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Revised techniques for the study of ant larvae

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Introduction

In 1960 we published "Techniques for the study of ant larvae." We referred to it in 1976 and again in 1986. In the intervening years three authors have cited it and the quality of their drawings suggest they have benefited from our suggestions. The poor quality of most other recent drawings suggest they have not seen our article. Perhaps a 1960 reference is too old to be caught in latter-day "literature searches." Or, if the searcher did find it, he was unwilling to take the trouble to follow the recommended techniques. And troublesome they are, especially if one does not have readily available the required apparatus and chemicals.

Preservation

Any preservative may be used for ant larvae if only the external anatomy is to be studied. We prefer 75% ethyl alcohol. Rubbing alcohol (70% ethyl, not isopropyl) may be used, but should be changed at least once. No special preparation is necessary; the larvae are simply dropped into the preservative. If the alcohol is too dilute or if the container has been overcrowded internal structures may be inadequately preserved, but the integument will still be usable for external anatomy. If large larvae are jostled the hairs may be broken.

Naturally if one plans to investigate also internal anatomy or histology, appropriate killing and fixing reagents are necessary.

If we have only one larva (or very few) of a species we make notes and side-view drawings before subjecting it to any additional techniques. This is simply good insurance.

Relaxation

Even dried larvae may be used for the study of external anatomy. Either of two methods of relaxing is recommended:

1. Two days in a 0.5% solution of trisodium phosphate. Transfer to 75% alcohol.
2. Two days (or less) in the following: 95% ethyl alcohol 280 ml; distilled water 230 ml; benzene 35 ml; ethyl acetate 95 ml. Transfer to 75% alcohol.

Dried larvae are handled with extreme care, since hairs are very likely to be broken in this condition.

Handling

The most convenient receptacle for the treatment of larvae is a staining dish (=embryological cup) 41 X 41 X 18 mm in outside dimensions. Minute specimens, however, are best treated in deep-well slides. Small specimens are transferred with a pipette, large specimens between the points of forceps (but without compression). Or, if one prefers, the larvae may be left in the same dish or deep-well slide; the old reagent is drawn off with a pipette and then replaced with the next reagent.

Special precautions

In case the cleaned integuments are flimsy, every possible precaution must be taken to prevent collapse. A collapsed integument is good for nothing but the head. To prevent collapse such integuments must be at all times completely immersed. If they are exposed to air or come in contact with the surface film they will collapse beyond repair. This collapse can probably be prevented by very gradual change of solutions; half of the old liquid is removed with a pipette and replaced by the new liquid with as little disturbance as possible. After a few minutes of gentle swirling, the process is repeated. After several repetitions practically all of the old liquid has been replaced.

Cleaning

The best cleaning reagent is potassium hydroxide solution (10 gm KOH in 90 ml water). While still in preservative the larvae are punctured with a dissecting needle or minute insect pin on the right side to permit the ready penetration of the cleaning solution. The number and locations of the punctures and the size of the needle depend upon the size of the larva. The specimen is left in the cleaning solution until the internal tissues are dissolved and only a transparent exoskeleton remains.

If cleaning is not complete by the end of a week, the following procedure may prove effective: transfer to 1% hydrochloric acid and leave for 15 minutes; 15 minutes in 95% alcohol; 15 minutes in carbolxylene; 15 minutes in 95% alcohol; then leave in KOH until clean. Some larvae contain droplets of opaque substances which are insoluble in KOH; these may later disappear in alcohol or xylene.

Staining

The exoskeletons of ant larvae are stained in a very dilute solution of acid fuchsin. We use the following formula: acid fuchsin 0.1 gm, concentrated hydrochloric acid 1 ml, distilled water 1 liter; a few thymol crystals are added to prevent mold. The cleaned exoskeletons are washed in water for 15 minutes and transferred to 2 ml of 1% HCL in a staining dish; 10 drops of the stain are added. The integuments are left in the stain overnight.

If overstained the integument can be completely decolorized by leaving in 1% KOH for several hours. It can then be washed in distilled water and restained.

Dehydration

After staining the exoskeletons are transferred to 95% ethyl alcohol (about 3 ml), in which they remain for about an hour. At the end of this period 2 drops of carbolxylene (=3 parts of xylene and 1 part melted phenol crystals) are added; after 5 or 10 minutes 2 more are added; then 4 drops twice; 8 drops twice; finally 16 drops. If the carbolxylene is added too rapidly the exoskeleton may collapse or become distorted. If water droplets separate from the alcohol, too much water has been carried over from the stain; the integuments are transferred to fresh 95% alcohol and the addition of carbolxylene is repeated.

Clearing

From the mixture of carbolxylene and alcohol the integuments are transferred to pure carbolxylene and left for 15 to 60 minutes; xylene is added gradually; then the integuments are transferred to pure xylene in a deep well slide (or if large, in a staining dish). Add a drop of thin Canada balsam every 15 minutes, allowing the xylene to evaporate meanwhile.

