ANATOMICAL, ENZYMATIC, AND MICROBIOLOGICAL STUDIES ON THE
DIGESTIVE SYSTEM OF PROSTEPHANUS TRUNCATUS (HORN)

Thesis submitted for the degree of
Doctor of Philosophy
at the University of Leicester

by

Manuel Vazquez-Arista
Department of Zoology
University of Leicester

1997
CHAPTER 1
1. GENERAL INTRODUCTION
   1.1 Prostephanus truncatus (Horn) (Coleoptera: Bostrichidae) 1
   1.2 Damage and Losses. 6
   1.3 Hosts 7
   1.4 Remarks 10
   1.5 General Objective 10
   1.6 Specific Objectives 10

CHAPTER 2
2. ANATOMICAL STUDIES OF THE ADULT AND LARVAL DIGESTION SYSTEM
   2.1 Introduction 11
      2.1.1 Foregut 12
      2.1.2 Midgut 12
      2.1.3 Peritrophic Membrane 13
      2.1.4 Endo-ectoperitrophic Circulation in the Midgut 14
      2.1.5 Hindgut 15
2.1.6 Excretory System 16
2.1.7 Cryptonephric System 17
2.1.8 Aims of the Anatomical Study 18

2.2 Materials and Methods 19
2.2.1 Insects 19
2.2.2 Preparation of Gut 19
2.2.3 Scanning Electron Microscopy (SEM) 19
2.2.4 Transmission Electron Microscopy (TEM) and Light Microscopy (LM) 19

2.3 Results 21
2.3.1 External Anatomy 21
2.3.2 Internal Anatomy 29

2.4 Discussion 48

CHAPTER 3

3. ENZYMATIC STUDIES OF THE ADULT AND LARVAL DIGESTION SYSTEM

3.1 Introduction 52
3.1.1 Glycosidases 55
3.1.2 Endopeptidases 55
3.1.3 Exopeptidases 57

3.2 Materials and Methods 60
3.2.1 Insects 60
3.2.2 Determination of pH 60
3.2.3 Passage Time of the Food Bolus 60
3.2.4 Crude Extracts of Insects 61
3.2.5 Determination of Protein 61
3.2.6 Determination of Amylolytic Activity 62
3.2.7 Determination of Proteolytic Activity 63
3.2.8 Detection of Amylolytic Activity by 64
    Electrophoretic Zymograms
3.2.9 Detection of Proteolytic Activity by 65
    Electrophoretic Zymograms

3.3 Results 66
3.3.1 Determination of pH and Passage 66
    Time of the Food Bolus
3.3.2 Determination of Amylolytic Activity 68
3.3.3 Determination of Proteolytic Activity 70
3.3.4 Detection of Amylolytic Activity by 72
    Electrophoretic Zymograms
3.3.5 Detection of Proteolytic Activity by 72
    Electrophoretic Zymograms

3.4 Discussion 75

CHAPTER 4

4. MICROBIOLOGICAL STUDIES OF THE ADULT AND LARVAL DIGESTION SYSTEM

4.1 Introduction 77
4.2 Materials and Methods 83
    4.2.1 Insects 83
4.2.2 Preparation of Gut Extracts 83
4.2.3 Isolation of Aerobic Cellulolytic Microorganisms 83
4.2.4 Gram Staining 84
4.2.5 Identification of Bacteria 84
4.2.6 Bacterial Growth under Different Conditions 84
4.2.7 Bacterial Growth Rate 85
4.2.8 Susceptibility to Antibiotics 85
3.2.9 Transmission Electron Microscopy 86
3.2.10 Methanogenic Microorganisms 86
4.3 Results 87
4.3.1 Isolation of Aerobic Cellulolytic Microorganisms 87
4.3.2 Gram Staining 87
4.3.3 Identification of Bacteria 93
4.3.4 Bacterial Growth under Different Conditions 101
4.3.5 Bacterial Growth Rate 102
4.3.6 Susceptibility to Antibiotics 103
4.3.7 Transmission Electron Microscopy 104
4.3.8 Methanogenic Microorganisms 109
4.4 Discussion 110

CHAPTER 5

5. GENERAL DISCUSSION 112

APPENDIX 122

REFERENCES 132
To my beloved wife Lupita and my adorable children Lagdi, Quiram, and Dacti
for their enormous support and understanding.
To my mother Francisca and my father Manuel who brought me up.

To my sister Lilia and her daughter Perla, my brother Jose Luis and family for their love and invaluable support.

To my mother-in-law Ma. de la Luz, my aunt Margarita, my uncles Pino and Arturo for their love and care about my family.

To my sister-in-law Clara, her husband Pedro, and their children Juan Manuel and Fransisco for their love and friendship.

To my brother-in-law Rafael, his wife Guadalupe and their children Paola, Rafael, and Ana Belen for their love.
‘For you must not think that in Man only the Art of the great Artificer is so great, ... but what creature soever you would dissect, you shall finde the like art and wisdome to appear in it. And such creatures as you cannot possibly dissect, will make you admire the more, the smaller they are.’

THOMAS MOUFFET, The Theater of Insects (translated by Edward Topsel, 1658).
ACKNOWLEDGEMENTS

I am very grateful indeed to my supervisor Professor R. H. Smith, Head of the Zoology Department, for his guidance and critical review of this manuscript. I am also indebted to my supervisor at the Research Centre and Advanced Studies (CINVESTAV) in Mexico, Dr A. Blanco-Labra for his advice and enthusiastic encouragement throughout this project.

My gratitude to Dr M. H. Walker for her constructive criticism on the anatomical study.

I would also like to thank the ODA/The British Council and CINVESTAV, without their financial support this project would not have been possible.

My thanks to Mrs E. M. Roberts and Mr S. C. Hyman from the E. M. laboratory for their invaluable help on Electron Microscopy techniques; Mr M. Ward for his interest in my work in the Environmental Biology laboratory.

My thanks also to Dr V. Olalde-Portugal from CINVESTAV for his recommendation on the microbiological study; Mrs E. Hinojosa and Mr R. Hernandez-Delgadillo from the National School of Biological Sciences of the National Polytechnic Institute, for their help in identifying the bacteria isolated from *P. truncatus*; Mrs N. A. Martinez-Gallardo from the Biochemistry laboratory at CINVESTAV, for her laboratory advice on the enzymatic study; and others too many to mention.
ABSTRACT

*Prostephanus truncatus* (Horn) is a pest of stored maize that has caused major problems since appearing in Africa, though it is regarded as a minor pest in Mexico and Central America. The ability of *P. truncatus* to feed and breed on a great variety of environments, raises the questions to whether this beetle can break down starch and cellulose. The digestive system of *P. truncatus* may explain its host range. The digestive systems of adults and larvae of three *P. truncatus* strains from different geographic regions (Mexico, Togo, and Tanzania) were the subjects of the present work.

The anatomical study of the digestive system of *P. truncatus* shows that the guts of adults and larvae were externally different and that these differences reflect different life spans and their different feeding habits. The internal anatomy, is similar, with the exception of the presence of peritrophic membrane and glycogen-like granules in the midgut of the adult stage. There is a cryptonephric system present in both adult and larval guts, but in different anatomical structures.

The enzymatic study shows that the adult stage has more amylolytic and proteolytic activity than that of the larval stage. The same isoenzyme bands of amylolytic activity were found in the adult and larval guts, but the slowest band was less dense in the larval instar. There were differences in the isoenzyme pattern of the proteolytic activity in the three strains studied and among both stages, which suggest that the insect strains from the different geographic regions may be of different origin.

The microbiological study shows the presence of cellulolytic bacteria in the digestive system of the strain from Tanzania. A microbial biofilm was detected in the hindgut (ileum) of *P. truncatus*, which may protect the gut bacteria. There are also methanogens which play an important role during the anaerobic hydrolysis of cellulose in
some wood-boring insects. The differences among the bacteria found in the digestive system of the three strains also suggest that they came from different geographic conditions.

The results are consistent with evidence from other work, which suggests that the infestations in East and West Africa may be independent and have different origins.
some wood-boring insects. The differences among the bacteria found in the digestive system of the three strains also suggest that they came from different geographic conditions.

The results are consistent with evidence from other work, which suggests that the infestations in East and West Africa may be independent and have different origins.
1. GENERAL INTRODUCTION

1.1 Prostephanus truncatus (Horn) (Coleoptera: Bostrichidae) 1

1.2 Damage and Losses 6

1.3 Hosts 7

1.4 Remarks 10

1.5 General Objective 10

1.6 Specific Objectives 10
1. GENERAL INTRODUCTION

1.1 Prostephanus truncatus

The Larger Grain Borer (LGB), Prostephanus truncatus (Horn) (Coleoptera: Bostrichidae), also known as the Greater Grain Borer (Figure 1.1), was for many years recognized as a minor pest of field and farm-stored maize restricted to parts of Mexico, Central and South America (Figure 1.2) (Delgado and Luna, 1951; Ramírez, 1960; Giles and Leon, 1975; Wright, 1984; Hoppe, 1986; Dobie, 1988), occasionally found in Brazil (Hodges, 1994), and the southern U. S. A. (Back and Cotton, 1922).

However, the LGB attracted attention when at the end of the 1970s it was introduced to Africa, presumably from Central America (Diané, 1993) or Mexico (Mushi, 1984; Nissen et al., 1991). There were apparently two separate outbreaks reported, from Tanzania, East Africa (Dustan and Magazine, 1981; Golob and Hodges, 1982), and Togo, West Africa (Krall, 1984; Harnisch and Krall, 1984; Krall, 1987). At present, these two countries are the nuclei of the spread of LGB in Africa, causing considerable damage in the farm-level storage of maize and cassava (Figure 1.3).

Horn (1878) named the species from two specimens obtained in California, as Dinoderus truncatus and Lesne (1897) gave it the genus Prostephanus. There are three species of Bostrichidae commonly or frequently infesting stored grains, they are: Rhyzopertha dominica (Fabricius), P. truncatus (Horn), and Dinoderus minutus (Figure 1.4). Other species of Dinoderus and several species of Heterobostrychus, Bostrychoplites, Apte, Sinoxylon, etc., are occasionally found on dried cassava or in bamboo and wood (Anonymous, 1991).

Most species of the family Bostrichidae are wood-borers and their activities can weaken the farmers' stores or domestic dwellings, which are frequently made of timber or bamboo, and provide hiding places for residual pest populations (Anonymous, 1991). Wood-borers often digest cellulose, which is a complex process and in insects is most often mediated by symbiotic cellulolytic microorganisms, although some species of the Anobiidae family, which belongs to the superfamily Bostrichoidea as LGB does
(Crowson, 1981, pp. 695-698), secrete their own cellulases (Martin, 1983). Nevertheless, Martin (1991) concluded that there is no complete evidence of the symbiotic independence among the species of the Anobiidae family that digest cellulose.

Figure 1.1. *Prostephanus truncatus* (Horn)

adult (life size 3.0-4.5 mm) and larva.
Figure 1.2. Distribution of Prostephanus truncatus in Mexico, Central and South America. The shaded area shows countries in which the pest has been recorded.
Figure 1.3. Distribution of *Prostephanus truncatus* in Africa.

The shaded area shows countries in which the pest has been recorded.
Figure 1.4. Dorsal view of common Bostrichidae in stored products. Arrows show the declivity of elytra. A) *Rhizopertha dominica* (life size 2.0-3.0 mm).

B) *Dinoderus* spp. (life size 2.5-3.5 mm).

C) *Prostephanus truncatus* (life size 3.0-4.5 mm)
1.2 Damage and Losses

*P. truncatus* is considered a primary and serious pest of farm-stored maize and dried cassava, although cobs may also be attacked in the field just before harvest by adults boring into the apex and crawling between the sheathing leaves (Hodges and Meik, 1984). LGB adults bore into a wide range of foodstuffs and some other materials such as wood (Shires, 1977).

There are few published records about the occurrence of *P. truncatus* in Central America and Mexico, though it is said to cause sporadic but high levels of maize damage in the rural areas of its native countries. In Central America, the overall damage attributable to LGB may be perceived as less than that caused by other primary pests, especially *Sitophilus* spp. (Coleoptera: Curculionidae), although systematic comparison of damage by the two insects has not been made. However, the potential for damage by *P. truncatus* is much greater than that by *Sitophilus* (Cowley et al., 1980; Cave and Wright de Melo, 1990; Böye, 1990).

In Mexico, the most numerous primary pests in rural maize stores were *S. zeamais* Motschulsky, *Sitotroga cerealella* (Oliver) (Lepidoptera: Gelechiidae) and *P. truncatus*. Nevertheless, LGB was more abundant in stores in the drier, temperate regions than in those more tropical (Tigar, et al., 1994a and 1994b). In the central highlands of Mexico maize losses reached over 14% during 10 months storage (Rios, 1991), whereas in the south of Mexico losses were reported of up to 30%, although according to the author this figure probably represents an over-estimate, due to the farmers' common mistake of confusing damage with losses (Rodriguez-Rivera and Herrera-Rodriguez, 1989).

In Nicaragua, weight losses up to 40% have been recorded from maize cobs stored on the farm for six months (Giles and Leon, 1975). Post-harvest losses of maize in small farms of Costa Rica, caused by both *S. zeamais* and *P. truncatus*, were between 3 and 8% in the course of 6 months, and reached 13% after 8 months (Böye, 1988). From Honduras, locally serious losses of over 30% have been reported, particularly associated with heavy LGB infestation (Hoppe, 1986), However, losses reached 8.5% and 31.6%
after eight months in successive years in an experimental store in Honduras (Novillo, 1991).

Severe damage and losses of stored foods (especially maize and cassava), attributable to LGB, have been well documented after it spread into Africa (Hodges, 1986). When compared with the damage caused by the more usual storage pests (e.g. *Sitophilus oryzae*, *S. zeamais* and *S. cerealella*), under similar circumstances, *P. truncatus* is obviously a very serious pest (Anonymous, 1991).

In Tanzania, maize cobs recently harvested showed 20% of LGB infestation, and after 5-6 months 80% (Hodges, 1984). Maize cobs in storage showed losses of up to 34% after 3-6 months and the weight loss in the region was 9% (Hodges et al., 1983). Also Keil (1988) reported severe losses, averaging 17.9% after six months and 41.2% after eight months.

Extensive damage was also reported in Togo, and in some maize stores there were holes bored by *P. truncatus* in 100% of the cobs after nine months in storage (Krall, 1984). Pantenius (1988) reported an average weight loss of 30.2% in maize stores after six months; prior to the appearance of LGB, the average loss in this region was 7.1%, and after eight months losses averaged 44.8%.

*P. truncatus* also causes extensive damage to dried cassava roots (manioc). In Tanzania losses of up to 70% in fermented and 50% in unfermented cassava were recorded after only four months of storage (Hodges et al., 1985).

### 1.3 Hosts

Initial work on LGB was concentrated on its role as a pest of stored products, especially maize and cassava roots. Because of the presence of *P. truncatus* away from centres of maize production (Herrera-Rodriguez et al., 1987 and 1991; Rees, 1990; Rios-Ibarra, 1991; Tigar et al., 1994a), it is believed that maize is not the only substrate for LGB reproduction.

*P. truncatus* has been recorded as reproducing on soft wheat, chickpeas and artificial grains of compacted cereal flour (Howard, 1983; Hodges, 1986). There is also evidence to suggest that LGB can reproduce on the stems of some woody plants
Nevertheleah, there are negative results in the search for possible host plants (Böye, 1988; Rios-Ibarra, 1991).

As the presence of *P. truncatus* is associated with dry environments (Tigar et al., 1994a, 1994b), and because *Prosopis* species (Leguminosae family) are particularly abundant in these places of Mexico, a search was made around maize fields near the Research Centre and Advanced Studies (CINVESTAV) looking for some evidence of the presence of LGB on the tree *Prosopis levigata*. The results obtained were that the beans of this plant provide a niche for a large number of different insects, but the only one that was found to produce damage by boring into its seeds was *Acanthoscelides obtectus* (Say) (Coleoptera: Bruchidae), which is a pest of stored beans (*Phaseolus vulgaris*) (personal observation).

However, it is possible that some reports result from misidentification of this pest in the field. For example, reproduction of a superficially similar bostrichid *Xylobiops parilis* (Lesne) (Fisher, 1950), in a young weakened stem of *Jacaranda mimosifolia* has been found (personal observation). This beetle has the typical cylindrical shape, but is larger and more active than LGB in its crawling and flight behavior. Its antennae, palpi, anterior coxae, legs (except anterior tibiae), and base of elytra are brownish yellow. It has 10-segmented antenna; first and second segments robust, first long, arcuate, second one-third as long as first; third to seventh segments short, compact, and transverse; last three segments forming a large, loose, compressed club, each with two round sensory depressions on each surface. Its pronotum is slightly wider than long, widest along middle; apical and posterior angles broadly rounded, the former usually with a small tooth near margin. Each elytron of this beetle has three costiform tubercles along anterior margin of apical declivity, the two inner tubercles short and obtuse at apices, the outer one long and usually spinose at apex (Figure 1.5).

Records of *P. truncatus* on unusual hosts need to be confirmed by expert identification.
Figure 1.5. *Xylobiops parilis* L. (Coleoptera: Bostrichidae) feeding on a weakened stem of *Jacaranda mimosifolia*

1.4 Remarks

From the above, electron microscope images showed the following: 

1. *P. truncatus* adult and larval digestive systems.
2. Enzymatic studies of the amylase and protease activities in *P. truncatus* adult and larval digestive systems.
3. Microbiological studies of the presence of cellulase enzymes in *P. truncatus* adult and larval digestive systems.

