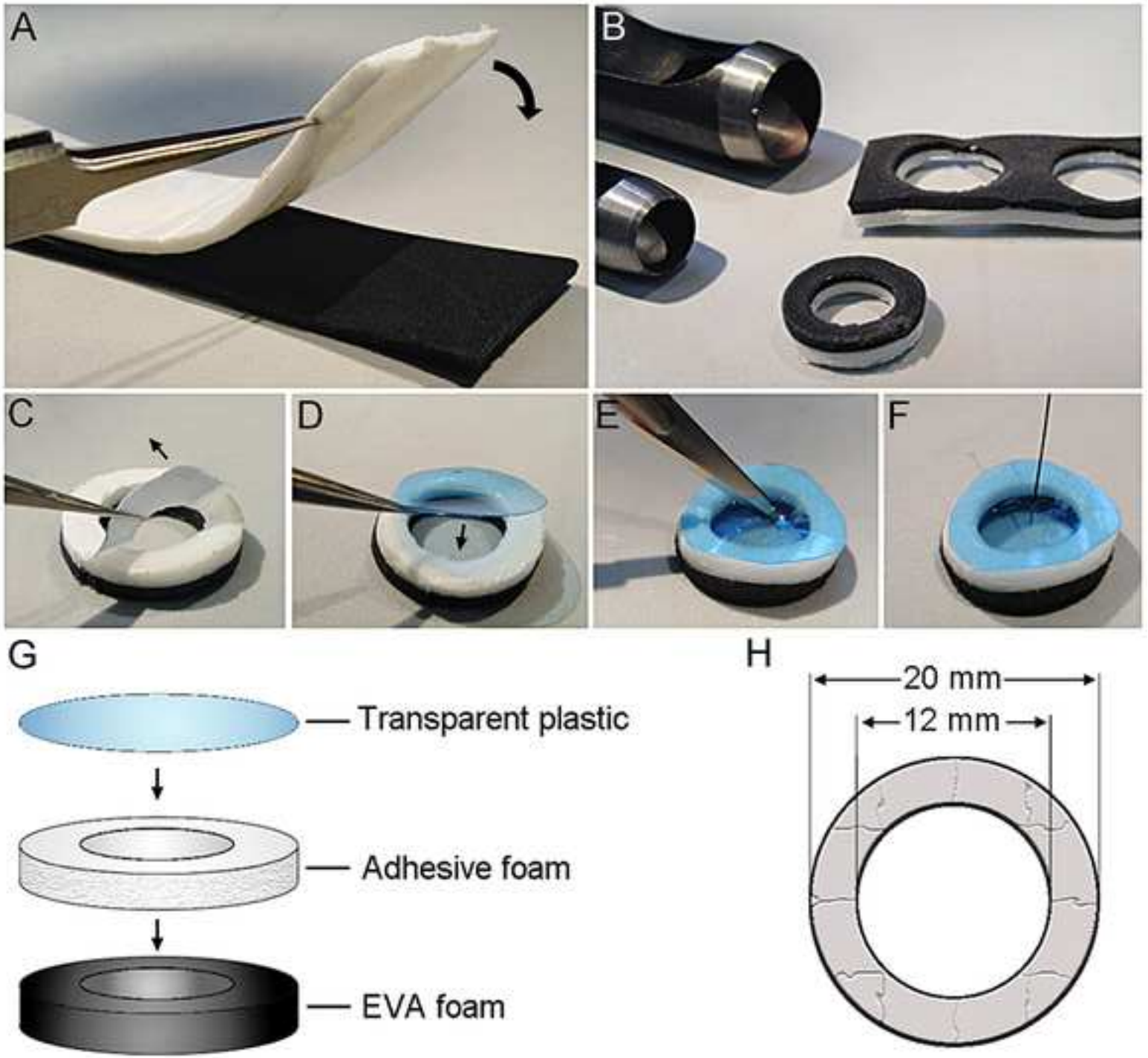
Figure



Figure

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