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Microsurgical Obstruction of Testes Fusion in Spodoptera litura

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TITLE:

Microsurgical Obstruction of Testes Fusion in *Spodoptera litura*

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KEYWORDS:

Fusion of testis; microsurgery; blockage; *Spodoptera litura*

SUMMARY:

Aluminum foil was microsurgically inserted between the testes of *Spodoptera litura* to obstruct the fusion of testis. The procedure includes freezing, fixing, disinfection, incision, placing the barrier, suturing, postoperative feeding, and inspection. This approach provides a method to interfere with tissue formation.

ABSTRACT:

Instead of using genetic methods like RNA interference (RNAi) and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated endonuclease Cas9, a physical barrier was microsurgically inserted between the testes of *Spodoptera litura* to study the impact of this microsurgery on its growth and reproduction. After inserting aluminum foil between the testes, insect molting during metamorphosis proceeded normally. Insect growth and development were not remarkably altered; however, the number of sperm bundles changed if testes fusion was stopped by the microsurgery. These findings imply that blocking testicular fusion can influence male reproduction capability. The method can be further applied to interrupt communication between organs to study the function of specific signaling

pathways. Compared to conventional surgery, microsurgery only requires freezing anesthetization, which is preferable to carbon dioxide anesthetization. Microsurgery also minimizes the surgery site area and facilitates wound healing. However, the selection of materials with specific functions needs further investigation. Avoiding tissue injury is crucial when making incisions during the operation.

INTRODUCTION:

Fusion is a common phenomenon in tissue or organ development. Examples include dorsal closure and thorax closure in *Drosophila*[1] and palate morphogenesis, neural tube morphogenesis, and heart morphogenesis in mice and chicken[2]. CRISPR and RNAi have been applied to investigate the roles of genes in the process of fusion[2-4].

Spodoptera litura (S. litura, Lepidoptera: Noctuidae) is a detrimental polyphagous pest that is widely distributed in tropical and subtropical areas of Asia, including China[4-6]. The wide distribution of *S. litura* is partly attributed to its powerful reproductive capability, which is relevant to gonad development. Male infertility is one approach to control this pest. As shown in the schematic figure of testicular structure, the testes are enclosed by the testicular sheath, including the external sheath (peritoneal sheath) and inner basal lamina. The basal lamina extends internally to form the follicular epithelium and separates the inner area of the testis into four chambers named follicles (**Figure 1**).

In the follicles, spermatogonia develop into spermatozoa after mitosis and meiosis, and then the spermatozoa in the sperm sacs align in the same direction to form sperm bundles[7]. During spermatogenesis, the primary spermatocytes differentiate into eupyrene sperms or apyrene sperms. Spermatocytes in the larval phase develop into eupyrene sperm with a long tail connected to a head of an elongated nucleus; these can fertilize eggs. Conversely, spermatocytes in the mid-pupal phase develop into apyrene sperm with a discarded nucleus; these sperm assist the survival, motion, and fertilization of eupyrene sperm[9,10]. The 6th day of the pupa is the period during which eupyrene and apyrene sperm bundles form.

[Place **Figure 1** here]

Testicular fusion occurs in most insects of the Lepidoptera order[11,12], especially in those species that are agricultural pests. Testicular fusion refers to a pair of testes growing bilaterally in the larval phase, approaching and adhering to each other, eventually integrating into a single gonad[11]. In *Spodoptera litura*, it happens during metamorphosis from the larval to the pupal stage. From day 1 of the 5th instar (L5D1) to day 4 of the 6th instar (L6D4), the pair of testes grows gradually in size, and the color turns light yellow from ivory-white. It becomes faint red as it reaches the prepupal phase (L6D5 to L6D6). Two bilateral symmetrical testes approach each other during the prepupal stage, fuse into one, and twist clockwise to produce a single testis in the pupal and adult phases[11]. This phenomenon does not occur in silkworms, which have considerable economic importance and have been domesticated for 5000 years[13]. Thus, it is assumed that the testes' fusion improves reproductive capability.

To determine the significance of *Spodoptera litura* testicular fusion, it is important to

investigate the effects of blocking the process. In this protocol, aluminum foil was microsurgically inserted between the testes to keep them separated, and the consequent changes in the development of the insects and their testes were studied.

PROTOCOL:

1. Insect rearing and maintenance

1.1. Culture the *Spodoptera litura* larvae in environmental simulation chambers with an artificial diet until they reach day 4 of the 6th instar (L6D4). Select male larvae when the worms enter the first day of the 6th instar (L6D0) based on the inverse triangle-shaped structure on the eighth abdomen[14].

NOTE: Larvae rearing and maintenance techniques were published previously[4,14].

2. Presurgical preparation

2.1. Trim the aluminum foil into rectangular pieces with rounded corners (1 mm x 2 mm, **Figure 2**).

2.2. Sterilize the surgery platform and related items (table surface, microscope, icebox, insect box, wax tray, pins, and thread) by spraying 75% alcohol on their surface and wiping them down.

2.3. Sterilize surgical tools (including the aluminum foil) with a high-pressure steam sterilizer for 30 min, and place them in a heating and drying oven at 120 °C.

2.4. Ensure that the operators wear clean laboratory clothes, surgical masks, and sterile gloves.

3. Microsurgical placement of a barrier between the testes

NOTE: The general work-flow is as follows: Freezing → Fixing → Disinfection → Incision → Barrier Placement → Suturing→ Postoperative Feeding and Inspection

3.1. Place male larvae (L6D4) on ice for 10–30 min to keep them anesthetized during the operation.

3.2. Place a larva on the wax tray with the dorsal side up, and then fix the head and the tail of the larva with pins and threads, showing the surgical area that is the dorsal surface on the 9th body segment (**Figure** 3A).

3.3. Disinfect the surgical area by applying 3% iodine tincture with a cotton swab to the epidermis (9th body segment), followed by 70% alcohol to remove the iodine (**Figure 3B**).

NOTE: Focus on the larva through coarse and fine adjustment of the surgical microscope (**Figure 3C**). Place the wax tray on a larger culture dish filled with ice to keep the anesthesia.

3.4. Make a 2 mm-long incision on the dorsal epidermis of the 9th body segment. Next, use a sterile cotton swab to remove any leaking hemolymph and fat bodies and obtain a clear view of the surgical area.

NOTE: It is important to avoid the heart during the procedure. This can be done by making the incision slightly next to the mid-line in the 9th body segment or at the joint between the 9th and 10th body segments to prevent the testes from popping out due to the larval internal pressure. While using the scalpel, make a vertical slit with the blade first (**Figure 4A**), and then turn it 45° towards the epidermis before evenly and continuously cutting through the epidermis (**Figure 4B**).

3.5. Use surgical tweezers to insert a piece of aluminum foil between the testes (**Figure 5**).

3.6. At the end of the surgery, close the incision to avoid infection, and allow the larvae to recover from the surgery.

3.6.1. Close the epidermis with a running suture (**Figure 6**).

3.6.2. Use a needle holder and surgical tweezers to tie a surgical square knot (requiring two opposing mirror-image simple knots (**Figure 6D,E**))

3.6.3. Use scissors to cut the excess suture from the loop tails, leaving a 2 mm thread behind.

3.7. After suturing, gently lay the larva in the rearing box and maintain them in a clean environmental simulation chamber. Continue observing the larvae.

NOTE: The wound stops leaking hemolymph, and the larvae gradually recover after the surgery. The worms continue to complete their metamorphosis.

[Place **Figure 2, Figure 3, Figure 4, Figure 5, Figure 6** here]

REPRESENTATIVE RESULTS:

The effects of microsurgery on *Spodoptera litura* growth and development

The microsurgery left a 2 mm-long wound on the dorsal larval epidermis that eventually stopped leaking hemolymph and healed. The larvae went through prepupal and pupal stages and eclosed, indicating that the microsurgery had no major impact on growth and development. When the larvae molted into pupae, the suture threads were discarded along with the epidermis. There were no obvious differences in the appearance of the pupae that did and did not undergo surgery. After eclosion, adult females successfully mated with the adult males previously operated on, resulting in fertilized eggs and hatching larvae (**Figure 7**).

