

Instructions for dissecting male genitalia of *Helicoverpa* (Lepidoptera: Noctuidae) to separate *H. zea* from *H. armigera*

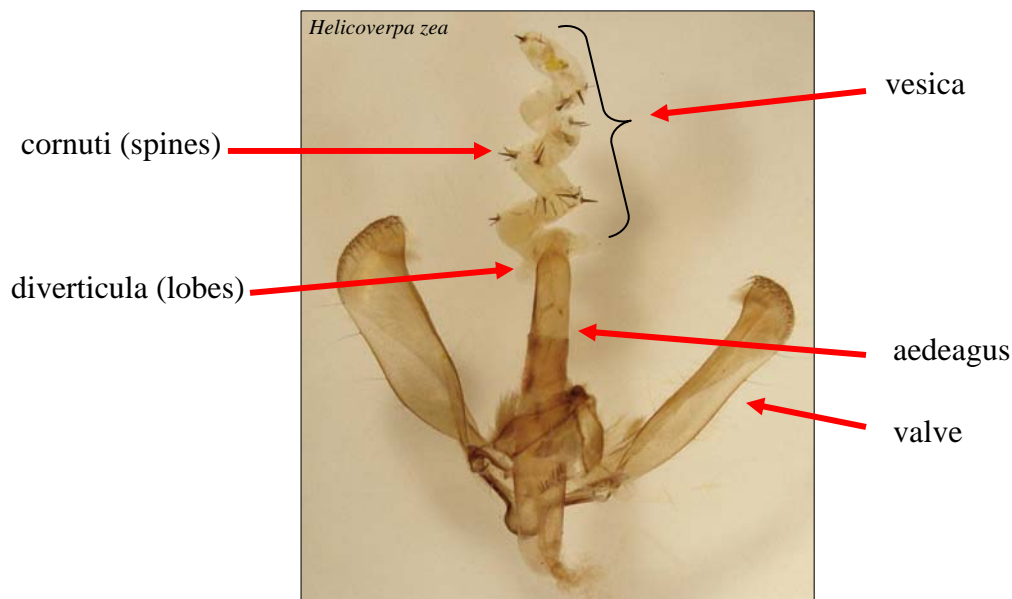
Introduction

The purpose of this handout is to guide you in the preparation and screening of specimens obtained with *Helicoverpa armigera* pheromone lures. These instructions will help you distinguish *Helicoverpa armigera*, the target, from the native *Helicoverpa zea*, which is strongly attracted to the lure. You can adapt the preparation techniques to the materials you have available in your work area.

Part 1 presents in detail and with illustrations one method and some alternatives for how to prepare specimens, including how to make the potassium hydroxide solution used to clear tissues. Part 1 also has a summary that can be posted by your preparation area.

Part 2 describes at length how to examine genitalic characters and one abdominal character to separate the two species.

Part 3 presents a short cut for separating *H. zea* from *H. armigera* using genitalic characters only.



Terminology of *Helicoverpa* genitalia

Instructions for Processing *Helicoverpa* Moths

Part 1: Preparing Specimens

1. **Heat.** Turn on the heating block or other heating unit available. If you plan to use a cold-clearing technique you can skip steps 1, 2, 8 and 10.

2. **Water bath.** Place a metal cooking pan with ~ ½ inch of tap water on the heating block. The water should not reach boiling point. Use the same temperature each time. A recommended temperature is 50°C. You can adapt the preparation techniques to the materials you have available in your work area using a different type of heater and pan for the water bath. Electric

heating pans and coffee mug warmers are some options.



Start warming the water.

3. **Sorting specimens.** Open the box or bag of specimens and drop the specimens on a clean white plastic tray, a sheet of paper, or table top. If the field samples are dry and dusty, wear a dust mask or use a fume hood to avoid breathing in moth scales. Sort specimens and separate those that appear to be *Helicoverpa* using general screening aids. Count the specimens and write the number on the specimen report. Process a single sample at a time (a sample is a set of specimens collected at the same site and date).