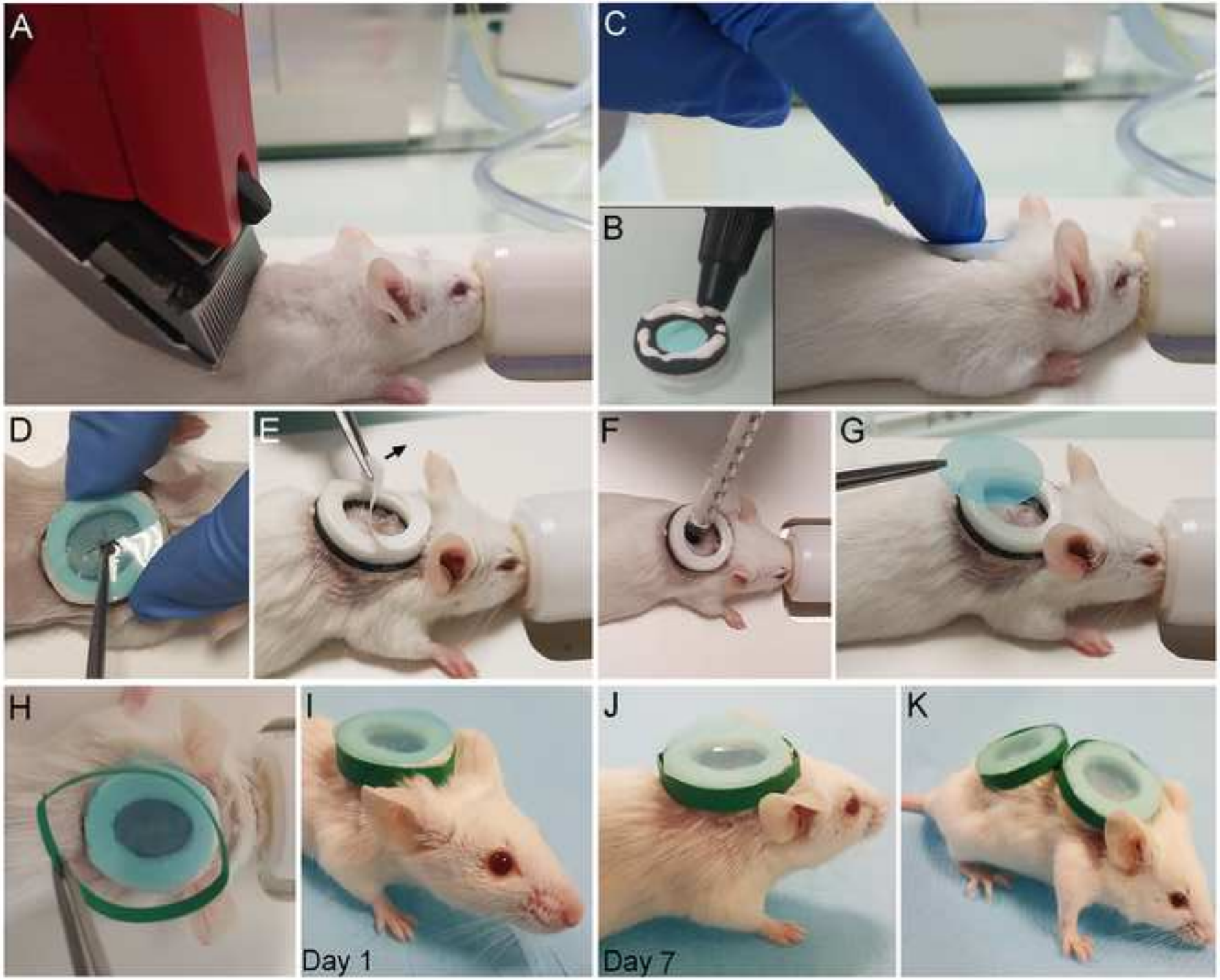

Figure

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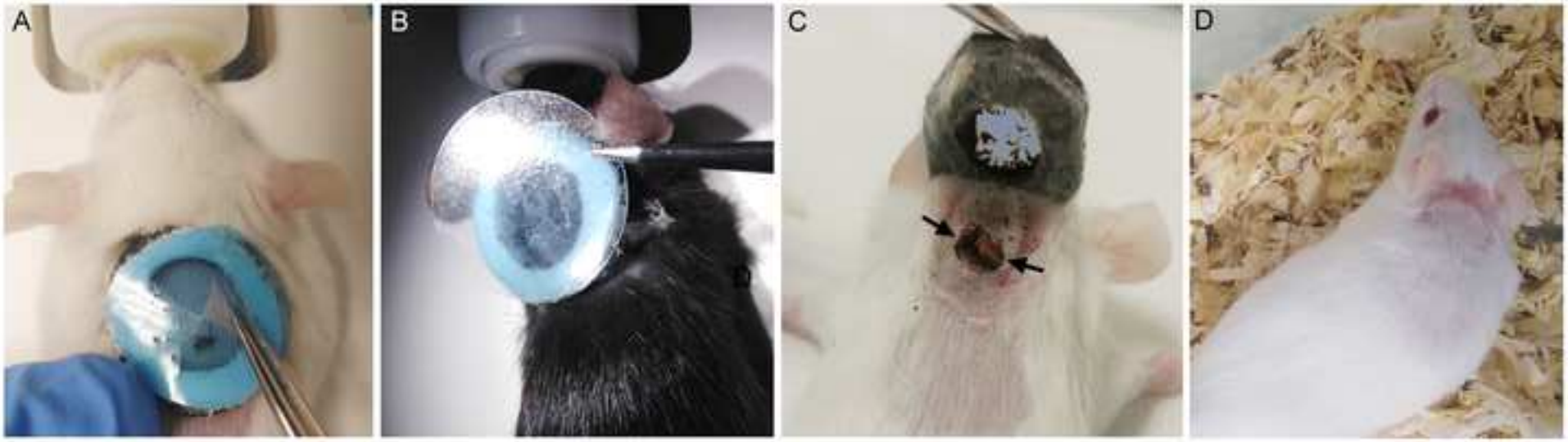

