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A Versatile Model Of Hard Tick Infestation On Laboratory Rabbits

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Corresponding Author:	Ladislav Simo French National Institute for Agricultural Research, UMR-BIPAR (Anses, ENVA, INRA) Maisons-Alfrot, Val-de-Marne FRANCE
Corresponding Author's Institution:	French National Institute for Agricultural Research, UMR-BIPAR (Anses, ENVA, INRA)
Corresponding Author E-Mail:	ladislav.simo@vet-alfort.fr;simo@k-state.edu
First Author:	Consuelo Almazán
Other Authors:	Consuelo Almazán
	Sarah Irene Bonnet
	Martine Cote
	Yoonseong Park
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Please consider the revised version of the manuscript entitled " ***A Versatile Model Of Hard Tick Infestation On Laboratory Rabbits*** ", by Almazan, C., et al., for publication in the Journal of Visualized Experiments (JoVE). 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 the hard ticks on the laboratory rabbits. The most significant change/improvement to the first submission is the Velcro-based openable capsule allowing convenient monitoring and sampling of the ticks during the feeding on the rabbits. In addition based on the reviewers recommendation we focused on emphasizing the technique itself by highlighting its simplicity and versatility and not the experimental animal welfare as was the case in the initial submission. This simple method using easily accessible materials may be used for a variety of different experimental settings when studying tick biology, and is expected to be adapted for use at tick research centers around the world.

Reviewers' constructive criticisms were greatly appreciated. Our manuscript word file document contains the "clean" (no tracking changes) version, followed by the "tracking changes version" in the same document. Also all our point by point answers to reviewers specific comments are in the separate file (Rebuttal document).

Please let me know if you have any questions.

Sincerely,

Dr. Ladislav Simo
Chargé de recherche de 1ère classe
L'Institut national de la recherche agronomique (INRA)
UMR BIPAR Anses, ENVA, INRA
22 rue Pierre et Marie Curie

94700 Maisons-Alfort
France
Tel: +33 1 49 77 46 52
Fax: +33 1 49 77 28 28

TITLE:

A Versatile Model of Hard Tick Infestation on Laboratory Rabbits

AUTHORS & AFFILIATIONS:

Consuelo Almazán[1], Sarah Bonnet[1], Martine Cote[1], Mirko Slovák[2], Yoonseong Park[3], Ladislav Šimo[1]

[1]L'Unité Mixte de Recherche (UMR) Biologie moléculaire et immunologie parasitaires (BIPAR), Institut national de la recherche agronomique (INRA), Ecole Nationale Vétérinaire d'Alfort, Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail (ANSES), Université Paris-Est, Maisons-Alfort, France

[2]Institute of Zoology, Slovak Academy of Sciences, Bratislava, Slovakia

[3]Department of Entomology, Kansas State University, Manhattan, KS, USA

CORRESPONDING AUTHOR:

Ladislav Šimo (ladislav.simo@vet-alfort.fr)

Phone: +33 783692721

EMAIL ADDRESSES OF CO-AUTHORS:

Consuelo Almazán (consuelo.almazan@anses.fr)

Sarah Bonnet (sarah.bonnet@vet-alfort.fr)

Martine Cote (martine.cote@vet-alfort.fr)

Mirko Slovák (slovak.mirko@gmail.com)

Yoonseong Park (ypark@ksu.edu)

KEYWORDS:

Ticks, feeding, rabbit, jacket, capsule, glue, animal recovery

SUMMARY:

We have developed a simple and versatile system to feed hard ticks on laboratory rabbits. Our non-laborious protocol uses easily accessible materials and can be adjusted depending on the requirements of the various experimental settings. The method allows comfortable monitoring and/or sampling of ticks during the entire feeding period.

ABSTRACT:

The use of live animals in tick research is crucial for a variety of experimental purposes including the maintenance of hard tick colonies in the laboratory. In ticks, all developmental stages (except egg) are hematophagous, and acquiring a blood-meal when attached to their vertebrate hosts is essential for the successful completion of their life cycle. Here we demonstrate a simple method that uses easily openable capsules for feeding of hard ticks on rabbits. The advantages of the proposed method include its simplicity, short duration and most importantly versatile adjustment to the needs of specific experimental requirements. The method makes possible the use of multiple chambers (of various sizes) on the same animal, which permits feeding of multiple stages or different experimental groups while reducing the overall

animal requirement. The non-irritating and easily accessible materials used minimizes discomfort to the animals, which can be easily recovered from an experiment and offered for adoption or reused if the ethical protocol allows it.

INTRODUCTION:

The hard ticks (Ixodidae) are well known as slow feeding arthropods and can be attached on a host for several days, or weeks, depending on the species and developmental stage[1]. These obligatory hematophagous arthropods are vectors of a wide variety of infectious agents, such as bacteria, protozoa, and viruses, and thus present a significant risk to humans and animal health[1]. When studying tick biology or evaluating new control methods, the establishment of an effective tick feeding system is crucial in order to effectively design the experiments and accomplish the goal(s) of the study. Recently, several artificial tick feeding systems (avoiding the use of live animals) have been developed[2–4] and they should be used whenever possible. However, these systems have not been able to completely replace tick feeding on live animals, and they are not suitable substitutes for many physiologic conditions required for scientific studies. Therefore, in some cases, the use of experimental animal hosts is crucial to guarantee the relevance of experimental results.

Laboratory New Zealand rabbits have shown to be the most suitable and accessible hosts for several ixodid tick species[5–9]. Two common strategies of tick feeding on rabbits have been frequently used: a) feeding on rabbit ears covered with cotton cloth or socks[6,7], and b) feeding in cotton bags[9], nylon bottles[10] or neoprene chambers[11] glued to the rabbit's back. The feeding on rabbit's ears is not an elegant system, because ticks (especially early stages, larvae or nymphs) may crawl and attach deep in the ear canal, which is uncomfortable for the animal and makes the monitoring of tick feeding and/or the recovery of engorged ticks difficult. This system is also limited to only two tick groups on ears fully covered by socks protected by Elizabethan collars, representing a significant discomfort for the animal. Other systems[9–11] are definitely more advanced and well suited for hard tick colony maintenance. However, they are limited in the number of experimental groups placed on the rabbit as well as in the modifiable sizes/shapes of the feeding chambers. In addition, these protocols often require hobbling the rear rabbit legs to avoid scratching and the use of Elizabethan collars to prevent grooming.

Herein we propose a simple, non-laborious and very effective method to feed multiple groups of hard ticks in closed chambers glued to the rabbit back covered by a jacket, eliminating the need for Elizabethan collars or hobbling during the experiment. Specifically, our system uses elastic capsules made from an ethylene-vinyl acetate (EVA) foam sheet covered by mosquito mesh and glued to the shaved rabbit back with fast solidifying (3 min) non-irritating latex glue. This technique allows the attachment of multiple capsules of desired size or shape, and few weeks after the experiment the rabbits are fully recovered. The system is suitable mainly for the nymphal and adult hard tick stages, but with a little modification it can be used for larvae feeding as well. The EVA foam-based methods for hard tick feeding may be adapted to other types of vertebrate hosts, for example sheep (which is shown as one of the alternatives in this paper).

PROTOCOL:

Note: In this study, rabbits were maintained in standard cages with food and water offered *ad libitum* 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 two experienced technicians for any abnormal skin reactions, health problems or complications. The experimental room was secured by framing the interior of the doorway with double-sided tape to avoid accidental escape of ticks. The method works best if two people work as a team, but it is possible to complete single handedly by one experienced person. The six month old, Rambouillet female sheep was kept at the Centre for Biomedical Research (CRBM) facilities at the National Veterinary School of Alfort (ENVA), where water and food were supplied *ad libitum,* and it was checked twice daily.