[Place **Figure 7** here]

The larvae went through the pupal stage and eclosed after the microsurgical placement of aluminum foil between the testes. Detailed results following this operation have been published previously[11]. Although the barrier stopped the testes from fusing in some larvae, most larvae underwent testicular fusion during larval to pupal metamorphosis.

In this research, individuals were grouped by three treatments: Experimental (Exp), Sham-operation (Ctl-sham), and no operation (Ctl). Individuals of the Exp group underwent microsurgery to insert a physical barrier, and their testes remained separated during the pupal and adult stages. Individuals of the Ctl-sham group underwent the same microsurgery; however, their testes were not blocked and fused for unknown reasons. The Ctl group contained the larvae that grew naturally without surgery; their two testes fused normally during the prepupal stage.

The microsurgery group contained two subgroups: larvae that underwent microsurgery to place a barrier between the two testes (Group A) and those that underwent microsurgery to remove one testis (Group B: left testis removed in Group B-1; right testis removed in Group B-2). **Table 1** shows the numbers of operation larvae, larval mortality rates, numbers of pupae, percentage of pupation, numbers of adults, percentages of adult emergence, percentages of successful mating, and percentages of successful operations in different groups. Group A includes larvae that underwent microsurgery to insert a barrier between the testes. The success of this procedure could only be determined after dissection, which is when they were further divided into the Exp and Ctl-sham groups.

As shown in **Figure 8**, the larval mortality rate was slightly higher in the surgical group, whereas the percentages of pupation, adult emergence, and successful mating were slightly lower in the surgical group than the control group. However, none of the differences were significantly different, indicating that the microsurgery did not markedly influence the growth and development of *Spodoptera litura* larvae.

[Place **Table 1** here]
[Place **Figure 8** here]

The influence of microsurgery on the number of sperm bundles of *Spodoptera litura*

Microsurgery was performed to insert a physical barrier to stop the testes fusion or remove unilateral testis in *Spodoptera litura.* Eupyrene and apyrene sperm bundles were counted to calculate the percentage of eupyrene sperm bundles on the sixth day of the pupal stage. The individuals were grouped by treatment, as described above. The numbers of sperm bundles (eupyrene sperm bundles, apyrene sperm bundles, and total) were significantly lower in the Exp group than in the Ctl-sham and Ctl groups. The mean number of eupyrene sperm bundles from two separated testes in the Exp group was 2082 ± 599. In the Ctl-sham and Ctl groups with fused testes, the number of eupyrene sperm bundles ranged from 4652 to 6200.

The number of apyrene sperm bundles in the Exp group was 1602 ± 703, while it ranged from 3299 to 4632 in the Ctl-Sham and Ctl groups. The total of sperm bundles in the Exp group was

3684 ± 985; it ranged from 9284 to 10832 in the Ctl-Sham and Ctl groups. Thus, the percentages of eupyrene sperm bundles ranged from 50% to 60%, with no significant differences among all three groups. **Figure 9** shows that when fusion is prevented, the amount of eupyrene and apyrene sperm bundles decreased, whereas the percentage of eupyrene sperm bundles was unchanged.

[Place **Figure 9** here]

After removing a unilateral testis of the larvae, the numbers of eupyrene and apyrene sperm bundles were counted to calculate the percentage of eupyrene sperm bundles on the sixth day of the pupal stage. The number of eupyrene and apyrene sperm bundles ranged from 1286 to 1638 and 720 to 850, respectively, which means the total number ranged from 2006 to 2488, corresponding to a eupyrene sperm bundle percentage of 63% to 65%. **Figure 10** shows that the number of sperm bundles decreased significantly after unilateral testis removal (reduced by 60% to 70%), without much influence on the percentage of eupyrene sperm bundles.

[Place **Figure 10** here]

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic diagram of the testicular structure of Lepidoptera insects[11].

Figure 2: Physical barrier prepared using aluminum foil (1 mm x 2 mm).

Figure 3: Before incision. (**A**) Fixing the larva. (**B**) Disinfection of the epidermis of the surgical area. (**C**) Performing surgery under the microscope.

Figure 4: Incision. (**A**) Slit the larvae vertically with the blade. (**B**) Turn the blade 45° toward the epidermis before cutting through.

Figure 5: Inserting the physical barrier (aluminum foil) between the testes.

Figure 6: Suturing. (**A**) Insert the needle. (**B**) Withdraw the needle. (**C**) Withdraw and clamp the needle. (**D**) Tie the first simple knot. (**E**) Tie the opposing mirror-imaged simple knot. (**F**) Cut excess suture thread.

Figure 7: *Spodoptera litura* Development after microsurgery. (**A**) Male larva at L6D4. (**B**) L6D4 larva immediately after surgery. (**C**) Pre-pupa (L6D6). (**D**) P0, the red arrow indicates the location of the surgery; the yellow arrow shows the discarded epidermis with suture thread. (**E**) Mating adults. (**F**) Eggs and hatched larvae from a female adult mating with a male that underwent surgery. Scale bars = 1 cm.

Figure 8: The influence of microsurgery on *Spodoptera litura* growth and development of (n ≥ 6).

Figure 9: The numbers of sperm bundles and percentages of eupyrene sperm bundles in different groups. (**A**) The number of sperm bundles in the Exp group was significantly lower than

in the Ctl-sham and Ctl groups. (**B**) The percentages of eupyrene sperm bundles were not significantly different among the three groups. Asterisk indicates a significant difference when compared with Ctl. $P < 0.05$, Mean ± SD ($n \geq 5$).

Figure 10: The numbers of sperm bundles and percentages of eupyrene sperm bundles after removing unilateral testis. (**A**) The numbers of sperm bundles in pupae that underwent unilateral testis removal were significantly different among the three groups (left and right testis removed in Microsurgery Group B-1 and Microsurgery Group B-2, respectively) (**B**) The percentage of eupyrene sperm bundles in pupae that underwent unilateral testis removal was not significantly different compared with Ctl. The asterisk indicates a significant difference compared with Ctl. $P < 0.05$, Mean ± SD ($n \geq 8$). Control group = no surgery, the testes fused naturally during the prepupal stage; Ctl-Sham group = operation unsuccessful and testes fused after microsurgery; Exp. Group = microsurgery performed to insert a physical barrier between the two testes.

Table 1: The effects of microsurgery on *Spodoptera litura* development. Microsurgery groups B-1 and B-2 underwent microsurgery to remove unilateral testis (left in Microsurgery Group B-1 and right in Microsurgery Group B-2). Note: Microsurgery groups A-1 to A-8 underwent microsurgery to insert a barrier between the testes; Microsurgery groups B1 and B2 underwent microsurgery to remove unilateral testis (left in Microsurgery Group B-1 and right in Microsurgery Group B-2); the rates and percentages are given as Mean ± SD. Asterisks indicate that the individuals in the group were dissected at the pupal stage, and there were no statistics on the number of adults, percentage of adult emergence, or percentage of successful mating; N indicates no data.

DISCUSSION:

After microsurgically obstructing testes fusion in *Spodoptera litura*, the number of sperm bundles decreased, which supported the hypothesis that this fusion is beneficial to the reproductive capability. Surgical manipulation has been used to study the physiological development of insects since the early 20th century. To determine whether the cranial nerve is regulated by insect metamorphosis, some researchers performed procedures such as ligation and decapitation on different insects (including *Rhodnius prolixus* of Hemiptera, *Lymantria dispar* of Lepidoptera)[15,16]. The process of decapitation involves removing the head with a scalpel, disinfection with antibiotics, and paraffin wax sealing of the wound after operation[17]. After extraction and transplantation of the prothoracic glands of *Bombyx mori*, the wound was sealed with paraffin wax[18]. However, the inevitable consequences of these conventional treatments are infection and a high mortality rate, which makes it difficult to analyze the physiological state during the late stages of development of the insect.