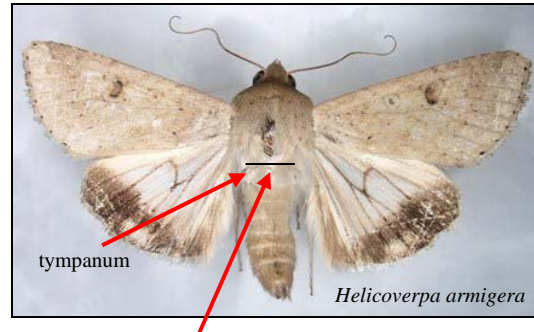


Clean surface



Dust mask use is recommended when processing dusty samples

4. **Removing abdomens.** Remove the abdomen from each *Helicoverpa* specimen with blunt forceps. If the specimens are dry and brittle, break each abdomen off at the base where it meets the thorax by pushing the abdomen to the side with the forceps while holding the thorax down with a finger or forceps. If the specimens are soft, separate the abdomen of each specimen by first pressing down at the base with broad forceps, then pulling it off carefully. You can find the base of the abdomen by looking behind the base of the hind wings, where you can notice the large tympana, which look like windows. The abdomen begins after the tympana. Keep all wings for now.



Break the abdomen off where the line indicates

5. **Labeling.** Place all abdomens from a single trap in a scintillation vial (20 ml glass vials with a wide mouth and screw top lids) and add a small paper label written with pencil or waterproof ink (example: 1, 2, 3, 4). Make matching notes on the specimen reports. For larger samples of 50 specimens or more, divide the samples, calling them 1a, 1b, 1c and 2a, 2b, 2c, 2d, for example, each with between 20

and 30 abdomens. Other types of glass vials can be used, as well as small open glass or ceramic containers.



label

Keep track of samples with small labels.

6. **Moistening.** Add alcohol to each vial to moisten the abdomens so they readily sink into the KOH. Keep them in alcohol less than 2 minutes. The specimens do not get damaged if they stay longer in alcohol but it becomes harder to evert the vesica. Isopropyl is fine for our purposes but ethanol is used for research purposes. This step can be skipped but if you do not moisten the abdomens, they will tend to float in the KOH.

7. **KOH***. Remove alcohol and add 10% KOH solution (see instructions on how to make the KOH solution). Fill each vial about halfway, that is, about 10 ml; you should use more for large samples but do not fill the vials to the top. Close the vials with the screw top lids, so that you reduce chances of a spill; do not close the vials tightly.



Prepare your KOH solution in advance. Fill half of the scintillation vial with 10% KOH*. Close the vial loosely with a lid. Wear lab gloves.

8. **Heating KOH***. Place all vials with labels inside the metal pan with the warm water and cover (although the cover is optional). Make sure the heat is set below the boiling point of the water (that is, less than 100 degrees Celsius); you can try 50°C or higher. Remember that vials are capped loosely.



45 minutes
50°C

9. **Clearing.** Keep specimens in the warm KOH* solution for 45 minutes. The potassium hydroxide macerates the tissues, and proteins and fats are removed, leaving chitinous structures such as the skin of the abdomen and the valves. If you do not want to use warm KOH, you can leave the abdomens (or even whole specimens) in cold KOH overnight or for 5 to 8 hours. If you leave specimens too long in the KOH solution, they will be damaged or destroyed.

10. **Cooling:** Remove vials from the warm water. You may want to use leather or cotton gloves, paper towels, or a cloth to grab the vials. Let vials cool down slowly, on the counter, for at least 10 to 30 minutes. However, specimens can be examined as soon as they come out of the heated water (with care because of the caustic KOH*). After this process, specimens should not be left in the KOH.



Remove vials from the warm water. Gloves are optional.

11. **Rinsing.** Remove KOH with a plastic pipette or a glass dropper and discard the used KOH. Wear eye shields (or glasses) and lab gloves because warm or hot 10% KOH* can permanently damage the eyes and irritate the skin. Add alcohol (or water) to rinse, removing it with a plastic pipette, and add water or alcohol again (alcohol is preferable). The specimens are ready for dissection.



Remove KOH and rinse samples.