Note: Our laboratory has received permission to use rabbits and sheep for tick feeding by the Ethics Committee for Animal Experiments ComEth Anses/ENVA/UPEC, Permit Numbers 01741.01 and 11/10/16-5B, respectively. Since we used only pathogen-free ticks in our experiments, all the rabbits used in this study were offered for adoption via the White Rabbit Association, Paris, France.

1. Preparation of the Capsules

1.1. Cut the desired size of the capsule from the EVA foam sheet (**Figure 1A**). Round the outer corners (**Figure 1B**) of the capsule to minimize accidental detachment when gluing it to the rabbit skin.

Note: The frame thickness of the capsule should be around 8 mm. Use a 5 mm thick foam sheet for the larvae, nymphs, and small tick adult species such as *Ixodes*. A 1 cm thickness foam sheet is suitable for large size adult ticks such as *Amblyomma* sp., *Hyalomma* sp., *etc.* The size of the capsule varies based on the experimental requirements. For example, for 20 *Ixodes* adult couples, 200 nymphs, or 1000 larvae, we use an inner capsule size of 5 x 5 cm, 6 x 7 cm or 7 x 9 cm, respectively.

1.2. Cut the 8 mm wide strips of self-adhesive hook tape (see **Table of Materials**) and stick them to the prepared EVA foam frame (**Figure 1C**).

1.3. Cut the same size strips from self-adhesive loop tape (see **Table of Materials**) and bind them to the hook sides attached to the EVA-foam frame (**Figure 1D**).

1.4. Cut the appropriate size of the fine mosquito mesh (mesh size less than 50 μm) to the size of the EVA foam frame and stick it to the self-adhesive loop (**Figures 1E and 1F**). Cut the overhangs if necessary.

Note: This type of capsule can be used to feed nymphs and adults of hard tick species, while a different sealing system of the capsules is needed for larval feeding (**Supplemental Figure 1**) to prevent accidental escape of the larvae via the fastened hook-and-loop side.

2. Preparation of the Rabbit before Tick Infestation

2.1. Shave the area of the rabbit back and sides to be used with clippers (**Figure 2A**).

2.2. Apply non-irritating latex glue to the entire surface of the prepared capsule and wait for 1 minute (**Figure 2B**).

2.3. Glue the capsule by pressing to the skin (especially at the corners) with the fingers for about 3 minutes (**Figures 2C and 2D**).

Note: When gluing more than one capsule, make sure to keep at least 5 mm space between them (**Figures 2E and 2F**). We usually avoid the region of the spine, but it may be used if needed.

2.4. Slightly lift the capsules to visually check their attachment to the skin. If non-attached regions are found, apply the glue using a spatula and press for another 3 minutes.

2.5. Apply protective tape to the rear paws of the rabbit to prevent jacket damage (**Figure 2G**).

Note: This step is optional and is mainly to prevent jacket damage, not damage to the tick capsule.

2.6. Put on the rabbit jacket by placing the front legs through the openings and tightening the neck well. Do not place the rear legs through the elastic enclosures at this step and leave the zipper open (**Figure 2H**).

3. Tick Infestation

3.1. Place the ticks into a plastic syringe (1 or 5 mL depending of the number of the individuals) with the needle-end cut and plugged with cotton (**Figure 1G**). If a small amount of ticks are to be infested, use forceps.

Note: For tick colony maintenance allow the engorged tick female(s) to lay eggs with subsequent hatching inside the syringe (5 or 10 mL) covered by the mosquito mesh wrapped by rubber band[12] to avoid laborious manipulation of the larvae at the time they are applied to the host (**Figure 1H**). Also, fully engorged larvae may be allowed to molt in the syringe (**Supplemental Figure 1I**; 5 or 10 mL) for direct infestation of the rabbit with nymphs.

3.2. Place the syringe deep into the capsule via the open corner and inoculate the ticks by pushing the syringe plunger. Slowly twist the plunger toward the rabbit skin to remove the remaining ticks attached to the plunger and simultaneously pull it out from the capsule (**Figure 2I**).

Note: If some of the individuals crawl out of the capsule, return them using forceps.

3.3. Close the capsule by refastening the hook-and-loop tape.

3.4. Place the rear legs of the rabbit into the rear elastic enclosures of the jacket and zip closed.

Note: Make that an index finger can fit between the neck of the jacket and the rabbit to ensure comfort and also to avoid chewing on the jacket.

3.5. Return the rabbit to the cage (**Figure 2J**).

Note: The time from the infestation to collection of replete ticks vary among different tick species and the developmental stages. For example, for *Ixodes scapularis* and *Ixodes ricinus*, the durations of feeding for adults, nymphs, and larvae are 6-9, 3-4, and 2-3 days, respectively. A list of references for 29 different hard tick life cycles under laboratory conditions can be found in Levin and Shumacher (2016)[9].

4. Collection and Monitoring of Ticks

4.1. Take the rabbit from the cage to the bench and unzip the jacket.

4.2. Gently restrain the rabbit with your hands. Open the capsule by unfastening the hook-and-loop tape (**Figures 2K and 2L**) and collect the ticks by brushing the engorged larvae (**Supplemental Figure 1**) or nymphs to a plastic dish or using forceps for adults (**Figure 2L**). If partially fed (not replete) ticks are required, use a tick twister or forceps to detach them.

Note: If maintaining the tick colonies please see the note in step 3.1. Maintain the engorged ticks in appropriate humid and temperature conditions according to the particular tick species.

4.3. If needed, refasten the hook-and-loop tape to close the capsule.

5. Recovery of the Rabbit

5.1. Remove the mosquito mesh completely from the capsule and leave the jacket on the rabbit (**Figure 2M**).

5.2. Wait 3-4 weeks and try to remove the capsule by gently trimming one of the corners (**Figure 2N**). If the capsule is still firmly attached, repeat this step one week later.

5.3. Remove the jacket and let the rabbit recover in the cage.

Note: Once the capsule is off, check the skin of the rabbit for abnormal reactions. Although normally no treatment is required, an emollient lotion can be used in case of irritation.

5.4. If the protocol and experiments allow, the recovered rabbit (**Figure 2O**) can be reused or offered for adoption.

Note: Rabbits have been shown to acquire tick resistance once exposed to repeated tick infestations[13]; therefore, reinfestations are not recommended unless the experiment requires.

REPRESENTATIVE RESULTS:

Here we propose for the first time a detailed step-by step method of hard tick feeding in EVA foam capsules applied to a shaved rabbit's back, covered by a jacket (**Figures 1 and 2**). This protocol is suitable for various types of experiments when different tick groups on the same host are required and can be also used for mass rearing of hard ticks. The tick feeding success in the laboratory mostly relies on the fitness of the tick individuals and suitability of the rabbit host for the particular tick species, rather than the technique itself. Our system using EVA-foam capsules glued to the rabbit back have been proven to be highly successful when feeding different developmental stages of various hard tick species (**Table 1**) and may also be adapted for other type of laboratory hosts, such as sheep (**Figure 3**).

The main advantages of this method are the simplicity, easily accessible materials (**Table of Materials**) and, most importantly, a comfortable open-close system allowing easy monitoring of the ticks during the feeding. In addition, this versatile method offers the possibility of a variety of different experimental settings based on the modifiable number, shape, and composition of the capsules on the host (**Figure 2D-F**), meeting the challenges of the particular study. The use of the highly effective, fast-drying, and non-irritating latex glue ensures that the capsule is firmly glued in three minutes and remains attached for at least three weeks. This procedure also allows complete recovery of the rabbit hosts after the experiments (**Figure 2O**).

FIGURE AND TABLE LEGENDS:

Figure 1. Preparation of the EVA foam capsule and ticks. A, B: Cutting the capsule from the EVA foam. **C:** Placing the strips of self-adhesive hook tape to the capsule. **D:** Binding the loop side strips and peeling the tape from the fasteners. **E, F:** Sticking the mosquito mesh to the adhesive strips. **G:** Example of freshly hatching larvae inside the syringe with the cut needle-end covered by the mosquito mesh held in place by a rubber band. **H:** Example of adult hard ticks inside the syringe (5 mL) with the cut needle-end covered by cotton.