Therefore, this protocol was designed to ensure a minimally invasive surgery done under the microscope to minimize the wound. Moreover, compared to carbon dioxide anesthetization, freezing anesthetization is more feasible and convenient. Aluminum foil, used as the obstructer, was cut into a size of 1 mm x 2 mm, an area equivalent to the space between the testes. Following microsurgery, the suture threads fall off with the early epidermis during molting,

allowing metamorphosis and development to proceed normally. The reproduction results suggest that successful microsurgery did not significantly influence insect development. When the testes did not fuse, the numbers of total, eupyrene, and apyrene sperm bundles were significantly lower than those in the Ctl group. These results indicate that male reproductive capability is affected by testes fusion. Assessment of sperm cell quality and vitality have different indexes and methods in various animals, including sperm acrosomal status in mammals[19], sperm motility[20], mitochondrial activity[21], plasma membrane integrity[22], and other markers[23,24] . Because of insects' unique sperm cell development, future studies need to examine changes in reproduction capability (mating, incubation[25]).

Critical steps in this protocol require particular attention to ensure reliable results. Avoidance of injury to other tissues is important when making the incisions. Second, the selection of the barrier material must be based on its nontoxic and sterile properties and lack of sharp boundaries. Finally, the incision was closed with a running suture and a surgical square knot, followed by sealing in the surgical area to effectively prevent postoperative infection. Operations on the insect's internal structure such as transplantation, extraction, and application of drugs can still be performed, followed by sealing in the surgical area.

High success rates require proficient skills, and this technique has some disadvantages. First, it is not efficient, as the operations are done one by one, due to which individual variation is inevitable. Preliminary studies showed that when using medical venous transfusion tubes, rubber diaphragms, absorbent beads, and dental materials to separate the testes, the outcomes were not as successful as expected. Moreover, the technique is not successful when the obstructer is adrift. Possible reasons for a decreased surgical success rate include the barrier slipping off when the insects move, shrink, molt, and re-organize organs during the prepupal phase. Alternatively, the aluminum foil can be inserted too close to the lubricous gut, causing the barrier to float away. Therefore, a suitable material should be further optimized.

Despite the limitations of microsurgery, it provides a method to obtain preliminary results about biological phenomena before establishing a transgenic model system. Sweeney and Waterson analyzed rid development in chick embryos by inserting tantalum foil blocks[26], while Wilde and Logan used aluminum foil as an impermeable barrier to study the role of retinoic acid signaling in the induction and subsequent initiation of fore- and hindlimbs[27]. In invertebrate *Spodoptera litura*, this microsurgery successfully enables normal worm growth and development, providing a way to study physiological phenomena.

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DISCLOSURES:
The authors have nothing to disclose.

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Figure

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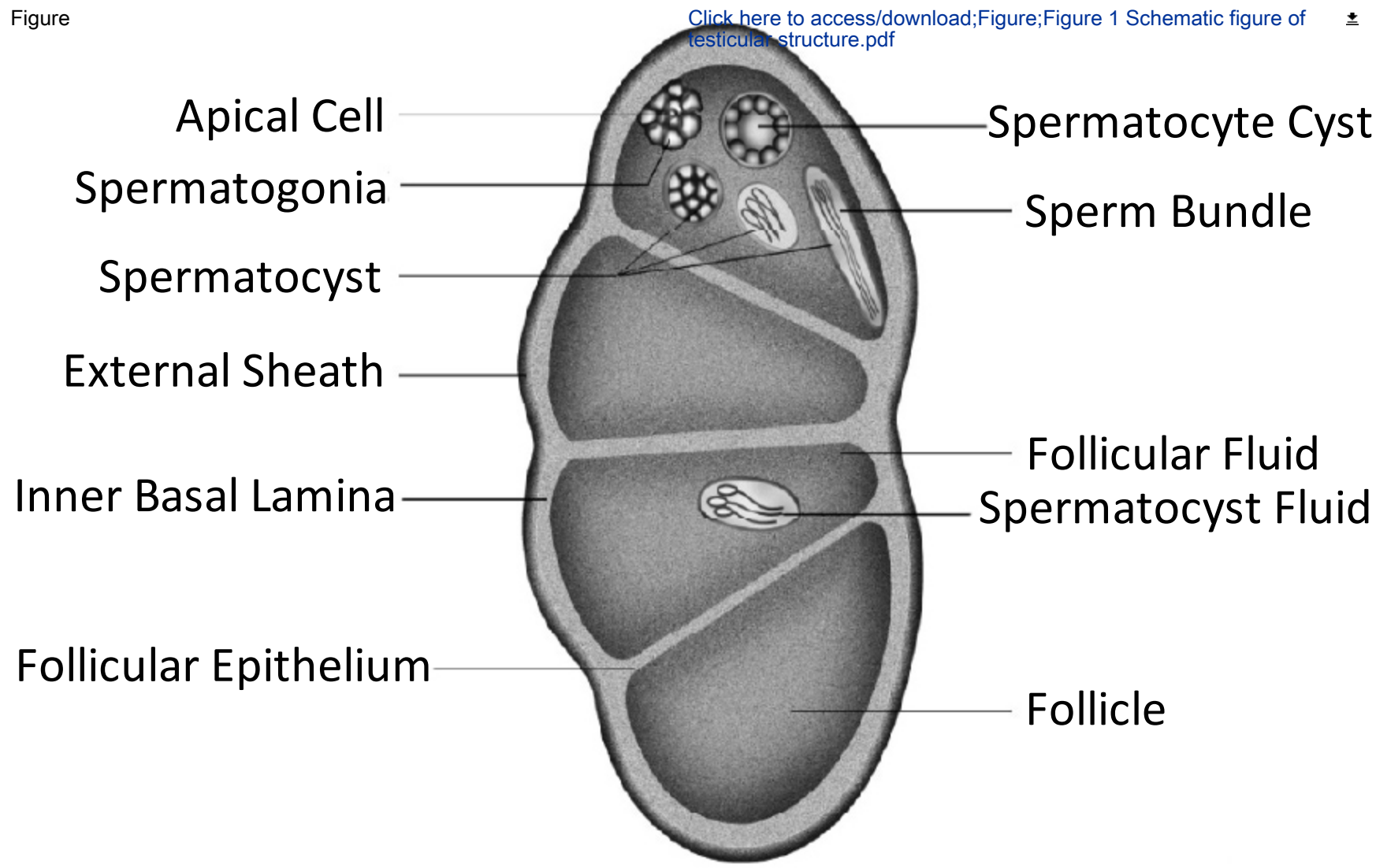

