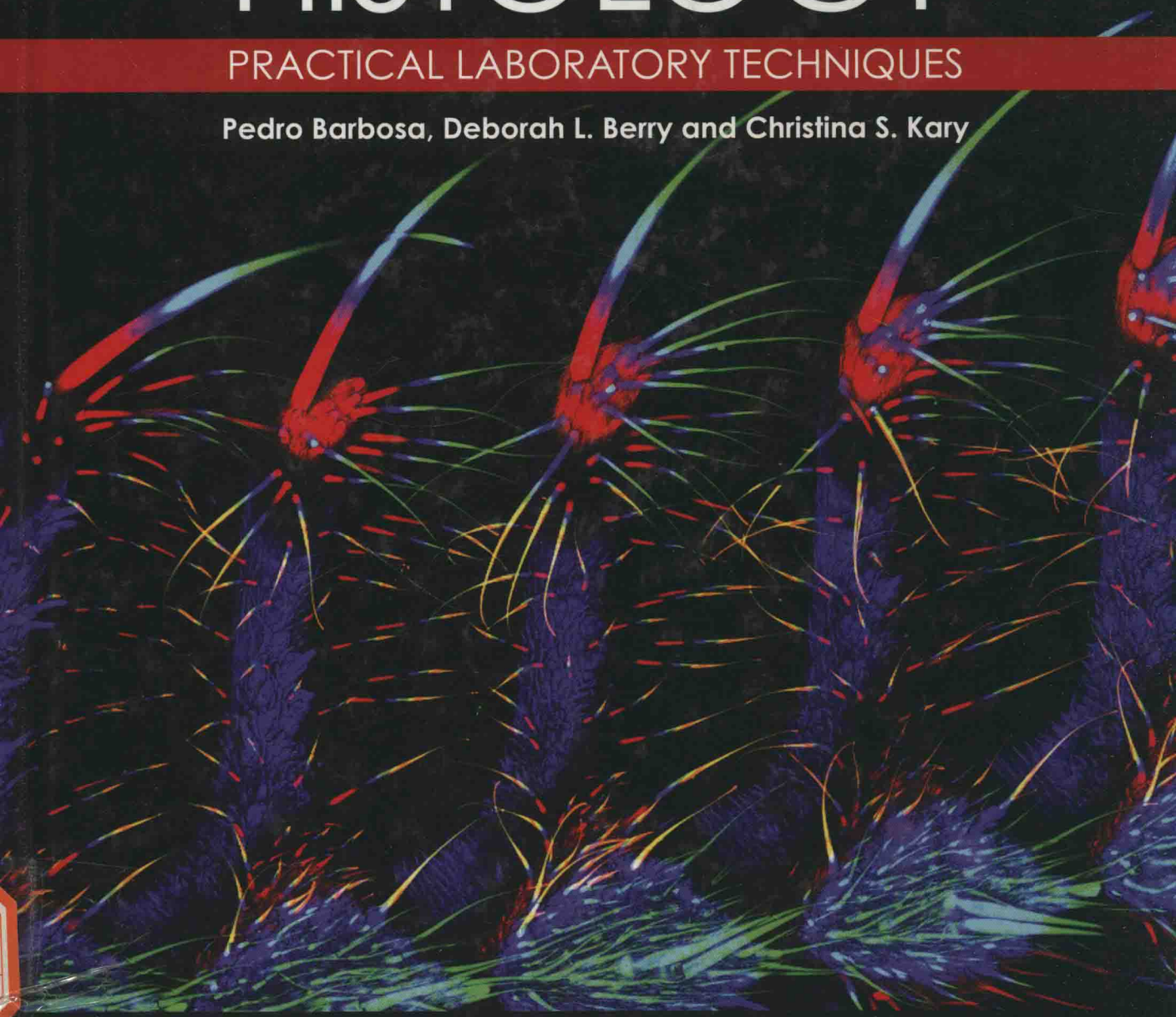
INSECT HISTOLOGY

PRACTICAL LABORATORY TECHNIQUES

Pedro Barbosa, Deborah L. Berry and Christina S. Kary



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Insect Histology

Practical Laboratory Techniques

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Preface

"The standard histological procedures of the zoologist do not as a rule give very successful results when applied to insects ... " (Van Heerden, 1945). If one adds to this statement that histological methods, entomological or otherwise, are a frustrating balance between science and art, one can understand the reluctance of entomologists to utilize histological techniques. The situation is additionally complicated by the fact that the techniques that have been formulated are scattered throughout various scientific journals and in general textbooks.