Figure 2. Gluing of the capsule to the rabbit, tick infestation/recovering and capsule removal. A: Shaved rabbit's back. **B:** Application of the glue to the prepared EVA foam capsule. **C, D:** Attachment of different size capsules to the rabbit. **E, F:** Dorsal view of a rabbit showing two or several chambers attached to the back, respectively. **G:** The tape is positioned around the rear feet. **H:** The jacket is applied starting from the front legs and neck, and the rear part is left open. Opening the corner of the capsule is also shown. **I:** Inoculation of ticks to the capsule via the open corner using the syringe. **J:** The jacket is zipped completely and rabbit is relocated to the cage. **K:** Monitoring the ticks during their feeding by opening the capsule. **L:** Collecting replete ticks using the forceps. **M:** Empty capsule after tick removal. **N:** Detachment of the capsule (after 3-4 weeks) from the rabbit. **O:** Fully recovered rabbit

Figure 3. EVA foam system adapted to sheep. A: Attachment of the foam capsules to a shaved and cleaned area in the lateral side of the sheep back. **B:** After infestation, the attached capsules are covered with a bandage (orthopedic stockinette), instead of a jacket.

Table 1. Engorgement rate of different developmental stages of hard tick species feeding on rabbits inside EVA-foam capsules. *Due to the fast molting process of *H. excavatum* immature stages, the engorged larvae (data not shown) were left to molt into nymphs that subsequently engorged in the same capsule.

DISCUSSION:

The most important step in this entire protocol is to glue the capsule firmly to the shaved skin. For this reason, constant pressure for at least 3 minutes is critical, especially at the corners. When inoculating the ticks into the capsule, it is important to apply them deep into the opposite corner from the open one to avoid tick escape during the sealing. When planning the experiments, make sure that all the capsules are covered by the jacket to avoid damage by chewing or scratching. Make sure that the neck region of the jacket is tight enough to prevent chewing but sufficiently loose that the rabbit remains comfortable.

One of the main advantages of the described technique is its simplicity and that modifications in terms of size and number of the capsules can be used. During our experiments, detachment of capsules from skin were not observed. However, occasional damage of the jacket (but not the capsule) by the rabbit may occur.

In rabbits, we have observed that, in the capsule, fully engorged-detached ticks (mainly immature stages such as larvae and nymphs) desiccate faster due to the temperature of the rabbit. For this reason, we suggest estimating the duration of the feeding period of particular tick species and stage, to plan engorged tick collection immediately after their detachment. Although our system has been tested for feeding of various hard tick species (**Table 1**), the rabbit is not a natural host for all hard tick species[14]. This limitation may be overcome by adapting this system to other animal hosts suitable for a particular hard tick species. Here we reported the use of the EVA foam system adapted to sheep to feed all the developmental stages of *I. ricinus* (**Figure 3**). In this particular case, the sheep needs to be shaved and washed with cotton impregnated with 70% ethanol to eliminate the oil present at the surface of the skin. Afterwards the same procedure as the one described for rabbits was followed, but instead of a jacket, a cotton bandage was used around the back as shown in **Figure 3**.

When developing this system, we paid special attention to minimize the amount of the materials used and steps of the procedure. Compared to other methods, we do not use anesthesia, rabbit collars, ear socks or hobbling of the rear legs[5–10]. In addition, the present protocol is not laborious, and no intensive training is required to become familiar with this technique. The EVA-foam based tick feeding system detailed in this study is expected to be used for a variety of different experiments when studying tick biology, host-vector-pathogen interactions, or evaluating different control measures like acaricides or vaccines. Our future direction will be adapting the EVA foam capsule feeding system to the mouse model to feed immature stages of hard ticks.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure

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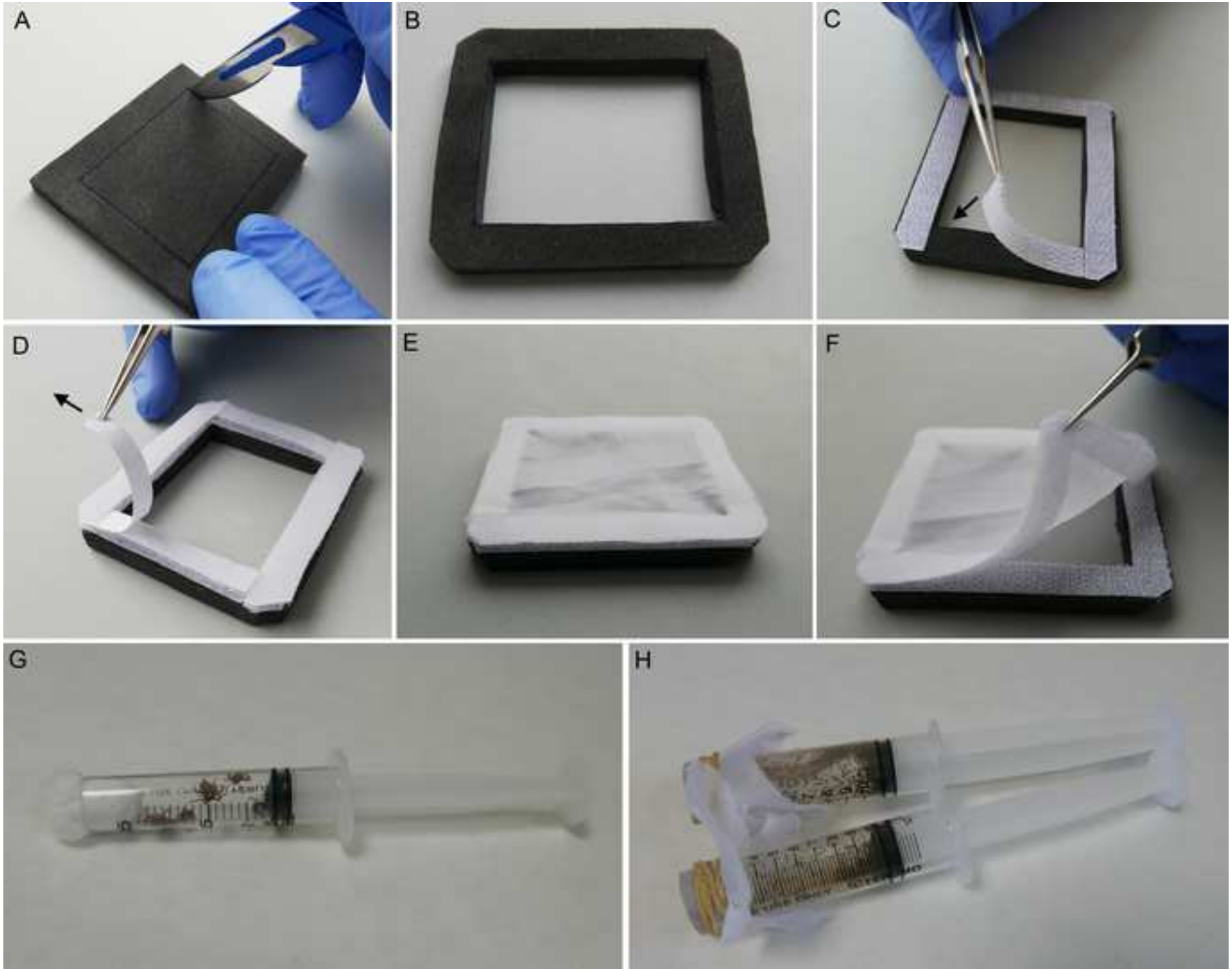