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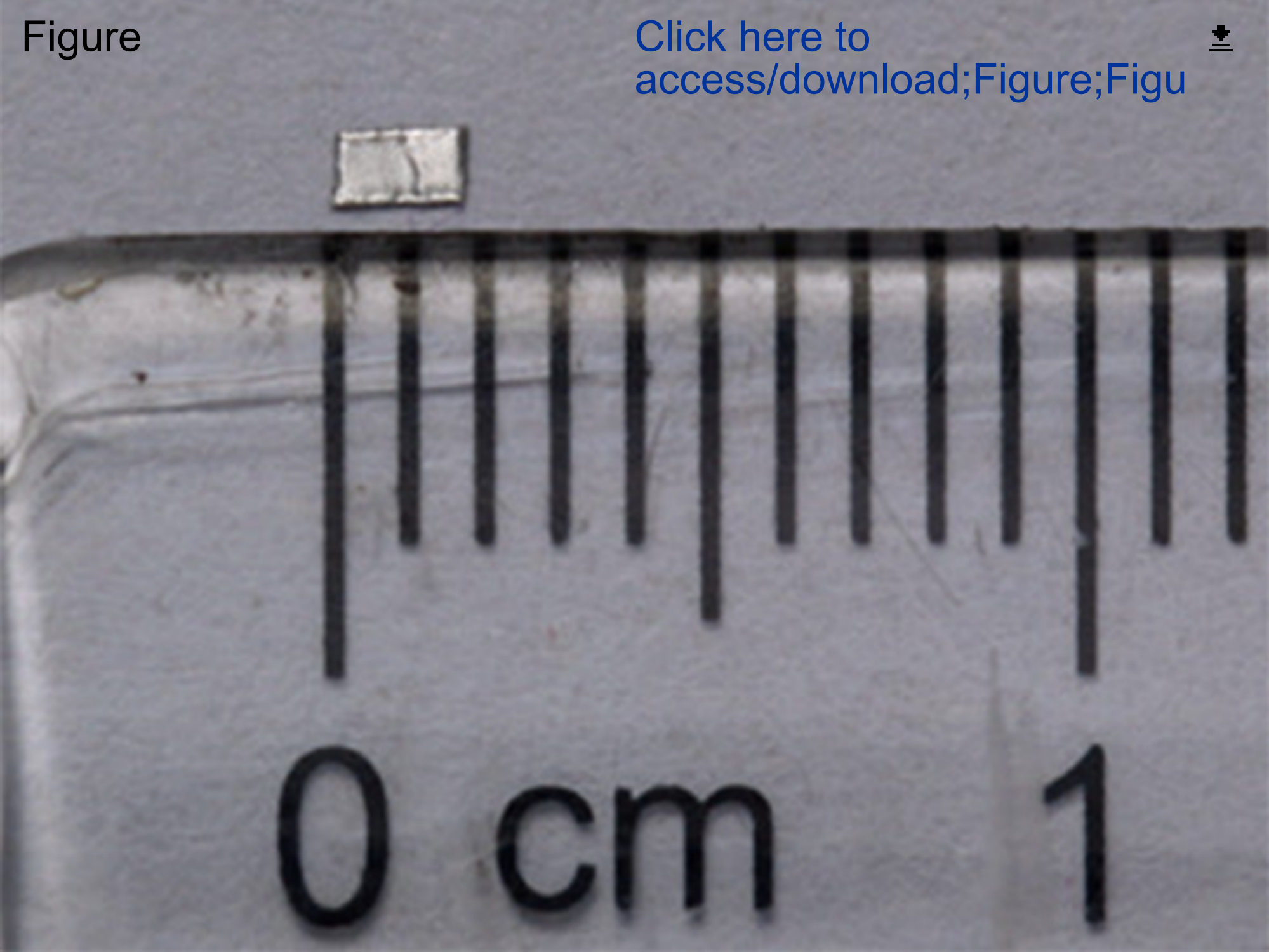

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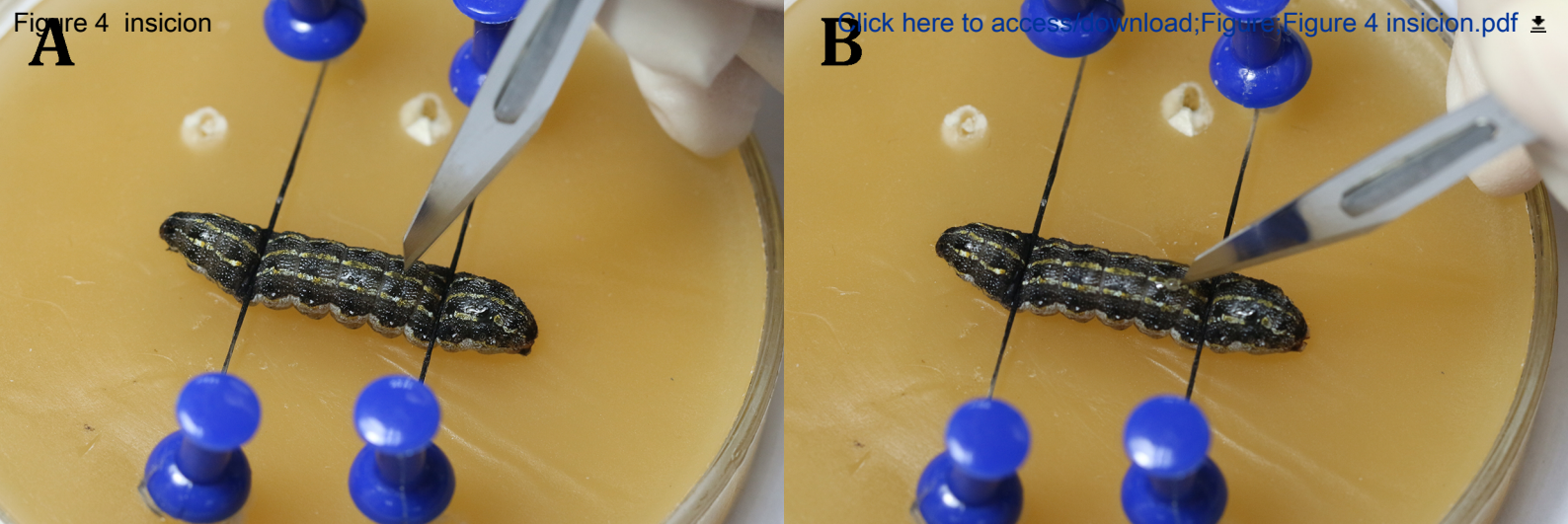

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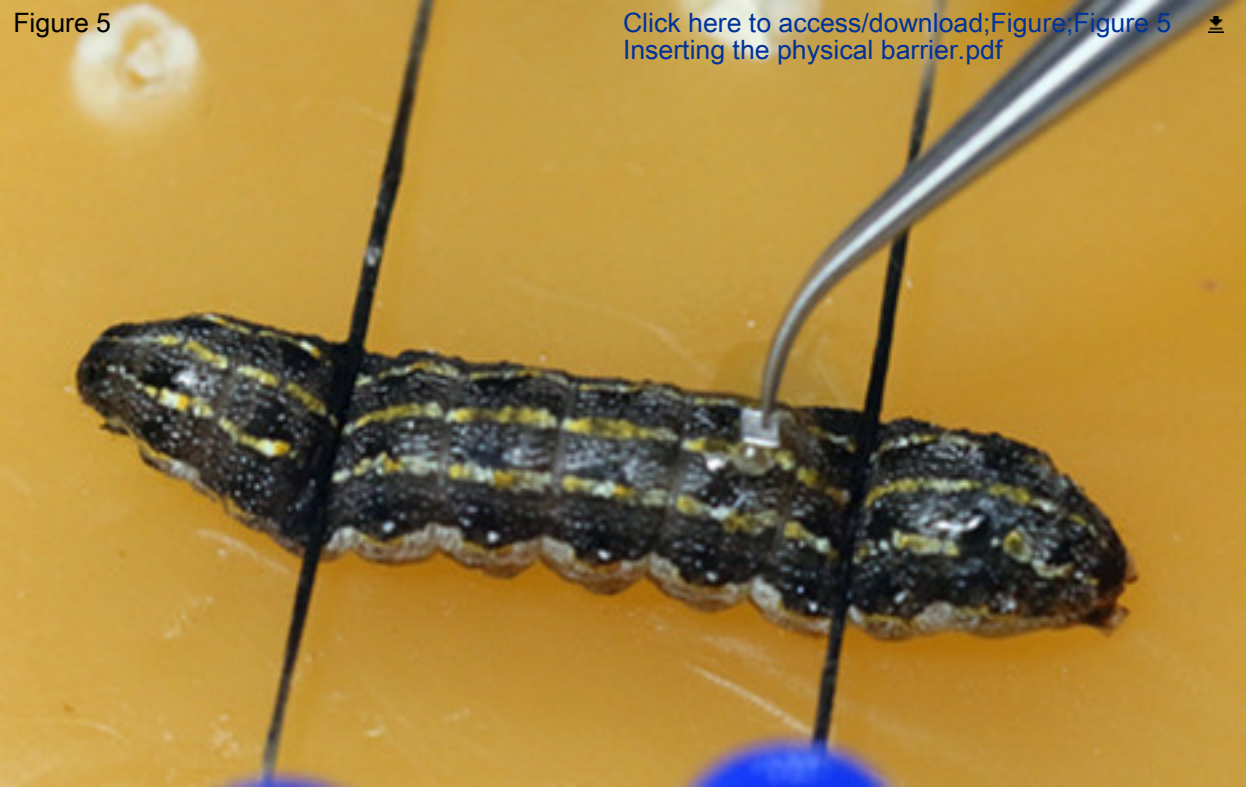