1.5 General Objective

In the present study it was examined whether the LGB digestive system, how its digestive enzyme system is formed, and finally whether LGB has a weakened stem of *Jacaranda mimosifolia*
1.4 Remarks

From the above facts, interesting questions emerge:

1. Was *P. truncatus* introduced into Africa from Mexico?
2. Do *P. truncatus* populations in Tanzania and Togo have the same origin?
3. Is *P. truncatus* able to digest both starch and cellulose?
4. Has *P. truncatus* the collaboration of some symbionts?
5. Is there a phylogenetic relationship with respect to cellulose digestion between species of the Anobiidae family?

1.5 General Objective

In order to obtain answers to the above questions, different aspects of the digestive system of adults and larvae were studied in insects from cultures which also have different origin: Mexico, Tanzania and Togo. These aspects involve the anatomy and function of the LGB digestive system, how its digestive enzyme system is formed, and finally whether LGB has the collaboration of symbionts.

1.6 Specific Objectives

To fulfil the general objective, three particular approaches were used:

1. Electron microscopy study of *P. truncatus* adult and larval digestive system.
2. Enzymatic studies of the amylase and protease activities in *P. truncatus* adult and larval digestive system.
3. Microbiological studies of the presence of cellulolytic symbionts in *P. truncatus* adult and larval digestive system.
## CONTENTS

2. ANATOMICAL STUDIES OF THE ADULT AND LARVAL DIGESTION SYSTEM

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1 Introduction</td>
<td>11</td>
</tr>
<tr>
<td>2.1.1 Foregut</td>
<td>12</td>
</tr>
<tr>
<td>2.1.2 Midgut</td>
<td>12</td>
</tr>
<tr>
<td>2.1.3 Peritrophic Membrane</td>
<td>13</td>
</tr>
<tr>
<td>2.1.4 Endo-ectoperitrophic Circulation in the Midgut</td>
<td>14</td>
</tr>
<tr>
<td>2.1.5 Hindgut</td>
<td>15</td>
</tr>
<tr>
<td>2.1.6 Excretory System</td>
<td>16</td>
</tr>
<tr>
<td>2.1.7 Cryptonephric System</td>
<td>17</td>
</tr>
<tr>
<td>2.18 Aims of the Anatomical Study</td>
<td>18</td>
</tr>
<tr>
<td>2.2 Materials and Methods</td>
<td>19</td>
</tr>
<tr>
<td>2.2.1 Insects</td>
<td>19</td>
</tr>
<tr>
<td>2.2.2 Preparation of Gut</td>
<td>19</td>
</tr>
<tr>
<td>2.2.3 Scanning Electron Microscopy (SEM)</td>
<td>19</td>
</tr>
<tr>
<td>2.2.4 Transmission Electron Microscopy (TEM) and Light Microscopy (LM)</td>
<td>19</td>
</tr>
<tr>
<td>2.3 Results</td>
<td>21</td>
</tr>
<tr>
<td>2.3.1 External Anatomy</td>
<td>21</td>
</tr>
<tr>
<td>2.3.2 Internal Anatomy</td>
<td>29</td>
</tr>
<tr>
<td>2.4 Discussion</td>
<td>48</td>
</tr>
</tbody>
</table>
2. ANATOMICAL STUDIES OF THE ADULT AND LARVAL DIGESTIVE SYSTEM

2.1 Introduction

The alimentary canal of all insects is formed from three separate elements: the stomodaeum and proctodaeum arise as invaginations of the embryonic ectoderm and form the fore and hindgut; the mesenteron, midgut, is endodermal in origin. These three elements join together to form a continuous tube late in embryonic development, although in a few species the gut remains or becomes occluded at one or two points (Chapman, 1985, pp. 165-211).

The alimentary canal is a tube of epithelium running a straight or convoluted course from the mouth to the anus. In the head it is connected to the body wall by muscles; elsewhere its coils are supported as a rule only by tracheal branches (Wigglesworth, 1977, pp. 476-552).

There are three main regions in the insect gut, with sphincters (valves) controlling food/fluid movement between regions (Figure 2.1.1)

![Diagram of the insect alimentary canal](image)

Fig. 2.1.1. Generalized insect alimentary canal showing division into three regions.

(After Dow, 1986).
The foregut (stomodeum) is concerned with ingestion, storage, grinding and transport of food to the next region, the midgut (mesenteron). Here digestive enzymes are produced and secreted and absorption of the products of digestion occur. The material remaining in the gut lumen together with the urine from the Malpighian tubules then enter the hindgut (proctodeum), where absorption of water, salts and other valuable molecules occurs prior to elimination of the feces through the anus.

2.1.1 Foregut

Each region of the gut displays several local specializations which are variously developed in different insects, depending on diet. Typically the foregut is subdivided into a pharynx, an oesophagus and a crop (food storage area); and in insects that ingest solid food there is often a grinding organ, the proventriculus (or gizzard). At the anterior end of the foregut, the mouth opens into a preoral cavity bounded by the bases of the mouth parts and often divided into an upper area or cibarium and a lower part or salivarium. In insects that store meals in the foregut, the crop may contain the greater portion of the food and is often capable of extreme distension, with a posterior sphincter controlling food retention. The gut epithelium is one cell layer thick throughout the length of the alimentary canal and rests on a basement membrane surrounded by a variably developed muscle layer.

2.1.2 Midgut

Both the foregut and hindgut have a cuticular lining, which is a chito-protein complex, whereas the midgut does not (Cohen, 1987). In generalized insects there are two main areas of the midgut: the tubular ventriculus and blindly ending lateral anterior diverticula
called caeca. Most cells of the midgut are structurally similar, being columnar with microvilli (finger-like protrusions) covering the inner surface. The midgut epithelium of most insects is separated from the food by a thin sheath called the peritrophic membrane. It consists of a network of chitin fibrils in a protein-carbohydrate matrix and is either delaminated from the whole midgut or produced by cells at the anterior of the midgut (Dow, 1986).

2.1.3 Peritrophic Membrane

The peritrophic membrane constitutes a very efficient high-flux sieve, which is perforated by pores allowing passage of small molecules while restricting large molecules, bacteria and food particles from directly accessing the midgut cells. Marcer and Day (1952) described three types of peritrophic membranes in insects: 1) the regular fibrillar network, with approximately hexagonal symmetry; 2) the honeycomb network (a honeycomb-like formation); and 3) the irregular meshwork, which is a formation of distinct superimposed layers of irregularly arranged fibrils. The number of layers that constitute this peritrophic membrane is variable, Brandt et al. (1978) demonstrated that the peritrophic membrane in the midgut of the moth larvae, *Orgyia pseudotsugata*, was formed by two layers. Becker et al. (1976) showed that the peritrophic membrane in the midgut of the blowfly, *Calliphora erythrocephala*, was formed by three layers. Lehane (1976) studying the stablefly, *Stomoxys calcitrans*, proved that the peritrophic membrane of this insect is a complex five layered structure. Chemically, peritrophic membranes have been shown to resemble the inner layers of the cuticle, in possessing both chitin and protein (Smith, 1968). This membrane is found in insects whose diets contain hard particles, as well as in many insects that have diets lacking rough particulate matter, though it is absent in insects that suck plant juices (Richards and Richards, 1977). In
some insects, all or most midgut digestion occurs inside the peritrophic membrane in the endoperitrophic space. In others only initial digestion occurs there and smaller food molecules then diffuse out into the ectoperitrophic space, where further digestion takes place (Terra, 1988). A final phase of digestion usually occurs on the surface of the midgut microvilli where certain enzymes are either trapped in a mucopolysaccharide coating or bound to the cell membrane. The major roles usually ascribed to the peritrophic membrane are: a) mechanical protection for midgut cells, which was once believed to be its principal function (Wigglesworth, 1977, pp. 476-552); b) a physical barrier for microorganisms (Richards and Richards, 1977); c) a device to help in the prevention of digestive enzyme excretion (Terra and Ferreira, 1981; Terra, et al., 1979); and d) a permeability barrier for digestive enzymes and products of digestion, leading to compartmentalization of digestion (Terra, 1990).

2.1.4 Endo-ectoperitrophic Circulation in the Midgut

Fluid containing partially digested food molecules and digestive enzymes is thought to circulate through the midgut in a posterior direction in the endoperitrophic space and forwards in the ectoperitrophic space, whereas the endogenous fluid such as water and soluble materials circulates forwards in the ectoperitrophic space, as indicated in Figure 2.1.2. This endo-ectoperitrophic circulation may facilitate digestion by moving food molecules to sites of final digestion and absorption or by conserving digestive enzymes that are removed from the food bolus before it passes to the hindgut (Gullan and Cranston, 1994).
2.1.5 Hindgut

The hindgut is often a simple tube, conveying waste materials from the midgut and Malpighian tubules to the anus, but in some species portions of this segment of the digestive tract play an important part in osmoregulation. This tube is lined, like the foregut, with a delicate cylinder of cuticle, and is often dilated posteriorly to form a rectum or rectal pouch. Typically, the beginning of the hindgut is defined by the entry point of the Malpighian tubules, often into a distinct pylorus forming a muscular pyloric sphincter, followed by the ileum, colon and rectum. The main functions of the hindgut are the absorption of water, salts and other useful substances from the feces and urine (Smith, 1968).
2.1.6 Excretory System

Excretion or the removal from the body of waste products of metabolism, especially nitrogenous compounds, is essential. It differs from defecation in that excretory wastes have been metabolized in cells of the body rather than simply passing directly from the mouth to the anus (sometimes essentially unchanged chemically). Production of insect urine or frass is the result of two intimately related processes: excretion and osmoregulation. The system responsible for excretion and osmoregulation is referred to loosely as the excretory system and its activities are performed largely by the Malpighian tubules and hindgut as outlined in Figure 2.1.3.

Fig. 2.1.3. Schematic diagram of a generalized excretory system (After Daly et al., 1978).
2.1.7 Cryptonephric System

Many larval and adult Coleoptera have a modified arrangement of the excretory system which is concerned either with efficient dehydration of feces before their elimination (in beetles) or ionic regulation (in plant-feeding caterpillars) (Gullan and Cranston, 1994). These insects have a cryptonephric system in which the distal ends of the Malpighian tubules are held in contact with the rectal wall by the perinephric membrane. Such an arrangement allows some beetles that live on a very dry diet, such as stored grain, to be extraordinarily efficient in their conservation of water. Water may even be extracted from the humid air in the rectum. In the cryptonephric system of the mealworm *Tenebrio molitor* (Coleoptera: Tenebrionidae), ions (principally potassium chloride, KCl) are transported into and concentrated in the six Malpighian tubules creating an osmotic gradient that draws water from the surrounding perirectal space and the rectal lumen (Gullan and Cranston, 1994), as shown in Figure 2.1.4.

![Diagram of Cryptonephric System](image)

Fig. 2.1.4. Cryptonephric system (After Bradley, 1965; Grimstone et al., 1968).
2.1.8. Aims of the Anatomical Study

The aims of this chapter are:

1. Compare the external morphology of the alimentary tract in the adult and larval stages of *P. truncatus* using scanning microscopy in order to find differences.

2. Compare the internal morphology of the differences using light, scanning and transmission microscopy.

3. Identify potential targets for the control of *P. truncatus*.
2.2 Materials and Methods

2.2.1 Insects

*P. truncatus* adults from a strain originally collected in Tanzanian were reared at 25 °C and 70 % relative humidity. *P. truncatus* larvae were developed using 20 adult insects on a mixture of 20 g of American yellow dent maize (grade No. 3) and 10 g of fine maize flour at the same temperature and humidity (Howard, 1983).

2.2.2 Preparation of Gut

Final instar larva and adult guts of *P. truncatus* from Tanzania were dissected in a cold drop of Ringer solution, pH 7.2. Any surrounding fat was removed.

2.2.3 Scanning electron microscopy (SEM)

The dissected guts were fixed overnight with 1 ml of 2.5 % glutaraldehyde/0.1M Sörensen phosphate buffer (V/V), pH 7.0, washed three times with 0.1 M Sörensen buffer, and post-fixed with 1 % OsO₄ in Sörensen buffer, pH 7.0, for 1 h, and then washed three times with double distilled water. Guts were dehydrated in increasing concentrations of acetone (30, 50, 70, 90 % for 30 min each and twice with 100 % for 45 min). Guts were then dried in a Balzers critical-point drier. Some midguts were carefully opened prior to Sputter coating with gold-palladium. Specimens were viewed with a Cambridge S100 scanning electron microscope operated at 25 kV.

2.2.4 Transmission Electron Microscopy (TEM) and Light microscopy (LM)

Dissected guts were divided into fore, mid, and hindgut. Each portion was fixed overnight with 1 ml of 2.5% glutaraldehyde/0.1M Sörensen buffer (vol:vol), pH 7.0. Each portion
was washed 3 times with 0.1M Sörensen buffer and post-fixed with 1% OsO₄ in Sörensen buffer for 1 h and then washed 3 times with double distilled water. Each portion was dehydrated in increasing concentrations of ethanol (30, 50, 70, 90, and 100%) for 30 min each and then washed twice using 1-2 epoxipropane. Portions of gut were embedded with Spurr low viscosity embedding medium and polymerised in an oven at 60°C for 16 h. The tops of blocks containing each portion of gut were cut and orientated to facilitate sectioning and identification, and then glued with Araldite onto the same blocks. Sections were cut using a Reichert-Ultracut ultramicrotome and fresh glass knives, which were made with the 7800 Knife-maker (LKB). 50-60 nm sections were collected on copper grids and stained with uranyl acetate and lead citrate (Griffin, 1990). A Siemens 102 transmission electron microscope was used at 80 kV. For LM observations, 0.5 μm sections were collected on clean slides and stained with 1% toluidine blue in 1% borax. These specimens were examined and photographed using an Olympus, BHS.
2.3 Results

2.3.1 External anatomy

*P. truncatus* adult and larval guts are made up of the three basic regions present in any insect alimentary canal, that is, fore, mid, and hindgut. Nevertheless, both adult and larval guts lack a crop in the foregut and gastric caeca at the anterior end of the midgut. The proximal ends of the six Malpighian tubules extended out into the body cavity from the posterior end of the midgut. The distal ends of these tubules in the *P. truncatus* adult are in contact with the rectum constituting its excretory or cryptonephric system, whereas in *P. truncatus* larvae they are in contact with a sac-like structure which is part of the hindgut.

SEM examination shows that there are notable differences in the external appearance of the mid and hindgut of *P. truncatus* adult and larva (Figures 2.5 and 2.6). In the adult the foregut is the shortest part of the gut (Figure 2.5). It has a simple tube-like structure and lacks a crop. The outer surface of the midgut is covered with regularly spaced rounded structures (Figure 2.5). The structures are the regenerative crypts (Borror et al., 1989; Crowson, 1981, pp. 695-698; Wigglesworth, 1977, pp. 476-552) and their function is to provide a continuous supply of new cells for the epithelial lining of the midgut. The Malpighian tubules are located at the posterior end of the midgut (Figure 2.5). The hindgut is the longest part of the gut and is generally convoluted (Figure 2.5). Its ileum and colon are tubular, although more complex than the foregut. The rectum is constituted by the rectal tube and the rectal sac which is seen in Figure 2.5. The external surface of the larval gut is less elaborated than the adult gut (Figure 2.6). The midgut appears to lack the regenerative crypts. The hindgut is also convoluted but at the end of the ileum and colon there is a sac-like structure (Figure 2.6).
Figure 2.5. SEM view of a *P. truncatus* adult gut. Note foregut (F), regenerative crypts (RC) of the midgut, Malpighian tubules (MT), ileum (I), and rectum (R).

Scale bar = 1 mm.
Figure 2.6. SEM view of a *P. truncatus* larva gut. There are no regenerative crypts in the midgut (M). Note Malpighian tubules (MT), ileum (I), colon (C), and sac-like (S) structure in the hindgut. Scale bar = 1 mm.
At higher magnification the regular arrangement of the regenerative crypts on the surface of the midgut is clearly seen (Figure 2.7). Associated with the regenerative crypts is a tracheal system, which runs as a series of parallel strings along its length (Figure 2.7). In the larva the tracheal system is seen overlaying the surface of the midgut (Figure 2.8).

Figure 2.7. SEM higher magnification view of a *P. truncatus* adult midgut.

Note regenerative crypts (RC), trachea (T). Scale bar = 100 μm.
Figure 2.8. SEM higher magnification view of a *P. truncatus* larval midgut.

There are no regenerative crypts. Note trachea (T). Scale bar = 50 μm.
Looking at the adult hindgut at higher magnification, its main sections (ileum, colon, rectal tube and rectal sac) are clearly observed (Figures 2.9). The surface of the tubular hindgut is covered by a series of ridges, while the surface of the rectal sac is smooth (Figure 2.9). Similar ridges are also observed in the ileum and colon. Running along the side of the rectum is a series of additional structures which may serve to attach the rectal tube and rectal sac (Figure 2.9). These structures have bulbous bases adjacent to the rectum and tapering ends that overlie the rectal sac. In the larva there is no rectal sac comparable to that of the adult gut, but Malpighian tubules are seen connecting at the apex of the sac-like structure of the hindgut (Figure 2.10). The same type of ridges seen on the surface of the adult hindgut is also observed in the larva (Figure 2.10).
Figure 2.9. SEM higher magnification view of a *P. truncatus* adult hindgut.

Note midgut (M), Malpighian tubules (MT), ileum (I), colon (C), rectal tube (RT), rectal sac (RS), additional structures linking rectal tube and rectal sac (arrowed).

Scale bar = 200 μm.
Figure 2.10. SEM higher magnification view of a *P. truncatus* larval hindgut.

Note ileum (I), rectal tube (RT), sac-like (S) structure. Scale bar = 100 μm.
2.3.2 Internal anatomy

As there were externally visible differences in the mid and hindgut of both *P. truncatus* stages, the study of the internal anatomy was concentrated on these differences.