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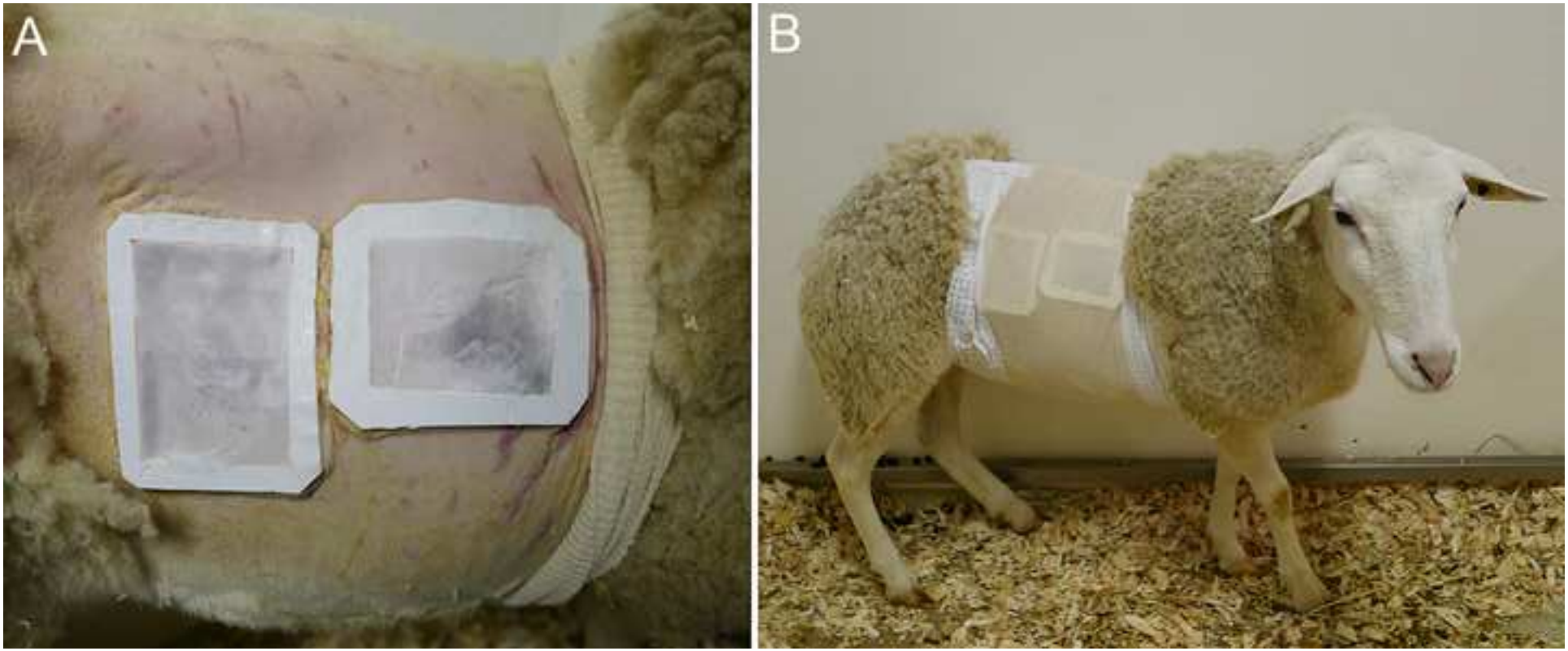

Table 1

Tick species	Number of infested ticks/Number of en	
	Larvae	Nymphs
Ixodes ricinus	-	692/557 (80.49%)
Ixodes scapularis	-	-
Dermacentor reticulatus	3550/3255 (91.7%)	900/803 (89.22%)
Rhipicephalus appendiculatus	3300/2822 (85.52%)	490/421 (85.91%)
Rhipicephalus pulchelus	-	1920/1831 (95.36%)
Amblyomma variegatum	332/225 (67.77%)	404/308 (76.24%)
Amblyomma americanum	-	140/134 (95.71%)
Hyalomma excavatum	1000*	510* (58.8%)

gorged ticks (%)
Females
670/592 (88.35%)
40/34 (85%)
323/305 (94.42%)
370/362 (97.83%)
282/257 (91.13%)
207/146 (70.53%)
31/27 (87.1%)
380/313 (82.36%)

Materials Table

Name of Material/ Equipment	Company
New Zealand Rabbits (2.5-3.5 kg)	Charles River
Rambouillet sheep	Local provider-tick free farm
EVA foam 5 mm thick	Cosplay Shop
Foam Sheet 9" X 12" 6 mm-White	Amazon
Full length rabbit jackets	Harvard Apparatus, Inc.
Non-toxic latex glue	Tear mender
Tubular cotton orthopedic stockinette	BSN Medical
Mosquito mesh	Loisirs Creatifs
Leukoplast	BSN medical S.A.S
Adhesive hook-and-loop tape	AIEX store
Fast drying glue	Fixtout

Catalog Number	Comments/Description
Strain Code 571	
Female 6 months old	
EVA-PE451kg (950mm x 450mm)	10 mm PE45kg foam from the Cosplay Shop may be used for the large adu
FOAMSHT6-20	6 mm-EVA foam ca be ordered via Amazon as an alternative to the foam f
620077- medium, 6270078 - large	
Fabric & Leather Adhesive	
9076 (12-15 cm wide)	
Very fine filter nylon mesh fabric	Any mosquito mesh, or curtain material with the mesh size less than 50 m
LF 72361-02	
AIEX 39.37 Feet/12m Hook and Loop Self Adhesive Tape Roll, 20 mm width, white colour	Fullfiled by Amazon
Superglue	

ılt tick species
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1 Alewife Center #200
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CORRESPONDING AUTHOR:

Name:	Ladislav Simo
Department:	Animal Health Department
Institution:	French National Institute for Agricultural Research (UMR-BIPAR)
Article Title:	A MODEL OF TICK INFESTATION EMPHASISING ON LABORATORY ANIMAL WELFARE
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The point-by-point responses to the editorial and reviewers comments:

Editorial comments:

General:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response: The spelling and the grammar has been checked across entire manuscript.

Protocol:

1. For each step, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

Response: The protocol has been modified based on the editorial comment.

Specific Protocol steps:

1. 4: What is a usual time scale from infestation to collection? Is there a maximum time?

Response: The following Note has been included to the step 3.4 in the protocol (Line 185-189):

NOTE: The time from the infestation to collection of the ticks vary among different tick species and their developmental stages. For example I. scapularis and I. ricinus duration of feeding for adults, nymphs and larvae is 6-7, 3-4 and 2-3 days respectively. The list of references for the 29 different hard tick life cycle under laboratory condition can be found in Levin and Shumacher (2016).

2. 5.2: What are usual abnormal reactions, and how are they treated? Also, Figure 3H does not exist.

Response: Although we have not experienced any abnormal reactions, in some cases the skin irritation after the capsule is removed may occur, in that case an emollient lotion can be used, we have included this in the manuscript. All the figures have been reordered and cited in the text.

Results:

1. Can you provide examples of outcomes from this procedure, in particular how its results compare to other methods? These can be from other publications, although reused Figures will need to be properly cited and copyright permission obtained (if necessary).

Response: We included/modified a paragraph in the results section giving the examples of the tick feeding success in our system. We did not provide the comparison with other methods because the feeding success does not relay on the technique but on the fitness of the ticks and suitability of the rabbit for the particular tick species as a host. The aim of this

study is to offer to the community the user-friendly system which can be used for different kind of purposes when studying tick biology or maintaining the tick colonies.

The following paragraph have been included/modified in the results (Line 235-241):

The tick feeding success in the laboratory mostly relies on the fitness of the individuals and suitability of the rabbit host for the particular tick species rather than the technique itself. This protocol has been proven for successful feeding of different live stages of several hard tick genera such as Ixodes, Dermacentor, Rhipicephalus, Hyalomma and Amblyomma. For example we observed 93.3% (n=240) engorgement rate of I. scapularis and I. ricinus adults. High success rate (data not shown) was observed also for the maintenance of colonies of these two species when feeding their developmental stages (Suppl. Fig. 1).

2. Figure 4 has not been referenced in the text.

Response: All the figures have been reordered and cited in the text.