Instructions for Processing *Helicoverpa* Moths
Steps for Preparing Specimens – a Summary

1. **Heat.** Turn on the heating block.
2. **Water bath.** Place a metal cooking pan with ~ ½ inch of tap water on the heating block.
3. **Sorting specimens.** Sort specimens and separate those that appear to be *Helicoverpa*.
4. **Removing abdomens.** Remove the abdomen from each *Helicoverpa* specimen with blunt forceps.
5. **Labeling.** Place all abdomens from a single trap in a vial and add a small paper label.
6. **Moistening.** Add alcohol to each vial to moisten each abdomen. Keep them in alcohol less than 2 minutes.
7. **Adding KOH*.** Add 10% KOH solution. Close the vials loosely.
8. **Heating KOH*.** Place all vials in the pan with the warm water.
9. **Clearing.** Keep specimens in the warm KOH* solution for 45 minutes.
10. **Cooling:** Remove vials from the warm water. Let vials cool down slowly, between 10 and 30 minutes.
11. **Rinsing.** Remove KOH* with a plastic pipette. Rinse with alcohol or water. Add alcohol. The specimens are ready for dissection.

Instructions for Processing *Helicoverpa* Moths

Preparing the 10% KOH solution

KOH* – potassium hydroxide

Option 1: Purchase 10% KOH solution

Option 2: Add 2 to 4 pellets of KOH to the vial or small dish with water in which you are processing a few specimens.

Option 3: Best choice: Prepare your own fresh 10% solution.

How to prepare the 10% KOH solution

-add 100 ml (milliliters) of water to a plastic Nalgene or glass bottle. Plain tap water can be used for screening purposes, but distilled or deionized water is normally used for research purposes.

-place a small plastic or glass dish on a balance.

-turn on the balance and select 'grams' for weight units.

-weigh 10 gr (grams) of pure KOH pellets (don't touch the pellets!).

-add the pellets to the plastic Nalgene or glass bottle with water, close with a lid, and gently swirl. Let this mix rest at least for 15 minutes or until the pellets are dissolved before using the solution.

-other quantities you can prepare are 20 gr KOH pellets to 200 ml of water, or 15 gr KOH pellets to 150 mls of water. Each ml of water weighs one gram, so you can also weigh the water in the balance. For example, you can measure 25 gr of pellets and add them to 250 gr of water.

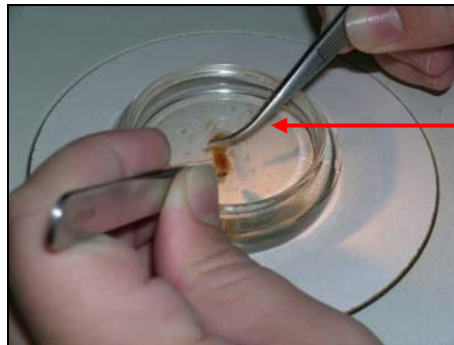
-Label the plastic or glass bottle with "10% KOH" to avoid accidents.

***Caution:** KOH is very caustic. Do not grab or hold the solid pellets with your fingers. Always transfer the pellets by shaking them into a dish or vial, using a metal or plastic spatula, or use forceps. Use gloves, especially when handling warm KOH solution. If some of the 10% KOH solution touches your skin, rinse your skin thoroughly with water. A splash of warm KOH solution can do permanent damage to your eyes. So, for eye protection, wear safety glasses when handling warm or hot KOH solutions. Read the MSDS (material safety data sheet) information for KOH that arrives when you order this chemical. Have emergency eye rinse bottles at hand.

Instructions for processing *Helicoverpa* moths

Part 2: Examining genitalic characters

1. Place one abdomen at a time into a glass dish with alcohol for microscope examination. In time, you can process several specimens at a time in one dish.
2. **Obtaining the genitalia.** Hold the abdomen at the base with straight forceps and press it gently with the round end of curved-tipped forceps from base to apex to extrude the entire genitalia, being very careful not to damage the aedeagus. A small camel hair brush can also be used instead of forceps to press on the abdomen. If the genitalia do not exit through the apex, gently grab the valves and pull. If the abdomen is inflexible, return it to KOH for further clearing. If the genitalia are obtained, but are not flexible, place the genitalia in the warm KOH* solution for 5 or 10 more minutes.