This book is designed to bring the procedures of insect histology to entomologists and others who utilize insects as experimental animals. Since no technique can be applicable to all insects, the techniques in this book are presented as guidelines. These basic methods can be easily modified to suit the characteristics of a particular insect or specific research problems.

It would be useless to present another book on the theory and use of histological methods, particularly since success in applying procedures is in part contingent upon practice and experience. In addition, there are already numerous sources of generalized information on the theoretical aspects of histological techniques. Instead, in this book the reader will fixatives, stains, procedures, and so on, which have been reported to be specifically applicable to insects. The book also presents information useful in dealing with histological problems encountered in insect tissues such as sclerotized chitin, yolk-laden eggs, chromosomes, genitalia, and so on.

* Van Heerden, H.P. 1945. Some histological methods of interest to entomologists. *Journal of the Entomological Society of South Africa* 8:157–161.

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Introduction

The *Manual of Basic Techniques in Insect Histology* is designed as a resource for those researchers who require basic procedures and information essential for the histological display of insects, in part or in total. Specifically, it can serve as a basic laboratory reference or as an essential supplement to complement lectures in courses which deal with insect histology.

This second edition of the book extends the original histological approaches into modern applications. The manual provides a comprehensive survey of fixation techniques which are crucial to all downstream histological preparations and applications. Preparations and techniques unique to insects are provided for advanced techniques such as immunohistochemistry, in situ hybridization, TEM, SEM and whole mount preparations.

In order to permit efficient use by the reader, the information in this book is presented in a readable and consistent format. Although there are divergences where necessary or where the information is not available, most of the book follows the same format. Finally, throughout the book, the amounts of all ingredients are designated by the term, parts (pt.). In compounds which occur as solid, parts equals grams, while in those compounds occurring as liquids, parts equals milliliters. All procedural information and recommendations for the use of particular methods in this manual are taken from the literature cited. In some instances, not as much information is available as one might desire. However, these materials and procedures were included since the characteristics and limitations of a technique are a function of the insect and experimental conditions.

In histology many chemicals are used that are harsh, corrosive, potential irritants, and some (such as Dioxane or Formaldehyde) may be carcinogenic. Like most chemicals they can be absorbed through the skin or inhaled; in some cases inhaled over a period of time. Thus, one must use common sense in developing lab practices and constant vigilance and care in order to keep chemicals off the skin, or avoid inhalation. And, when in doubt, use the hood. A small amount of planning and thought can avoid a great deal of trouble and regret. Thus, safety glasses or goggles and shield, proper gloves, laboratory coat and apron, adequate ventilation, and a class B extinguisher should be used or available in the lab. Always seek expert advice when in doubt.

About the companion website

This book is accompanied by a companion website:

www.wiley.com/barbosa/insecthistology

This website includes:

- Powerpoints of all figures from the book for downloading
- PDFs of tables from the book

Contents

1 Problems of sclerotized chitin: Softening insect cuticle

1.1 Introduction

The softening and processing of heavily sclerotized specimens for subsequent histological preparations is one of the major problems in insect histology. Many approaches to the solution of this problem have been suggested. Attempts to soften and otherwise alter sections with sclerotized chitin have been incorporated at every procedural level of histological methods. Suggestions have been made for changes in fixation, clearing, mounting, and embedding. Others have also attempted prefixation, postfixation, premounting, presectioning, and so on, as additional steps geared towards improving the quality of sections.

Aside from the more detailed procedures and specific compounds that are recommended in the following pages there are other simple general methods recommended. These techniques represent basic procedures that have been used independently or in conjunction with other methods. One of the most widely used procedures is the treatment of insect specimens with sodium or potassium hydroxide. These chemicals soften sclerotized portions of specimens and dissolve the soft internal tissues. They are generally used either cold or warm at a 10% concentration. These substances are also frequently used in the preparation of insect specimens for taxonomic study.

