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A PROCEDURE FOR EXAMINING THE GENITALIC MUSCULATURE OF LEPIDOPTERA

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ABSTRACT. The functional morphology of the genitalia (characteristics of the sclerotized parts and the presence and position of the associated musculature) has been the basis of recent phylogenies of Tortricidae and several other groups of Lepidoptera. Examination of this musculature can be difficult. Procedures for fixing muscles and preserving specimens for future preparation, for dissecting, cleaning and staining the genitalia, and for treating the preparation for viewing are presented. Using these methods, one can stain muscles selectively, minimize handling during cleaning to reduce the potential of physical damage, and view the musculature through transparent sclerotized parts.

Additional key words: functional anatomy, dissection, staining, male genitalia.

The sclerotized parts of the genitalia often are the best or only means of identifying species of Lepidoptera and can provide important characters for determining taxonomic relationships. The associated functional musculature has been used as another source of data on phylogenetic relationships, particularly of higher taxa in Lepidoptera in recent decades.

Forbes (1939) described differences in the male genitalic musculature among six species in five families of Lepidoptera that he examined and two other species illustrated by Snodgrass (1935:fig. 308). Utilization of these characters in taxonomic studies of Lepidoptera was proposed by Stekol'nikov (1965), who examined males of five and females of two species of Noctuidae. Stekol'nikov (1967a, 1967b) constructed a phylogeny of butterflies based on the functional morphology of the genitalia and discussed evolutionary trends in the genitalia of primitive Lepidoptera. An ensuing series of papers by Kuznetsov and Stekol'nikov (1981, 1984, 1985) proposed the higher classifications of various groups of Lepidoptera based largely upon genitalic musculature of the males, and others (e.g., Razowski 1981) have employed these characters in studies of Tortricidae and other Lepidoptera.

Workers wishing to investigate these conclusions further and those intending to use their system to assign troublesome genera to tribe or subfamily may have difficulty preparing specimens for examining musculature, as there is little published information on the methodology. The following procedure, derived by trial and error and from the suggestions of colleagues, may greatly simplify this task. We worked with Tortricidae, and the techniques should be applicable to all Lep-

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idoptera although we did not attempt dissection of smaller moths such as leaf miners. With this procedure, much of the fatty tissue is dissolved, so handling time during cleaning is minimized. The sclerotized parts turn transparent which enables one to view the internal muscles and their connections.

Preparation of Lepidopterous Genitalic Musculature

The best preparations of genitalic musculature result from proper preservation of specimens, from careful dissection, cleaning, and staining, and from treating the stained, dissected genitalia for viewing. Sectioning with a microtome may be necessary for minute Lepidoptera, but the following procedure was effective on a species of *Diactenis,* one of the smallest tortricid moths. Although it was not tried, the procedure should be satisfactory for examination of female musculature as well.

Some internal muscles and their attachment points are seen more easily when the valvae are spread. The valvae of some specimens will spread automatically when killed or immersed live in the preservative, or they can be forced open by squeezing the pregenital segments prior to preservation in fluid. However, using our procedure, the sclerotized parts of the genitalia become transparent, and attachment points can be determined. Consequently, most preparations were of specimens with the valvae closed, because it was easier to position them for dorsal, ventral, or lateral viewing.

Preservation of the specimen: Musculature of dried, pinned specimens can be observed, but better preparations are obtained from specimens preserved in fluid soon after capture. Specimens placed directly into 70% alcohol (we used either isopropanol or ethanol) are usually satisfactory, but the muscle tissue may deteriorate slowly, and older specimens may be unusable.

We obtained better results by first immersing the specimen in Kahle's fluid for 12–24 hours to fix the muscles. Peterson (1964:67–68) and Borror et al. (1976:736) give different, but equally effective recipes for Kahle's fluid.

The moth can be immersed directly into Kahle's fluid; then a few drops of 70% alcohol should be added to enhance wetting. Alternatively, the specimen can be dipped into 70% alcohol or cellusolve (ethylene glycol monoethyl ether) for a few seconds until soaked, then transferred to Kahle's fluid. When it is impractical to preserve specimens immediately, muscles of those killed in dry cyanide vials can be similarly fixed if treated soon after capture.