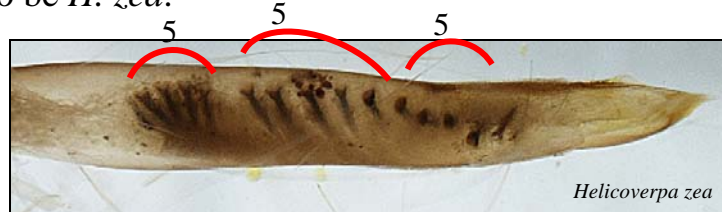
Use round forceps to press on the abdomen

3. **Examining the valves.** Grasp each valve on its side with forceps and gently open them. If each valve is long and thin, has no projections, and has a row of inward-curved spines at the apical margin, it is most likely *Helicoverpa*. If these spines are lacking, the specimen cannot be in the genus *Helicoverpa*.
4. **Removing the valves.** To examine the aedeagus, first remove the valves. Hold the aedeagus at the base with one set of forceps and pull the valves (together) with the other forceps, detaching the valves from the aedeagus.



Aedeagus still attached to the abdomen, and the valves removed

5. **Counting cornuti.** Count the number of cornuti (=spines) inside the aedeagus. They can be seen through the wall of the aedeagus. The cornuti are arranged in sets, so do not count individual spines. If you count 12 cornuti sets or less it could be *H. armigera*, and if you count 15 or more sets, and definitely more than 12 sets, it is probably *H. zea*. If the aedeagus has no cornuti, or very few, the specimen is probably aberrant and sterile; it is considered to be *H. zea*.



In this image you can see at least 15 sets of cornuti

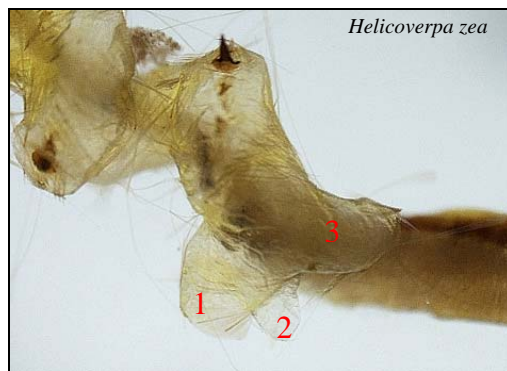
6. **Everting the vesica.** Examination of the vesica is essential for final screening for *Helicoverpa armigera*. The vesica is a membranous structure inside the aedeagus. You can skip Step 6 and go to Part 3 if you do not have a fine-needle syringe. Fill half of a syringe with alcohol, which, for our purposes, can be 75% ethanol or isopropyl alcohol, but researchers generally use 99% isopropyl alcohol to evert the vesica. Evert the vesica by inserting the needle into an opening on the side near the base of the aedeagus while holding the aedeagus at the base with forceps; then, press the plunger. If the vesica is in good condition, it may evert successfully and completely, although all that is needed is a partial eversion. If the vesica, or any other membrane of the aedeagus, is damaged or brittle, it will not evert.



The vesica is everted as it is pushed through the apex of the aedeagus

Syringes: You can buy micro-fine U-100 insulin syringes from Fisher Scientific or other companies, ½ cc volume, 0.36 mm x 13 mm needle. Its sharp tip can be rounded by sanding on a metal or emery board. You can also use a glass syringe fitted with a size 30 needle (Pogue 2004).

7. Counting the diverticula. If you clearly see three contiguous lobes (diverticula) at the base of the vesica, the specimen is *Helicoverpa zea*. If you are only able to see one or two lobes, rotate the aedeagus since some lobes may be ventrally produced and may not be noticed at first. If only one lobe is definitely observed, examine other characters. It is advisable to have some *H. zea* for reference as well as for practice.