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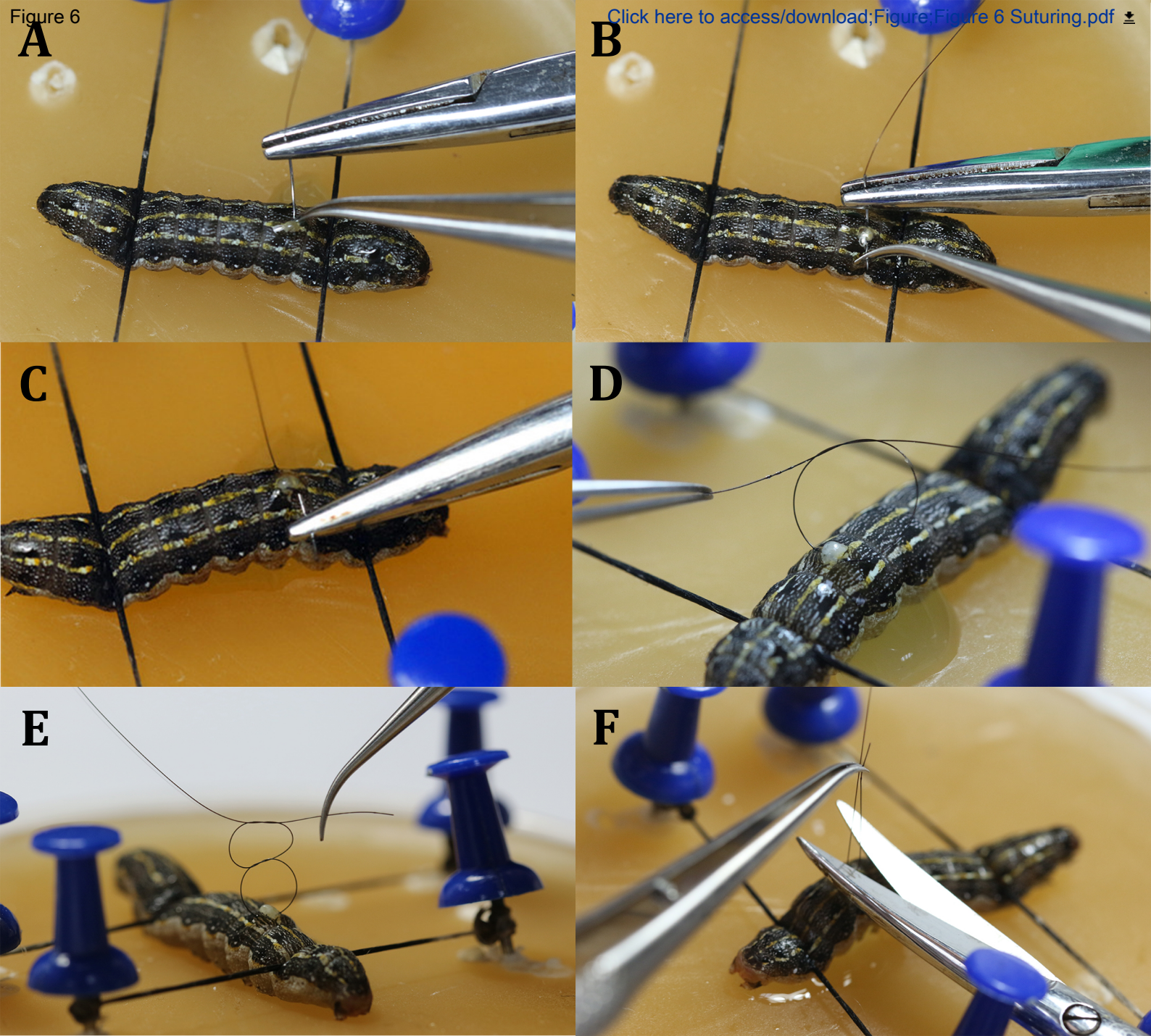

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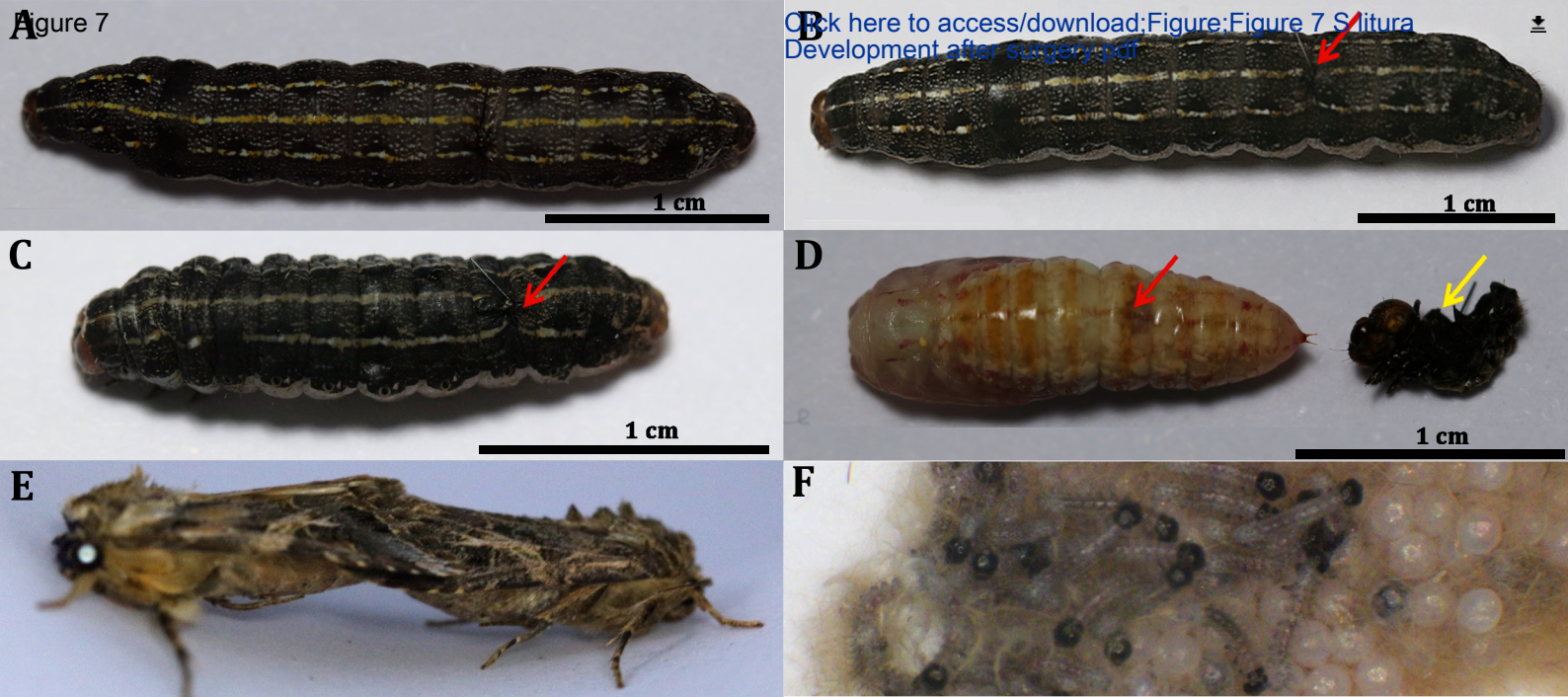


