

# Chapter 2

## Methods of Collecting and Studying Microinsects

### 2.1 Introduction

Microinsects are one of the least thoroughly studied groups of insects and quite objective reasons are largely associated with their microscopic body size, difficulty in collecting the material, technical and methodological difficulties of studying samples, and others.

The current renaissance of insect morphology is due to new methods that have become available to researchers (Friedrich et al. 2014). Though many of the new approaches provide good results in studying the microinsects, such studies often require modification in methods for adjusting them to the specifics of microinsects. In the past few years, morphology of the microinsects is the the best-studied field of knowledge (Polilov 2015), but other aspects of the biology of microinsects remain almost unstudied.

### 2.2 Collecting Methods

Microinsects have extremely diverse modes of life and occur almost ubiquitously. Some miniature insects are free-living at all the stages of their development, e.g., beetles of the families Ptiliidae and Corylophidae, which live in all kinds of substrates such as forest litter, decaying wood, polypore, or feces of various animals in both adult and larval stages, while some are parasites of plants, e.g., many thrips. The smallest insects are called as parasitoids, where their larvae develop inside eggs of other insects. Some microinsects are nidicolous pests of stored products, e.g., psocopterans of the family Liposcelididae. Based on different modes of life found in microinsects, the methods used to collect them also strongly differ depending on the target group of insects.

Small coleopterans are usually collected by sifting the substrate through a soil sieve with a mesh size of 0.7–2 mm, and processing the substrate in eclectors or windowpane trapping (for more details, refer Suter 1966). Miniature hymenopterans are most successfully collected by the following methods: aerial sweeping, yellow traps, pyrethrum spraying, Malaise traps, and hatching from infested hosts (for more details, refer Noyes 1982). Thrips that live on plants and other parasites of plants can be collected by sweeping and shaking off from plants (for more details, refer Stannard 1968). The other thrips and small psocopterans are collected by the methods identical to those used to collect microcoleopterans.

## 2.3 Fixation and Storage of Material

A lot of methods are used to fix biological samples and many of them are applicable to microinsects. The choice of the fixative depends on the intended further usage of the material and expected methods for studying the samples.

For faunistic or taxonomic purposes, microinsects are usually fixed and preserved in 70–80 % ethanol, in which they are immersed immediately once captured. The traditional method of killing and subsequent storage on cotton beds is impractical for such small insects, because they often crumple and can be strongly damaged during manipulations. For the purpose of studying the DNA of the collected samples, the material is stored in 100 % ethanol at  $-20^{\circ}\text{C}$ .

External morphology can also be studied using the fixed material as described above; this method of fixation is good for preserving most structures. Finer work, especially in larvae with soft integuments, is preferably done using histological fixatives described as follows.

Two groups of fixatives are used for studying anatomy (internal morphology), one for histological purposes and the other for immunolabeling. In spite of their small size, the integument of microinsects is poorly permeable or not permeable to fixatives; therefore, it is preferable to perforate such samples prior to fixation and increase the duration of fixation. The most widely used histological fixatives are Brodsky's fixative (FAE: 80 % ethanol: 37 % formaldehyde [PFA]: acetic acid = 2:1:0.3) and the Duboscq-Brazil fixative (Alcoholic Bouin : 95 % ethanol : saturated water solution of picric acid : 37 % formaldehyde : acetic acid = 4:3:2.2:0.8). The duration of fixation is 2–12 h, depending on the size of the specimen and intactness of its integument. After fixation, the material can be stored in 70–80 % ethanol. For immunolabeling, buffered formalin is usually used (4 % PFA, 0.1 M phosphate buffer pH 7.2–7.4), with an admixture of a detergent (usually 0.05–0.3 % Triton X-100) added to accelerate the penetration of the fixative into the specimen and increase the wettability of the integument. The material is fixed for 3 h at room temperature or overnight at  $4^{\circ}\text{C}$ . After fixation, the samples are stored in the same buffer with 0.05 % sodium azide. Another fixative quite suitable for fixation of microinsects for immunolabeling is Dent's

fixative (methanol : dimethylsulfoxide = 4:1); the material is usually fixed overnight at 4 °C. After fixation using Dent's fixative, the material can be preserved for a long time in methanol at -20 °C.

Fixation for studying ultrastructural organization is often complicated by the slow penetration of fixatives through the integument (or even holes in it) and by the nonwettability of the integument in the majority of microinsects. The most widely used method is fixation in buffered glutaraldehyde (2.5 % GA, 0.1 M sodium cacodylate buffer pH 7.2) for 2–10 h at 4 °C and subsequent postfixation in osmium oxide solution (2 % OsO<sub>4</sub>, 0.1 M sodium cacodylate buffer pH 7.2) for 2–10 h at 4 °C. Prior to fixation, the integument must be perforated. This method of fixation is suitable for many but not for all microinsects and purposes; if the result is poor, it is better to use simultaneous fixation in 1 % OsO<sub>4</sub> and 1 % GA in 0.1 M sodium cacodylate buffer, pH 7.2, for 1 h at 4 °C or fixation in a mixture of 2 % GA, 2 % PFA, and 2 % DMSO in the same buffer for 2–10 h at 4 °C and postfixation in 2 % OsO<sub>4</sub> in the same buffer. The material can be stored in the buffer or, after dehydration, in 100 % ethanol, at 4 °C.