3 lobes: *H. zea*

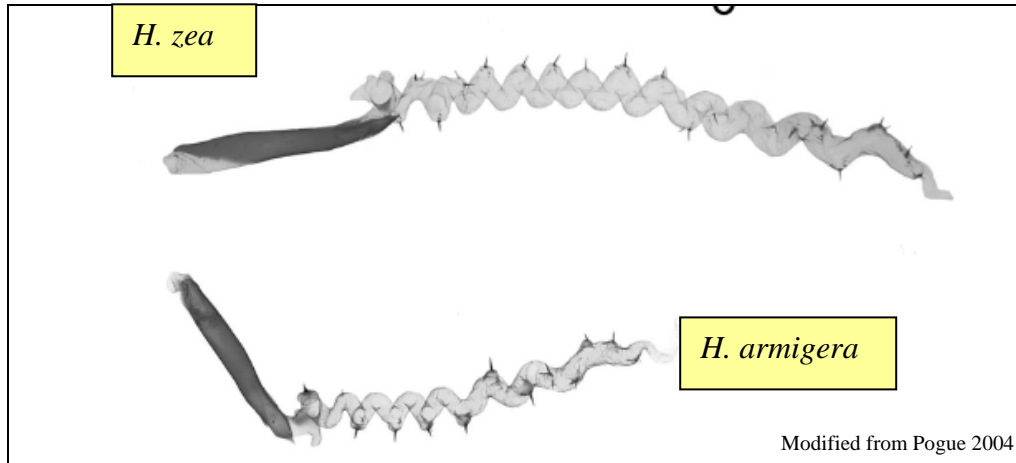
1 lobe: *H. armigera*



Helicoverpa armigera has only one lobe at the base of the vesica while *H. zea* has 3 lobes.

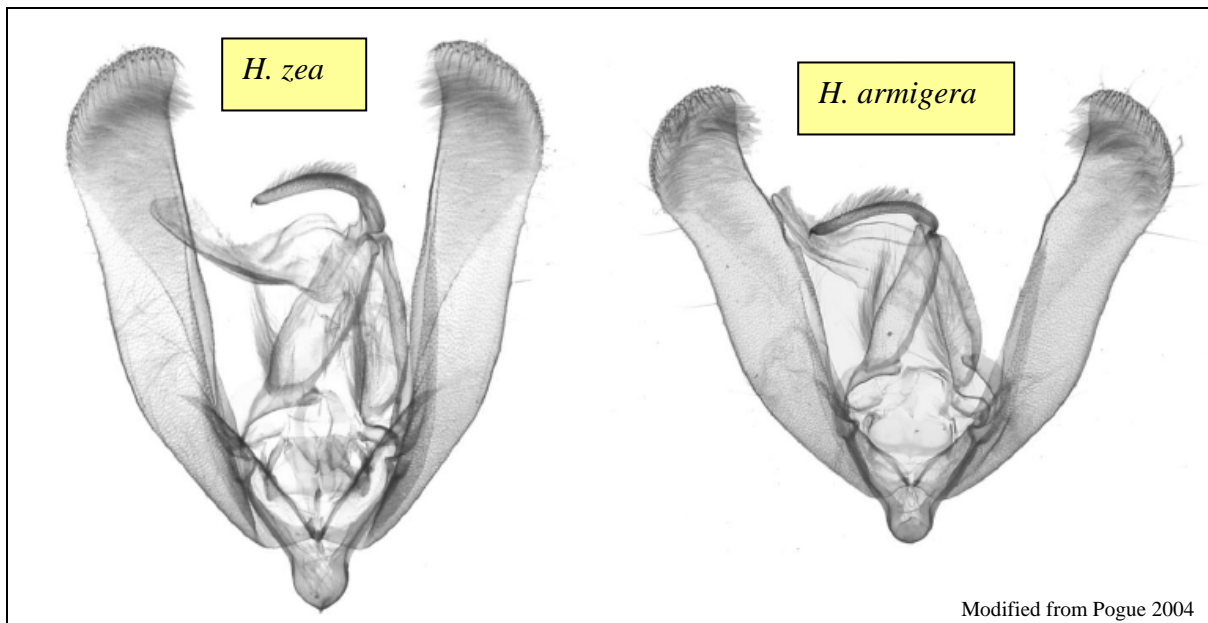
8. Examining other characters. Refer to Pogue (2004) for more details.