LM views of the transverse sections of the adult midgut indicate that the epithelial lining is greatly folded giving its lumen a convoluted profile (Figure 2.11). These columnar epithelial cells have their nuclei located basally, and apically they have a microvillous border (Figure 2.11 and 2.12). Within the cytoplasm of these cells are numerous densely staining granules (Figure 2.11 and 2.12). Surrounding the midgut are the regenerative crypts, each of which contains cells that are in the process of mitosis (Figure 2.12). Some cells of the regenerative crypts are seen in direct contact with the epithelium lining the lumen of the midgut (Figure 2.11). The outer wall seems to consist of a thin outer epithelium, a thin muscle layer beneath which is a convoluted line that completely encircles the adult midgut (Figure 2.13).

Using TEM on a transverse section of the adult midgut, it is possible to distinguish the convoluted line which lies between the thin muscle layers and the basement membrane of the epithelial cells surrounding the whole midgut (Figure 2.14). Looking at higher magnification this convoluted line in the adult midgut seems to be composed of glycogen-like granules (Figure 2.15). This line of glycogen-like granules extends out from the midgut and completely surrounds the regenerative crypts (Figure 2.16). In the thin outer epithelium of the midgut tracheae are present (Figure 2.17).
Figure 2.11. LM view of a transverse section of *P. truncatus* adult midgut.

Note regenerative crypt (RC), columnar cell (CC), lumen (L), microvilli (MV), mitotic cell (arrowed). Scale bar = 100 μm.

Figure 2.12. LM higher magnification view of a transverse section of *P. truncatus* adult midgut. Note regenerative crypt (RC), convoluted line (asterisk), columnar cell (CC), microvilli (MV), lumen (L), dense granule (DG), mitotic cell (arrowed). Scale bar = 50 μm.
Figure 2.13. LM higher magnification view of a transverse section of *P. truncatus* adult midgut. Note regenerative crypt (RC), convoluted line (asterisk), columnar cell (CC), dense granules (DG), mitotic cell (arrowed). Scale bar = 50 µm.

Figure 2.14. TEM view of a transverse section of *P. truncatus* adult midgut.

Note outer basal lamina (OBL), epithelial layer (EL), muscle (MU), convoluted line (asterisk). Scale bar = 2 µm.
Figure 2.15. TEM higher magnification view of a transverse section of *P. truncatus* adult midgut. Note glycogen-like granules (GG), Muscle (MU).

Scale bar = 0.5 μm

Figure 2.16. TEM view of a transverse section of part of a regenerative crypt of *P. truncatus*. Note layer of glycogen-like granules (GG), mitotic cell (arrowed). Scale bar = 5 μm.
Figure 2.17. TEM view of a transverse section of part of a regenerative crypt of *P. truncatus* muscle (MU) and the edge of the midgut wall. Note convoluted layer of glycogen-like granules (GG), tracheole (T). Scale bar = 5 μm.
LM views of transverse sections of the larval midgut indicate a much simpler organization than in the adult (Figure 2.18). The lumen is approximately tubular and the epithelial cells lack the dense staining granules seen in the adult epithelial cells. There are no regenerative crypts extending out from the surface, but developing crypts are observed as clusters of cells located around the periphery, the cytoplasm of which is less densely stained, (Figure 2.18). At higher magnification, the larval midgut transverse section shows a fine outer basal lamina and a few cells clustered around the periphery (Figure 2.19). The epithelial cells are in formation presenting a non-uniform profile. The convoluted line of glycogen-like granules observed in the adult midgut is absent in the larval stage. (Figure 2.19).

Using TEM on a transverse section of the larval midgut the absence of the glycogen-like granules was confirmed (Figure 2.20). The larval midgut wall is composed of a thin outer basal lamina, a thin epithelial layer containing tracheae, a thin muscle layer, and a thin inner basal lamina. (Figure 2.20).
Figure 2.18. LM view of a transverse section of *P. truncatus* larva midgut.

Note clustered cells forming a crypt (arrowed), columnar cell (CC), lumen (L). Scale bar = 100 μm.

Figure 2.19. LM higher magnification view of a transverse section of *P. truncatus* larval midgut. Note clustered cells forming a crypt (arrowed), columnar cell (CC), microvilli (M), lumen (L). Scale bar = 30 μm.
Another important difference found between the adult and larva was the presence of peritrophic membrane in the adult midgut. Using SEM the peritrophic membrane is seen as a mesh network overlaying the tips of the microvilli of the columnar epithelial cells (Figures 2.21). This mesh network appears to be composed of several layers (Figure 2.22). This peritrophic membrane is also seen using TEM on a transverse section of the adult midgut (Figure 2.23). In contrast, there is no such mesh network overlaying the microvilli in the larval midgut (Figure 2.24).
Figure 2.21. SEM view of a cut face of *P. truncatus* adult midgut, showing the peritrophic membrane (PM) mesh overlaying the tips of the microvilli (MV), columnar cells (CC). Scale bar = 5 μm.
Figure 2.22. SEM higher magnification view of a cut face of *P. truncatus* adult midgut, showing the multiple fibrous layers of the peritrophic membrane (PM). Scale bar = 5 µm.
Figure 2.23. TEM view of a transverse section of *P. truncatus* adult midgut.

Note microvilli (MV), peritrophic membrane (arrowed).

Scale bar = 1 μm.
Figure 2.24. SEM view of a cut face of *P. truncatus* larval midgut.

There is no peritrophic membrane present, microvilli (MV). Scale bar = 20 μm.
During the TEM study of the internal anatomy along the digestive system of *P. truncatus* adult and larva, the presence of bacterial fauna in the hindgut (ileum) was detected and these results are presented and discussed in the Microbiological study (Chapter 4).

The rectum in the adult gut is constituted of the rectal tube and the rectal sac (Figure 2.9) and in the larval gut of the rectal tube and its sac-like structure (Figure 2.10). Transverse sections of the adult rectum show that the rectal tube has a large luminal space that is lined by a thick cuticle (Figure 2.25), which varies from 5.0 to 10.0 μm in thickness (Figure 2.26). Beneath the cuticle is a thin epithelial layer (Figure 2.26). The outer wall surrounding the rectal tube is thick and is composed of distinct muscle blocks (Figures 2.25 and 2.26). The rectal sac contains the distal ends of the Malpighian tubules. As there are six Malpighian tubules in the adult and there are more than six profiles seen in transverse sections (Figure 2.25), this indicates that the Malpighian tubules must be folded within the rectal sac. Each Malpighian tubule is surrounded by a basal lamina and is lined by an epithelial layer with numerous microvilli extending into the lumen of the tubule (Figure 2.27). The basal surfaces of these epithelial cells consist of numerous plicae containing mitochondria. In glancing sections numerous profiles of mitochondria surrounded by membrane are seen (Figure 2.28). Tracheae are seen between the Malpighian tubules (Figure 2.27). The outer wall of the rectal sac or perinephric membrane has a fibrous appearance (Figure 2.27), which by TEM is seen to be composed of an outer basal lamina beneath which lie stacks of membranes enclosing thin cell processes that run around the periphery of the rectal sac (Figure 2.29). Joining the base of the rectal tube and the rectal sac are the additional structures seen in SEM (Figure 2.25). Near the base of these structures to one side of the rectal sac is a small opening, the
leptomphragma, which connects the perinephric system with the haemolymph (Figure 2.25) (Borror et al., 1989; Crowson, 1981, pp. 76-118; Wigglesworth, 1977, pp. 553-592).

Figure 2.25. LM transverse view of the rectum of a *P. truncatus* adult.

Note rectal tube (RT), rectal sac (RS), perinephric membrane (PNM), Malpighian tubule (MT), leptomphragma (arrowed), epithelium of the rectal sac (ERS), lumen (L), cuticle (CU), muscle (MU), additional structures (asterisk). Scale bar = 100 μm.
Figure 2.26. TEM view of transverse section of *P. truncatus* adult rectal tube. Note cuticle (CU), epithelial layer (EL), muscle (MU). Scale bar = 5 μm.

Figure 2.27. LM higher magnification view of the rectal sac of a *P. truncatus* adult hindgut. Note Malpighian tubule (MT), perinephric membrane (PNM), lumen (L), tracheole (T). Scale bar = 50 μm.
Figure 2.28. TEM view of a glancing section of the basal plicae of the epithelial cells of a *P. truncatus* Malpighian tubule in the rectal sac.

Note mitochondria (MI), membrane (arrowed). Scale bar = 1 μm.

Figure 2.29. TEM view of transverse section of a *P. truncatus* adult rectal sac.

Note outer basal lamina (OBL), epithelial layer (EL), stacks of membranes (SM), inner basal lamina (IBL), microvilli (MV). Scale bar = 1 μm.
In sections of the larval hindgut the ilium/colon is seen to have a highly folded cuticular lining and a very muscular wall. The lumen is very constricted. In comparison the rectal tube again has a thick cuticular lining and muscular wall, but its lumen is not so restricted (Figure 2.30). Within the sac-like structure many profiles of Malpighian tubules are seen around the periphery, again indicating that the tubules are folded (Figure 2.30). The microvilli of the epithelial lining of these Malpighian tubules appear much shorter than those seen in the adult (Figure 2.31). Also their lumens are more open than in the adult (Figures 2.30 and 2.31). The wall of the sac or perinephric membrane is not fibrous, but is composed of a single epithelial layer and an outer basal lamina (Figure 2.31). Using TEM this perinephric membrane is composed of a thin outer basal lamina with a small number of microvilli on the outer surface, a single epithelial layer, and a thin inner basal lamina (figure 2.32). Within this epithelium, tracheae are present (Figure 2.31). Within the centre of the sac is an additional structure that is covered by a basal lamina, within which are two layers of epithelial cells surrounding a central lumen with a cuticular lining (Figures 2.30 and 2.31).
Figure 2.30. LM transverse view of the sac-like structure of a *P. truncatus* larval hindgut. Note perinephric membrane (PNM), Malpighian tubule (MT), epithelium (E), lumen (L), cuticle (CU), rectal tube (RT), muscle (MU), ileum/colon (arrowed). Scale bar = 100 μm.

Figure 2.31. LM higher magnification view of the sac-like of a *P. truncatus* larva hindgut. Note perinephric membrane (PNM), Malpighian tubule (MT), epithelial cells (EC), lumen (L), tracheole (T), cuticle (CU). Scale bar = 50 μm.
Figure 2.32. TEM view of transverse section of a *P. truncatus* larval perinephric membrane. Note outer basal lamina (OBL), epithelial layer (EL), inner basal lamina (IBL), Malpighian tubule (MT). Scale bar = 5 μm.
DISCUSSION

The alimentary canal is the most universal conspicuous internal organ of beetles and considerably longer than the body, with the result that the hindgut and often the midgut, are thrown into loops. The gut structure varies greatly with the different foods and feeding habits of beetles. Crowson (1981, pp. 76-118) stated that, in beetles where both adults and larvae are feeding on solid food there are no major anatomical differences in the alimentary tract; if there are differences, these may be relatively slight.

Externally, in _P. truncatus_ there are some differences between the adult and larval alimentary tract. There are regenerative crypts covering the adult midgut of _P. truncatus_, these crypts are suppliers of new cells to the epithelial lining of the midgut, but they are absent in the larval stage. This difference may be explained by the different life spans of the two stages. As the adult lives for about a year at optimum conditions of temperature and relative humidity, it needs these structures to maintain an active digestive system. In contrast the larval stage lives only 16-17 days at 32°C and 70% r. h. on ground maize (Bell and Watters, 1982).

Internally, the adult midgut of _P. truncatus_ has a peritrophic membrane which is absent in the larval stage. This fact could be again related to their life spans and feeding habits. The larva of _P. truncatus_ live inside the whole maize grain and for only short periods, whereas the adult has to cope with different types of food materials over a long time and the midgut cells may have to be protected from damage by the peritrophic membrane. The peritrophic membrane covering the microvilli lining in the midgut of _P. truncatus_ adult seems to be formed by several layers of superimposed fibers, similar to the irregular meshwork mentioned by Marcer and Day (1952).
Another internal difference between both stages is the presence of glycogen-like granules in the adult midgut of *P. truncatus*. These granules may be necessary for the adult stage because, in living a long time, adults may need an energy source in periods of starvation, or these granules may be used as a fuel during flying. Large deposits of glycogen in flight muscle as well as the depletion of these reserves during flight indicate that in many insects, especially Diptera, glycogen provides a major vehicle for storage of flight energy, which can be rapidly mobilized to meet the metabolic requirements of the muscle (Sacktor, 1975).

The cryptonephridial system has been a subject of study for a very long time (Dufour, 1834 and 1943; Mobüsz, 1897), but these authors have mainly considered the arrangement of the tubules coincidentally with studies of the gut. However, Marcus (1930), Poll (1932, 1935), Conet (1934), Lison (1937a, b), and Patton and Craig (1939) have dealt exclusively with the cryptonephric tubules in Coleoptera and have tried to describe in detail the structure and nature of these tubules.

In *Rhyzopertha dominica* (Bostrichidae), like *P. truncatus*, the Malpighian tubules extend from the base of the midgut and their distal ends are located in a rectal sac adjacent to the rectal tube. Inside the rectal sac are the twisted loops of the reassociated Malpighian tubules, and the whole structure is supported by the fine sclerotised bars at the sides, as in *Dermestes* spp. (Saini, 1964). *P. truncatus* has the same cryptonephric system and it was demonstrated that this system in the adult stage is located in the rectal sac, while in the larval stage it is located in the sac-like structure. The multiple layers of the cell processes that form the perinephric membrane in the *P. truncatus* adult may be derived from the outer epithelial layer seen surrounding the sac-like structure in the larval stage. The function and/or fate of the cuticle-lined central part of this larval sac-like structure is unknown.
The role of the leptophragma located in the rectal sac of the adult hindgut is obscure, although it is possible that, as in *Tenebrio*, leptophragma allows entry of potassium chloride, unaccompanied by water, to the Malpighian tubules (Borror et al., 1989; Crowson, 1981, pp. 76-118; Wigglesworth, 1977, pp. 553-592).

The peritrophic membrane is absent in the midgut of the final larval instar of *P. truncatus*, which suggests that the peritrophic membrane is formed at the end of the final larval period and during the pupal period, as happens with *Plodia interpunctella* (Ferkovich et al., 1981). This finding suggests that inhibition of the chitin synthetase in *P. truncatus* would prevent biosynthesis of its peritrophic membrane and could be a control method for this beetle. Chitin synthetase has been inhibited in *Tribolium castaneum* using either peptidyl nucleosides as polyoxin D and uridine 5'-diphosphate (Cohen and Casida, 1980), or benzimidazoles (Cohen et al., 1984). Chitin biosynthesis has also been inhibited in *Plodia interpunctella* using benzoylphenyl ureas such as diflubenzuron (Ferkovich et al., 1981). In *T. castaneum* and *P. interpunctella*, larvae feed and develop completely on grain fragments and flour left by the primary pests. *P. truncatus* is a primary pest and its larval and pupal stages spend their whole time inside maize. For this reason *P. truncatus* cannot easily be controlled successfully with the chemicals cited above because the larvae are protected from application of chemicals. Inhibition of chitin synthetase could be carried out by plant proteins such as hevamine (Terwisscha et al., 1978) or microbial proteins from, for example, *Mucor rouxii* (López-Romero et al., 1978) or *Candida albicans* (Braun and Calderone, 1979). One possibility is to produce transgenic plants by genetic engineering in order to provide them with specific insect-resistance. These transgenic maize plants could be more or less resistant to several other internal seed feeders such as *Sitophilus* spp. *P. truncatus*, however, might be one of the most vulnerable pest species because the adults consume substantially larger quantities of maize.
than other species when tunnelling (Cowley et al., 1980) and would be likely to suffer more abrasion if the gut of the peritrophic membrane were incomplete or absent. Transgenic maize seeds could be made available to farmers and would carry none of the risks associated with conventional chemical control of pests of stored grain.
CONTENTS

3. ENZYMATIC STUDIES OF THE ADULT AND LARVAL DIGESTION SYSTEM

3.1 Introduction 52

3.1.1 Glycosidases 55

3.1.2 Endopeptidases 55

3.1.3 Exopeptidases 57

3.2 Materials and Methods 60

3.2.1 Insects 60

3.2.2 Determination of pH 60

3.2.3 Passage Time of the Food Bolus 60

3.2.4 Crude Extracts of insects 61

3.2.5 Determination of Protein 61

3.2.6 Determination of Amylolytic Activity 62

3.2.7 Determination of Proteolytic Activity 63

3.2.8 Detection of Amylolytic Activity by Electrophoretic Zymograms 64

3.2.9 Detection of Proteolytic Activity by Electrophoretic Zymograms 65

3.3 Results 66

3.3.1 Determination of pH and Passage Time of the Food Bolus 66

3.3.2 Determination of Amylolytic Activity 68
3.3.3 Determination of Proteolytic Activity

3.3.4 Detection of Amylolytic Activity by Electrophoretic Zymograms

3.3.5 Detection of Proteolytic Activity by Electrophoretic Zymograms

3.4 Discussion
3. ENZYMATIC STUDIES OF THE ADULT AND LARVAL DIGESTIVE SYSTEM

3.1 Introduction

The major part of the food requiring digestion by insects consists of polymers, which include: 1) starch and cellulose, formed by glucose units linked by α and β bonds, respectively; 2) hemicellulose, which is a mixture of β-bonded monosaccharide polymers; and 3) proteins, which are chains of amino acids.

The digestive processes occur in three phases: initial, intermediate, and final. Initially a decrease in molecular weight of polymeric food molecules occurs through the action of polymer hydrolases, such as amylase, cellulase, hemicellulase, and trypsin. The resulting oligomers then undergo hydrolysis by polymer hydrolases or are hydrolyzed by oligomer hydrolases. The products of this phase are dimers or small oligomers such as maltose, cellobiose, and dipeptides derived from starch, cellulose, and proteins, respectively. In the final digestion dimers are split into monomers by dimer hydrolases (Terra, 1990).