Mounting

When the balsam in the deep-well slide attains the same consistency as that which is being added, a large drop of the latter is placed on a clean slide and the exoskeleton is transferred to it. At this time medium to large specimens are surrounded--or at least flanked-- by supports of some sort. We use fragments of broken slides which are about the same thickness as the depth of the specimen. If it obvious that the exoskeleton will not remain in the desired position, supports of more fragment of broken slides or fragments of cover slip may be placed under the low part.

Positioning

With needles **dipped in xylene** the integument is now arranged in the proper position for drawing (discussed below). Since the balsam must be soft at this stage, the specimen will rarely remain in precisely the desired position; hence the slide is allowed to rest flat in a covered container (e.g., a petri dish) for a day.

That evening the position is checked again under a stereomicroscope and if the specimen needs moving, needles--again **dipped in xylene**--are used to reposition it. If the balsam is too firm a drop of xylene is put on it and let stand a few minutes before using the needles. If it is apparent that the specimen will not remain in the desired position, more supports may be inserted at this time. It is sometimes necessary to rearrange a specimen on half a dozen successive days. If more balsam is needed to cover a specimen in the desired position it is best to wait until the specimen has dried for several days or the specimen may move again when fresh balsam is added. Once a specimen has remained in the desired position for 48 hours the technique is considered finished.

All specimens are mounted in side view and the length measured. Later some specimens are softened with xylene and the heads removed and

mounted in full-face view, with the remainder of the exoskeleton ventral side up (or, if the head is applied to the ventral surface, the whole exoskeleton is mounted ventral side up). Other kinds of mounts are made whenever necessary.

For a side view of an entire exoskeleton with the left side up and in strict profile, i.e., the middorsal line, the midventral line, the middle of the anus and the middle of the labium are all in the same plane. A good check is to be sure that the left spiracle of each somite is directly above the right spiracle of the same somite. It is conventional to show the entire larva viewed from the left side. Actually it doesn't matter which side is up. If for any reason (e.g., damaged left side) an integument is mounted with the right side up, it is a simple matter to reverse the original drawing.

The exoskeleton of the head is mounted in full-face (i.e., anterior) view. To accomplish this the uppermost border of the occiput is set in the same plane as the lowermost part of the ventral surface of the labium. An imaginary line drawn though the center of the head divides the left and right portions equally.

When the head is applied to the ventral surface of the body (i.e., no neck), it is often possible to get it in the correct position by mounting the entire integument ventral side up. In most genera, however, it is necessary to cut off the head (either alone or with a portion of the prothorax) and mount it separately.

It is often necessary to prop up the ventral part of the head on fragments of cover slip, but only very large heads require retaining walls.

After the head is removed, the remainder of the integument is mounted with the ventral side up by following the procedure for the entire integument (see above).

Instars

When we had young larvae we have usually described them, but we rarely designated the instar. To determine accurately instar, sex and caste usually requires larvae not available to us nor the collector.

For the reliable identification of instar we require: a first-instar larva inside an egg ready to hatch; a second-instar larva inside a first-instar ready to moult; a third-instar larva inside a second instar larva ready to moult; etc.; and finally a mature larva. Maturity is proved by a prepupa, which will reveal all characters of a mature larva except shape. For further confirmation a worker pupa or a worker is desirable. If the worker caste is polymorphic a prepupa of each size is required. Mature sexual larvae can be recognized only from prepupae ready to moult.

In a monomorphic worker caste we might be tempted to extrapolate an instar if the preceding and succeeding stages were well established.

Covering

It is not necessary to cover a mount if it is to be studied at low magnifications. In fact, covering is avoided whenever possible; if the balsam is soft (and it may take years for the interior of a thick covered mount to harden), the weight of the cover slip will set up currents which are likely to move the specimen out of the desired position. For study under an oil immersion objective (X970) the desired part of the integument is detached, flattened and covered.

If the surface of hardened balsam becomes opaque, a drop of xylene should be spread over the surface.

Reversibility

It is reassuring to realize that, **after the integument has been cleaned**, every step is reversible. The same may be said of any series of steps and even of the entire technique from the cleaned exoskeleton to dried balsam. Only one precaution is necessary: hydration (i.e., from 95% alcohol to stain) must be gradual.

Perfectionism

The most important advice we can give to beginners is to avoid perfectionism in making mounts of ant larvae. Not only is perfectionism harmful to the technician (frustration, waste of time), but it can be very damaging to the material. Whenever the conscientious worker has an almost-good-enough preparation, there is always the temptation to try to adjust it a little bit more. Since the balsam must be a little thicker than usual to hold the specimen in the new position, the risk of damage is therefore proportionately increased and the damage may well be irreparable.

It is far better to stop short of perfection in mounting and resort to correction of the position of the slide. Before drawing we make any slight correction that may be necessary by propping up an edge or a corner or an end of the slide.

Storage

Prepared slides of ant larvae are always stored flat in the horizontal position. To store on edge is to risk displacement of the specimen, since the interior of a thick balsam mount requires years to harden.

Never stack slides lacking cover glasses.