3. Figure 2L is not described in the Legend. ok

Response: All the figures have been reordered and cited in the text.

References:
1. Please ensure references have a consistent format.
Response: All the references have been corrected to same format.

Table of Materials:
1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

Response: The recommended mesh diameter of the mosquito mesh has been included (Line 124-125):

Cut the appropriate size of the fine mosquito mesh (mesh size less than 50 µm) to the size of the EVA foam frame and stick it to the self-adhesive Velcro loop side (Figs. 1E, F).

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
The title might not be representing the reality since infesting an animal with ticks is not indeed promoting the animal welfare. Something like "less damaging or less harmful" would be more accurate.

Response: We have changed the manuscript title to : *"A Versatile Model Of Hard Ticks Infestation On Laboratory Rabbits"*

Major Concerns:
The use of "non-irritating" materials, as far as possible and with the limitations already known, rather than the protocol in this manuscript contributes mainly to the overall idea, tick infestation and animal welfare. The use of "non-irritating" materials mostly the glue determine the damage/toxicity not only to the animal but to the ticks in every single protocol of animal tick infestation. Besides obviously the management of the animal. Using the glue once the ticks were put on the capsule is a GREAT disadvantage.

Response: We have substituted the glue with the Velcro system which allows comfortably reopen and close the capsule during the tick feeding period and does not represent any risk of the vapor or skin irritation to the ticks or rabbit during the infestation. The latex glue (solidifying in 3 minutes), for attaching the capsule to the rabbit is absolutely non-toxic (guaranteed by developers) and we did not observed any harm or abnormal reaction to this product. Base on this change the manuscript has been modified in order to emphasize the simplicity and versatility of this techniques rather than emphasizing the non-irritating materials.

Following this comment we have changed/substituted the 1.2 step in the protocol (Line 120-126):

1.2 Glue the mosquito mesh on the capsule using fast drying cyanoacrylate glue (superglue hereafter) and leave one corner open for the later tick infestation (Fig 1 C).

To

1.2. Cut the 8 mm wide strips of the self-adhesive Velcro hook side and stick them to the prepared EVA foam frame (Fig. 1 C). Cut the same size strips from the Velcro loop side and bind them to the hook sides attached to the EVA-foam frame (Fig. 1D).

1.3. Cut the appropriate size of the fine mosquito mesh (mesh size less than 50 µm) to the size of the EVA foam frame and stick it to the self-adhesive Velcro loop side (Figs. 1E, F). Cut the overhangs if necessary.

Also the steps 4.1 and 4.2 has been modified to (Line 193-203):

4.1 *Take the rabbit from the cage to the bench and unzip the jacket.*

4.2 Gently restrain the rabbit with the hands. Open the capsule by unfastening the Velcro (Fig. 2 K, L) and collect the ticks by brushing the engorged larvae (Suppl. Fig. 1) or nymphs to a plastic dish, and using forceps for adults (Fig. 2L). If partially fed (not replete) ticks are required, use a tick twister or forceps to detach them.

NOTE: If maintaining the tick colonies please see the NOTE in Step 1.1. of the protocol. Maintain the engorged ticks at more than 95% humidity and 21 ˚C.

4.2 *If needed, refasten the Velcro to close the capsule.*

And the following note has been removed :

NOTE: Make sure to prevent the tick from sticking to the tape. It is also possible to reseal the capsule with another layer of the mosquito mesh. However, in this case we recommend using another glue than superglue as the vapors of the superglue may affect the ticks.

Nothing is mentioned about the animal handling when sampling ticks. This would consider the overall stress to the animal when infesting with ticks. It assumes that the animal is going to stay still and not promoting the ticks to drop off the thin capsule (lines 268 and 269).
-The animal is restrained physically.

Response**:** Following this comment we included more detailed description of the animal handling and sampling the ticks in the protocol (Line 193-203).

4. Collection of ticks

4.3 *Take the rabbit from the cage to the bench and unzip the jacket.*

4.2 Gently restrain the rabbit with the hands. Open the capsule by unfastening the Velcro (Fig. 2 K, L) and collect the ticks by brushing the engorged larvae (Suppl. Fig. 1) or nymphs to a plastic dish, and using forceps for adults (Fig. 2L). If partially fed (not replete) ticks are required, use a tick twister or forceps to detach them.

NOTE: If maintaining the tick colonies please see the NOTE in Step 1.1. of the protocol. Maintain the engorged ticks at more than 95% humidity and 21 ˚C.

4.4 *If needed, refasten the Velcro to close the capsule.*

Minor Concerns:
Some sizes/times are mentioned like if the authors provide irrefutable evidence of the impossibility of using other sizes/values; for example in line 105, 111, 112, 127, 133.

Response: Here we understand the point of the reviewer. We use here imperative mood as JoVE requires. The sizes and values (unless specifically explained) are approximate and that is why we included the terms like: for example…, or should be…to give the users option to modified if needed.

Figure 2F, 2K, 2L are omitted in the text.
Response: All the figures have been reordered and cited in the text.

Another concern that is not mentioned is the limited use of this protocol in experiments with multiple sampling times.

Response: The capsule open-close system has been improved (Fig 1.). This Velcro-based system allows reopen/refasten the capsule as much times as needed. The protocol has been

changed appropriately as mentioned above in the major concerns of this reviewer.

Line 178. Assuming the ticks are already off the animal even though is not clearly mentioned, section 4 is collection of ticks.

Response: The section 4 of the protocol has been elaborated more in details as mentioned above.

Line 243-245. This is always the case in every single protocol of animal tick infestation.

Response: We understand the comment and have modified the paragraph (Line 299-203). One original sentence left for readers who are not familiar with the tick biology. In addition we are referring here to another possible host using our system to give the users option to change the host if needed.

Modified paragraph Line 299-203:
Although our system has been tested for feeding of various hard tick species, the rabbit is not a natural host for all hard tick species[13]. This limitation may be overcome by adapting this system to other animal hosts suitable for a particular hard tick species like sheep, where the EVA foam system was adapted to feed all the developmental stages of I. ricinus (Fig. 3).

Line 253-254. The feasibility of using EVA foam would depend of the animal size.

Response: The EVA foam system is adaptable to any animal size which is great advantage of this technique. It means the feasibility does not depends of the animal size.

Line 278. Studies regarding host-vector-pathogen interactions would require in many cases the use of the natural animal host involving the use of bigger animals. The use of the jacket or something equivalent is mandatory. For many animal host this will be an issue.

Response: Here we refer to the general EVA-foam feeding based system and not the jacket as explained in the text when EVA-foam system has been adapted to the sheep model where instead of jacket the elastic bandage has been used in instead of jacket. To void confusions and be more specific about the EVA-foam capsule system, we have changed the sentence (Line 318-320):

This method is expected to be used for the variety of different experiments when studying tick biology, host-vector-pathogen interactions, or evaluating different control measures like acaricides or vaccines.

To:

The EVA-foam based tick feeding system detailed in this study is expected to be used for the variety of different experiments when studying tick biology, host-vector-pathogen interactions, or evaluating different control measures like acaricides or vaccines.

-

Reviewer #2:

Manuscript Summary:

As it is said, 'there are more ways than one to skin a cat," so are there more ways than one for feeding ticks on an animal. A significant number of publications had described various methods with different degree of detail, including a manual published by Levin and Schumacher in 'Experimental and Applied Acarology' 2016. Improvement of "old" techniques and development of new ones is an ongoing process and publication of these developments should be encouraged as it increases the "toolbox" available for researchers' consideration and choosing. However, advantages of one tick-feeding method over others is often just a matter of opinion and the most important/frequent consideration is which of those methods is more familiar for a particular investigator and his/her Institution. Therefore, I am quite surprised that the authors of the reviewed manuscript decided to advertise their method as something far superior that others. In my opinion, such advertisement is both UNFOUNDED and poses a risk of undermining long-established approved protocols used by other researchers.