After fixing the muscles, the specimen can be dissected immediately or retained in 70% alcohol. Specimens transferred from Kahle's fluid

to 70% alcohol were in excellent condition more than five years after preservation. Prolonged immersion in Kahle's fluid or preservation in 95% or absolute alcohol makes the muscles brittle and gives them a greater tendency to detach from the sclerotized structures.

A few preparations were made from dried, pinned specimens by removing the abdomen and soaking it in warm water until the viscera softened. The muscles of previously dried specimens are inelastic, sometimes shrunken and contorted, and easily detached, and they do not stain as well as those of specimens preserved in fluid, so results vary.

Dissection and staining: Remove the abdomen from the specimen and place it in 70% ethanol. Shallowly insert the tip of each of two pair of No. 5 jeweler's forceps, one on each side of the pleuron, into the intersegmental membrane anterior to the last or next to last visible pregenital abdominal segment, and carefully peel away the integument. Continue to remove the integument of the pregenital segments from the genitalia until none can be removed without risking damage to the genitalic muscles.

Large agglomerations of fat will inhibit staining, but at this time attempt to remove only the larger looser globules atop the base of the aedeagus to avoid damaging the muscles.

Place the excised genitalia in a drop or two undiluted van Giesen's muscle stain (1 part of 2–3% acid fuchsin and 9 parts of saturated picric acid) on a stain plate for 3–10 minutes or until stain begins to penetrate but does not completely stain the internal muscles of the tegumen. Next, soak the preparation in 70% alcohol for several hours to allow stain to penetrate internal muscles while washing away excess.

After soaking in alcohol, muscles of a properly stained preparation will be red throughout, fat will be paler, and the sclerotized parts should be mostly unstained. Some staining of the sclerotized parts is unavoidable, especially the aedeagus, some of the more membranous parts, and around the margins of other structures.

If too much stain was removed, the exposed muscles on the aedeagus will have lost much of their color while the internal muscles remain red. Place such preparations briefly into a drop of stain then wash off the excess in 70% alcohol.

If understained, stain will not have penetrated the internal muscles, and the staining-soaking step should be repeated. Sometimes, particularly in previously dried specimens, some muscles will not stain well, and additional attempts will only stain the sclerotized structures.

Subsequent steps will remove some additional stain, so slight overstaining is acceptable. If the sclerotized portions are stained excessively, additional soaking in clean 70% alcohol may eventually remove the excess. If still overstained, immerse the preparation in hydrogen per-

oxide solution, then restain if necessary. Final cleaning follows the next step.

Transfer the preparation to cellusolve and soak for 10 hours or longer. The solvent will dehydrate the preparation and dissolve much of the fat and cause much of the remainder to agglomerate into easily removable globules. The dehydrated remnants of pregenital segments and unwanted extrinsic muscles can be abraded from the genitalia easily with forceps or fine probes, and most of the remaining fat globules can be teased free of the preparation with fine probes. Smaller traces of fat will dissolve or turn transparent in the next step, so it is not necessary to risk damage by trying to remove small bits trapped between muscles.

Preparation for viewing: Place the cleaned preparation in methyl salicylate (oil of wintergreen) for viewing. From about 30 minutes to about five days following immersion, the sclerotized structures will be sufficiently transparent to view the internal muscles, yet will retain enough pigmentation to determine the attachment points of the muscles.

With prolonged immersion in methyl salicylate, the preparation becomes increasingly brittle and more easily damaged during handling, and the sclerotized parts slowly darken, presumably due to infusion of the stain. Partial darkening may help identify sclerotized structures, but after about five days, some musculature may be difficult to see through the sclerotized parts. A darkened preparation can be bleached in hydrogen peroxide after being washed of methyl salicylate in cellusolve then 70% alcohol. It can be restained and treated for viewing as before, but it will be more brittle and somewhat inferior overall.

We positioned preparations for viewing with glass chips in the depression of a culture slide filled with methyl salicylate. A camera lucida attached to a binocular dissecting microscope facilitated sketching the preparation. When necessary, a compound microscope was used to determine musculature attachment points accurately.

After examination, the preparation was washed free of methyl salicylate in cellusolve, then bathed in 70% alcohol. It was preserved in 70% alcohol with the remainder of the specimen. In the alcohol, the stain slowly leaches from the preparation. It can be restained and cleared for re-examination, but will be of lesser quality than after the first treatment.

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