Figure

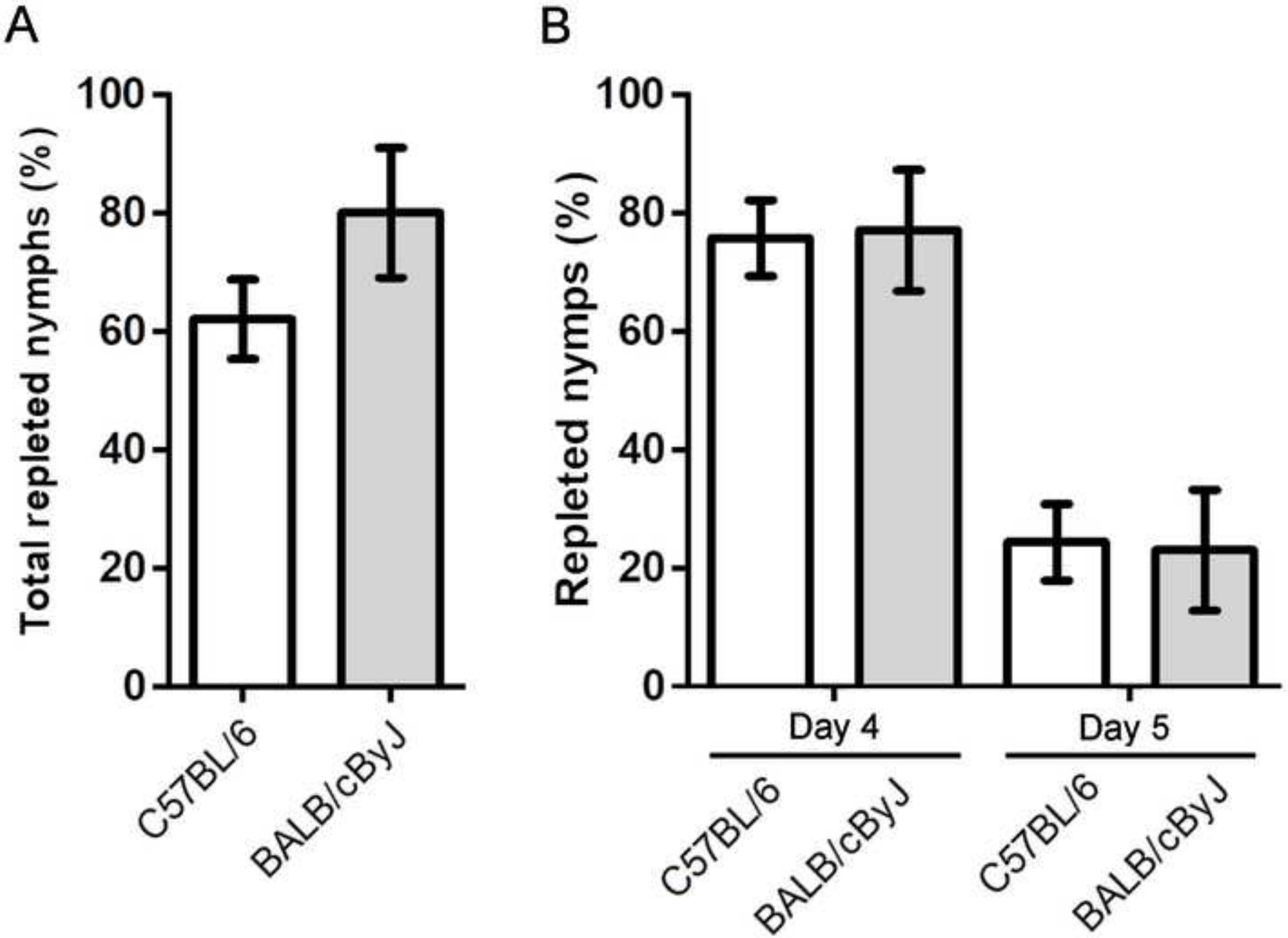

Table of Materials

Name of Material/ Equipment	Company	Catalog Number
EVA-foam 2 mm thick, (low density)	Cosplay Shop	EVA-45kg (950/450/2 mm)
Heat Shrink Tubing Electric Wire Wrap Sleeve	Amazon	B014GMT1AM
Mice BALB/cByJ	Charles River	Strain code 627
Mice C57BL/6	Charles River	Strain code 664
No-toxic Latex Glue	Tear mender	Fabric & Leather Adhesive
Punch Tool Hand Art Tool	Amazon	B07QPWNGBF
PVC Binding Covers Transparent	Amazon	B078BNLSNP
Self Adhesive Pad Sponge Double Coated Foam Tape	Amazon	B07RHDZ35J
Transparent seal stickers (20 mm diameter circles)	Amazon	B01DAA6X66

Comments/Description

It can be ordered also via Amazon (ref. no. B07BLMJDXD)
Different diameters of Heat Shrink Tubing are available via Amazon.

Also available also via Amazon (ref. no. B001RQCTUU)
Saled by amazon as Leather Working Tools 1-25mm Round Steel Leather Craft Cutter Working for Belt Strap
Any transparent PVC sheet of ticknes between 0.150 mm to 0.180 mm is suitable

Saled by amazon as 2 Rolls Double Sided Foam Tape, Super Strong White Mounting Tape Foam

- **Point-by-point responses to the editorial and reviewer comments for the manuscript JoVE61430. All answers are in red color.**

- **Please note that manuscript with "tracking changes" is at the end of this document.**

Editorial comments:

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

Entire manuscript has been proofread for spelling and grammar issues.

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

We have corrected entire manuscript according these requirements.

3. Please ensure that the long Abstract is within 150-300 word limit and clearly states the goal of the protocol.

The long abstract has 153 words and clearly states the goal of the protocol.

4. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."

We corrected original steps 3.3, 3.5, 4.1, 4.2, 5.2 and 5.4 and moved non imperative sentences to the NOTES. For this reason the step 5.4 does not exist anymore. In current version all the steps are in imperative tense.

5. The Protocol should contain only action items that direct the reader to do something.

Following the previous correction all the steps contain only the action directing the readers to do something.

6. Please ensure that individual steps of the protocol should only contain 2-3 actions sentences per step.

We divided each of the steps 3.1 and 3.2 in order to achieve max. 3 sentences per step as recommended.

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