Specifically, authors name the following as advantages of their method (lines 268-269): "Compared to other methods, we do not use anesthesia, rabbit collars, ear socks or hobbling of the rear legs. This method significantly decreases the stress of the rabbits, and they can be fully recovered after the experiment." I have to disagree with most of these assessments, especially regarding the "Emphasis on Laboratory Animal Welfare"

Response: We appreciate the comment of the reviewer. There are several ways to feed ticks on animals, and our intention with this manuscript was never to claim superiority, but to share with the researchers in this field a simplified and versatile method and compare it with existing methods as required by the JoVE policy. The tick feeding protocol by Levin and Schumacher in 'Experimental and Applied Acarology' 2016 is definitely advantageous however comparing to our method is laborious, takes a long time and its limit in the number of capsules placed on the animal. In addition we strongly believe that avoiding, E-colar, hobbling and anesthesia in our protocol significantly simplify the entire procedure and reduces the discomfort of the used animals. The rabbits in our research move freely in the cage, have easy access to the water and food and have comfortable sleep during the night or when resting, which is not the case when use the E-colar or hobbling the rear legs. However we agree with the reviewer that we do not have measurable evidence about the rabbit stress lowering. For this reason we have modified the text (original 258-262) in the part pointed out by the reviewer “The significance of the method with respect to existing/alternative methods.“

When developing this system, we paid special attention to minimize the stress of the experimental animals. Compared to other methods[6,8,9,10], we do not use anesthesia, rabbit collars, ear socks or hobbling of the rear legs. This method significantly decreases the stress of the rabbits, and they can be fully recovered after the experiment.

To (Line311-315):

When developing this system, we paid special attention to minimize the amount of the materials used and steps of the procedure. Compared to other methods, we do not use anesthesia, rabbit collars, ear socks or hobbling of the rear legs[5,6,7,8,9,10]. In addition, the present protocol is not laborious, and no intensive training is required to become familiar with this technique.

Another change has been made in “Introduction”:

Two common strategies of tick feeding on rabbits have been frequently used: a) feeding on rabbit ears covered with cotton cloth or socks[7], and b) feeding in cotton bags glued to the rabbit’s back[9]. The feeding on rabbit’s ears is not an elegant system, because ticks (especially early stages, larvae or nymphs) may crawl and attach deep in the ear canal what is uncomfortable for the animal and makes difficult the recovery of the engorged ticks. This system is also limited only to two tick groups on ears fully covered by socks and protected by Elizabethan collars, representing a significant discomfort for the animal. The second system, where ticks are placed in cotton stockinette or neoprene cells glued to the shaved rabbit back, is more advanced but the protocols are quite laborious often involving Elizabethan collar to prevent grooming, hobbling the rear legs to avoid scratching, or even sedating or anesthetizing the animals during manipulations[10].

Has been changed to (Line: 70-75):

Two common strategies of tick feeding on rabbits have been frequently used: a) feeding on rabbit ears covered with cotton cloth or socks[6,7], and b) feeding in cotton bags[9], nylon bottles[10] or neoprene chambers[11] glued to the rabbit’s back. The feeding on rabbit’s ears is not an elegant system, because ticks (especially early stages, larvae or nymphs) may crawl and attach deep in the ear canal which is uncomfortable for the animal and makes the monitoring of tick feeding or/and the recovery of engorged ticks difficult. This system is also limited only to two tick groups on ears fully covered by socks and protected by Elizabethan collars, representing a significant discomfort for the animal. Other systems[9,10,11] are definitely more advanced and well suited for hard tick colony maintenance. However, they are limited in the number of experimental groups placed on the rabbit as well as in the modifiable sizes/shapes of the feeding chambers. In addition these protocols often require anesthetizing or sedating the animals during manipulation, hobbling the rear legs to avoid scratching, and the use of Elizabethan collar to prevent grooming.

Major Concerns:

1-ANESTHESIA.

In other published methodologies, sedation is used only to alleviate stress of the rabbit during the shaving procedure and to keep it calm while bags or chambers are glued to the skin. After that, no sedation is needed or recommended for the duration of tick feeding and collection. The method described here still requires shaving of the rabbit's hare but, instead of sedating the animal, authors recommend using physical restraint by a second person. This physical restraint can be quite prolonged: at least 3-5 minutes for shaving + approximately 20 minutes for gluing 4 chambers (5 min each) with additional time as needed if chamber(s) do not attach properly. That is at least 25-30 minutes of complete physical restraint of an easily spooked animal during an unusual and stressful procedure.

Laboratory Animal Welfare regulations require that "… for animal care, treatment, and practices in experimental procedures to ensure that animal pain and distress are minimized, including adequate veterinary care with the appropriate use of anesthetic, analgesic, TRANQUILIZING DRUGS, or euthanasia". As a member of Animal Use Committee, I would not approve such a stressful procedure to be conducted without appropriate sedation, especially considering that all this stress can be easily alleviated with simple and safe anesthesia.

Response: With the respect to the reviewer we do not agree with this comment. Simply we did not find a need to use anesthesia in our procedure. The reviewer is referring that the procedure may takes 30 minutes for shaving the rabbit or gluing the capsules. In reality several capsules may be glued simultaneously whish reduce the time of the process. Despite this fact the rabbits are calm. The protocol highlighted in the first comment of this reviewer Levin and Schumacher in 'Experimental and Applied Acarology' 2016 takes 3 hour just for the glue to completely solidify (in our case 3 minutes). The design of our EVA foam capsule, plus the latex glue allows it to be attached quickly that anesthetized animals is absolutely not required. In addition not every animal facility possess the anesthesia equipment's. Also the anesthesia or tranquilizing drugs in the rabbit system may negatively affect the tick feeding. In contrary with the reviewer who would not allowed this procedure without anesthesia the ethical comity of our institution (and former ethical comity in USA , Kansas State University where this methods has been used) warmly approved this protocol.

2-USE OF EVA-FOAM CHAMBERS.

The previously published techniques use various hard-sided chambers (usually with screw top closer) or elastic fabric bags for containment of feeding ticks on animals. The reviewed manuscript suggests using suggests using elastic chambers instead. The difference in the duration of feeding within a single cohort of ticks can be 3 days for larvae and ~7 days for adults. So, it is usually recommended that ticks be removed from those containers as soon as they complete their engorgement and detach from the skin. This is necessary to both save engorged ticks from desiccation or being damaged by the animal as well as to minimize additional irritation to the animal cause by unattached ticks crawling inside the container on already irritated skin. Therefore, all previously suggested bags and chambers are designed for easy REPEATED access to ticks inside. As authors of the reviewed manuscript correctly point out (lines 259-260), "engorged-detached ticks (mainly immature stages such as larvae and nymphs) desiccate faster due to the temperature of the rabbit when left" on an animal. That is why engorged larval and nymphal ticks must be collected, removed from the animal twice daily. Elastic chambers made out of foam does not seem to allow frequent repeated reopening of the chamber for tick collection. This will result in higher tick mortality - DECREASED EFFICIENCY OF ANIMAL USE, as well as extra irritation to the animal itself.

On the other hand, I am not sure what happens to ticks that had not yet completed their engorgement by the time when feeding chambers are opened. Are those ticks left on the rabbit? Or, are they allowed to escape from an opened chamber?

Response: We agree with the comment and we have improved our system with the Velcro and as is mentioned for the reviewer 1. We have improved the capsule open-close system with the Velcro (Figure 1). This system allows comfortably reopen/refasten the capsule and collect/monitor the ticks. The text has been changed appropriately to this modification (has been already answered in the case of the reviewer no. 1).