- a. The vesica of *H. zea* is much longer than the vesica of *H. armigera*.



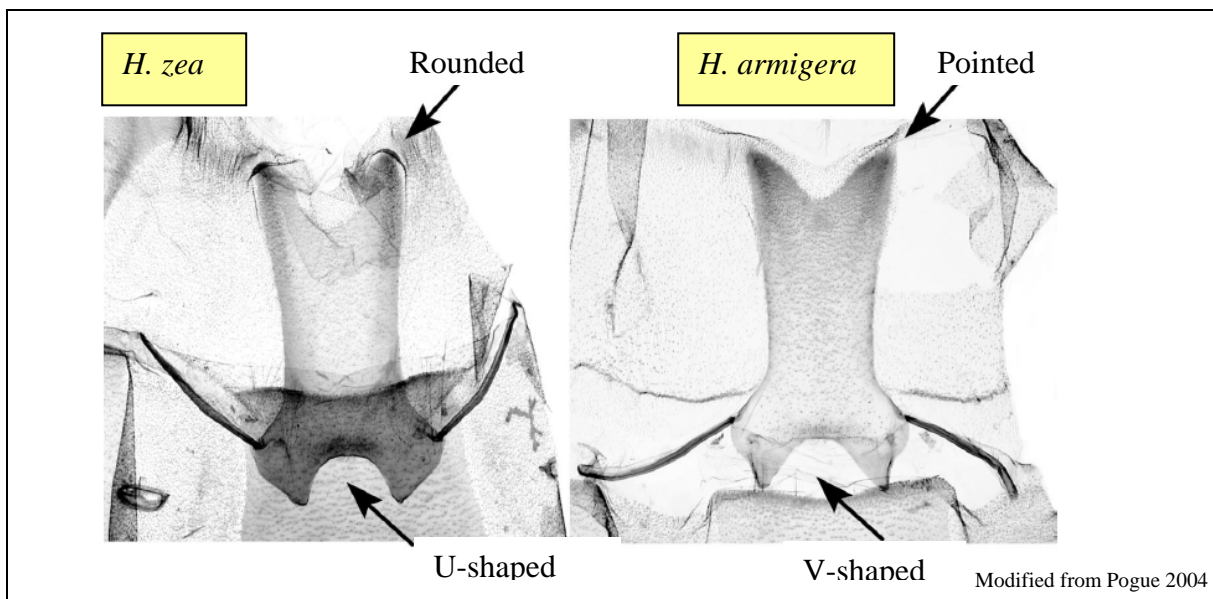
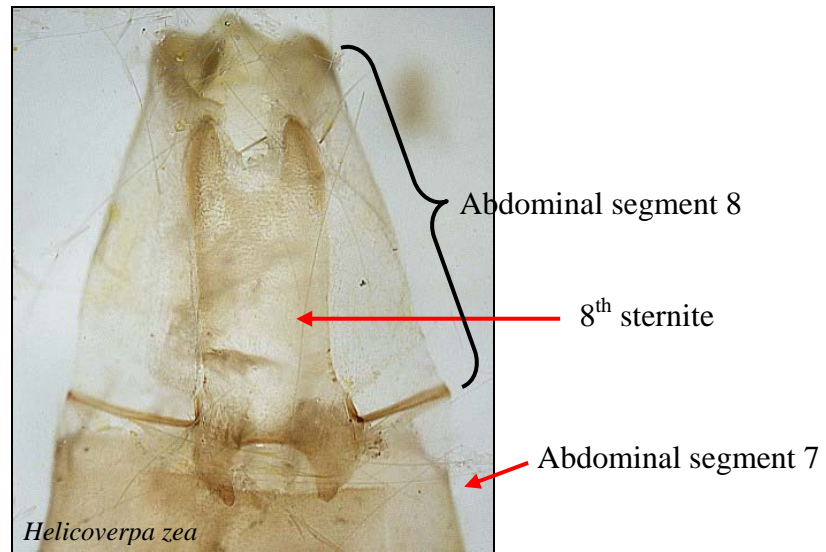
Fully everted vesicae.