Nutrients in complex form such as proteins, carbohydrates and lipids, are often found in the diet of insects, and must be reduced in size in order to enable their incorporation via semipermeable membranes in the alimentary epithelium. Insects as a group are eminently adapted to a wide range of diets, some extremely marginal (Applebaum, 1985).

It is now firmly established that the basic molecular structure and features of hydrolysis by vertebrate digestive enzymes are highly conserved in evolution, with the kinetic characteristics a corollary of slight differences in amino acid sequence.
superimposed on a common basic structure. Insects are no exception to this rule (Dadd, 1970; House, 1974).

It has been considered that the rate of production and secretion of midgut enzymes in so-called continuously feeding insects, such as grain feeding insects, did not vary, while in discontinuous feeders, as exemplified by blood-sucking or predatory insects, taking large meals at long intervals, enzymes were produced on demand (Chapman, 1985, pp. 213-240). However, as Dadd (1970) points out, even insects in continuous contact with their food do not feed continuously and the evidence now shows that enzyme production is linked with feeding in all insects, though some enzyme activity persists in some species in the absence of feeding.

In *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) larvae, some protease activity was already present after ecdysis before feeding began, but the level increased and, in the final instar, subsequently declined so that none was present in the prepupa or early pupa. The level of activity increased after adult emergence even in the absence of food, but it was elevated following feeding (Dadd, 1956). Midgut amylase activity also increased after emergence in the absence of food (Jankovic-Hladni et al., 1978).

The order Coleoptera is able to exploit any type of organic material occurring on land or in fresh water which serves as food. The diversification within the order in feeding habits and digestion is almost as great as that in the foods themselves. We also find beetles that range from almost completely omnivorous habits to a high degree of host or food specificity. In the majority of beetles, the adults are relatively long-lived and feed actively, sometimes on the same kind of food as their larvae, but often on something quite different (Crowson, 1981, pp. 160-183).

The midgut is the principal source of digestive enzymes in Coleoptera, and also one of the main sites for the absorption of digested material from the gut contents.
midgut contents of adult or larval beetles tend to have a more alkaline, or less acid, reaction than do those of the fore- or hind-gut. Table 3.1 shows the pH values in different parts of the gut for some Coleoptera which are considered to be primary and secondary pests of stored grains (Crowson, 1981, pp. 160-183).

The time taken by food to pass right through the alimentary canal has been recorded for a number of species in the laboratory. Passage time probably varies with the temperature and the physiological state of the insect. Passage time periods of the Coleoptera order of a few hours and up to 2 days have been reported, with an apparent tendency to longer times in larger species (Crowson, 1981, pp. 160-183). Efficient digestion depends not only on the level of enzyme activity, but also on the period for which food is retained in the gut and subjected to enzyme activity. The optimum retention time will also be affected by the availability of food and the nutritional requirements of the organism, and regulatory mechanisms exist which ensure that the passage of food through the gut is adjusted to meet these varying conditions (Chapman, 1985, pp. 213-240).

<table>
<thead>
<tr>
<th>Species</th>
<th>Foregut pH</th>
<th>Midgut pH</th>
<th>Hindgut pH</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Trogoderma parabile</em></td>
<td>5.2 - 6.0</td>
<td>7.2 - 8.0</td>
<td>3.6 - 4.6</td>
</tr>
<tr>
<td><em>T. versicolor</em></td>
<td>5.2 - 6.8</td>
<td>c. 6.8</td>
<td>3.6 - 4.6</td>
</tr>
<tr>
<td><em>Rhyzopertha dominica</em></td>
<td>6.8</td>
<td>anterior 5.2 - 6.8 \ posterior 7.0 - 7.2</td>
<td>anterior 3.6 - 4.6</td>
</tr>
<tr>
<td><em>Cryptolestes turcicus</em></td>
<td>3.6 - 4.6</td>
<td>5.2 - 6.8</td>
<td>4.6 - 5.2</td>
</tr>
<tr>
<td><em>C. ferrugineus</em></td>
<td>4.6 - 5.2</td>
<td>5.2 - 6.0</td>
<td>4.6 - 5.2</td>
</tr>
<tr>
<td><em>Oryzaephilus mercator</em></td>
<td>4.6 - 5.2</td>
<td>anterior 6.0 - 6.8 \ posterior 5.2 - 6.0</td>
<td>anterior 5.2 - 6.8 \ posterior 4.6 - 5.2</td>
</tr>
<tr>
<td><em>O. surinamensis</em></td>
<td>5.2 - 6.0</td>
<td>7.2 - 7.6</td>
<td>anterior 4.6 - 5.2 \ posterior 4.6 - 5.2</td>
</tr>
<tr>
<td><em>Tribolium confusum</em></td>
<td>4.6 - 5.2</td>
<td>anterior c. 6.8 \ posterior c. 5.2</td>
<td>anterior 3.6 - 4.6</td>
</tr>
<tr>
<td><em>T. castaneum</em></td>
<td>5.2</td>
<td>7.2 - 7.6</td>
<td>3.6 - 4.6</td>
</tr>
<tr>
<td><em>Sitophilus granarius</em></td>
<td>c. 5.2</td>
<td>anterior 6.8 - 7.0 \ posterior c. 8.4</td>
<td>4.6 - 5.2</td>
</tr>
</tbody>
</table>

Table 3.1. pH gut values of some stored grain pests (Crowson, 1981, )
The description and definition of digestive enzymes mentioned here is based on the nomenclature recommended by the International Union of Biochemistry (Enzyme Nomenclature, 1978). Enzymes are classified and divided into groups on the basis of the type of reaction they catalyze, and enzyme nomenclature therefore defines that group of enzymes displaying the same catalytic property.

3.1.1 Glycosidases
Glycosidases are broadly defined as hydrolyzing O-glycosyl bonds. The majority of published data usually note the presence of a variety of enzymes, based on activity on substrates that may be more or less specific and usually from complex enzyme mixtures.

Carbohydrates are presumably absorbed only in the form of monosaccharides in insects, as in other animals. Certainly most insects possess enzymes capable of breaking many oligosaccharides and polysaccharides down to their component sugars. Amongst the glycosidases, the α-amylase (EC 3.2.1.1; 1,4-α-D-glucan glucanohydrolase) has received more intensive scrutiny. This enzyme hydrolyses 1,4-α-D-glucosidic linkages in polysaccharides containing three or more 1,4-α-D-linked D-glucose units (Krishna, 1955).

3.1.2 Endopeptidases
Endopeptidases are defined as proteolytic enzymes cleaving internal peptide bonds not adjacent to amino- or carboxy-termini and exhibiting various degrees of amino acid specificity. Proteases are grouped according to the structure of their active centre and according to amino acid specificity. Insect endopeptidases belong to one of the following three groups:

1. Serine proteases (EC 3.4.21), having an active centre of serine and histidine.
2. Cystein proteases (EC 3.4.22), Having a cysteine in the active centre.

3. Carboxyl proteases (EC 3.4.23), in which an acid residue is involved in the catalytic process.

Among the first group, trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1) have been identified in numerous insects as the major components of the digestive fluids and they are sometimes recorded as trypsin-like and chymotrypsin-like enzymes. In most cases these tryptic and chymotryptic activities are described within the context of the complete digestive protease complement and their identification is based on hydrolysis of specific substrates and on their inhibition by protease inhibitors exhibiting various degrees of specificity to known vertebrate serine proteases. Casein is commonly used as a substrate for determination of total protease activity and the B-chain of oxidized insulin occasionally in order to determine bond specificity. The synthetic substrates used in order to identify trypsin include:

a) p-tosyl-L-arginine methyl ester (TAME)

b) α-N-benzoyl-L-arginine ethyl ester (BAEE)

c) α-N-benzoyl-DL-arginine-p-nitroanilide (BAPNA)

Identification is often corroborated by the use of specific trypsin inhibitors, which may be synthetic products or of natural origin.

Among the second group, cathepsin B (EC 3.4.22.1) is an intracellular lysosomal acid protease extensively studied and described in mammalian systems. It exhibits endopeptidase action on proteins and both esterolytic and amilolytic activity on synthetic substrates, especially those derived from arginine (Barret and McDonald, 1980).

Among the third group, pepsin (EC 3.4.23.1) is apparently restricted to cyclorrhaphous larvae and has been positively identified in the larval midgut of Calliphora vicina by its activity on N-carbobenzoxy glutamyl-L-tyrosine and N-acety-L-phenylalanyl
diiodotyrosine, and by inhibition with diphenylhydrazine (Pandola and Greenberg, 1975).

3.1.3 Exopeptidases

Exopeptidases remove terminal amino acids from either the carboxyl end (carboxypeptidases) or from the amino end (aminopeptidases) and usually, but not always, exhibit a broad specificity for terminal amino acids. Exopeptidase action is an essential element in the reduction of dietary macromolecules to primary units capable of being absorbed through the midgut epithelium. Surprisingly little has been published on the properties of insect exopeptidases compared to the amount of information now available on endopeptidases. In most case aminopeptidases (EC 3.4.11; α-aminoacylpeptide hydrolase) and metallocarboxidases (EC 3.4.17) have been noted incidentally as part of the complement of digestive proteases. The broad overall specificity seems to indicate that leucyl substrates are preferentially cleaved by insect aminopeptidase (Applebaum et al., 1964).

Initial investigations of insect digestive proteolysis were based on methods developed for vertebrate digestive studies. As a consequence, enzymes similar to pepsin, trypsin, and chymotrypsin were the first to be described in insects. In general, it was concluded from these studies that insect digestive proteases have similarities with vertebrate systems in both their nature and extracellular location (House, 1974; Law et al., 1977).

The order Coleoptera is an extremely large and diverse group of organisms; as a consequence, it is difficult to make generalizations concerning the protease distributions in either this order or in insects in general (Houseman and Thie, 1993).

Table 3.2 and Table 3.3 show the enzymes of some stored grain Coleoptera.
<table>
<thead>
<tr>
<th>INSECT</th>
<th>ENZYME</th>
<th>pH</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Tenebrio molitor</em></td>
<td>Proteinases</td>
<td>6.2-6.4 (1)</td>
<td>Birk, et al., 1962</td>
</tr>
<tr>
<td><em>Tribolium castaneum</em></td>
<td>Amylases</td>
<td>4.4-5.7 (1)</td>
<td>Krishna &amp; Saxena, 1962</td>
</tr>
<tr>
<td></td>
<td>α-Galactosidases</td>
<td>3.6-6.0 (1)</td>
<td>Krishna &amp; Saxena, 1962</td>
</tr>
<tr>
<td></td>
<td>β-Galactosidases</td>
<td>4.0-9.8 (1)</td>
<td>Krishna &amp; Saxena, 1962</td>
</tr>
<tr>
<td></td>
<td>β-Glucosidases</td>
<td>4.5-8.5 (1)</td>
<td>Krishna &amp; Saxena, 1962</td>
</tr>
<tr>
<td></td>
<td>Proteinases</td>
<td>5.2-7.0 (1)</td>
<td>Krishna &amp; Saxena, 1962</td>
</tr>
<tr>
<td></td>
<td>Proteinases</td>
<td>6.5-6.9 (1)</td>
<td>Birk, et al., 1962</td>
</tr>
<tr>
<td></td>
<td>Amylases</td>
<td>4.6-5.2 (1)</td>
<td>Applebaum, 1964</td>
</tr>
<tr>
<td></td>
<td>Proteinases</td>
<td>5.5-6.0 (1)</td>
<td>Murdock, et al., 1984</td>
</tr>
<tr>
<td></td>
<td>Amylase</td>
<td>4.5 (2)</td>
<td>Sandoval-Cardoso, 1991</td>
</tr>
<tr>
<td></td>
<td>Proteinase</td>
<td>3.0 (2)</td>
<td>Blanco-Labra, 1996</td>
</tr>
<tr>
<td><em>Tribolium confusum</em></td>
<td>Proteinases</td>
<td>6.5-6.9 (1)</td>
<td>Birk, et al., 1962</td>
</tr>
<tr>
<td><em>Trogoderma spp.</em></td>
<td>Amylases</td>
<td>4.6-7.6 (1)</td>
<td>Krishna, 1955</td>
</tr>
<tr>
<td></td>
<td>Proteinases</td>
<td>4.6-7.6 (1)</td>
<td>Krishna, 1955</td>
</tr>
<tr>
<td><em>Rhyzopertha dominica</em></td>
<td>α-Amylase</td>
<td>3.4 (2)</td>
<td>Baker, 1991</td>
</tr>
<tr>
<td></td>
<td>α-Amylase</td>
<td>3.5 (2)</td>
<td>Baker, 1991</td>
</tr>
<tr>
<td><em>Prostephanus truncatus</em></td>
<td>Proteinases</td>
<td>7.2, 7.6, 8.1 (2)</td>
<td>Sandoval-Cardoso, 1991</td>
</tr>
<tr>
<td></td>
<td>Proteinases</td>
<td>8.5, 9.0 (2)</td>
<td>Houseman, et al., 1993</td>
</tr>
<tr>
<td><em>Sitophilus granarius</em></td>
<td>Proteinase</td>
<td>7.0 (1)</td>
<td>Baker, 1982</td>
</tr>
<tr>
<td><em>S. oryzae</em></td>
<td>Proteinases</td>
<td>4.0, 10.0 (1)</td>
<td>Baker, 1982</td>
</tr>
<tr>
<td></td>
<td>Amylase</td>
<td>5.0 (1)</td>
<td>Baker, 1983</td>
</tr>
<tr>
<td></td>
<td>Amylases</td>
<td>4.5-5.0 (2)</td>
<td>Baker, 1987</td>
</tr>
<tr>
<td></td>
<td>Proteinase</td>
<td>6.8 (2)</td>
<td>Liang, et al., 1991</td>
</tr>
<tr>
<td><em>S. zeamais</em></td>
<td>Proteinases</td>
<td>4.0, 10.0 (1)</td>
<td>Baker, 1982</td>
</tr>
<tr>
<td></td>
<td>Amylase</td>
<td>4.75 (1)</td>
<td>Baker, 1983</td>
</tr>
<tr>
<td></td>
<td>α-Glucosidase</td>
<td>5.5 (2)</td>
<td>Baker, 1991</td>
</tr>
<tr>
<td></td>
<td>β-Glucosidases</td>
<td>5.2, 5.7 (2)</td>
<td>Baker, 1991</td>
</tr>
<tr>
<td></td>
<td>β-Galactosidase</td>
<td>4.6 (2)</td>
<td>Baker, 1991</td>
</tr>
<tr>
<td></td>
<td>β-Fructosidases</td>
<td>5.5, 6.3 (2)</td>
<td>Baker, 1991</td>
</tr>
<tr>
<td></td>
<td>α-Galactosidase</td>
<td>5.1 (2)</td>
<td>Baker, 1991</td>
</tr>
<tr>
<td></td>
<td>Amylase</td>
<td>4.5 (2)</td>
<td>Sandoval-Cardoso, 1991</td>
</tr>
<tr>
<td></td>
<td>Proteinase</td>
<td>2.5 (2)</td>
<td>Sandoval-Cardoso, 1991</td>
</tr>
<tr>
<td></td>
<td>Proteinases</td>
<td>5.5, 7.5 (2)</td>
<td>Houseman, et al., 1993</td>
</tr>
</tbody>
</table>

(1) larva or gut extracts.
(2) enzyme purified or partially purified.

Table 3.2. Enzymes studied in some Coleoptera pests of stored cereals.
<table>
<thead>
<tr>
<th>INSECT</th>
<th>ENZYME</th>
<th>pH</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Callosobruchus</em></td>
<td>Proteinase</td>
<td>5.0 (1)</td>
<td>Murdock, et al., 1984</td>
</tr>
<tr>
<td><em>maculatus</em></td>
<td>Proteinase</td>
<td>5.4 (1)</td>
<td>Gatehouse, 1985</td>
</tr>
<tr>
<td></td>
<td>Proteinase</td>
<td>5.0 (2)</td>
<td>Kitch, et al., 1986</td>
</tr>
<tr>
<td></td>
<td>Amylases</td>
<td>5.2-6.0 (2)</td>
<td>Campos, et al., 1989</td>
</tr>
<tr>
<td></td>
<td>Proteinases</td>
<td>5.5, 6.0 (2)</td>
<td>Campos, et al., 1989</td>
</tr>
<tr>
<td></td>
<td>Proteinase</td>
<td>4.0 (1)</td>
<td>Silva, et al., 1991</td>
</tr>
<tr>
<td></td>
<td>Proteinase</td>
<td>3.3 (2)</td>
<td>Silva, et al., 1991</td>
</tr>
<tr>
<td><em>Zabrotes subfasciatus</em></td>
<td>Amylases</td>
<td>4.5, 5.0 (2)</td>
<td>Lemos, et al., 1990</td>
</tr>
<tr>
<td></td>
<td>Proteinases</td>
<td>3.5, 5.5 (2)</td>
<td>Lemos, et al., 1990</td>
</tr>
<tr>
<td><em>Acanthoscelides</em></td>
<td>Proteinases</td>
<td>5.5, 6.5 (1)</td>
<td>Wieman, et al., 1988</td>
</tr>
<tr>
<td><em>oblectus</em></td>
<td>Proteinases</td>
<td>4.5, 7.0 (2)</td>
<td></td>
</tr>
</tbody>
</table>

(1) larva or gut extracts.
(2) enzyme purified or partially purified.

Table 3.3. Enzymes studied in some Coleoptera pests of stored pulses
3.2 Materials and Methods

3.2.1 Insects

Insects were selected at random from stock cultures and the strains of *P. truncatus* were kept as described in Appendix 1.