Preserved larvae may be stored indefinitely in 75% ethyl alcohol. The only precaution required is to prevent drying by evaporation through a faulty seal. (Dried larvae may be relaxed by methods given above, but the material is never as good as that kept moist.)

Summary of technique

1. Puncture integument
2. 10% KOH until clean
3. Distilled water--15 minutes
4. Transfer to 1% HCl
5. Add 10 drops of acid fuchsin: leave overnight
6. 95% alcohol for an hour
7. Add carbolxylene gradually
8. Carbolxylene 15-60 minutes
9. Xylene
10. Add thin balsam gradually
11. Transfer to balsam on slide
12. Arrange in desired position
13. Examine daily: rearrange as often as necessary until desired position is maintained for 48 hours

Descriptions

We have generally followed this plan in writing our specific descriptions of ant larvae: body shape; leg, wing and gonopod vestiges; spiracles; integument; body hairs; head shape; antenna; head hairs; labrum; mandible; maxilla; maxillary palp; galea; labium; labial palp; opening of sericteries; hypopharynx. To facilitate taking notes we have prepared and used a form with blank spaces under each of the above headings.

When a larva is to be described the prepared slides are studied under a stereomicroscope for low magnifications and a microscope for high magnifications.

Measurements are made by comparison with the squares of an eyepiece reticule (=net micrometer) which has been calibrated with a stage micrometer.

Drawings

All our drawings of ant larvae are made with the aid of a microscope. A reticule (=net micrometer) is placed in the eyepiece. This superimposes a grid of squares on the image of the object. The drawing of the object is made on coördinate paper. The size of squares used is determined by the desired enlargement of the drawing. If coördinate paper is not available or if the squares are not of the desired size, it is not difficult to construct a coördinate grid on plain paper.

When the eyepiece of the grid is properly placed with reference to the image and the coördinate system is established on the paper, we are ready to start drawing.

The dimensions and proportions of the drawing are established by short marks across the lines of the coördinate system in the appropriate places. The main outline is constructed by joining these intersections with freehand lines. Structures inside or outside the main outline are treated in approximately the same way, but more freehand drawing is required because fewer squares are involved and smaller parts are likely to be wholly inside a single square.

The pencil drawing is covered with plastic drawing film. The fine details are drawn with a 00 or 000 Rapidograph pen. The outline is added with a coarser pen. In inked drawings lines should never cross. Those representing nearer objects are solid; for the more distant objects the lines are broken at the crossing.

In the case of bilaterally symmetrical structures (e.g., head in anterior view) only half is drawn, with vertical matching marks above and below the outline. After this half (with its matching marks in blue pencil) has been inked the pencil drawing is drawn over carbon paper with the carbon surface turned toward the back of the drawing. The matching marks are indicated. The pencil drawing is then turned over and the carbon copy is inked.

Most of our drawings are orthographic projections of opaque objects (even though the integuments studied are actually transparent). Only structures of the near side of the plane of the outline are drawn. For example, in a side view of a larva the outline represents the imaginary middorsal and midventral lines; hairs and other parts attached below the plane of these two lines are not shown. To our rule of assumed opacity a few exceptions are made, e. g., dark-colored structures overlaid by transparent tissues (e.g., mandibles).

The drawings should be twice the desired dimensions of the published figures. We have found 80 mm to a be convenient length for a drawing of the entire larva. The plane of projection includes

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the middorsal line (imaginary), the midventral line (imaginary), the middle of the anus and the middle of the bottom of the labium. It is conventional to view the larva from the left. But if for some reason it has been drawn from the right, the original drawing (on coördinate paper) can be reversed by reversing the inked film when mounting.

The head is drawn in full-face (i.e., anterior) view. The plane of projection passes through the dorsalmost border of the head and the ventral border of the ventral surface of the labium. The head drawing must be bilaterally symmetrical. A convenient maximum width is 50 mm.

The left mandible is drawn in anterior view. The plane of projection passes through the apex and the anterior condyle. The lateral and medial condyles must be in the same plane. A convenient length for the drawing for the mandible is 60 mm.

Photographs

Photographs of ant larvae under light microscopes are occasionally useful, but the "state of the art" nowadays is SEM (scanning electron microscope). SEM is apparently the "buzz-word" used as bait in applications for grants. It proves that the applicant is on the research bandwagon. If he gets the grant, then he must spend part of it for expensive SEMs, which may be worthless or even misleading; they may be unnecessary or even irrelevant to the applicant's thesis, but the editor will not know.

SEMs are useful for showing minute details of a small surface area, but for low magnifications of ant larvae we regard them as abominations. An ant larva is white and opaque, but an SEM will turn it into a ghost--black and translucent.

We still maintain that the external anatomy of an ant larva is best portrayed by a good drawing.

Literature cited

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ERRATA

In volume 3, no. 3, an error in the captions of two photos has been noted. In the article, "A Revision of the South American Hairstreak Butterfly Genera *Tergissima* and *Femniterga* (Lycaenidae: Theclinae), by K. Johnson, the caption for figure 2, page 197, refers to figure 5, page 201. The figures were reversed by the printer as the pages were made up.