Figure 8

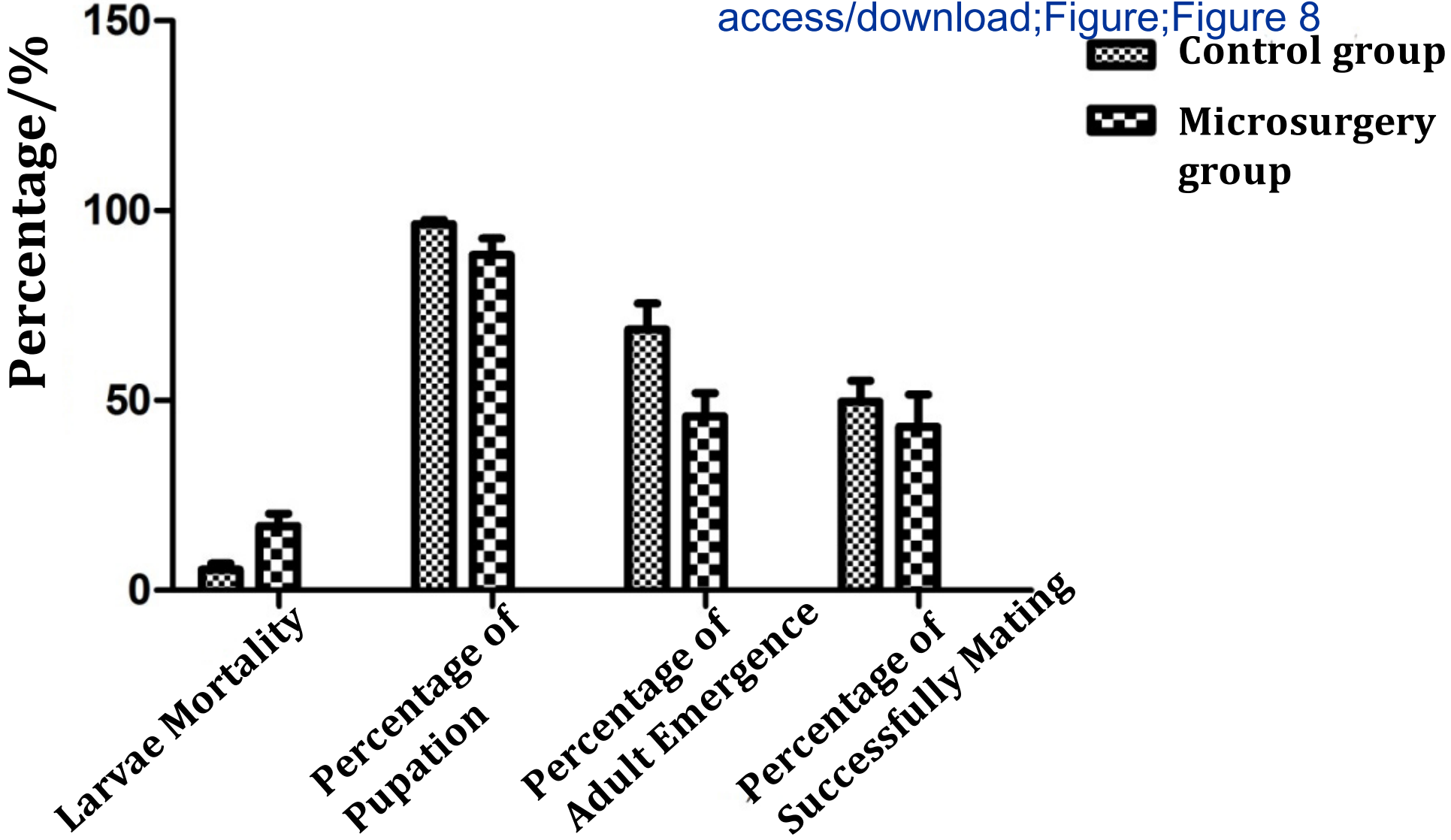


Figure 9

Click here to access/download;Figure;Figure 9 The Nos of sperm bundles of ESB.pdf