The use of hypochlorite of soda is another alternate for softening chitin. It is suggested for the preparation of all stages, that is, larvae, pupae, and adults. The insect is usually placed in boiling hypochlorite of soda (about 25% in distilled water). It is usually left in the solution for about 24 hours or more. A third, widely used approach is the use of tenerals or newly moulted specimens. In this way, the specimens are used before the cuticle has hardened.

The elimination of certain chemical agents which tend to harden insect tissues can also be helpful. Occasionally, it is best merely to avoid long exposures to hardening compounds. For example, to avoid excess hardening, short exposures or avoidance of the higher concentrations of ethanol will aid in preventing its hardening effects. The use of n-butyl or t-butyl alcohol as a substitute dehydrating agent may avoid the hardening of tissues. Similarly, prolonged exposure to certain chemicals or fixatives containing chemicals such as acidified dichromate, mercuric chloride or chromic acid is not recommended. Prolonged heating may also cause unwanted brittleness. The choice of clearing agent may also be a key factor in brittleness of tissue preparations. Thus, the use of clearing agents other than xylene or similar compounds will result in

Insect Histology: Practical Laboratory Techniques, First Edition.
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Fig. 1.1 Beetles have a hardened cuticle. (Source: © Michal Grabowski. http://commons.wikimedia.org/wiki/File:Xylena_exsoleta.jpg#filehistory /CC BY-SA 3.0.) See plate section for the color version of the figure.

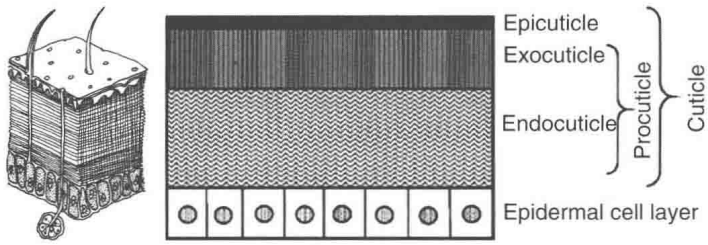

Fig. 1.2 Components of the cuticle. Procuticle – polysaccaride chitin and cross-linked proteins involved in sclerotization.

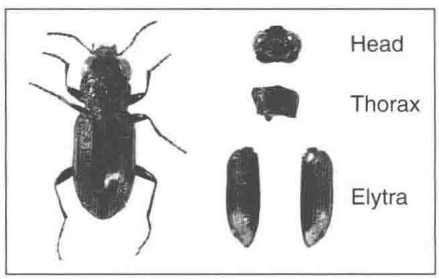

Fig. 1.3 The most sclerotized parts of beetles. (Source: Ellis 2000. Reproduced with permission of Elsevier.)

improved preparations. Finally, excessively high temperatures and prolonged periods of infiltration in wax may be another source of troublesome tissue hardening.

Another widely used procedure involves the puncturing of insect specimens before placing them in a fixative. This allows complete penetration of the fixing agent. Care must always be taken not to damage particular areas of interest on the specimen. The following procedure was suggested as an alternative to the puncturing of specimens.

1.1.1 *Gottlieb's technic [1]*

Application:
Recommended for the histological preparation of insect larvae, pupae, and adults.

Formula:
Solution A: Relaxing fluid
Drosophila Ringer's with magnesium sulphate (4%) added.

Solution B: Chrome alum fixative

Chrome Alum	3 pt
Formaldehyde (40%)	30 pt

Proprionic Acid	2 pt
Distilled Water	238 pt
Dimethyl Sulfoxide	25 pt

Procedure:

1. Rinse specimen in Ringer's solution and place in warmed solution A for 2 to 5 min.
2. Transfer to warmed solution B in a covered dish (on hot plate) for no more than 5 min.
3. Keep specimen in dish and remove from hot plate for 10 to 15 min.
4. Rinse in distilled water and dehydrate.
5. Dehydration must be slow and gentle.
6. Three methods of dehydration are recommended:
   a. Dioxane.
   b. Graded ethanol series with a benzene clearing agent.
   c. Graded tertiary butyl alcohol series.
7. In graded ethanol series a slow transfer to benzene is required before infiltration by using trichloropropane.
8. In the dioxane procedure, the following steps are necessary:
   a. Several changes of 50% dioxane for one day.
   b. Several changes of 100% dioxane for 2 to 3 days.
9. Infiltration is as follows:
   a. Transfer to solutions of increasing paraffin concentrations for 24 to 36 hr.
   b. Transfer to two baths of pure paraffin for 12 hr each.