We checked all the steps and feel that all of them answer the "how" question.

8. 2: Age sex strain of mice used. Do you check the depth of anesthesia?
We specified the sex, strain and age of mice in the first NOTE of the Protocol. Our technique does not represent any painful procedure and only proper restraining is necessary in order to glue the capsule and deposit the ticks. In addition we used inhalation anesthesia for 5 minutes maximum for each mouse and animals recover very quickly without any problems. However, we included additional sentence into the step "2" regarding the isoflurane level parameters in order to avoid animal killing by too high anesthesia level.

9. 3.1: is it one per capsule?

We included a NOTE with the maximum nymphs per capsule recommended.

10. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

We highlighted in grey background the filmable content of the protocol. In our case is entire protocol which does not exceed 2.5 pages.

11. Please ensure that the result is described with respect to your experiment, you performed an experiment, how did it help you to conclude what you wanted to and how is it in line with the title.

Our results describe well the experiment and reflect the purpose of it as well as are in great alignment with the title.

12. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e.

“This figure has been modified from [citation].”

Our manuscript does not contain any previously published material.

13. Please write the discussion in paragraph style only. No bullet points.

The bullets points were removed from the discussion.

14. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage, (YEAR).] For more than 6 authors, list only the first author then et al.

All the references were corrected as required.

15. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please sort the table in alphabetical order.

The excel table of materials used has been reorder alphabetically and contains all required information.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
The manuscript reads well and all critical steps necessary to replicate the method are well described.

Major Concerns:
none

Minor Concerns:
Numbers of nymphs shown in figure 4B do not add up with Figure 4A; please recalculate or better explain in case I misunderstood the data;

The data has been recalculated and new image 4A,B is submitted with appropriate explanation in the legend. In addition the (n) number increased due the inclusion of new data we have achieved during the manuscript review process.