3.2.2 Determination of pH

The pH of guts of adults and larvae from strains originating from Mexico, Tanzania and Togo was determined using diets consisting of maize flour with specific pH dyes (Sinha, 1959; Terra et al., 1979; Bignell and Anderson, 1980; Espinoza-Fuentes and Terra, 1987), as follows:

1) Maize (cacahuazintle race) (5 g) was finely ground and homogeneously mixed with 0.1 % pH indicator, using an A10 kinematic mill. The pH indicators used were: Bromophenol blue (pH 3.0-4.6) (yellow-blue), Congo red (pH 3.0-5.0) (Blue-violet), Litmus (pH 4.5-8.3) (red-blue), and Methyl red (pH 4.2-6.2) (pink-yellow). Each mixture was separately put in small glass containers.

2) Adults and larvae (20 of each) were separately put into each glass container. The two stages were left for feeding and then dissected (Appendix 1) after 180 min. The colour of the foregut, midgut, and hindgut was immediately recorded.

3.2.3 Passage Time of the Food Bolus

The passage time of the food bolus through the gut was determined using maize flour diets with Evans blue dye (Espinoza-Fuentes and Terra, 1987), as follows:

1) Maize (cacahuazintle race) (5 g) was finely ground and thoroughly mixed with 0.1 % Evans blue.
2) The two stages of *P. truncatus* (40 of each) were separately put into glass containers with the above mixture. They were dissected (Appendix 1) after 30 min of feeding at several intervals (5 min up to 15, and then every 10 min). The colour of the gut contents was changing with the dye according to their pH and this was always clearly visible.

3.2.4 Crude Extracts of Insect.

Crude extracts of entire adult and larval guts with their contents were prepared in 50 µl insect Ringer solution (Appendix 2). They were separately homogenised with a Potter-Elvehjem homogeniser and immediately centrifuged at 10,000 g for 3 min. The resulting supernatants were stored at -80 °C.

3.2.5 Determination of Protein.

Protein content was measured by the method of Bradford (1976), as follows:

1) Crude extracts were separately prepared using 25 adult and larval guts and the final volume adjusted to 200 µl with insect Ringer solution (Appendix 2).

2) A standard curve was prepared with several dilutions of protein standard (1 mg bovine serum albumin in 1 ml deionized water) from 2 to 20 µg/ml and using the DU-50 Beckman Spectrophotometer with the Quant II Linear program at 595 nm.

3) Five µl of each crude extract was placed in a clean and dry test tube and adjusted to 0.4 ml with deionized water. A test tube with 0.4 ml deionized water was used as a blank.

4) 0.1 ml Dye Reagent Concentrate (Appendix 2) was added to both test tubes and immediately shaken avoiding excess foaming.

5) Absorbance at 595 nm was measured.
3.2.6 Determination of Amylolytic Activity.

The amylolytic activity, as α-Amylase activity, of *P. truncatus* adult and larval guts in the strains from Mexico, Tanzania and Togo was determined by the Hopkins and Bird method (1954), which uses starch as a substrate as follows:

1) Gut extracts used were those prepared for the protein determination.

2) The amount of extract used for the amylolytic activity was adjusted so as to give a constant protein content. For *P. truncatus* adults in strains from Mexico and Togo the gut extract used was 6 µl, and 5 µl for the Tanzanian strain. In the case of larvae, the gut extract used was 40, 50, and 30 µl for the strains from Mexico, Togo, and Tanzania respectively.

3) The DU-50 spectrophotometer was adjusted to zero at 580 nm with a test tube containing 1.2 ml of 0.04 M succinic acid, pH 6.5 and 5 ml acidic I-KI solution (Appendix 2).

4) A starch control was prepared using 600 µl of 0.125 % starch (Appendix 2), 1.2 ml of 0.04 M succinic acid buffer, pH 6.5, and 5 ml acidic I-KI solution (Appendix 2), in a test tube. Absorbance at 580 nm was measured (S absorbance).

5) Enzyme activity was determined with the above amount of gut extracts in test tubes, adjusting to 1.2 ml with 0.04 M succinic acid buffer, pH 6.5 plus 600 µl of 0.125 % starch and incubated for 3 min at 30°C. Then 5 ml acidic I-KI solution was added and absorbance was immediately measured at 580 nm (E absorbance).

6) Amylolytic activity of adult and larval guts for each of the different strains of *P. truncatus* was calculated by the formula:

\[
\text{Activity/ml} = \frac{(S \text{ absorbance}) - (E \text{ absorbance})}{3 \times (\text{amount of gut extract in ml})}
\]
3.2.7 Determination of Proteolytic Activity.

The protease activity, as a trypsin activity, of *P. truncatus* adult and larval guts was determined by the Schwertz and Takenaka method (1955), which uses BAEE as substrate as follows:

1) Gut extracts used were those prepared for the protein determination.

2) The amount of extract used for the proteolytic activity was adjusted so as to give a constant protein content. The gut extract used for *P. truncatus* adults from the strains collected in Mexico and Togo was 10 µl, and 13 µl for the Tanzanian strain. For larvae the gut extract used was 30, 40 and 30 µl for the strains from Mexico, Togo and Tanzania respectively.

3) The DU-50 spectrophotometer was calibrated with 2.8 ml of 0.15 M Tris-(hydroxy-methyl)aminomethane (Tris) buffer, pH 8.1 (Appendix 2) and 50 µl of 0.014 M of BAEE (4.98 mg/ml 0.15 M Tris buffer) at 253 nm using the Kinetics program.

4) Enzyme control was determined with the above amount of gut extracts in a test tube containing 2.8 ml of 0.15 M Tris buffer, pH 8.1 and then 50 µl of 0.014 M of BAEE. Absorbance was measured at 253 nm (E absorbance).

5) The proteolytic activity was calculated by the formula:

\[
\text{Activity/ml} = \frac{(E \text{ absorbance})}{0.01} \times (\text{amount of gut extract in ml})
\]

* one unit of trypsin is defined as the increment of 0.01 absorbance at 253 nm.
3.2.8 Detection of Amylolytic Activity by Electrophoretic Zymograms.

The α-Amylase activity was determined using agarose-starch gels according to Baker (1983) as follows:

1) Crude extracts were prepared by using 50 adult guts and 50 larval guts, each one in 50 μl insect Ringer solution. The sample to be used for electrophoresis was made by adding 10 μl of 0.04 M succinic acid, pH 6.5 to 10 μl of the above extracts. From these solutions, 15 μl / well (39-45 μg protein) for the polyacrylamide gel electrophoresis (PAGE) and 5 μl / well (11-15 μg protein) for the zymograms were finally used.

2) Polyacrylamide gel electrophoresis was carried out at 4 °C and 20 mA according to Laemmli (1970). Resolving and stacking gels of 12 % and 4 % respectively (Appendix 2) were used. Electrode buffer, pH 8.3 (Appendix 2) was also used. Electrophoresis was stopped when the solution of crude extracts (5 and 15 μl) : sample buffer (Appendix 2) (1:1) had reached the bottom of the gel.

3) The agarose-starch gel was prepared with 0.5 % agarose and 0.5 % starch (Appendix 2) in 0.04 M succinic acid, pH 6.5 and heated until solution clarification. This solution was poured into an appropriate glass tray to gel and stored in the fridge. This gel must be activated by incubation with the succinic acid buffer for 30 min at room temperature prior to its use.

4) An amylolytic activity zymogram was prepared by placing the polyacrylamide gel (5 μl / well) on the agarose-starch gel, avoiding bubble formation. Both gels were covered and incubated for 120 min at 30 °C. The polyacrylamide gel was taken out, the agarose-starch gel washed with deionized water and then stained with a I - KI solution (Appendix 2) for 10-20 min.

5) Protein bands were detected from the electrophoresis gel (15 μl / well) by silver staining according to Heukeshoven and Dernick (1985). Gel fixation was carried out
overnight with ethanol-acetic acid solution (Appendix 2), twice washed with 10 % ethanol (Appendix 2) for 10 min, and three times with deionized water for 10 min. The gel was covered with 0.1 % AgNO3 solution (Appendix 2) for 30 min and then washed with deionized water for 10-20 sec. The development was done with a 3 % Na2CO3 solution (Appendix 2) and 0.02 % formaldehyde (37 %) solution (Appendix 2) until protein bands appeared and immediately stopped with 1 % acetic acid solution (Appendix 2) for 15 min. Finally, the gel was washed three times with deionized water for 10 min.

### 3.2.9 Detection of Proteolytic Activity by Electrophoretic Zymograms.

The proteinase activity was determined using casein as substrate according to Garcia-Carreño (1993) as follows:

1) Crude extracts were prepared as those for the electrophoretic zymogram for amylolytic activity.

2) Electrophoresis was carried out as the electrophoretic zymogram for amylolytic activity, but instead of 12 % resolving gel, 10 % was used (Appendix 2).

3) A proteolytic activity zymogram was prepared by placing the polyacrylamide gel (5 μl / well) into an appropriate glass tray with a 2 % casein solution (Appendix 2) for 30 min at 5 °C, and then incubating for 90 min at 25 °C. Finally, the gel was washed with deionized water and immediately fixed and stained in a one-step process by immersing the gel in staining solution containing 40% ethanol, 10% acetic acid, and 0.1% Coomassie brilliant blue for 120 min. To destain and improve the contrast of the clear zones indicating proteinase activity, gel was washed with 40% ethanol-10% succinic acid solution for 120 min.

4) Protein bands were detected from the electrophoresis gel (15 μl / well) as indicated in the electrophoretic zymogram for amylolytic activity detection.
3.3 Results

3.3.1 Determination of pH and Passage Time of the Food Bolus

The pH and passage time of the food bolus through the gut of *P. truncatus* adults and larvae from strains from Mexico, Tanzania and Togo are shown in Figures 3.1 and 3.2 respectively. There were no differences between the insect strains.

The colour of the lumen of the foregut, midgut, and fore-hind gut, using methyl red, was a fairly uniform strong yellow, and showed no colour at all with the other pH indicators. This colour indicates that these regions of the gut have pH 6.2. The rest of the gut (mid- and hind-hindgut), presented a strong pink colour with methyl red and a strong blue colour with bromophenol blue. Both colours indicate that the pH of these regions are between 4.2 and 4.6.

The total passage time through the whole *P. truncatus* adult gut was 159.3 ± 4.6 (mean of three replicates ± s.d) minutes. Now, considering the pH regions detected, the passage time through the foregut, midgut, and fore-hindgut was 63.3 ± 3.6 min, however for the mid- and hind-hindgut was 96.0 ± 3.4 min. The total passage time through the whole *P. truncatus* larva gut was 145.3 ± 2.3 min, where 50.7 ± 1.8 min was for the foregut, midgut, and fore-hindgut, and 94.3 ± 1.3 min for the mid- and hind-hindgut.

Thus, the only difference observed between adults and larvae was that the passage time of the food bolus from the foregut to the end of the fore-hindgut was faster in larvae than in adults.
Figure 3.1. Digestive tract of *Prostephanus truncatus* adult. The pH of the fore and midgut was 6.2 while that of the mid- and hind-gut was 4.2-4.6.

The passage times are means of 3 replicates (± s.d).

Figure 3.2. Digestive tract of *Prostephanus truncatus* larva. The pH of the fore and midgut was 6.2 while that of the mid- and hind-gut was 4.2-4.6.

The passage times are means of 3 replicates (± s.d).
3.3.2 Determination of Amylolytic Activity

α-Amylase activity of adult presented the same pattern for the three strains. Adults had more amylolytic activity than larvae and this relationship was uniform for the three strains studied. The adult guts showed about three times more α-amylase activity than that of larvae (Figure 3.3). However, there was a significant difference between the three strains in the α-amylase activity of larvae only (Figure 3.7). The strain from Togo shown had about 20 % lower activity (Table 3.6).

The statistical analysis of this data was done using the computer package "Minitab". One-way Anova was carried out for adults (Tables 3.4 and 3.5) and larvae (Table 3.6 and 3.7). Data were square-root transformed in order to stabilise variance.

Figure 3.3. α-Amylase activity per gut of *P. truncatus* adults and larvae.

A unit of enzymatic activity (U Act) was defined as the decrease in one unit of absorbancy at 580 nm. Data are the average of three repetitions, each of 25 guts.
Table 3.4. ANOVA Table of means on α-amylase for adult strains.

<table>
<thead>
<tr>
<th>STRAINS</th>
<th>N</th>
<th>MEAN</th>
<th>S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEXICO</td>
<td>3</td>
<td>0.193</td>
<td>0.011</td>
</tr>
<tr>
<td>TANZANIA</td>
<td>3</td>
<td>0.200</td>
<td>0.010</td>
</tr>
<tr>
<td>TOGO</td>
<td>3</td>
<td>0.183</td>
<td>0.021</td>
</tr>
</tbody>
</table>

Table 3.5. ANOVA Table for Square root on α-amylase for adult strains.

<table>
<thead>
<tr>
<th>SOURCE</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>Prob.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td>2</td>
<td>0.000422</td>
<td>0.000211</td>
<td>0.95</td>
<td>n.s*</td>
</tr>
<tr>
<td>Error</td>
<td>6</td>
<td>0.001333</td>
<td>0.000222</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>8</td>
<td>0.001756</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* No significant difference

Table 3.6. ANOVA Table of means on α-amylase for larval strains.

<table>
<thead>
<tr>
<th>STRAINS</th>
<th>N</th>
<th>MEAN</th>
<th>S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEXICO</td>
<td>3</td>
<td>0.057</td>
<td>0.003</td>
</tr>
<tr>
<td>TANZANIA</td>
<td>3</td>
<td>0.061</td>
<td>0.002</td>
</tr>
<tr>
<td>TOGO</td>
<td>3</td>
<td>0.049</td>
<td>0.003</td>
</tr>
</tbody>
</table>

Table 3.7. ANOVA Table for Square root on α-amylase for larval strains.

<table>
<thead>
<tr>
<th>SOURCE</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>Prob.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td>2</td>
<td>0.0002216</td>
<td>0.0001108</td>
<td>14.88</td>
<td>0.005*</td>
</tr>
<tr>
<td>Error</td>
<td>6</td>
<td>0.0000447</td>
<td>0.0000074</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>8</td>
<td>0.0002662</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Significant difference

Table 3.7. ANOVA Table for Square root on α-amylase for larval strains.
3.3.3 Determination of Proteolytic Activity

Trypsin activity was higher in adults, as was found for α-amylase activity in the three strains. The trypsin activity per adult gut was similar for the Mexican and Togo strains, but the Tanzanian strain had 20 % lower activity. However, the trypsin activity for the larval strains was completely different (Figure 3.4).

There were highly significant differences between the three strains and between the adult and larva stages (Table 3.9). The statistical analysis of this data was done using the computer package "Minitab". Balanced Anova was carried out for adults and larvae (Table 3.8 and 3.9). Data were square-root transformed to stabilise variance.

Figure 3.4. Proteolytic activity per gut of *P. truncatus* adults and larvae.

A unit of enzymatic activity (U Act) was defined as the amount of enzyme that catalyzed an increase of 0.01 absorption units per min at 253 nm.

Data are the average of three repetitions, each of 25 guts.
Table 3.8. ANOVA Table of means on proteolytic activity for adult and larval strains.

<table>
<thead>
<tr>
<th>STRAINS</th>
<th>ADULTS</th>
<th>LARVAE</th>
<th>MEAN</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEXICO</td>
<td>3.21</td>
<td>2.19</td>
<td>2.70</td>
</tr>
<tr>
<td>TANZANIA</td>
<td>2.59</td>
<td>1.26</td>
<td>1.93</td>
</tr>
<tr>
<td>TOGO</td>
<td>3.19</td>
<td>1.50</td>
<td>2.34</td>
</tr>
<tr>
<td>TOTAL</td>
<td>3.00</td>
<td>1.65</td>
<td>2.32</td>
</tr>
</tbody>
</table>

Table 3.9. ANOVA Table for Square root on proteolytic activity for adult and larval strains.

<table>
<thead>
<tr>
<th>SOURCE</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>Prob.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td>2</td>
<td>1.7902</td>
<td>0.8951</td>
<td>1505.79</td>
<td>0.000***</td>
</tr>
<tr>
<td>Stage</td>
<td>1</td>
<td>8.1608</td>
<td>8.1608</td>
<td>1.4E+04</td>
<td>0.000***</td>
</tr>
<tr>
<td>Strain.Stage</td>
<td>2</td>
<td>0.3373</td>
<td>0.1687</td>
<td>283.71</td>
<td>0.000***</td>
</tr>
<tr>
<td>Error</td>
<td>12</td>
<td>0.0071</td>
<td>0.0006</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>17</td>
<td>10.2954</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

***, Highly significant difference
3.3.4 Detection of Amylolytic Activity by Electrophoretic zymograms

The zymograms of amylolytic activity and the electrophoretic pattern of protein bands for adults are shown in Figure 3.5 and for larvae in Figure 3.6. Zymograms of amylolytic activity for both, adult (Figure 3.5A) and larva (Figure 3.6A), have two well defined bands of α-amylase activity in all the three strains. The main difference found in these zymograms was that in larvae, the upper band is thinner relative to the lower band than in the adult. There is no difference between insect strains in the electrophoretic protein pattern among adults (Figure 3.5B) and larvae (Figure 3.6B).

3.3.5 Detection of Proteolytic Activity by Electrophoretic zymograms.

The zymograms of proteolytic activity and electrophoretic proteins pattern of adults are shown in Figure 3.7 and for larvae in Figure 3.8. Zymograms of the adult gut (Figure 3.7A) and the larval gut (Figure 3.8A) presented several bands of proteolytic activity, which are different between the three insect strains. The Mexican P. truncatus adult and larval guts presented four well-differentiated bands of proteolytic activity (Figures 3.7A3 and 3.8A1 respectively), the strain from Togo three (Figures 3.7A2 and 3.8A2), and the Tanzanian strain two (Figures 3.7A1 and 3.8A3). These differences are most clear in the larval zymogram (Figure 3.8A), although there is no difference in the electrophoretic protein pattern between adults (Figure 3.7B) and larvae (Figure 3.8B).
Figure 3.5. A) Amylolytic activity bands of *P. truncatus* adult guts. B) Electrophoretic pattern of protein bands of *P. truncatus* adult guts. (1) (4) Mexican, (2) (5) Togo, and (3) (6) Tanzanian strains.