3-USE OF RABBIT JACKETS INSTEAD OF E-COLLARS

Although it is difficult to judge the rabbit's preference for one device over another, e-collars are safe and animal-friendly devises used in veterinary practice all over the World for a wide variety of animal species from small birds to sheep and goats. In our experience, rabbits adjust to collars quickly with no changes in demeanor, behavior, food and water intake, or any other signs of discomfort. Applicability of rabbit jackets depends on their availability

(they cost $100-150 each) and whether or not they can be used in combination with "traditional" (more accessible) screw top capsules or fabric bags.
Hobbles recommended in other techniques are replaced here with taping over rabbits' rear paws - equal substitution.

Response: Although E-collars may be very useful, we prefer to use jackets since in our observations that jackets are more comfortable for the animal and are better protection of the capsules containing the ticks. In addition the use of the jackets eliminate the use of the rear legs hobbling which is in our case substituted by taping the nails of the rear paws. The taping the paws (which is an optional step in our protocol) allows normal walking of the rabbit which is not the case of the hobbling. In summary in our observations the jackets are doing great job in protecting the capsules and minimizing the discomfort of the animals.

4-RECOVERY
In our experience, rabbits are quite capable of removing bags and capsules themselves within a few days after being allowed to groom themselves. Complete recovery of skin lesions or the site of tick attachment is natural process that does not depend on a specific tick-feeding technique.

Response: We are agree with your comment, however, the use of materials are non-irritant, the damage of the skin is reduced, thus the recuperation takes less time. To let rabbit to groom the capsule (as mentioned by the reviewer) is not a good idea while there is a risk of eating the EVA foam. As we mentioned in the protocol in our non-irritating glue system the capsule drop of after 3-4 weeks naturally under the rabbit jacket. So the rabbit is not exposed to the risk of eating/swallowing it.

Overall, I do not have any objections to publication of a yet another tick-feeding technique AS LONG AS CLAIMS OF SUPERIORITY ARE REMOVED.

Response: Here again, our intention has not been to claim superiority. We were trying to compare our technique with existing methods. However taking into account the reviewers points, we have change the sound of the manuscript in several places (detailed above) to avoid this impression. In addition great advantage of this technique is the use of the openable Velcro system-sealed capsule which has been included to the revised version of this manuscript.

Reviewer #3:
Manuscript Summary:
The manuscript describes the feeding of ticks on laboratory animals using feeding chambers made with novel materials which propose some advantages. The text is overall well written in a clear and understandable way, presenting details to enable the methods to be repeated by other researches. Specific comments about the text are written below.

Major Concerns:
The main flaw of the work is that authors need to include the methodology described by Bouchard and Wikel (2005) in the introduction and discussion. Their methodology also uses chambers made with material other than cotton bags or cloths which are more similar to the chambers made in the present work. Bouchard and Wikel suggest using 1.5 mL Eppendorf®-

style tubes to feed nymphs on mice and wide-mouth Nalgene® 500 mL bottles to feed adult tick couples on rabbits, both fixed at the back of the host. The caps of the chambers are glue-free and easy to open, which is advantageous in situations when you have to daily check the ticks and an advantage in comparison to the limitations described in lines 172-176 and 258-263. (Bouchard, K.R., Wikel, S.K., 2005. Care, Maintenance, and Experimental Infestation of Ticks in the Laboratory Setting, In: Marquardt, W.C. (Ed.) Biology of Disease Vectors. Elsevier, San Diego, pp. 705-712.)

Response: The suggested references have been included**.** A advantageous open/close system has been improved in our EVA foam capsule that can be opened when required (Figs. 1, 2). This issue has been addressed in the case of the specific comments for the reviewers 1 and 2 (please see above).

Minor Concerns:
-Another work that is worth citing was published by Levin and Schumacher (2016). Although similar to others (2 and 9), this is a methodology paper giving details on blood feeding using cotton stockinettes. This work is an appropriate citation for statements in lines 64 and 192. (Levin, M.L. & Schumacher, L.B.M., 2016. Manual for maintenance of multi-host ixodid ticks in the laboratory. Exp Appl Acarol, 70: 343-367)

Response: Thank you for your comment. We have included the appropriate reference of Levin and Schumacher, 2016.

Although the welfare of the animals is improved in comparison to the methodology of using bags in the ears, I don't see significant improvements in animal welfare in comparison to methodologies that use chambers at the back of the rabbit. These are subjective comparisons (elizabethan collar x jacket and a protective tape applied to the rear paws; anesthesia x physical restraint) all with advantages and disadvantages in terms of animal welfare. Therefore, the term "welfare" should be removed from the title.

Response: We agree with the comment and we have changed the manuscript title to : *"A Versatile Model Of Hard Ticks Infestation On Laboratory Rabbits"*

Line 51 -53 - Remove reference 2; it is not appropriate for this statement. Troughton et al described tick life cycle in the laboratory and not pathogen transmission.

Response: The reference no. 2 has been removed.

Line 119 - Do you really need to completely shave the back of the rabbit? Shearing the fur completely seems to me a negative point regarding animal welfare as it can potentially hurt the animal. We have seen that just trimming the hair close to the skin is better than completely shaving the rabbit's back. Trimming instead of shaving prevents irritation of the skin and the remaining fur helps the keeping the chamber fixed and also accelerates the recovery of the animal.

Response: In our experience, shaving the rabbits does not hurt nor irritate the skin. If only trimming is done, the fur may affect the visibility of larvae or nymphs and complicates the collection of engorged ticks. In addition to properly glue the capsule shaving is necessary. By shaving the rabbit with the clippers we have not observed any irritation.

Line 155 -159 - How long after the chamber is glued to the back of the rabbit do you need to wait before introducing the tick? Usually at least a gap of 24 hours is needed for the glue to dry completely before introducing the ticks to avoid glue odors and ticks getting glued inside the chamber.

Response: The non-irritant latex glue is completely dry after 3 mins (mentioned in protocol); therefore, the animal can be infested immediately. After 3 minutes solidification the glue does not have any odor to irritate the rabbit or the ticks. I addition there is not risk to glue the tick while the glue is completely dry. This step is actually one of the important advantages of our proposed method detailed in this manuscript.

Did authors have any problem with excess humidity inside the chambers? Usually excess humidity can kill ticks and the present method seems to impair water evaporation due to the two layers formed by the mosquito mesh and the rabbit jacket.

Response: No excess of humidity is observed with this method. The rabbit jacket along the mosquito mesh has enough porosity to allow flow of evaporation. Specifically exactly opposite is the true and the replete tick have to be monitored twice daily to avoid death due desiccation caused by the temperature of the rabbit.

-Line 218- Authors should include figure "L" in the legend. Last image should be K and L.

Response: All the figures have been reordered and cited in the text.

Reviewer #4:

Manuscript Summary:

The authors of this paper present a method for feeding different life stages of ticks on rabbits, potentially simplifying maintenance of tick colonies and blood feeding for experiments.

Major Concerns:

-Can the authors can provide any data on tick feeding success of this method? What percentage of ticks fed? Mortality data would also be useful: we have no idea how useful this technique is compared to other methods. The authors do provide the caveat that ticks may be vulnerable to desiccation with this method, but more information would be helpful.

Response: Here we would like to mentioned that the message of the manuscript is not to highlight better engorgement results comparing to the other protocols while still we are dealing with the rabbit system which is commonly used in tick research laboratories. Here we offer simple and versatile methods to feed hard ticks on the laboratory rabbits which can be adopted by several laboratories around the world.

Following this comment, we have included the percentage of the *I. scapularis* and *I. ricinus*

ticks that successfully completed the feeding using our system (Line 235-244):

The tick feeding success in the laboratory mostly relies on the fitness of the individuals and suitability of the rabbit host for the particular tick species rather than the technique itself. This protocol has been proven for successful feeding of different live stages of several hard tick genera such as Ixodes, Dermacentor, Rhipicephalus, Hyalomma and Amblyomma. For example we observed 93.3% (n=240) engorgement rate of I. scapularis and I. ricinus adults. High success rate (data not shown) was observed also for the maintenance of colonies of these two species when feeding their developmental stages (Suppl. Fig. 1). This EVA foam capsule system may also be adapted for other type of laboratory hosts, such as sheep (Fig. 3).