The protective tape from Fig 2H-K is missing in the list of used materials.

Information regarding the protective band has been included to the material table.

Please highlight that this protocol can be only applied when all welfare and safety measures are met in the laboratory / at the research institute.

The notice was included in the first NOTE of the Protocol.

Reviewer #2:
Manuscript Summary:
Manuscript is well written and is of interest to tick researchers.

Major Concerns:
Fig. 3C- What is the big lesion on mouse back? Is that a wound due to tick feeding?

The reviewer mistakes the attached ticks in Fig. 3C with the lesion. For this reason we added arrows to the figure pointing the attached ticks and explain it in the figure caption. There is no lesion in Figure 3C.

Minor Concerns:
Needs copy-editing.

Entire manuscript has been checked for copy editing.

Line 138- Please delete to

“to” has been deleted.

Line 231- please delete individual.

“individual” has been deleted

How many mice were used per strain? The N in Fig. 4 represents number of tick nymphs but there is no indication how many mice were used.

We included the information regarding the number of mice per strain in the legend of the figure 4.

TITLE:

A capsule-based model for immature hard tick stages infestation on laboratory mice

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KEYWORDS:

Ticks, infestation, feeding, nymphs, larvae, mice, capsule, glue, animal recovery

SUMMARY:

In this study, we developed a feeding system for nymphal and larval stages of hard tick using a capsule attached to laboratory mouse. The feeding capsule is made from flexible materials and remains firmly attached to the mouse for at least one week and allows comfortable monitoring of tick feeding.

ABSTRACT:

Ticks are obligatory blood feeding parasites at all stages of development (except eggs) and are recognized as vectors of various pathogens. The use of mouse models in tick research is critical for understanding their biology and tick-host-pathogen interactions. Here we demonstrate a non-laborious technique for the feeding of immature stages of hard ticks on laboratory mice. The benefit of the method is its simplicity, short duration, and the ability to monitor or collect ticks at different time points of an experiment. In addition, the technique allows attachment of two individual capsules on the same mouse, which is beneficial for a variety of experiments where two different groups of ticks are required to feed on the same animal. The non-irritating and flexible capsule is made from easily accessible materials and minimizes the discomfort of the experimental animals. Furthermore, euthanasia is not necessary - mice recover completely after the experiment and are available for re-use.

INTRODUCTION:

Ticks are important vectors of several pathogens and represent a serious risk to animal and human health[1]. Setting up an effective feeding system is crucial when studying their biology, tick-host-pathogen interactions, or establishing effective control measures. Currently, several artificial feeding systems which avoid the use of live animals are available for ticks[2-4] and these should be utilized whenever experimental conditions allow. However, in various experimental settings these systems fail to appropriately mimic the specific physiologic features and the use of live animals is necessary to achieve relevant results.

Laboratory mice are commonly used for the study of many biological systems and are routinely utilized as hosts for feeding ticks[5-9]. The two most common methods of feeding immature ticks on mice include free infestations and the use of confinement chambers attached to the mouse. Free infestations are primarily used for larval stages and engorged ticks are allowed to drop to an area where they can be recovered. Confinement chambers are usually composed of acrylic or polypropylene caps which are glued to the mouse's back. The first technique is an effective natural system for tick feeding but does not allow close monitoring during the experiment because the individual ticks are dispersed on different parts of the host body. Additionally, engorged ticks that drop to a recovery area can become contaminated with feces and urine[10-14] that may severely affect the tick fitness or they can be damaged or eaten by the mouse if there is no separation between the animal and the recovery area[15]. Chamber-based systems allow the confinement of ticks to a defined area, however the gluing process is laborious and the caps are often weakly adherent to the glue and thus they often detach during the experiment[16-19]. The caps are also stiff, uncomfortable, and lead to skin reactions which prevent the re-use of the mice and necessitates their euthanasia after the experiment.

In our previous study we successfully developed an effective system using chambers made of ethylene-vinyl acetate (EVA) foam for feeding ticks on laboratory rabbits[20]. Herein, we adapted this system to a mouse model and propose a simple and clean method to feed immature hard tick stages in closed capsules made from EVA-foam. Specifically, our system uses elastic EVA-foam capsules glued to the shaved mice back with fast drying (3 minutes), non-irritating latex glue. This technique allows firm and long lasting attachment of capsules to the experimental mouse as well as effective tick infestation/collection during entire course of the experiment. The flat capsule is made from flexible materials and does not impede manipulation of the mouse for blood collection or other purposes. The system is suitable mainly for the nymphal tick stages, but with slight modification it can be used for feeding larvae as well. The method can be completed by one single experienced person and extensive training is not required.