Figure 3.6. A) Amylolytic activity bands of *P. truncatus* larva guts. B) Electrophoretic pattern of protein bands of *P. truncatus* larva guts. (1) (4) Mexican, (2) (5) Togo, and (3) (6) Tanzanian strains.
Figure 3.7. A) Proteolytic activity bands of *P. truncatus* adult guts. B) Electrophoretic pattern of protein bands of *P. truncatus* adult guts. (1) (6) Tanzanian, (2) (5) Togo, and (3) (4) Mexican strains.

Figure 3.8. A) Proteolytic activity bands of *P. truncatus* larva guts. B) Electrophoretic pattern of protein bands of *P. truncatus* larva guts. (1) (4) Mexican, (2) (5) Togo, and (3) (6) Tanzanian strains.
3.4 Discussion

The pH values obtained in the digestive system of *P. truncatus* are broadly similar to those found in other primary and secondary pests of stored grains (Table 3.1). The pH of the digestive tract is almost neutral in the midgut (6.5) and acid in the hindgut end (4.2-4.6). Therefore, as most of the digestion process is performed in the midgut, this means that most of the enzyme activity is going to take place at around pH 6.5. Other authors detected differences between the foregut and midgut (Table 3.1) maybe because they were using more accurate methods of determining pH.

The passage time of the food bolus in *P. truncatus* also agrees with the general periods of a few hours recorded in other Coleoptera, depending on the developmental stage of the insect (Crowson, 1981c). Passage time of food in the larval gut is faster than in the adult stage. The difference found in time is basically located in the insect foregut, midgut, and fore-hindgut (ileum), where digestion takes place and this difference was about 20%. This could be a consequence of differing adult and larval feeding habits; whereas the larva is only in contact with the internal structure of the maize, which is fundamentally starch and proteins from the endosperm and embryo, the adult has to deal with other carbohydrates and proteins from the pericarp and attached cells. Most of the food retention time in the *P. truncatus* adult and larva guts is in the mid-, and hind-hindgut (colon and rectum), where the excretory system is located. For *P. truncatus*, which is able to develop in dry environments, this fact could be important since it allows the insect to remove the majority of water present in the digested bolus. It is, of course, true that rapid passage through the fore and midgut cannot continue if the hindgut is full. The volume of food material will decrease because of digestion and absorption, but the slower passage time through the hindgut may limit the throughput of food.
Enzyme activity is present in insects before feeding begins, increasing its level during food consumption but decreasing in the last larval instars, so this activity is not present in the pupal stage. The level of activity increases after adult emergence even in the absence of food, but is elevated following feeding (Dadd, 1956; Jancovic-Hladni et al., 1978). Amylolytic and proteolytic activities seem to be in agreement with the above statement with respect to the high enzyme levels in the adult guts of the three strains studied. This fact, and the slower passage time of the food bolus through the adult midgut, suggest that there may be more efficient digestion in the adult. The larger enzyme activity of the adult may be related to its special destructive capacity, which was already demonstrated by Cowley et al. (1980) when tunneling was measured in comparison with other pests of stored maize.

The differences in proteolytic activity detected in the zymograms among the *P. truncatus* strains could indicate that they are strains of different origin. Consequently, the introductions of this insect into East and West Africa could have different origins. It is not possible to make a definitive statement on the basis of comparison of single populations of three geographical strains, but the interpretation of the difference found here is consistent with results obtained by Nissen et al. (1991) using a different methodology.
CONTENTS

4. MICROBIOLOGICAL STUDIES OF THE ADULT AND LARVAL DIGESTION SYSTEM

4.1 Introduction ................................................. 77

4.2 Materials and Methods ...................................... 83
   4.2.1 Insects .................................................. 83
   4.2.2 Preparation of Gut Extracts ......................... 83
   4.2.3 Isolation of Aerobic Cellulolytic Microorganisms .... 83
   4.2.4 Gram Staining .......................................... 84
   4.2.5 Identification of Bacteria ............................. 84
   4.2.6 Bacterial Growth under Different Conditions ....... 84
   4.2.7 Bacterial Growth Rate ................................ 85
   4.2.8 Susceptibility to Antibiotics ........................ 85
   4.2.9 Transmission Electron Microscopy .................. 86
   4.2.10 Methanogenic Microorganisms ....................... 86

4.3 Results ...................................................... 87
   4.3.1 Isolation of Aerobic Cellulolytic Microorganisms .... 87
   4.3.2 Gram Staining .......................................... 87
   4.3.3 Identification of Bacteria ............................. 93
   4.3.4 Bacterial Growth under Different Conditions ....... 101
   4.3.5 Bacterial Growth Rate ................................ 102
4.3.6 Susceptibility to Antibiotics

4.3.7 Transmission Electron Microscopy

4.3.8 Methanogenic Microorganisms

4.4 Discussion
4. MICROBIOLOGICAL STUDIES OF THE ADULT AND LARVAL DIGESTIVE SYSTEM

4.1 Introduction

The intestinal contents of most insects support the proliferation of a variety of bacteria, yeasts and protozoa. Specific forms observed usually reflect a fortuitous profile of contamination by common microorganisms in a non-sterile environment. Most of the work done on the significance of intestinal microflora has dealt with their contribution to nutrition and in particular to the supply of vitamins or other essential primary nutrients (Wigglesworth, 1977, pp. 476-552).

Less attention has been devoted to their contribution to digestive processes, although it is possible that some of the reported luminal enzymes may originate in intestinal flora. Insects that feed on specialized diets, which may be refractive to digestion or nutritionally deficient, are more likely to exploit the degradative potential of microorganisms or their ability to synthesize essential dietary components and supply them to the host. Particular attention has been given to the digestion of cellulosic substrates, and the participation of intestinal microorganisms in their biodegradation (Applebaum, 1985).

Cellulose, the major component of most lignocellulases, is a linear homopolymer of β-1,4-linked glucose. Enzymatic hydrolysis of cellulose to glucose occurs by the action of cellulase, a general term referring to a mixture of enzymes with different activities and specificities (Breznak and Brune, 1994).

In white rot fungi and probably in insects, the cellulase system consists of a mixture of three classes of soluble extracellular enzymes: (1) endo-β-1,4-glucanases, which randomly cleave β-1,4-glucosidic bonds along a cellulose chain; (2) exo-β-1,4-
glucanases, which cleave cellobiose units from the non-reducing end of a cellulose chain; and (3) β-1,4-glucosidases, which hydrolyze cellobiose and water soluble celloextrans (oligosaccharides) to glucose, which can be used to meet the carbon and energy requirement of an organism (Martin, 1991).

There is a diversity of symbiotic associations between animals and microorganisms that ranges from the adventitious and loosely commensal to absolutely specific, one to one, host/symbiont coexistences, often involving intricate anatomical and behavioral specializations for ensuring transfer of symbionts from generation to generation (Koch, 1967). Many of these insects cannot survive without their symbionts, and this has frequently been shown to entail a nutritional dependence by the insect host on its endogenous microbes (Brooks, 1963).

Many symbiont-dependent insects can be placed in the following specialized and nutrient-limited categories: (1) eaters of wood; (2) plant sap suckers; (3) insects which feed only on blood in all developmental stages; (4) feeders on dried seeds, cereals or other desiccated organic tissues; (5) scavengers on oddities such as hair, feathers, old honeycomb, etc.; and perhaps (6) many saprophytic feeders (Dash et al., 1984).

Wood is an unpromising food for several reasons. The chief constituents of wood are cellulose (40-62 % of the dry weight) and lignin (18-38 %). Hemicellulose, a mixture of polysaccharides, both hexosans and pentosans, come next. Pentosans (6-23 %) yielding xylose and arabinose on hydrolysis, occur chiefly in the cell walls; hexosans (2-14 %) yielding glucose, are chiefly energy-reserve material. Starch ranges from 0 to 5.9 %; sugar, expressed as glucose, from 0 to 6.2 %; and protein from 1.1 to 2.3 % (Wigglesworth, 1977, pp. 476-552).

Wood is high in cellulose content and is potentially a good source of energy-producing carbohydrate; however, the preponderant dead heartwood is a parsimonious
donor of protein, other amino nitrogen compounds, or vitamins. Most animals lack the
digestive enzymes (lignases, cellulases and cellobiases) needed to liberate for absorption
the metabolically-utilizable glucose monomers from lignocellulose. Although a scattering
of insects in several orders secrete cellulolytic enzymes *sui generis*, the majority of wood-
eaters gain their nourishment only through the mediation of microorganisms (Dadd,
1985). This may be simply a matter of using breakdown and metabolic products resulting
from the decay of wood by a wide and non-specific flora of bacteria and fungi, as for
lucanid and scarabid beetle larvae, some of which have a bacterial fermentation chamber
type of gut and utilize acetic acid, like ruminant mammals (Bayon and Mathelin, 1980).
Some wood-infesting insects may seed their burrows with specific symbiotic fungi, which
they line with a mycelial pasture that is probably their main food (Norris and Baker, 1968;
Norris, 1979).

Many wood-eating insects utilize cellulolytic enzymes, which are either
endogenously secreted or produced by symbiotic microorganisms such as protozoa,
bacteria and fungi (Parkin, 1940; Spiller, 1951; Becker, 1977; Yamin, 1981; Breznak,
1982; Dasch et al., 1984; Cruden and Markovetz, 1987; Orpin and Anderson, 1988;
Coughlan, 1992). Also, there are some insects such as the lower termites that rely on a
combination of the two possibilities (Hogan et al., 1988). Other wood or foliage-eating
insects are completely non-cellulolytic and may derive no benefit from the cellulose
consumed in their diets (Martin, 1983). Instead they rely for their nutrition on the
degradation of other polysaccharides such as starch.

Among wood-eating insects, the lower termites all depend nutritionally on a
complex symbiotic microbiota, typically comprising several species of ciliate protozoa,
bacteria (both free in the digestive tract and symbiotic within the symbiotic protozoa and
including nitrogen-fixers), characteristic spirochaetes whose elimination soon results in
the insect’s death, and various fungi that are probably adventitious or commensal. The contribution of these several microbes to the nutrition of termites is still far from clear (Breznak, 1975; Breznak, 1982; Eutik et al., 1978), but it seems well established that both bacteria and many of the large ciliates digest and metabolize wood to acetic acid for use by the termites (Honigsberg, 1970; Mannesmann, 1972; Thayer, 1976); the bacterial flora includes species capable of nitrogen fixation (Breznak et al., 1973; Benemann, 1973; French et al., 1976; Potrikus and Breznak, 1977), thereby providing amino acids to increase the lack of supplies available to termites directly from wood.

Although high levels of methanogenesis occur only in anoxic environments, such as swamps and marshes, or in the rumen, the process also occurs in habitats that normally might be considered oxic, such as forest and grassland soils. In such habitats methanogenesis occurs in anoxic microenvironments, for example, in the midst of soil crumbs, in the mammalian intestinal tract (producing from 80 to $10^2$ g/year), in the guts of wood-eating insects (producing from 25 to $150 \times 10^2$ g/year), and in most other anoxic habitats. In the termite hindgut, endosymbiotic methanogens are thought to benefit their protozoan hosts by consuming $\text{H}_2$ generated from glucose fermentation by cellulolytic protozoans (Brock et al., 1994, pp. 623-691).

The symbiotic relationships of beetles may conveniently be divided into ectosymbiosis and endosymbiosis; in the latter case the symbiotic microorganisms function inside the beetle's body, while in the former case they do not. The usual functions of endosymbiotic microorganisms in beetles are to synthesize some nutritionally essential substances that are lacking in their diet, or else to make possible the utilization of some components of the food that the beetle would be unable to digest (Crowson, 1981, pp. 519-558).
Some of the clearest examples of insect nutritional dependence on symbionts have been demonstrated with various stored-products beetles that normally contain extracellular yeast located in mycetomes, which are outgrowths of the midgut (Pant et al., 1960; Pant and Gabrani, 1963; Pant and Kapoor, 1963; Jurzitza, 1969a, 1969b, 1974). Interestingly, natural strains of *Sitophilus granarius* are known both with and without symbionts, though all possess mycetomes (Lum and Baker, 1973). Since defaunated individuals of many of these normally symbiont-containing beetles appear to grow perfectly well so long as the diet contains ample nutrients of all classes (Baker and Lum, 1973), it seems likely that the advantage to the insect carrying symbionts lies solely in the provision of nutrient supplements that allow the insect to thrive with a much wider dietetic range than would otherwise be possible.

Cockroaches, which are omnivores, in addition to a heavy crop and midgut population of non-specific microorganisms, possess intracellular bacteroids in the mycetomes of the body. Roaches whose bacteroids have been largely eliminated by antibiotic treatment grow poorly on the usual dog-food stock diet, but can be grown apparently normally if this is supplemented with various yeast or liver products, which are thought to provide short-chain peptides that optimize growth (Brooks and Kringen, 1972).

Three types of wood feeding are recognized in beetle larvae: (1) Larvae able to utilize only the cell contents and perhaps part of the polysaccharides intermediate between starch and the hemicelluloses: Lyctidae and Bostrichidae. (2) Larvae using the cell contents and the carbohydrates of the cell wall as far as the hemicelluloses: Scolytidae. (3) Larvae able to use all the carbohydrates of the cell wall including cellulose: Anobiidae and most Cerambycidae. Where cellulose is not attacked the insects become increasingly dependent on the starch and sugar in the wood.
Cellulose digestion has been shown in 78 species of insects from 20 families representing 8 orders, though in all but three cases, cellulolytic activity is associated with symbiotic microflora. Taxa in which symbiotic cellulolytic capacity is common include three Coleoptera families: Anobiidae (furniture beetles and death watch beetles), Buprestidae (metallic wood borers), and Cerambycidae (long-horned beetles) (Martin, 1991). The Anobiidae family belongs to the Bostrichoidea superfamily, which also includes the Bostrichidae family (Crowson, 1981, pp. 695-698). The Anobiidae and Bostrichidae families are related phylogenetically, and it was of interest to determine whether these phylogenetic similarities also extended to their ability to support microorganisms that digest cellulose.

In this section, microorganisms in the gut were investigated and characterised in order to evaluate their possible contribution to digestion of cellulose. Characterisation included evaluating whether they might have cellulolytic activity under anaerobic conditions, producing methane as in lower termites (Brock et al., 1994, pp. 623-691). The microflora in the three geographical strains of *P. truncatus* were also compared.
4.2 Materials and Methods

4.2.1 Insects

*P. truncatus* strains were kept and developed as described in Appendix 1.

4.2.2 Preparation of Gut Extracts

Fifteen final instar larvae (30 days old) and 14 day old adults per strain were dissected (Appendix 1) in a cold drop of Ringer solution, pH 7.2 (Appendix 2). Fat tissue was physically removed. Whole guts (fore, mid, and hindguts) were thoroughly rinsed 3 times with cold, sterile distilled water and then homogenized in a sterile Potter homogenizer in 1 ml of sterile distilled water. Rinses and homogenization were done in a biological safety cabinet.

4.2.3 Isolation of Aerobic Cellulolytic Microorganisms

A loopful of gut homogenates was inoculated in Petri plates containing carboxymethyl cellulose (10 g/litre) as a source of carbon; Dubos solution, pH 6.0 (Appendix 2); and agar (18 g/litre), (Pramer and Schmidt 1965). This operation was repeated to obtain separation of the microorganism strains. Pure cultures were obtained from the isolated strains by inoculation in both the above culture medium and nutrient agar (23 g/litre) (pH 6.0). These pure cultures were used for further assays. The streak-plate method (Appendix 3) was used for inoculation. Microorganisms were conserved, as stock cultures, by inoculation of a pure culture loopful in test tubes with inclined nutrient agar (23 g/litre). Petri plates and test tubes were incubated at 29 ± 1°C under aerobic conditions (Brock et al., 1994, pp. 89-127).
4.2.4 Gram Staining

This technique is used to differentiate Gram-positive from Gram-negative bacteria and it is the first step for their identification (Appendix 3).

4.2.5 Identification of Bacteria

In order to identify any organism, it is necessary to perform different biochemical tests. These tests measure the presence or absence of enzymes involved in the catabolism of the substrate or substrates added to a differential medium. This medium has an indicator, usually a dye, which allows the differentiation between various chemical reactions carried out during growth. Identification procedures or criteria have been developed selecting a specific battery of tests, based on whether they are Gram-negative or Gram-positive bacteria (Brock et al., 1994, pp. 479-505). To identify the groups and genera of the different bacteria isolated from *P. truncatus* guts, the Cowan and Steel (1979) criteria were selected, whereas the criteria of Balows et al. (1992) were used for the species classification. References of the biochemical tests carried out for the identification of the bacteria into species are given in Table 4.2.

4.2.6 Bacterial Growth under Different Conditions

The two pH values found in the adult and larval guts in section 3 were about 6.5 (fore, mid, and the first third of hindgut) and about 4.5 (rest of hind-gut). A loopful of each stock culture was therefore inoculated and then incubated in Petri plates containing nutrient agar at pH 4.5 and 6.5. Inoculation was done by the streak-plate method, and incubation was maintained at 29 ± 1°C under both aerobic and anaerobic conditions. Anaerobic condition was achieved by using an anaerobic jar. This jar is an airtight
container where oxygen from its atmosphere is removed by a chemical reaction once Petri plates or test tubes are placed inside (Brock et al., 1994, pp. 479-505).