The plastic syringe method used for tick infestations could be better described. It seems the ticks would get easily damaged by the syringe plunger pushing them out or a few might remain in the barrel of the "needle" area. Perhaps a few additional sentences describing this process would clarify this part of the procedure for the reader.

Response: Tit seems that the reviewer misunderstood that the “needle area” of the plunger is completely cut from the syringe. By this way no damage of the ticks have been observed (for all life stages). A figure (Fig 1H,G) has been included to the manuscript for the clarification.

Minor Concerns:
-The "mosquito mesh" could be described in more detail. Types of cloth can vary considerably in mesh size and is a crucial detail when dealing with the minute larval stages. Is this material known by any other name (curtain material, etc.)? Perhaps the pore size of the cloth could be included in the manuscript.

Response: The recommended mesh diameter of the mosquito mesh has been included (Line 124-125):

Cut the appropriate size of the fine mosquito mesh (mesh size less than 50 µm) to the size of the EVA foam frame and stick it to the self-adhesive Velcro loop side (Figs. 1E, F).

Line 186: How should one "treat appropriately"? Is there a protocol that could be referenced (perhaps the animal use protocol)?

Response: Although we did not observed any abnormal reaction to the glue or EVA-foam capsule itself. For easier understanding we have included the sentence (Line: 214-2016):

Note: Once the capsule is off, check the skin of the rabbit for abnormal reactions. Although normally no treatment is required, an emollient lotion can be used to treat irritation of the skin’s rabbit.

The quality of photos in figure 2 is not good, at least in the version I received. This may be a result of pdf conversion and not the fault of the authors, but I would suggest that high

quality photos be used in the final version.

Response: We submitted high resolution images and as the reviewer assumed the quality level decreased due to the pdf conversion.

Also, there are several grammatical errors or confusing syntax in this manuscript. I have listed a few below that should be corrected, but this manuscript would benefit from having a native English speaker proofread it.

Response: The English grammar has been corrected across the entire manuscript.

-The title is grammatically incorrect. I would recommend this revision: "A Model of Laboratory Tick Infestation Emphasizing Animal Welfare"

Response: We have changed the manuscript title to: "*A Versatile Model Of Hard Ticks Infestation On Laboratory Rabbits.*"

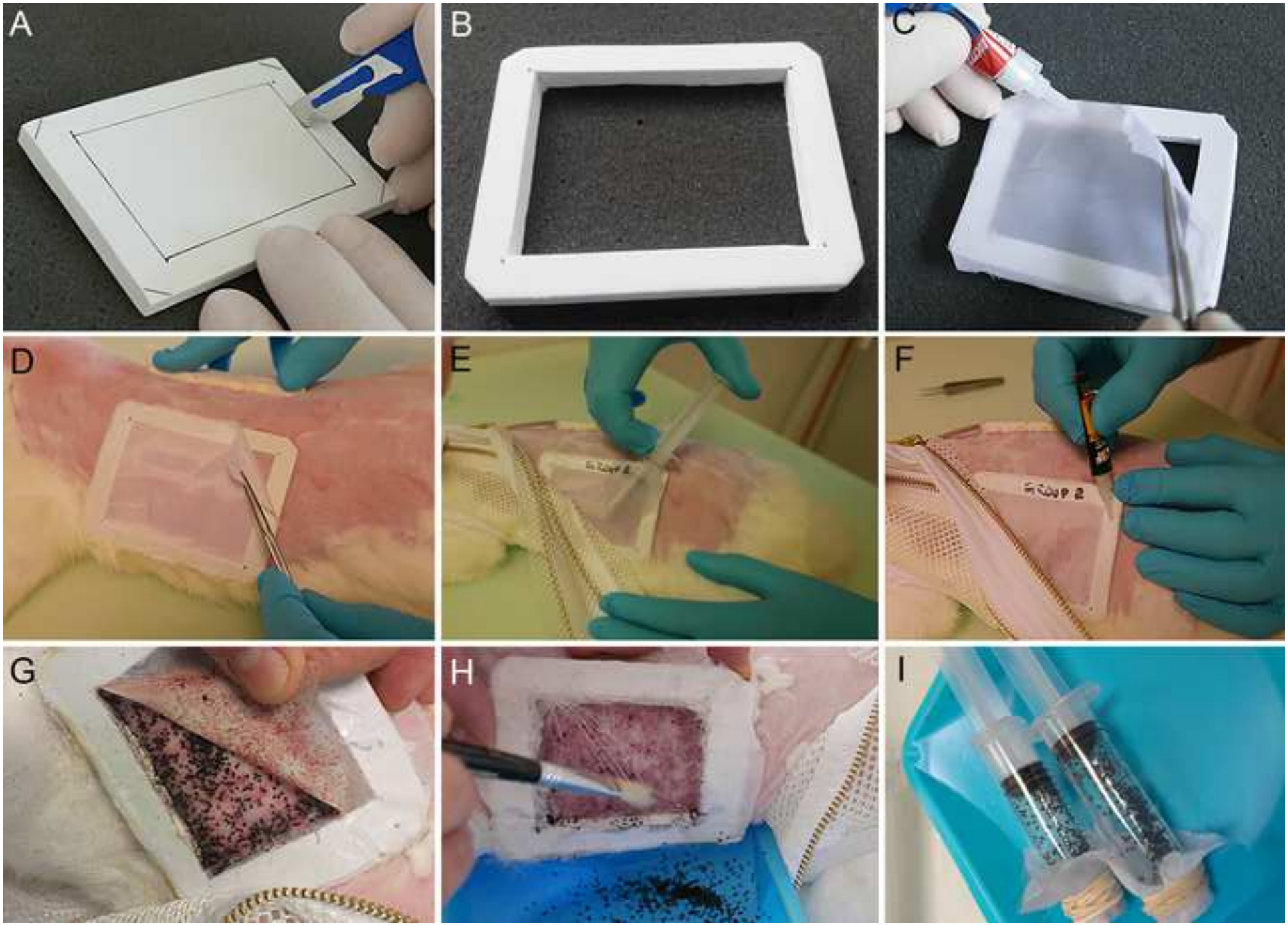
-Line 38: "...when attached to their vertebrate hosts..."
Response: has been corrected.
-Line 60: Spelling error: should be "guarantee"
Response: has been corrected.
-Line 66: "...what is uncomfortable..." should be "...which is uncomfortable...". Also, "makes the recovery of engorged ticks difficult." makes more sense grammatically.
Response: has been corrected.
-Line 186: "abnormal reaction" should be "abnormal reactions".
Response: has been corrected.
-Line 193: "during the manipulation..." should be "during manipulation"
Response: has been corrected.
-Line 195: "...applied to a shaved rabbit's back..."
Response: has been corrected.
-Line 204: "...other types of..."
Response: has been corrected.

Additional changes/modifications:

1. Due to the improvement of the capsule open-close system, the Figures composition (along their panels) has been completely changed and new figures have been included.
2. One supplementary figure (Suppl. Fig. 1) has been included describing the feeding of the tick larval stages using EVA-foam capsules.
3. The Material table has been slightly modified.
4. Due inclusion of new references the citations order along the list of references have been changed.

Supplemental File (Figures, Permissions, etc.)

Click here to download Supplemental File (Figures, Permissions, etc.) Suppl. Fig 1.tif



Supplementary Figure 1. Modified EVA-foam capsule system to feed the larval stages of the hard ticks. A, B: Cutting the capsule from the EVA foam. C: Gluing the mosquito mesh to the capsule, note that one corner is left open D: Attached capsule on the rabbit. E: Inoculation of ticks to the capsule via the open corner. F: Sealing of the open corner with superglue. G: opening the capsule with scissors or scalpel. H: Collecting the replete larvae using a paintbrush. I: Placing the engorged larvae in the plastic syringe with the needle-end cut and covered with mosquito mesh held in place with a rubber band.