PROTOCOL:

NOTE: In this study, 10 - 12 weeks old female experimental mice (strain C57BL/6 and BALB/cByJ) were maintained in standard cages with food and water offered *ad libitum* (Green line ventilated racks at -20 Pa, Tecniplast) at the French Agency for Food, Environmental and Occupational Health & Safety (ANSES) accredited animal facilities in Maisons-Alfort, France. Animals were

monitored twice daily by experienced technicians for any abnormal skin reactions, health problems or complications. Please note that this protocol can be only applied when all welfare and safety measures are met in the laboratory.

NOTE: Our laboratory has received permission to use mice for tick feeding by the Ethics Committee for Animal ExperimentsComEth Anses/ENVA/UPEC, Permit Numbers E 94 046 08. For the endpoint, the animals were exposed to CO_2 for 9 minutes in two phases of 4 and 5 minutes each one.

1. Preparation of the Capsule

1.1 Stick the EVA-foam (2 mm thick) and the adhesive double sticky foam together (Figure 1A).

1.2 Using a 20 mm diameter leather hole punch, cut a circle from the ~~attached~~ ~~sticked~~ foam pieces. Then, using a 12 mm diameter hole punch, cut the interior to create the double foam circle (Figure 1B).

NOTE: The frame thickness of the capsule should be greater than 3 mm in size to guarantee sufficient surface for the gluing process to the host skin (see below).

1.3 Peel the protective paper strip from the adhesive double sticky foam (Figure 1C) and attach a transparent circular plastic of 20 mm diameter (Figure 1D).

NOTE: If feeding larvae, do not remove the protective paper strip from the adhesive foam and follow directly to the step 2 in the protocol and glue the double foam ring, including protective paper strip to the mouse.

1.4 Make a ~ 1cm slit in the transparent plastic (Figure 1E).

1.5 Create at least 10 small holes with an entomological pin (Figure 1F) to allow excessive moisture evaporation during the experiment.

NOTE: The capsule (Figure 1G) has a total height of 4 mm (2 mm EVA ~~-~~ foam together with 2 mm adhesive foam) can be used to feed nymphs and larvae of all of the hard tick species. The capsule size (Figure 1H) of 20 mm outer diameter is suitable for most of the mouse strains, but can be modified if necessary.

2. Preparation of the Mice before Tick Infestation

2.1 Anesthetize mouse with isoflurane. Once anesthetized, place mouse to the manipulation pad and attach to a nose-cone for the continuous isoflurane supply (Figure 2A). Monitor the breathing rate and reduce isoflurane level when less than 80 breaths per minute.

NOTE: Prior the manipulation, label the individual mouse by tattooing or radio-frequency identification chip if necessary. It is recommended to keep the individual mice in separate cages to avoid capsule damage by biting.

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2.2 Shave the anterior part of the mouse from behind the shoulder blades up to the area just behind the ears (Figure 2A).

NOTE: The shaved area should be greater than the capsule surface.

2.3 Apply non-irritating latex glue to the entire EVA-foam site of the prepared capsule and wait for 1 min (Figure 2B).

2.4 Glue the capsule to the mouse back by slight 3 minutes constant pressure with the ~~index~~ finger(s) (Figure 2C), especially on the left and right sides ~~of~~ of the capsule. Slightly lift the capsule to visually check its attachment to the skin. If non-attached regions are found, apply more glue using a spatula and press for another 3 minutes.

3. Tick Infestation

3.1 For nymph infestation, introduce the individual nymphs into the capsule via the cut made in step (1.4) (Figure 2D).

NOTE: We recommend to deposit up to 20 *Ixodes* nymphs per one capsule.

3.2 Slightly squeeze the capsule from two sides to allow the transparent plastic to bend for easier introduction of individual nymphs using fine dissection forceps (Figure 2D). Push the individual nymphs via the cut inside the capsule. Once inside turn the forceps in 90 degree and pull out the forceps to deposit ticks inside the capsule.

3.~~2~~ 3 For larvae infestation remove the paper slip ~~(see NOTE in step 1.3)~~ f~~o~~rom the attached capsule (Figure 2E). ~~The individual larvae should be placed into a 1 ml syringe with cut end plugged by piece of cotton (Figure 2F).~~ Place the syringe, containing larvae (Figure 2F), directly inside the capsule and deposit ticks by pushing the syringe plunger, ~~gently~~ Gently turn the plunger~~it~~ towards ~~to~~ the skin to remove the remaining ~~ticks~~larvae attached ~~to the plunger~~.