Note:

1. Graded alcohols series consist of the following: 10–70% (in 10% steps), to 85% (in 5% steps), to 100% (in 2.5% steps).
2. A complete schedule of dehydration solutions and a timetable is available on Table 1 of Gottlieb (1966).
3. Either t-butyl alcohol or dioxane is recommended to avoid excess hardening.

1.2 General Methods

The following are general methods, fixatives, and softening agents recommended for the softening of histological preparations with extensive sclerotized chitin.

1.2.1 *Cox's technic [2]*

Application:

Recommended for small insects and insect parts, e.g., beetle elytra.

Formula:

Solution A:

Potassium Hydroxide (10%)

Solution B:

Acetic Acid (33%)

Procedure:

1. Fix insect specimen.
2. Transfer to solution A for 24 hr.
3. Wash in water for 6 hr.
4. Transfer to solution B for 24 hr.

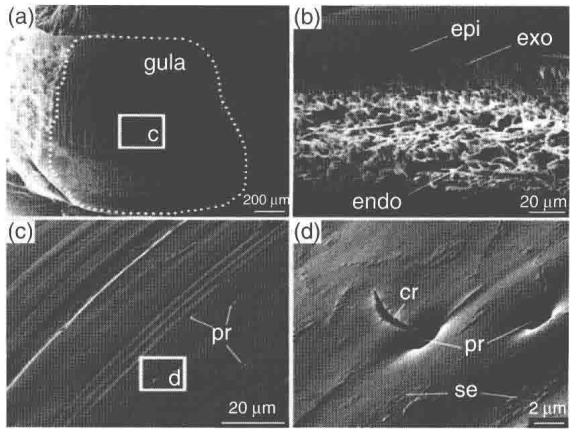

Fig. 1.4 Components of the cuticle – SEM images of the dry gula (a plate which in most insects supports the basal part of the labium). (a,c,d) Surface of the gula. (b) Cross fracture of the gula cuticle showing the epicuticle (epi), exocuticle (exo) and endocuticle (endo). Fibers of the outer part of the exocuticle are oriented perpendicular to the surface but are parallel in the deeper layers of the exocuticle and in the endocuticle. Pores (pr), dried organic substances (se) and cracks (cr) can be seen on the cuticle surface. Rectangles c and d, indicate parts of the sample magnified in c and d, respectively. (Source: Barbakadze *et al.* 2006. Reproduced with permission of the authors.)

5. Wash in water for 6 to 8 hr.
6. Dehydrate, embed, and mount.

1.2.2 *Eltringham's method II [2]*

Formula:

Solution A:

Sodium Hypochlorite (6%)

Solution B: Fixative

Water	250 pt
Picric Acid	2.6 pt
Nitric Acid	10 pt

Procedure:

1. Fix insect specimen.
2. Wash in running water for 4 hr.
3. Transfer to solution A for 60°C for 36 hr.
4. Wash in water for 4 hr.
5. Transfer to solution B at 60°C for 6 days.
6. Boil in alcohol (70%) for 1 min.
7. Let stand in alcohol (70%) for 1 min.
8. Dehydrate, clear in cedar-oil and mount.

1.2.3 *Verdcourt's nitric-ethanol [3]*

Application:

Recommended for the softening elytra of Coleoptera.

Formula:

Solution A:

Ethyl Alcohol	3 pt
Nitric Acid (concentrated)	1 pt

1.2.4 *Schultze's [3]*

Application:
Recommended for the softening elytra of Coleoptera.

Formula:

Nitric Acid (concentrated)	2 pt
Potassium Chlorate	1 pt

Note:
1. When warmed, this substance softens in a few minutes, but is harsh and must be watched.

1.2.5 *Modified Schultze's [3]*

Application:
Recommended for the softening elytra of Coleoptera.