4.2.7 Bacterial Growth Rate

A loopful of each stock culture was suspended and mixed in 3 ml of sterile nutrient broth and incubated overnight. From these solutions, 0.5 ml ($\approx 10^3$ to $10^4$ bacteria) was inoculated into nephelometric flasks with 50 ml of sterile nutrient broth and incubated with agitation (100 rpm) at 29 ± 1°C under aerobic conditions. Optical density was determined every 4 h by using a Klett Summerson Photocolorimeter with a red filter (600 nm) (Brock et al., 1994, pp. 321-360).

4.2.8 Susceptibility to Antibiotics

Two loopfuls of each stock culture were suspended and mixed in 1 ml of sterile distilled water. From these solutions, 0.5 ml was inoculated in Petri plates with nutrient agar and uniformly distributed with a sterile glass spreader (Appendix 3). A commercial multidisks kit (Sanofi Diagnostics Pasteur, S. A., Mexico) was used to test susceptibility of the bacteria to antibiotics. Each multidisk contains 12 antibiotics against Gram-positive and Gram-negative bacteria. Petri plates with the disks were left in refrigeration for about 30 min and then incubated for 24 h at 29 ± 1°C under aerobic conditions (Brock et al., 1994, pp. 479-505). This test was performed twice. The diameter of inhibition haloes was measured and compared with the table provided in the kit (Appendix 3). The antibiotics tested were amikacin (30 mcg), ampicillin (10 mcg), cephalothin (30 mcg), ceftriaxone (30 mcg), chloramphenicol (30 mcg), dicloxacilin (1 mcg), enoxacin (10 mcg), erythromycin (15 mcg), gentamicin (10 mcg), netilmicin (30 mcg), penicillin (10 U), and sulfamethoxazole-trimethoprim (25 mcg).
4.2.9 Transmission Electron Microscopy

Guts dissected were divided into fore, mid, and hindgut and prepared for TEM as described in section 2.24.

4.2.10 Methanogenic Microorganisms

Fresh adult and larval hindguts of *P. truncatus* from Tanzania were put separately on a slide and gently pressed with a glass cover. This preparation was immediately observed using an Olympus Fluorescence Microscope with integrated camera, BH2 model, and 420 nm wave length. The coenzyme F$_{420}$, which is associated with anaerobic breakdown of cellulose, fluoresces at 420 nm.
4.3 Results

4.3.1 Isolation of Aerobic Cellulolytic Microorganisms

Nine strains of bacteria were isolated from the guts of *P. truncatus*. Five were from the Tanzanian strain and were named Z1 (from the adult stage), Z2 (from the larval stage), and Z3, Z4 and Z5 (from the adult stage). Two were from the Mexican strain, M6 (from the adult stage) and M7 (from the larval stage). The last 2 were from the strain from Togo, G8 (from the adult stage) and G9 (from the larval stage). As these bacteria had slow growth in carboxymethyl cellulose medium (7 days), they were inoculated in nutrient agar, growing faster (2 days). From this culture medium the nine bacteria were again inoculated in carboxymethyl cellulose and their ability to grow in this medium had not changed. Then, the bacterial growth under different conditions, bacterial growth rate, and susceptibility to antibiotics were carried out using the nutrient agar medium.

4.3.2 Gram Staining

Eight Gram-negative (Z1, Z2, G8, G9, M7, Z3, Z4, and Z5) (Figures 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, and 4.8 respectively) and one Gram-positive (M6) (Figure 4.9) bacteria were isolated and their purity confirmed by determining their shape, size, and cell aggregation.
Figure 4.1. Strain Z1 (Gram-negative rods) isolated from *P. truncatus* adult guts of the strain from Tanzania.

Figure 4.2. Strain Z2 (Gram-negative rods) isolated from *P. truncatus* larva guts of the strain from Tanzania.
Figure 4.3. Strain G8 (Gram-negative rods) isolated from *P. truncatus* adult guts of the strain from Togo.

Figure 4.4. Strain G9 (Gram-negative rods) isolated from *P. truncatus* larva guts of the strain from Togo.
Figure 4.5. Strain M7 (Gram-negative rods) isolated from *P. truncatus* larva guts of the strain from Mexico.

Figure 4.6. Strain Z3 (Gram-negative rods) isolated from *P. truncatus* adult guts of the strain from Tanzania.
Figure 4.7. Strain Z4 (Gram-negative rods) isolated from *P. truncatus* adult guts of the strain from Tanzania.

Figure 4.8. Strain Z5 (Gram-negative rods) isolated from *P. truncatus* adult guts of the strain from Tanzania.
Figure 4.9. Strain M6 (Gram-positive cocci) isolated from *P. truncatus* adult guts of the strain from Mexico.
4.3.3 Identification of Bacteria

The results of the biochemical tests carried out on the bacteria isolated from the three strains of *P. truncatus* for identification as far as genus or group (Cowan and Steel, 1979) are shown in Table 4.1. The genus or group identified and the references used for further identification to species are in Table 4.2.

<table>
<thead>
<tr>
<th>INSECT STAGE</th>
<th>Adult</th>
<th>Larva</th>
<th>Adult</th>
<th>Larva</th>
<th>Adult</th>
<th>Larva</th>
<th>Adult</th>
<th>Adult</th>
<th>Adult</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Z1</td>
<td>Z2</td>
<td>G8</td>
<td>G9</td>
<td>M6</td>
<td>M7</td>
<td>Z3</td>
<td>Z4</td>
<td>Z5</td>
</tr>
<tr>
<td>Form</td>
<td>Rod</td>
<td>Rod</td>
<td>Rod</td>
<td>Rod</td>
<td>Cocci</td>
<td>Rod</td>
<td>Rod</td>
<td>Rod</td>
<td>Rod</td>
</tr>
<tr>
<td>Motility</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Aerobic growth</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Anaerobic growth</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Oxidase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glucose (Acid)</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>-</td>
<td>O</td>
<td>-</td>
<td>O</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>O</td>
<td>F</td>
</tr>
<tr>
<td>Resistant to acid</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Spore formation</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Code used: Zs, strains from Tanzania; Gs, strains from Togo; Ms, strains from Mexico; +, positive; -, negative; O, Oxidation; F, Fermentation

Table 4.1. Biochemical tests for determining genus and groups of the bacteria isolated from *P. truncatus*.  

93
Table 4.2. Microbial genera and groups identified of the bacteria isolated from *P. truncatus*.

The specific battery of biochemical tests for the *Alcaligenes* genus (Z1 strain) is shown in Table 4.3 and the species identified was *faecalis*.

<table>
<thead>
<tr>
<th>TESTS</th>
<th>Z1 STRAIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrate reduction</td>
<td>-</td>
</tr>
<tr>
<td>Respiration with nitrates</td>
<td>+</td>
</tr>
<tr>
<td>Urease</td>
<td>-</td>
</tr>
<tr>
<td>Gelatine hydrolysis</td>
<td>-</td>
</tr>
<tr>
<td>Starch hydrolysis</td>
<td>+</td>
</tr>
<tr>
<td>Glucose</td>
<td>-</td>
</tr>
<tr>
<td>Manitol</td>
<td>-</td>
</tr>
<tr>
<td>Carboxymethyl hydrolysis</td>
<td>+</td>
</tr>
</tbody>
</table>

Species Identified: *faecalis*

Code used: Z1, strain from Tanzania; +, positive; -, negative

Table 4.3. Biochemical tests for the species identification of the *Alcaligenes* genus isolated from *P. truncatus* adults.
The specific battery of biochemical tests for the *Pseudomonas* genus (Z2 strain) is shown in Table 4.4. The species identified was *fluorescens* var. *cellulose*.

<table>
<thead>
<tr>
<th>TESTS</th>
<th>Z2 STRAIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth at 42°C</td>
<td>-</td>
</tr>
<tr>
<td>Growth in NaCl (6.5%)</td>
<td>+</td>
</tr>
<tr>
<td>Growth at 4°C</td>
<td>+</td>
</tr>
<tr>
<td>Gelatin hydrolysis</td>
<td>+</td>
</tr>
<tr>
<td>Pigmentation:</td>
<td></td>
</tr>
<tr>
<td>Piocianin</td>
<td>-</td>
</tr>
<tr>
<td>Pioverdin</td>
<td>+</td>
</tr>
<tr>
<td>Brown</td>
<td>-</td>
</tr>
<tr>
<td>Acid Production (Hugh-Leifson):</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>+</td>
</tr>
<tr>
<td>Lactose</td>
<td>-</td>
</tr>
<tr>
<td>Sacarose</td>
<td>+</td>
</tr>
<tr>
<td>Maltose</td>
<td>+</td>
</tr>
<tr>
<td>Manitol</td>
<td>+</td>
</tr>
<tr>
<td>Growth in MacConkey</td>
<td>+</td>
</tr>
<tr>
<td>Citrate utilization</td>
<td>+</td>
</tr>
<tr>
<td>Starch hydrolysis</td>
<td>-</td>
</tr>
<tr>
<td>Casein hydrolysis</td>
<td>+</td>
</tr>
<tr>
<td>Carboxymethyl hydrolysis</td>
<td>+</td>
</tr>
<tr>
<td>Species Identified</td>
<td><em>fluorescens</em> var. <em>cellulose</em></td>
</tr>
</tbody>
</table>

Code used: Z2, strains from Tanzania; +, positive; -, negative

Table 4.4. Biochemical tests for the species identification of the *Pseudomonas* genus isolated from *P. truncatus* larvae.
The specific battery of biochemical tests for the Flavobacteria group (G8 and Z4 strains) is shown in Table 4.5. The strain G8 belongs to the Flavobacteria of the group D and *Flavobacterium* genus, whereas the strain Z4 corresponds to the Flavobacteria of the group C. Table 4.6 shows the tests carried out for the strain Z4 to identify it to the *multivorum* species.

### Table 4.5. Biochemical tests for the group and genus identification of the Flavobacteria isolated from *P. truncatus* adults.

<table>
<thead>
<tr>
<th>TESTS</th>
<th>G8 STRAIN</th>
<th>Z4 STRAIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pigmentation (yellow)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Indole</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Gelatin hydrolysis</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>-</td>
<td>O</td>
</tr>
<tr>
<td>Carboxymethyl hydrolysis</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Group and Genus</td>
<td>Flavobacteria of the D group, <em>Flavobacterium</em> sp.</td>
<td>Flavobacteria of the C group</td>
</tr>
<tr>
<td>Group</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Code used: G8, strain from Togo; Z4, strain from Tanzania; +, positive; -, negative; O, oxidation

Table 4.5. Biochemical tests for the group and genus identification of the Flavobacteria isolated from *P. truncatus* adults.

### Table 4.6. Biochemical tests for the genus and species identification of the Flavobacteria isolated from *P. truncatus* adults.

<table>
<thead>
<tr>
<th>TESTS</th>
<th>Z4 STRAIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manitol</td>
<td>-</td>
</tr>
<tr>
<td>Gelatin hydrolysis</td>
<td>-</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>-</td>
</tr>
<tr>
<td>Nitrite reduction</td>
<td>-</td>
</tr>
<tr>
<td>Urease</td>
<td>-</td>
</tr>
<tr>
<td>Genus and Species Identified</td>
<td><em>Flavobacterium multivorum</em></td>
</tr>
</tbody>
</table>

Code used: Z4, strain from Tanzania; -, negative

Table 4.6. Biochemical tests for the genus and species identification of the Flavobacteria isolated from *P. truncatus* adults.
The specific battery of biochemical tests for the *Acinetobacter* genus (G9 strain) is shown in Table 4.7. The species detected were probably *calcoaceticus* or *iwoffii*. Therefore, the strain G9 was identified as *Acinetobacter* sp. There was also an identification problem with the *Streptococcus* genus (strain M6) as is shown in Table 4.8. The species detected were here probably *faecalis* or *faecium*. Therefore, the strain M6 was identified as *Streptococcus* sp.

![Table 4.7. Biochemical tests for the species identification of the *Acinetobacter* genus isolated from *P. truncatus* larvae.]

<table>
<thead>
<tr>
<th>TESTS</th>
<th>G9 STRAIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth at 42°C</td>
<td>-</td>
</tr>
<tr>
<td>Gelatin hydrolysis</td>
<td>-</td>
</tr>
<tr>
<td>Urease</td>
<td>-</td>
</tr>
<tr>
<td>Citrate</td>
<td>-</td>
</tr>
<tr>
<td>Glucose (Acid)</td>
<td>+</td>
</tr>
<tr>
<td>Nitrates reduction</td>
<td>+</td>
</tr>
<tr>
<td>Carboxymethyl hydrolysis</td>
<td>-</td>
</tr>
<tr>
<td>Species Identified</td>
<td>Probably <em>calcoaceticus</em> or <em>iwoffii</em></td>
</tr>
</tbody>
</table>

Code used: G9, strain from Togo; +, positive; -, negative

Table 4.8. Biochemical tests for the species identification of the *Streptococcus* genus isolated from *P. truncatus* adults.

<table>
<thead>
<tr>
<th>TESTS</th>
<th>M6 STRAIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth at 45°C</td>
<td>+</td>
</tr>
<tr>
<td>Hemolysis</td>
<td>α</td>
</tr>
<tr>
<td>Growth in NaCl (6.5%)</td>
<td>+</td>
</tr>
<tr>
<td>Gelatin melting</td>
<td>+</td>
</tr>
<tr>
<td>Lactose</td>
<td>+</td>
</tr>
<tr>
<td>Citrate</td>
<td>-</td>
</tr>
<tr>
<td>Manitol</td>
<td>+</td>
</tr>
<tr>
<td>Carboxymethyl hydrolysis</td>
<td>-</td>
</tr>
<tr>
<td>Species Identified</td>
<td>Probably <em>faecalis</em> or <em>faecium</em></td>
</tr>
</tbody>
</table>

Code used: M6, strain from Mexico; +, positive; -, negative
The specific battery of biochemical tests for the *Klebsiella* genus (M7 strain) is shown in Table 4.9. The species detected was probably *pneumoniae*. Therefore, the strain M7 was identified as *Klebsiella* sp.

<table>
<thead>
<tr>
<th>TESTS</th>
<th>M7 STRAIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactose (MacConkey)</td>
<td>-</td>
</tr>
<tr>
<td>Simmons citrate</td>
<td>-</td>
</tr>
<tr>
<td>Voges-Proskauer</td>
<td>+</td>
</tr>
<tr>
<td>Methyl red</td>
<td>-</td>
</tr>
<tr>
<td>TSI medium:</td>
<td></td>
</tr>
<tr>
<td>H₂S</td>
<td>-</td>
</tr>
<tr>
<td>Gas</td>
<td>-</td>
</tr>
<tr>
<td>LIA medium:</td>
<td></td>
</tr>
<tr>
<td>H₂S</td>
<td>-</td>
</tr>
<tr>
<td>Gas</td>
<td>-</td>
</tr>
<tr>
<td>MIO medium:</td>
<td></td>
</tr>
<tr>
<td>Motility</td>
<td>-</td>
</tr>
<tr>
<td>Indole</td>
<td>-</td>
</tr>
<tr>
<td>Ornithin</td>
<td>-</td>
</tr>
<tr>
<td>Carboxymethyl hydrolysis</td>
<td>-</td>
</tr>
<tr>
<td>Genus and species identified</td>
<td><em>Klebsiella, probably pneumoniae</em></td>
</tr>
</tbody>
</table>

Code used: M7, strain from Mexico; +, positive; -, negative

Table 4.9. Biochemical tests for the genus and species identification of the Enterobacteria isolated from *P. truncatus* larvae.
The specific battery of biochemical tests for the *Chromobacterium* genus (Z3 strain) is shown in Table 4.10. The species detected was *violaceum*.

<table>
<thead>
<tr>
<th>TESTS</th>
<th>Z3 STRAIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth at 4°C</td>
<td>-</td>
</tr>
<tr>
<td>Growth at 37°C</td>
<td>+</td>
</tr>
<tr>
<td>Carboxymethyl hydrolysis</td>
<td>+</td>
</tr>
<tr>
<td>Species identified</td>
<td><em>violaceum</em></td>
</tr>
</tbody>
</table>

Code used: Z3, strain from Tanzania; +, positive; -, negative

Table 4.10. Biochemical tests for the species identification of the *Chromobacterium* genus isolated from *P. truncatus* adults.

The specific battery of biochemical tests for the Cardiobacteria group (Z5 strain) is shown in Table 4.11. The genus identified was *Capnocytophaga*.

<table>
<thead>
<tr>
<th>TESTS</th>
<th>Z5 STRAIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indole</td>
<td>-</td>
</tr>
<tr>
<td>Nitrates reduction</td>
<td>-</td>
</tr>
<tr>
<td>Glucose</td>
<td>+</td>
</tr>
<tr>
<td>Lactose</td>
<td>-</td>
</tr>
<tr>
<td>Manitol</td>
<td>-</td>
</tr>
<tr>
<td>Carboxymethyl hydrolysis</td>
<td>-</td>
</tr>
<tr>
<td>Genus identified</td>
<td><em>Capnocytophaga</em></td>
</tr>
</tbody>
</table>

Code used: Z5, strain from Tanzania; +, positive; -, negative

Table 4.11. Biochemical tests for the genus identification of the Cardiobacteria isolated from *P. truncatus* adults.
Finally, Table 4.12 shows the identification of the bacteria isolated from the digestive system of adults and larvae of the three strains of *P. truncatus*. The Tanzanian strain presented three bacterial strains: *Alcaligenes faecalis* (Z1), *Pseudomonas fluorescens* var. cellulose (Z2), and *Chromobacterium violaceum* (Z3), with the cellulose degradation capacity.