NOTE: We recommend to place larvae into a 1 ml syringe with cut end plugged by piece of cotton prior the experiment.

3.4 Once the larvae are deposited onto the skin, close the capsule by attaching the transparent plastic (Figure 2G).

~~NOTE: We recommend to place~~ ~~The individual larvae should be placed into a 1 ml syringe with cut end plugged by piece of cotton (Figure 2F)~~ ~~prior the infestatioexperimentn.~~

3.~~3~~ 45 Apply the protective plastic~~/rubber~~ band around the capsule (Figure 2H). ~~This step greatly improved the durability of the capsule for the entire duration of the experiment (Figure 2I and J).~~

NOTE: The protective plastic band ~~This step~~ greatly improved the durability of the capsule for the

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entire duration of the experiment (Figure 2I and J). We have also succeeded in attaching two capsules to one individual mouse (Figure 2K). In this case, a minimum of 3 mm space between the capsules is required and the shaved area should be increased appropriately.

3.~~3~~ ~~56~~ Return the mice to the cage.

4. Collection of Ticks

4.1. Anesthetize the mouse as in step 2.1 above.

4.2 Make a cross cut (Figure 3A) to the plastic with a scalpel.

NOTE: This cross shaped cut enables easy ~~removal~~ collection of engorged ticks or ~~and~~ detachment of the feeding ticks if necessary. ~~(Figure 3A)~~

4.3. If needed, reclose the capsule by sticking an adhesive plastic patch to the transparent plastic (20 mm diameter, Figure 3B). ~~If collection of ticks at multiple time points is desired, the same sticky plastic patch can be used.~~

NOTE: If collection of ticks at multiple time points is desired, the same sticky plastic patch can be used. If the protocol requires, you may also euthanize the mouse, remove the capsule and collect/detach the ticks (Figure 3C).

5. Recovery of the mice

5.1 Keep the mice in cage for one additional week.

5.2 Let the capsule detach naturally. ~~In our experience it takes about 8-9 days for them to fall off.~~

NOTE: In our experience it takes about 8-9 days for capsules ~~them~~ to fall off. Once the capsule is off, check the skin of the mice for abnormal reactions. Although normally no treatment is required, an emollient lotion can be used in case of irritation. If the ethical protocol allows, the recovered mice (Figure 3D) can be reused for another tick infestation or different experiment(s).

~~5.4 If the ethical protocol allows, the recovered mice (Figure 3D) can be reused for another tick infestation or different experiment(s).~~

REPRESENTATIVE RESULTS:

We propose the detailed step-by step method for feeding immature hard ticks stages in EVA-foam capsules applied to a mouse's back (Figure 2). This non-laborious protocol is suitable for various types of experiments when precise tick monitoring and collection is required. The main advantages of this method are its simplicity, easily accessible cost-effective materials (Table of Materials) and short duration. Our system has been successfully used in various mouse strains with moderate to high engorgement success rate of nymphal stages of *Ixodes ricinus* ticks (Figure

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4). In addition we succeeded in attaching two capsules to one mouse individual (Figure 2K) allowing to us to feed two different groups of ticks on the same animal. The use of the highly effective, fast-drying, and non-irritating latex glue ensures that the capsule is firmly attached within ~~three~~ 3 minutes. In addition, the capsule remains attached for at least one week (Figure 2J) which is sufficient time for engorgement of most of the immature hard tick species[21-24]. Due to the capsule elasticity, further manipulation of the mouse for blood collection or other purposes is very convenient. This procedure also allows complete recovery of the mice after the experiments (Figure 3D) giving the opportunity to reuse the animals and avoid euthanasia.

FIGURE AND TABLE LEGENDS:

Figure 1. **Preparation of the EVA ~~–~~foam capsule. (A)** Attachment of EVA ~~–~~foam (black) and adhesive double sticky foam (white). **(B)** Cutting 20 mm diameter outer and 12 mm inner circle using leather hole punches. **(C)** Removal of the paper protection tape from the adhesive double sticky foam. **(D)** Attachment of the transparent plastic to the capsule. **(E)** Cutting the slit in the transparent plastic with a scalpel. **(F)** Creation of holes using an entomological pin in the plastic. **(G-H)** Schematic drawing of the different parts of the capsule and dimensions.