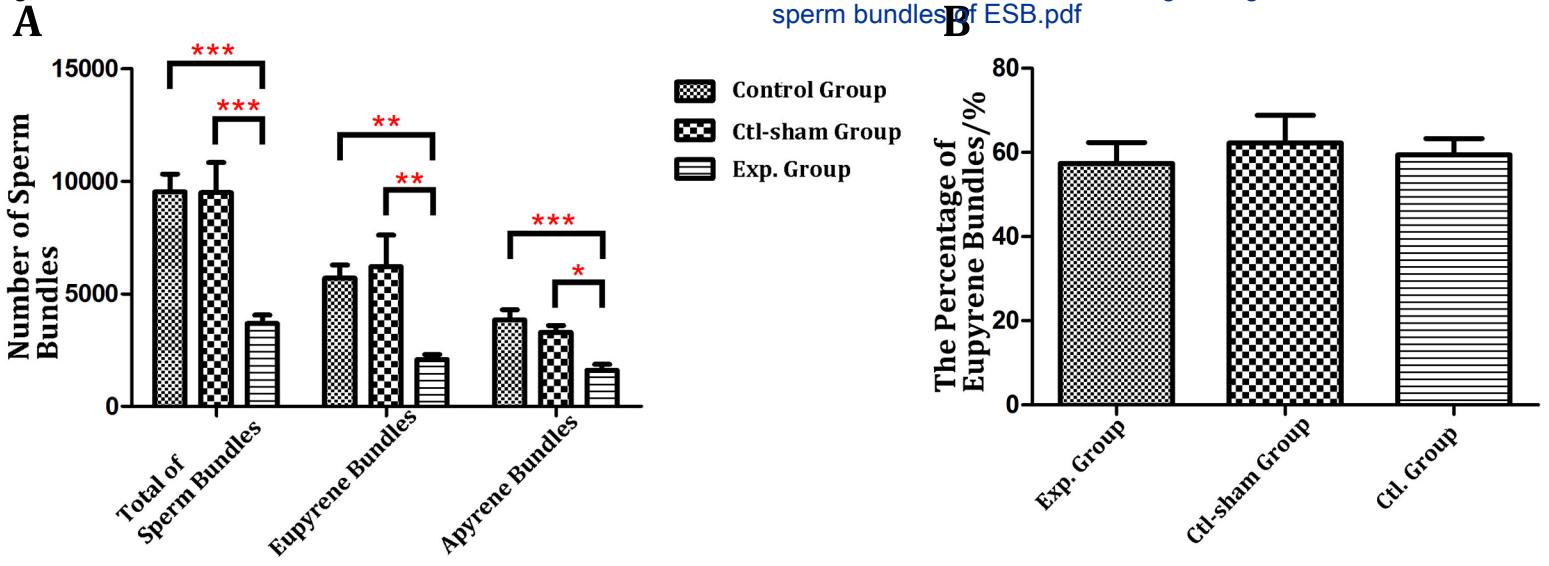


Figure 10

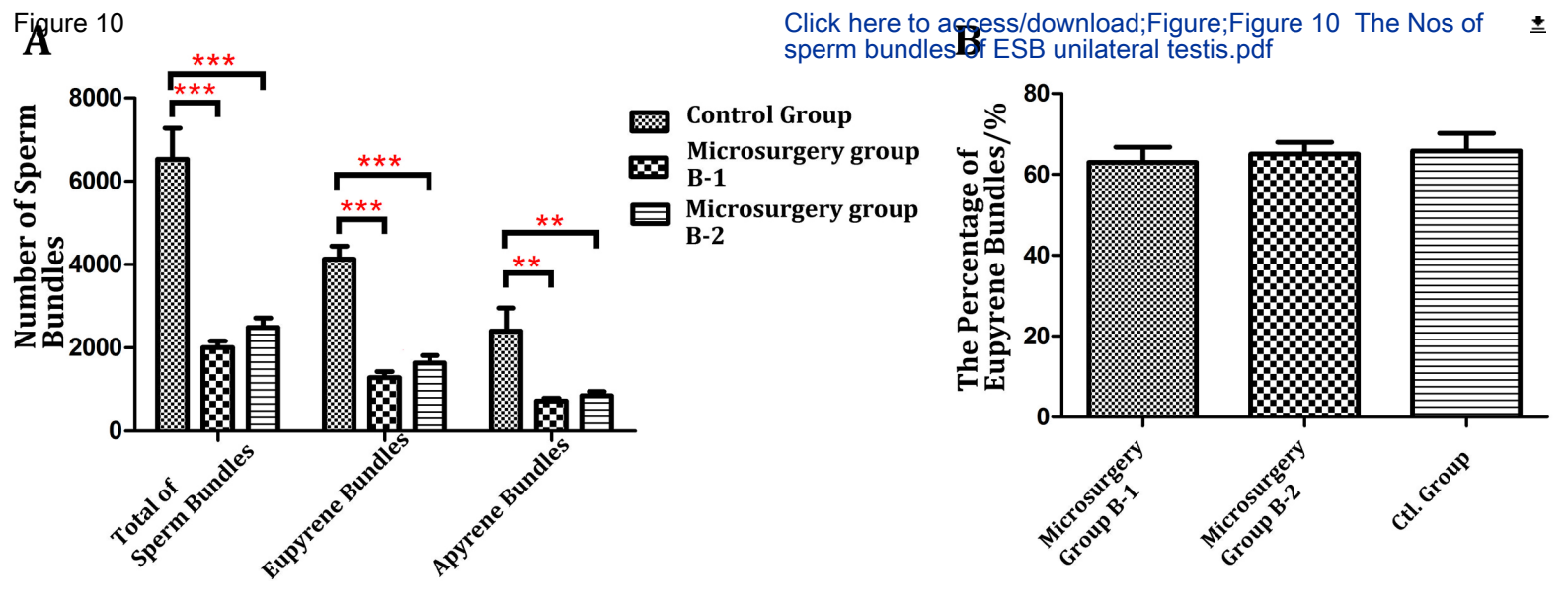


Table 1. The effects of microsurgery on the development of S.

Group	Number of Larvae	Larval Mortality Rate/%	Number of Pupae	Percentage of Pupation/%	Number of Adult	Percentage of Adult Emergence/%
Microsurgery group A-1*	79	35.4	39	76.5	N	N
Microsurgery group A-2*	117	12.8	102	100	N	N
Microsurgery group A-3*	73	13.7	57	90.5	N	N
Microsurgery group A-4	101	4	97	96	29	29.9
Microsurgery group A-5	176	20.1	140	79.5	28	20
Microsurgery group A-6	434	12.4	376	98.9	209	55.6
Microsurgery group A-7	260	10.8	135	58.2	66	48.9
Microsurgery group A-8	49	24.5	37	100	21	56.8
Microsurgery group B-1	117	29.1	71	85.5	30	42.3
Microsurgery group B-2	188	6.9	172	98.3	115	66.9
Average of Microsurgery Group (mean± SD)	159	17±10.1	123	88.3±13.7	71	45.8±16.3
Control Group 1*	40	17	37	100	N	N
Control Group 2	300	0	281	93.7	184	65.5
Control Group 3	354	11	305	96.8	127	41.6
Control Group 4	679	2.7	638	96.5	534	83.7
Control Group 5	448	4.2	399	93	232	58.1
Control Group 6	490	7.1	448	98.5	355	79.2
Average of Control Group (mean± SD)	385	5.4±6.2	351	96.4±2.7	286	65.6±15.1

litura.

Percentage of Successful Mating/%	Percentage of Successful Operation/%
N	10.3
N	11.8
N	10.5
N	26.9
44.4	25
26.8	14.3
47	48.4
81	58.8
23.3	N
35.7	N
43±20.8	25.8±18.5
N	N
N	N
N	N
41.2	N
60	N
48	N
50±9.5	N

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use

Answer : the manuscript was proofreaded thoroughly by ourselves and the company (www.newbridgetranslation.com.cn). The abbreviation has been defined.

2. Please provide an email address for each author.

Sure

3. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g. "we", "you", "our" etc.).

Answer : Most personal pronouns have been rewritten.

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." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Answer : The protocol section was written in the imperative tense. The text that cannot be written in the imperative tense was added as a "Note."

5. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. 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. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Answer:Details were added to our protocol steps

6. After including a one line space between each protocol step, highlight up to 3 pages of protocol text for inclusion in the protocol section of the video. This will clarify what needs to be filmed.

Answer: One line space between each protocol step was added.

7. Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate Figure Legend.

Answer : A scale bar information did not make when took images and it is hard

to add,especially for those took by photo camera. We are sorry for this.

8. Please ensure that the references appear as the following: [Lastname, F.I. LastName, F.I. LastName, F.I. Article Title. Source (italics). Volume (bold) (Issue), FirstPage–LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references. Please do not abbreviate journal names, and use title case for journal names.

Answer : We followed the instruction above to format the references

9. Please include a table of the essential supplies, reagents, and equipment, which is the Table of Materials. 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 Materials Table alphabetically by the name of the material.

Answer : Separate Table 1 was added to included the above information.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The manuscript is novel in nature and has the potential for implications which the author describes. Although the results were negative, they are still important. However, there are a number of concerns with the manuscript including important missing details in the protocol and poor reporting of statistical numbers. The manuscript should be reworked and include some of the following recommendations prior to recommending this manuscript for publication.

Answer:Thanks for the reviewer' suggestion. Statistical information was added

Major Concerns:

Protocol: Were all microsurgeries done by the same surgeon? This would be an important form of bias if not.

Answer:Yes, all microsurgeries were done by the same surgeon.

Results: The authors reported "averages" throughout, however this is difficult to interpret while reading and if means are being reported, they should include standard deviation so that the reader may properly interpret the results while reading.

Answer:Thank you for the comments and suggestions! We have already added SD in the Result and Table 2. This is a good point that we missed out.

Discussion: This section is lacking a nuanced message, or since the results were negative - which is fine, it should include areas to improve future research in this area.

Answer: We had performed microsurgeries one by one on more than one

thousand worms for past more than 3 years. Our study showed that testes fusion is benefical for male reproduction. The point is we will use other approach such as CRISPR/Cas9 to study the function of testes fusion, we have currently worked on.

Minor Concerns:
Intro: At the end of this section the authors should include their Hypothesis as well as explanation to support their hypothesis formulation.
Answer: Thank you for your suggestions. We have already added hypothesis at the end of introduction.

Discussion: The first paragraph should rehash the important findings and then subsequently go into the discussion on the relevance of these findings. This section is somewhat disorganized and should be reworked to read easier.
Answer: Thank you for your suggestions. We have added the important finding in the first paragraph of discussion. However, we discussed the pros and cons, applications and improvement of the microsurgery, because the journal focus on the technology to study.