- b. The valve on *H. zea* is usually 5 mm or longer, while on *H. armigera* it is usually less than 5 mm in length.



The valves on *H. zea* are longer than the valves on *H. armigera*.

- c. The 8th abdominal sternite (=ventral tergite or plate) has rounded posterior apices (tips) and a U-shaped anterior margin in *H. zea*, while this sternite has pointed posterior apices and a V-shaped (with apex flattened) anterior margin in *H. armigera*.



The 8th abdominal sternite differs in shape on the anterior and posterior margins in the two species. The anterior margin is at the bottom of these images, and the posterior margin is toward the top.

Instructions for processing *Helicoverpa* moths
Part 3: Screening Instructions – the Short Cut

1. Place the KOH-cleared abdomen in a glass dish with water or a weak solution (40%) of ethyl alcohol and place the dish at a dissecting microscope with good light.
2. Grasp the abdomen at the base with straight forceps and gently press it with the back side of curved-tipped forceps or with a small brush from the base to the apex to extrude the genitalia. Try not to detach the genitalia from the abdomen. If the abdomen is solid, return it to warm KOH* for further clearing. If the genitalia are obtained, but are not flexible, place the genitalia in the warm KOH solution for 5 or 10 more minutes.



Photos 1-3: Pressing the genitalia out of the abdomen of *Helicoverpa zea* with the round end of curved forceps.

3. Grasp each valve on its side with the forceps and gently open them to verify the specimen is *Helicoverpa*. Notice the row of downward-curved spines at the apex of each valve.



Photo 4: Opening the genitalia of *Helicoverpa zea*.

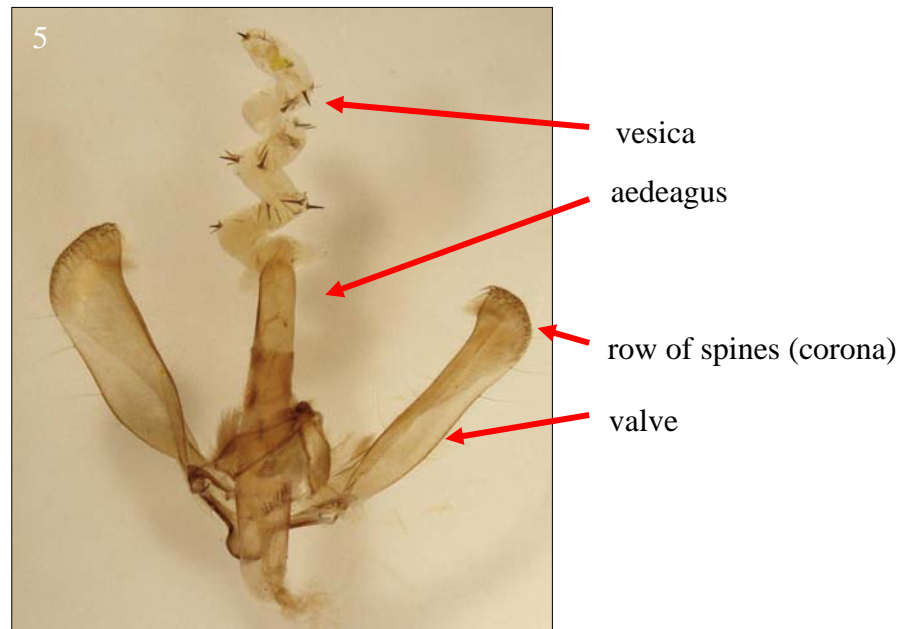


Photo 5: Genitalia of *Helicoverpa zea*. Note the row of spines at the apex of each valve. The vesica is partially everted. The spines on the vesica are called cornuti.

4. Detach the valves by holding the aedeagus at the base while pulling the valves over the aedeagus. The aedeagus should still be attached to the abdomen, but this short cut technique can still work if detached, although not as reliably. Before proceeding to Step 5, count the number of cornuti sets. In *H. zea*, you can see 15 or more sets of cornuti inside the aedeagus, while in *H. armigera* you can usually see only 12 sets or less.