Formula:

Nitric Acid (concentrated)	2 pt
Potassium Chlorate	1 pt

Note:
1. Softening occurs within 5 to 6 days, however, after about 12 days specimens become deformed.
2. Wash well in water after use.

1.2.6 *Verdcourt's technic [3]*

Application:
1. Recommended for the softening and histological preparation of hard chitinous insect tissues.
2. Recommended as particularly useful for softening and preparating slides of the elytra of coleopterans.

Procedure:
1. Place in softening agent (see Verdcourt's Nitric-ethanol, Schultze's or Modified Schultze's).
2. Wash well in water.
3. Dehydrate in alcohol.
4. Place in 1:1 alcohol and ether mixture for 2 days.
5. Transfer through 4, 8, and 10% solutions of celloidin for 3 days in each.
6. Prepare blocks as usual and harden in two changes of chloroform for 2 days.
7. Clear in cedarwood oil for several weeks.
8. Section.
9. Transfer to 1:1 alcohol and chloroform mixture.
10. Transfer to xylene.
11. Mount in balsam.

1.2.7 *Eltringham's method III [2]*

Formula:
Solution A: Sodium Hypochlorite

Solution B: Kleineberg's Fluid

Water	250 pt

Picric Acid	0.75 pt
Sulfuric Acid	5 pt

Procedure:

1. Wash in running water.
2. Transfer to solution A at 60°C for 24 hr.
3. Wash in water.
4. Place in solution B and boil for 1 min.
5. Let stand in solution B at 60°C for 4 days.
6. Place in alcohol (70%) and boil.
7. Dehydrate for several hours.
8. Clear in cedar-oil and mount.

1.2.8 *Eltringham's method IV [2]*

Application:
Recommended for use with live material.

Formula:
Solution A: Sodium Hypochlorite

Solution B: Kleineberg's Fluid

Water	250 pt
Picric Acid	0.75 pt
Sulfuric Acid	5 pt

Procedure:

1. Kill in solution A and boil for 10 min.
2. Let stand in solution A at 60°C for 4 days.
3. Place in alcohol (70%) and boil.
4. Dehydrate for several hours.
5. Clear in cedar-oil and mount.

1.2.9 *Henning's II [4]*

Application:
Recommended for general use as a softener of sclerotized chitin.

Formula:

Water	86 pt
Absolute Alcohol	128 pt
Mercuric Chloride	8 pt
Picric Acid	0.3 pt
Chromic Acid	0.2 pt
Nitric Acid	36 pt

1.2.10 *Kingsbury and Johannsen's fixative [5]*

Application:
Recommended for use in the prevention of excess hardening of insect tissues.

Formula:
Solution A: Perenyi's

Water	165 pt
Alcohol (90%)	75 pt
Chromic Acid	0.5 pt
Nitric Acid	10 pt

Solution B: Working Fixative

Water	185 pt
Mercuric Chloride	7.5 pt
Solution A	65 pt

1.2.11 *Murray's [4, 6]*

Application:
Recommended for softening formol-fixed insect material.

Formula:
Solution A:

Phenol	5 pt
Chloral Hydrate	5 pt

Solution B: Carnoy and Lebrun's

Absolute Alcohol	8 pt
Acetic Acid	8 pt
Chloroform	8 pt
Mercuric Chloride (to saturation)	6 pt

Procedure:
1. Primary fixation occurs in 10% formalin (in 8% sodium chloride).
2. Secondary fixation occurs in solution B.
3. Transfer to solution A for 12 to 24 hr.
4. Clear in chloroform, xylol, or carbon disulphide and mount.

1.2.12 *Sinha's fixative [7]*

Formula:

Picric Acid (saturated in 90% ethanol)	75 pt
Formalin	25 pt
Nitric Acid (concentrated)	8 pt

Procedure:
1. Fix specimens for 4 to 6 days.

1.2.13 *Modified Henning's [5]*

Formula:

Picric Acid (sat. aqueous solution)	6 pt
Sublimate (sat. solution in 60% alcohol)	12 pt
Chromic Acid (1/2% aqueous solution)	8 pt

1.2.14 *Frenzel's fluid [8]*

Formula:

Nitric Acid	1 drop
Mercuric Chloride (half-sat. solution in 80% alcohol)	1–2 pt