Reviewer #2:
Manuscript Summary:
Comments to authors
Journal of Visualized Experiments
JoVE62524
Obstructing the fusion of testis by microsurgery in Spodoptera litura.
The authors examined the effects of surgical blockage of testes fusion on eupyrene and apyrene sperm production in Spodoptera litura. The authors state in the text that "Results following the insertion of the aluminum foil to prevent testicular fusion have been published previously"(138-139). I do not know why they re-examined and described the already known results. It is necessary to describe the past results in detail particularly because the quoted paper is written in Chinese, and to clarify the difference from this study. Although I read the previous paper, it was a review article, and it seems likely that this study described them in detail. These citations are not correct because they confuse the reader. It should be written that previous paper is properly cited and detailed in this study. Therefore, the title should be altered as mentioned above.
Answer: Thank you for your comments and suggestions. That citation is a review while this article focus of method with representative results. In order to make it clear, we cited that paper.

The authors seem to use sham control as an example of surgery where the testes did not fuse, but I think sham control is just about damaging the epidermis. It is understandable that the testes are located near the back of the

larva and can be damaged during surgery, but once you get used to it, you should not be able to damage the testes during a small incision in the epidermis. Alternatively, fasting for a few hours and then surgery may reduce the possibility of damaging the midgut.
Answer: The key of sham control is not about damaging the epidermis but to exclude the influence of inserting the aluminum foil into the larvae. The damage of the epidermis can be recovered soon, but the effect of imbedding an artificial object in the larva remain unknown. Only in this way can we confirm that the changes were completely cause by the failure of fusion.

Certainly, the freezing method will be effective in minimizing larval bleeding and preventing infection from bacteria and the like. However, as shown in Table 1, the rate of successful adulthood may be low, and I do not think this is superior to the conventional surgical methods.
Moreover, the carbon dioxide anesthesia method is easier than this method, opposed to the author's opinion. However, I admit that this method has the potential to be used in various experiments in the future.
Answer: Both freezing method and carbon dioxide anesthesia method can anesthetize the larvae in short times, but freezing method is preferable due to the following reasons. 1. Larvae narcotize by carbon dioxide wake up sooner than freezing method, and microsurgeries take time. 2. During microsurgery, we can low down the temperature by placing an ice plate under the wax tray, which will do good to anesthetize the larvae. But using carbon dioxide throughout the surgery seems unpractical.

The results of spermatogenesis after surgery are interesting and original. However, please also mention the appearance of the testes on the 6th day of the pupal stage. Is the testis hemispherical or spherical? Is the testes size half the size of control? Also, I am not sure why the authors investigated spermatogenesis only at this time.
Answer: Thank you for your comments and suggestions. We investigated spermatogenesis on the 6th day of the pupal stage that's because the testis is fully fused as the biggest one, and that is the day when eupyrene sperm bundles and apyrene sperms bundle are forming mostly.

I can understand that surgery reduces the number of apyrene sperm, but it is wondering why the number of eupyrene sperm also decreases. Eupyrene spermiogenesis begins prior to apyrene spermiogenesis, and previous papers have reported that apyrene spermatogenesis is active during the pupal stage. By the 6 the day of the pupal stage many eupyrene sperm should have already been produced. In any case, it should be discussed more in the discussion.
In addition, the differentiation of eupyrene sperm and apyrene sperm and the timing of spermatogenesis should be explained in an introduction.
Answer: The differentiation of eupyrene sperm and apyrene sperm and the

timing of spermatogenesis have been explained in the introduction. Eupyrene spermiogenesis begins at the late stage of last larvae and around prepuae stage, blocking testis fusion may also affect the formation of eupyrene sperm and led to the decrease of eupyrene sperm number.

The whole sentences, especially the discussion, is difficult to understand and should be rewritten. English is not good, so I recommend the authors to be improved the text by an English native speaker.

Answer: We apologized for that we are not good at English written. The article was copy-edited by a Australia expert Dr. Lindsay Reese.

Major revision

I had already mentioned above. I do not repeat here the same thing.

Minor revision

1. Why used capital letters? In Summary.

Answer:Thanks for your suggestions. The letters have already been corrected.

2. How to use testes and testes. Did the authors insert the foil into the approaching part of the pair of testes? If so, the authors should use the plural testes.

Answer:Thanks for your suggestions. We have already corrected.

3. In the section of Protocol, why used the imperative form? Is it a style of this Journal?

Answer:YES.The imperative form is required by the Journal.

4. Line 22. Please add "treated and untreated after the insect. Also, please describe the details on the results of sperm production. Abstract should be rewritten.

Answer:Considering this journal focus on the method, we just presented representative results and emphasized the pros and cons, applications and improvment of microsurgery.

5. Line 24 or other: Capability is better than capacity.

Answer: We have already corrected. Thank you!

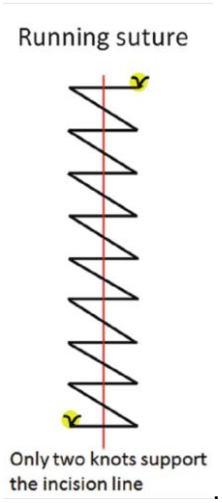
6. Line 27: Why is freezing anesthetization preferable than carbon dioxide anesthetization? Please explain it here.

Answer: Both freezing method and carbon dioxide anesthesia method can anesthetize the larvae in short times, but freezing method is preferable due to the following reasons. 1. Larvae narcotize by carbon dioxide wake up sooner than freezing method, and microsurgeries take time. 2. During microsurgery, we can low down the temperature by placing an ice plate under the wax tray, which will do good to anesthetize the larvae. But using carbon dioxide throughout the surgery seems unpractical.

7. Line 50: Please use "have" but not "has". As the word "Sperm" is a collective noun, "sperms" is not appropriated.
Answer: We have already corrected. Thank you!

8. Line 66: Physically?
Answer: We have already corrected. Thank you!

9. Line 112: What does "a continuous running suture" mean?
Answer: A running suture is more accurate definition, it means using a continuous suture material and works on alternating sides of the opening to pull the edges together to promote healing.
(reference website: https://www.infobloom.com/what-is-a-running-suture.htm#text=The%20running%20suture%20is%20a%20medical%20procedure%20used,to%20pull%20the%20edges%20together%20to%20promote%20healing; https://www.medscape.com/answers/1824895-32071/what-are-advantages-and-disadvantages-of-a-simple-running-suture-technique)



Running

suture(https://www.researchgate.net/figure/Conventional-running-sutures-and-interrupted

-sutures-as-compared-with-STRATAFIX-Yellow_fig1_321207128)

10. Lines 119-120: until dissection
Answer: We have already corrected. Thank you!

11. Line 180: Eupyrene sperm / eupyrene sperm and apyrene sperm * 100

Answer: We have already corrected. Thank you!

12. Line 182: eupyrene sperm bundles
Answer: We have already corrected. Thank you!

13. Line 213: inserting aluminum foil or the physical barrier (aluminum foil)
Answer: We have already corrected. Thank you!

14. Line 221: Spodoptera litura. Please do not omit the genus name.
Answer: We have already corrected. Thank you!

15. Line 240: N should be change to zero. This is easy to understand if we see the table.
Answer: The statistic from adult period was missing in individuals that were dissected at pupal stage. Also there's some statistic missing during the research. I am concerned that changing N to zero may mislead the reader since the table is describing numbers and percentages.

16. Line 264: "declined" is not correct. apyrene sperm bundles were smaller than
Answer: We have already corrected. Thank you!

17. Line 265: Please use" results" but not conclusion
Answer: We have already corrected. Thank you!

18. Line 332: Reference 11. The study of fusion of testis in Lepidoptera insects is correct.
Answer: We have already corrected. Thank you!

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Author(s):	Qianqian Ma#; Xiaolin He#; Yucheng Liu; Baozhu Jian; Meixin Chen; Qiong Wu; Qili Feng; Ping Zhao*; Lin Liu*

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