Figure 2. **Gluing the capsule to the mice and tick infestation. (A)** Shaving mouse's back anterior part. **(B)** Application of the latex glue to the EVA ~~–~~foam side of the capsule. **(C)** Attachment of capsule to the mouse. **(D)** Placing the ~~tick~~ nymph in the capsule via the cut in the transparent plastic. **(E)** Peeling the paper protection tape from the adhesive double sticky foam before larvae infestation. **(F)** Injections of larvae inside the capsule using a cut syringe. **(G)** Closing the capsule with the transparent plastic ~~to the capsule~~. **(H)** Placing a protective plastic ~~/rubber~~ band around the capsule. **(I)** Mouse with the attached capsule - 1st day. **(J)** Mouse with the attached capsule - 7th day. **(K)** Mouse with two capsules attached.

Figure 3. Tick collection and mouse recovery. (A) Cutting cross-shape opening for tick collection. **(B)** Resealing the capsule with adhesive plastic patch. **(C)** Euthanized mouse and capsule removal. Arrows show the attached ticks. ~~-~~**(D)** Recovered mouse after dropped off capsule.

Figure 4. Engorgement success and feeding duration of *Ixodes ricinus* nymphs feeding on mice. (A) Total percentage of engorged nymphs in C57BL/6 (n=15) and BALB/cByJ (n= 5) mice ~~strains~~. **(B)** Duration of nymph engorgement in C57BL/6 and BALB/cByJ mice ~~strains~~. The (n) ~~"n"~~ numbers ~~(n)~~ in panel A refer to the total nymphs infested.

- **DISCUSSION:**

- **Critical steps in the protocol**

The most critical step in the protocol is firm gluing of the capsule to the mouse skin. Therefore, the latex glue should be homogenously applied to the entire EVA ~~-~~foam surface of the capsule

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and constant pressure for 3 minutes should be applied, especially ~~on~~to the left and right side of the capsule~~, should be applied for 3 minutes~~. We also recommend placement of the capsule as far forward on the back as possible to avoid its removal by the mouse using its rear paws. In our experiments only the adhesion of the EVA ~~-~~foam and latex glue to the mouse skin has been validated and we can't guarantee achievement of same results using different materials.

- **Modifications and troubleshooting of the method**

During our experiments, detachment of the capsule from the skin within first seven days was not observed. We strongly recommend protecting the outer surface of the capsule using the plastic~~/rubber~~ band (Figure 2H). If the protective ~~strip~~ band is damaged over the course of tick feeding, it can be replaced with a new one. The diameter of the capsule can be modified for different mouse strain sizes. We suggest monitoring the feeding ticks at least twice daily and to collect engorged ticks immediately after detachment to avoid their desiccation.

- **Limitations of the method**

The number of infested ticks is limited by the capsule diameter as well as the host size. In our experiments we used maximum of 20 nymphs or 100 larvae of *I. ricinus* for one mouse ~~individual~~. For the larger size ticks such *Amblyomma* or *Hyalomma* sp., etc the number of infested ticks should be reduced to avoid harm to the host from blood loss[19,26,27]. Therefore this technique is not suitable for the maintenance of tick rearing colonies, where large numbers of ticks are required to feed. For this purpose, larger hosts like rabbits or sheep are recommended[27] to reduce overall animal requirement.

- **The significance of the method with respect to existing/alternative methods**

Our technique is suitable for various types of experiments where a mouse model is required and it is necessary to keep ticks in enclosed area for easy collection and/or monitoring of their biological parameters. Compared to other techniques[10-18], this simple protocol greatly reduces the overall anesthesia time (approximately 5 minutes) per mouse and the fast drying, non-irritating latex glue does not cause harm to the animal. The highly adhesive EVA ~~-~~foam capsule protects the tick feeding area and minimizes the risk of lost, damaged or eaten ticks as reported in free infestation systems[10-15]. The great advantage of the proposed technique is the flat-shape capsule and its firm long-lasting attachment to the skin allowing easy manipulation with the mouse if required. Special attention has been paid on usage of elastic and non-irritating materials to reduce the discomfort to the experimental animals allowing complete recovery of the mouse host after experiment (Figure ~~4C~~3D).

- **Future applications or directions of the method**

The method is expected to be used for a variety of the experiments when studying tick-host-pathogen interactions, tick manipulation of host immune systems, evaluating different tick control measures or tick biology.

ACKNOWLEDGMENTS:

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We acknowledge the technical assistance of Alain Bernier French National Institute of Agricultural Research (INRAE), and Océane Le Bidel (ANSES). The study was supported by the DIM One Health - *Région Île-de-France (Acronym of the project: NeuroPaTick)*. The mice were purchased by ANSES. Dr. Jeffrey L. Blair is acknowledged for reviewing the earlier version of the manuscript.

DISCLOSURES:

The authors have nothing to disclose.

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