Photo 6: Aedeagus of *Helicoverpa zea*. Count the number of sets of cornuti, not individual cornuti (spines). In this image you can see 17 or 18 sets of cornuti.

5. Hold the aedeagus at the base with straight forceps and very gently press it from the base to the apex with the back of curved forceps or with a small brush. The vesica will usually partially evert at this point if the aedeagus has not been damaged. You can repeat this step a couple of times if necessary. If the aedeagus is damaged, partial or complete eversion of the vesica will not be possible.

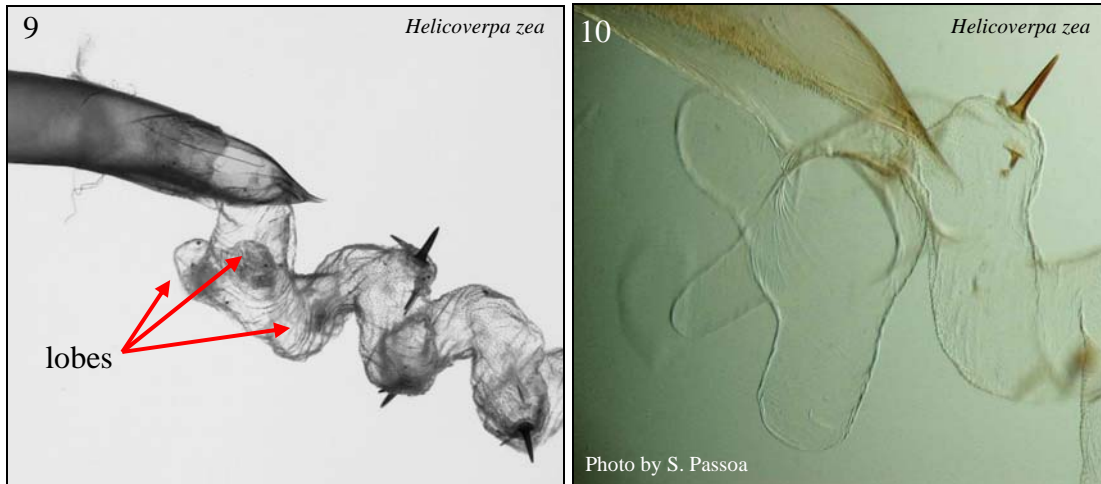
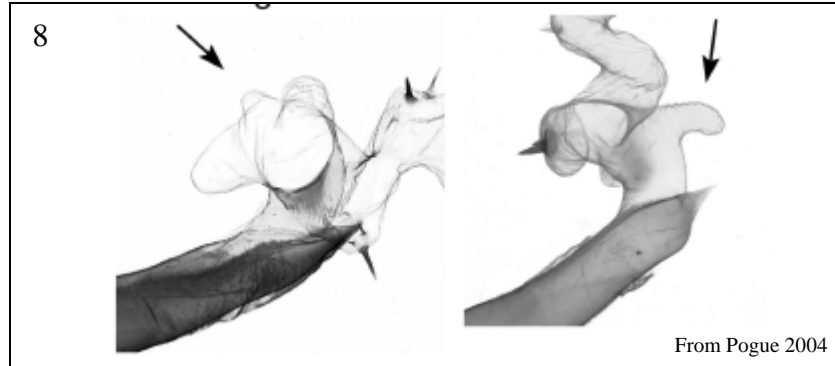


Photo 7: Pressing the aedeagus of *H. zea* to partially evert the vesica. Notice the valves have been removed. The lobes at the base of the vesica are visible and are marked with an arrow.

6. If you clearly see three contiguous lobes (diverticula) the specimen is *Helicoverpa zea*. Take time to examine the base of the vesica. If you are only able to see one or two lobes, rotate the aedeagus since some lobes may be ventrally produced and may not be noticed at first. If you only see one lobe, you can repeat Step 5 if the aedeagus has not been damaged.

3 lobes: *H. zea*

1 lobe: *H. armigera*



Photos 8 to 10: Count the number of lobes at the base of the vesica. *Helicoverpa armigera* specimens have only one lobe while *H. zea* specimens have three lobes.

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