## 2.4 Methods of Studying the Structure of Microinsects

### 2.4.1 *Optical Microscopy*

It is most convenient to study external morphology for morphological, diagnostic, or taxonomic purposes using total preparations or preparations of particular parts of bodies under transmission light microscopes. Prior to making the preparations, the samples are cleared with a solution of an alkali, hydrogen peroxide, or lactic acid. The concentration, duration, and temperature of exposure to clearing liquids are adjusted depending on the size of the sample and pigmentation of the integument. After clearing, temporary preparations in glycerol or permanent preparations in a permanent mounting medium are made. Larvae are often studied using phase-contrast or differential interference contrast (DIC).

Because of their extremely small size, microinsects are seldom dissected for studying their anatomy using histological methods, confocal microscopy, and tomography.

Because of small size and relatively strong integument of microinsects, classical histology methods based on paraffin embedding are not used in their anatomical studies. Sections are obtained from specimens (fixed and dehydrated in a series of alcohols of increasing concentrations) by embedding them in special mounting media (usually Araldite or Epon). The blocks obtained by this procedure are cut into sections of about 0.5–2 µm thick using a diamond or glass knife. Sections can be stained, without removing the mounting medium, with toluidine blue and pyronin, azure and eosin, methylene blue, or other universal histological stains.

Confocal microscopy is rarely used for studying the structure of microinsects, because its usage is hindered by the nonpermeability for large molecules and strong autofluorescence of their cuticle. The study of particular organs and body parts is complicated by their minute size. It is also impossible to obtain sections of microinsects by freezing or using soft mounting media, which are usually used in confocal microscopy, because insect integument is too hard. One exception in using the confocal microscopy is the study of skeletal structures and musculature, because of the strong autofluorescence of these structures; fixed material is usually decolorized with hydrogen peroxide, dehydrated, cleared in benzyl alcohol/benzyl benzoate (Murray's clear) or methyl salicylate, and finally autofluorescence is studied at wavelengths of 488–546 nm (for more details, refer Zucker 2006; Smolla et al. 2014).

### 2.4.2 *Electron Microscopy*

Scanning electron microscopy (SEM) is a very convenient and efficient method for studying the external morphology. For SEM studies, fixed samples have to be dehydrated, critical point-dried or hexamethyldisilazane-dried, glued to metal mounts with glue or double-sided tape, and sprayed with metal (for more details, refer Bolte 1996).

The ultrastructural organization of cells and tissues in microinsects is successfully studied using transmission electron microscopy (TEM). On the one hand, small size complicates the preparation and fixation of samples, on the other hand entire samples can fit into ultrathin sections. Samples are usually embedded by the standard method: fixed samples are dehydrated and embedded on a mounting medium (usually Epon), and the blocks obtained by this procedure are cut into ultrathin sections with an ultratome and transferred to special blends; the sections are usually additionally contrasted with uranyl acetate or lead citrate by the standard method.

Methods of 3-D studies of ultrastructural organization of cells and tissues have been actively developed over the past few years. Until recently, the only chance to study the spatial organization at the cellular and subcellular levels was to analyze series of ultrathin sections under a transmission electron microscope, but now a whole range of new methods are available; these include using focused ion beam and scanning electron microscopes (FIB-SEM), serial block-face SEM, nanotomography, and others. The key problem in the study of 3-D ultrastructural organization of biological systems is the preparation of samples, which provides quality for fixation, contrasting, and preservation of the sample up to its deepest layers. Protocols are currently developed that allow studying an entire mouse brain (Mikula and Denk 2015) or fruitfly brain (Hayworth et al. 2015) at the subcellular level, but these protocols either cannot be used for an entire organism or for an entire head detached from the body, because they cannot provide quality in a sample that consists of heterogeneous tissues. Three-dimensional electron

microscopy is also very promising for studying structure and ultrastructure of the microinsects, because in the near future it will allow to obtain comprehensive data in their 3-D organization at the cellular and subcellular levels.

### 2.4.3 Tomography

Tomography ( $\mu$ -ct) is one of the most efficient and widely used modern methods for studying the anatomy of insects, because it allows obtaining comprehensive 3-D data on the external and internal morphology of samples without destroying them (Friedrich et al. 2014). Samples for tomography are critical point-dried or immersed in a liquid (usually ethanol); if required, samples can be additionally contrasted with heavy metals (e.g., osmium) or iodine (Metscher 2009). Though tomography yields good results for microinsects, modern desktop tomographs have insufficient resolution (effective resolution about 1  $\mu$ m per pixel), while synchrotron-based tomographs are very expensive to use and still only approaching the resolution of ordinary light microscopes.

### 2.4.4 3-D Computer Modeling

3-D modeling has long become an integral part of morphological studies; it is also widely used in studies of microinsects. A wide range of 3-D modeling software is currently available including both free packages such as Reconstruct or IMOD, and large commercial packages such as Bitplate Imaris or Visage Amira, which provide everything that is needed for imaging, segmentation, and analysis of large sets of morphological data.

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