<table>
<thead>
<tr>
<th>Origin of <em>P. truncatus</em></th>
<th>Stages</th>
<th>Code used</th>
<th>Identified bacteria</th>
<th>Cellulolytic activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tanzania</td>
<td>Larva Z2</td>
<td><em>Pseudomonas fluorescens</em> var. cellulose</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Adult Z1</td>
<td><em>Alcaligenes faecalis</em></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Adult Z3</td>
<td><em>Chromobacterium violaceum</em></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Adult Z4</td>
<td><em>Flavobacterium multiborum</em></td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Adult Z5</td>
<td><em>Capnocytophaga</em> sp.</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Mexico</td>
<td>Larva M7</td>
<td><em>Klebsiella</em> sp.</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Adult M6</td>
<td><em>Streptococcus</em> sp.</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Togo</td>
<td>Larva G9</td>
<td><em>Acinetobacter</em> sp.</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Adult G8</td>
<td><em>Flavobacterium</em> sp.</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.12. Identification and cellulolytic activity against carboxymethyl cellulose of the bacteria isolated from larval and adult guts of *P. truncatus*.
4.3.4 Bacterial Growth under Different Conditions

Out of the five bacteria isolated from the larva and adult guts of the Tanzanian strain, *Alcaligenes faecalis*, *Chromobacterium violaceum*, and *Pseudomonas fluorescens* var. cellulose were able to grow at both pHs (4.5 and 6.5) and under aerobic and anaerobic conditions. The other two, as well as those from Mexico and Togo, only grew at pH 6.5 and under aerobic conditions. Therefore, this would mean that the three strains named above are facultative bacteria, having the ability to live in any part of the gut (fore, mid, and hindgut), whereas the other bacterial strains seems to be restricted to live in the mid, and in the first third of the hindgut or ileum.
4.3.5 Bacterial Growth Rate

The different nature of the nine bacteria isolated from the three strains of *P. truncatus* was demonstrated by their different growth rate (Fig. 4.10).

![Graph showing bacterial growth rate](image)

Fig. 4.10. Growth rate of the bacteria isolated from larva and adult guts of *P. truncatus*.

- Z1, *Alcaligenes faecalis*;
- Z2, *Pseudomonas fluorescens* var. cellulose;
- Z3, *Chromobacterium violaceum*;
- Z4, *Flavobacterium multiborum*;
- Z5, *Capnocytophaga* sp.;
- M6, *Streptococcus* sp.;
- M7, *Klebsiella* sp.;
- G8, *Flavobacterium* sp.;
- G9, *Acinetobacter* sp.
4.3.6 Susceptibility to Antibiotics

Susceptibility to antibiotics of the nine bacteria isolated from the guts of the *P. truncatus* strains is presented in Table 4.13. *Alcaligenes faecalis* (Z1), *Pseudomonas fluorescens* var. cellulose (Z2), and *Chromobacterium violaceum* (Z3) are resistant to more of the antibiotics tested.

Table 4.13. Susceptibility to antibiotics of the bacteria isolated from larva and adult guts of *P. truncatus*. R, resistant; I, intermediate; S, sensitive. Key to antibiotics (limits of the diameter of inhibition in mm): AK, amikacin (R, ≤14; S, ≥17); AM, ampicillin (R, ≤11; S, ≥14); CP, cephalothin (R, ≤14; S, ≥18); CF, ceftriaxone (R, ≤13; I, 14-20; S, ≥21); CL, chloramphenicol (R, ≤12; S, ≥18); DC, dicloxacillin (R, ≤10); EN, enoxacin (R, ≤14; S, ≥18); ER, erythromycin (R, ≤13; S, ≥18); GE, gentamicin (R, ≤12; S, ≥15); NT, netilmicin (R, ≤12; I, 13-14; S, ≥15); PE, penicillin (R, ≤28; S, ≥29); and ST, sulfamethoxazole-trimethoprim (R, ≤10; S, ≥16).
4.3.7 Transmission Electron Microscopy

The electron microscopic study demonstrated that the bacteria present in the *P. truncatus* guts were not randomly distributed, but they were specifically embedded in a microbial biofilm (Figures 4.11 to 4.15), which is built up on the cuticular surface of the first third of the hindgut or ileum and protects the normal flora of the gut from adverse environmental conditions and from biological and chemical antibacterial agents, having their own microenvironment (Costerton et al., 1995). The term microenvironment is defined by microbial ecologists as the actual place where a microorganism lives (Brock et al., 1994, pp. 623-691). Some of the bacteria present in the hindgut seem to be undergoing binary fission (Figure 4.13) and others are at the end of this process (Figure 4.14). There are also bacteria showing an invagination of the plasma membrane like either arrays of thicker membrane whorls or arrays of fine membrane whorls (Figures 4.11 to 4.15), similar to those named as mesosomes by Fitz-James (1960). These structures (only one per bacterium) appear to be more common for Gram-positive bacteria and they are usually involved in cell sporulation (Highton 1972). However, Rucinsky and Cota-Robles (1974) showed in *Chromobacterium violaceum* that there are as many as four mesosomes. Also, there is now fairly general agreement that mesosomes are present in both Gram-positive and Gram-negative bacteria, and that the biochemical functions and physiological importance of bacterial mesosomes remain a mystery still (Greenawalt and Whiteside, 1975).
Fig. 4.11. TEM view of a transverse section through the hindgut (ileum) of *P. truncatus*.

Note bacteria embedded in their microbial film (MB), lumen (L), mesosome-like structures (arrowed). Scale bar = 1 μm.
Fig. 4.12. TEM view of a transverse section through the hindgut (ileum) of *P. truncatus*.

Note bacteria embedded in their microbial film (MB), lumen (L), mesosome-like structures (arrowed). Scale bar = 1 μm.
Figure 4.13. TEM view of a transverse section of the hindgut (ilium) of *P. truncatus*. Bacterium during binary fission. Note mesosome-like structures (arrowed).

Scale bar = 0.5 μm.

Figure 4.14. TEM view of a transverse section of the hindgut (ilium) of *P. truncatus*. Bacterium after binary fission. Note mesosome-like structures (arrowed).

Scale bar = 0.5 μm.
Figure 4.15. TEM view of a transverse section of the hindgut (ilium) of *P. truncatus*.

Bacterium. Note mesosome-like structure (arrowed). Scale bar = 0.3 μm.
4.3.8 Methanogenic Microorganisms

Adult and larval hindguts of *P. truncatus* from Tanzania showed microorganisms with the typical colour of methanogens (pale green), because of the high content of the fluorescent coenzyme F₄₂₀ (Figure 4.16). These kinds of microorganisms are associated with the methanogenic process of the anaerobic cellulose decomposition by cellulolytic bacteria (Brock et al., 1994, pp. 623-691).

Figure 4.16. Fluorescence of a natural methanogens community colonizing the *P. truncatus* adult and larval guts from the Tanzanian strain.
4.4 Discussion

This study shows the presence of particular cellulolytic bacteria (*Alcaligenes faecalis*, *Pseudomonas fluorescens* var. cellulose, and *Chromobacterium violaceum*) in the hindgut of *P. truncatus* from the strain originally collected in Tanzania, but not in the other two insect strains. To our knowledge, this is the first time that cellulolytic bacteria have been found in the larger grain borer, in any other Bostrichid, or any other pest of stored grain.

Different organisms were identified from the adult and larval guts of the three strains of *P. truncatus*. The three insect strains were reared on the same kind of maize prior to and during the experiment, which suggests that the bacteria reported are not due to contamination, but were present when the beetles were collected from the wild.

Bacteria isolated from the adult and larval guts of *P. truncatus* which were not able to digest cellulose during their identification, could be due to the presence of some molecules of carbon liberated into the culture medium during its sterilization from the carboxymethyl cellulose.

Bacteria identified with cellulolytic activity are facultative aerobes with the ability to survive at any pH conditions of the gut, that is, in the fore, mid, and hindgut. The other bacteria identified (*Flavobacterium multiborum*, *Capnocytophaga* sp., *Streptococcus* sp., *Klebsiella* sp., *Flavobacterium* sp., and *Acinetobacter* sp.) may be restricted to the fore, mid, and in the first third of the hindgut or ileum.

Electron microscopy studies show that bacteria in the gut of *P. truncatus* are not transitory along the gut, but are embedded in a microbial biofilm at the ileum. This microbial biofilm has natural resistance to surfactants, phagocytosis, and antibiotic therapy (Costerton et al., 1995). Therefore, according to Koch (1967), this biofilm could be the
means that LGB has for transferring its bacteria from generation to generation, but how does it work?

Cellulolytic bacteria could help to explain why populations of this insect are able to survive in a wide range of different plant environments, because they are able to feed on different types of carbohydrates, including starch and cellulose. This ability allows them successfully to establish as pests in a large variety of crops and also allows them to maintain populations on different hosts from one maize season to the next.

There are important implications of the presence of cellulolytic bacteria in *P. truncatus* on maize storage. Farmers in many African countries as well as some places in Mexico and Central America, store their maize in wooden structures, which can serve as refuges for *P. truncatus*, making it difficult to use preventive or corrective actions against this beetle.

There is also evidence that *P. truncatus* can live and breed in trees or other hosts long distances from stored maize (Herrera et al., 1989 & 1991; Rees et al., 1990; Rios, 1991; Tigar et al., 1994a). The new results found here suggest that the presence of cellulolytic bacteria in one of the three insect strains could play an important role in expanding host range. This raises two questions: Why are cellulolytic bacteria not found in all strains? and how are bacteria present in a biofilm in the ileum transmitted to young insects?.

It is interesting that the Tanzanian strain, shown here to contain cellulolytic bacteria, also showed a different pattern of proteolytic isoenzymes revealed by electrophoresis. We do not know if the proteolytic activity is produced by the insect or associated with gut microflora, but this additional difference in the Tanzanian strain could support the suggestion that *P. truncatus* may have been introduced into East and West Africa from different sources and is consistent with the findings of Nissen et al. (1991).
5. GENERAL DISCUSSION

The spread of *P. truncatus* from some parts of America to both East and West Africa has brought it to the attention of scientists from different parts of the world and it has been the subject of a large number of studies (Bøye, 1988 and 1990; Cave, et al., 1990; Cowley et al., 1980; Delgado and Luna, 1951; Detmers, 1988; Detmers et al., 1990; Diané, 1993; Dobie, 1988; Dunstan et al., 1981, Giles and Leon, 1975; Golob and Hodges, 1982; Hardie and Whiley, 1992; Harnisch and Krall, 1984; Herrera-Rodríguez et al., 1987 and 1991; Hodges et al, 1983, Hodges, 1984 and 1986; Hodges and Meik, 1984; Hodges et al., 1985; Houseman and Thie, 1993; Howard, 1983; Keil, 1988; Krall, 1984 and 1987; Mushi, 1984; Nan’a yo et al., 1993; Nissen et al., 1991; Novillo, 1991; Pantenius, 1988; Ramírez, 1960; Ramírez et al., 1993; Rees, 1990; Rios-Ibarra, 1991; Rodriguez-Rivera and Herrera-Rodríguez, 1989; Sandoval-Cardoso, 1991; Shires and Xavier-Filho, 1991; Tigar et al., 1994a and 1994b; Wright, 1984). This beetle has a broad distribution in Mexico and Central America and is found in a range of different climatic conditions, crops different from maize and even in regions away from crops, though it is generally considered to be a minor pest of stored maize in the rural areas of Mexico (Delgado and Luna, 1951; Ramírez, 1960; Giles and Leon, 1975; Wright, 1984; Hoppe, 1986; Dobie, 1988). In Mexico *P. truncatus* can exist in the company of other primary pests of stored maize, such as *Sitophilus zeamais/oryzae* and *Sitotroga cerealella*, although its highest levels have a strong connection with very dry climatic conditions (Herrera et al., 1989 and 1991; Rees et al., 1990; Rios, 1991; Tigar et al., 1994a and 1994b).

In Africa this beetle has been introduced by accident, probably from either Mexico (Mushi, 1984; Nissen et al., 1991) or Central America (Diané, 1993) producing high levels of maize losses in African rural areas. *P. truncatus* has become a dangerous pest of...
maize and dry cassava in Africa and it has even been detected in the wooden structures made for storing maize and for dwellings. In these conditions, the other primary pests of maize, *Sitophilus* spp. and *Sitotroga cerealella*, have progressively been replaced by *P. truncatus* (Dustan and Magazini, 1981; Golob and Hodges, 1982; Krall, 1984; Harnisch and Krall, 1984; Krall, 1987; Anonymous, 1991).

Among the Bostrichidae, there are many species that are considered to be wood borers, such as *Xylobiops parilis* L. (Figure 1.5), which resembles LGB in appearance and is normally found in the fauna of Guanajuato state, Mexico (Fisher, 1950). However, there is no evidence that *X. parilis* or other Bostrichids digest cellulose, nor that digestion may be carried out by the enzymes secreted by insects or with the collaboration of symbionts (Martin, 1991). The host range of *P. truncatus* will be affected by its ability to digest cellulose, and it was therefore important to consider whether LGB is able to digest cellulose using either its own enzyme capacity or with the collaboration of cellulolytic microorganisms.

There is good evidence that only three insects, two higher termites and one Australian cockroach, have cellulose digestion independent of symbionts, and most insects that have the capacity to digest cellulose have been considered to be dependent on symbionts, such as protozoa, bacteria or fungi (Martin, 1991). In these studies it was found that *P. truncatus* has the ability to digest cellulose, not through its own enzymatic cellulolytic system, but via symbiotic bacteria in the hindgut.

Another fact that supports the idea that cellulose digestion may take place in the hindgut of *P. truncatus* is the presence of methanogenic microorganisms (Figure 4.16). Methanogens are known to be present in the mammalian intestinal tract, in the guts of wood-eating insects, and in most anoxic habitats, producing methane (CH₄) from the
hydrogen (H₂) and carbon dioxide (CO₂) liberated during cellulose hydrolysis (Brock, et al., 1994, pp. 623-691).

The anatomical study of the digestive system of *P. truncatus* showed that there are external differences between adults and larvae, which feed on the same host but with different feeding habits (adults have to perforate the pericarp, while larvae do not). The presence of the regenerative crypts in the midgut of the adult (Figures 2.5 and 2.7), but not the larva (Figures 2.6 and 2.8), is explicable because this beetle in the adult stage has to be more prepared for a long span of life, which in appropriate conditions could be a year, while the span of the larval stage is short compared to the adult. The adult stage needs to cope with long-term nutritional requirements and has to rely on its regenerative crypts for the constant renovation of its midgut epithelial cells.

With the exception of the regenerative crypts in the adults (Figures 2.11, 2.12, and 2.13), the convoluted line of glycogen-like granules surrounding the midgut (Figures 2.15, 2.16, and 2.17), and the peritrophic membrane (Figures 2.21, 2.22, and 2.23), both adult and larva have the same internal structure with different degrees of development. The peritrophic membrane probably prevents damage to the epithelial cells of the adult midgut by pericarp fragments.

The absence of the peritrophic membrane in the midgut of the larval instar (Figures 2.18 and 2.19) may be considered as a potential weakness in LGB and could be used in a novel control strategy. For example, the peritrophic membrane is composed mainly of chitin, and genetically transformed plants of maize could be used to produce chitinase, either natural or microbial, to inhibit the synthesis of the peritrophic membrane during the larval stage. The adult would then suffer damage to the gut through abrasion by either fragments of grain pericarp or another hard material. These genetically-transformed plants of maize could also have natural protection against other pests with
the same larval feeding habits, such as *Sitophilus* spp. and *Sitotroga cerealella*, providing that their larval stage lacks peritrophic membrane.

The role of the glycogen-like granules, absent in the larva, is unknown, although they could be there to meet the energy requirements of the adult when food is scarce or to be associated in the metabolic processes of the flight muscle (Sacktor, 1975). The flight of an insect involves very rapid oxidation of respiratory fuels by the flight muscle; clearly, in order to maintain very high rates of respiration, the flight muscles must be well supplied with both oxygen and the appropriate fuel, such as glycogen, trehalose, and glucose (Bailey, 1975).

The excretory system in the hindgut is present in both adult (Figure 2.9) and larval (Figure 2.10) guts, though in different structures. The presence of this excretory system or cryptonephric system in LGB may explain why this beetle survives in very dry conditions, as in the north of Mexico, and is present in higher proportions than competitors when the climate conditions in temperate zones, as in the central part of Mexico, become more dry. This system is dealing with certain chemical substances, such as uric acid or urates (Cochran, 1975), which may explain the lowest pH values (4.2-4.6) found in this part of the hindgut and, therefore, the absence of microorganisms in that region.

The enzymatic study shows that there are no differences between the pH values of the *P. truncatus* adult and larval guts (Figures 3.1 and 3.2). There are two well defined pH regions in both; the first with pH 6.2 includes the fore, mid and the first third of the hindgut, while the rest of the hindgut has pH 4.2-4.6. This means that most of the enzymatic activity is carried out at around pH 6.2 in both insect stages. The pH values found in the gut of LGB are in accordance with those reported for other pests of stored grains, such as *Rhyzopertha dominica, Trogoderma versicolor, Oryzaephilus mercator,*
and *Tribolium confusum* (Table 3.1). Thus, most of the 10 species considered in Table 3.1 have in their hindgut pH values similar to those found in the hindgut of LGB. The pH differences in the fore, mid, and hindgut among the different species could be due to the methodology used.

The total passage time of the food bolus in the digestive system of *P. truncatus* was different among the adult and larval guts (Figures 3.1 and 3.2), being faster in the larval stage. This difference was mainly observed in the pH 6.2 region, where digestion takes place. The epithelial cells are less developed in the larval midgut (Figures 2.18 and 2.19), and the digestion process in the adult may be more efficient, taking more time to digest food of different chemical nature.

The passage time of the food bolus through the secretory system of *P. truncatus* was about the same for both adult and larval stages (Figures 3.1 and 3.2). As the perinephric system is important in extracting traces of water present in the bolus digested, LGB could take more time in carrying out this function than in its digestion process. The cuticle which lines the rectal tube appears relatively thicker (Figures 2.25, 2.26, 2.30, and 2.31), which may prevent damage because of the dryness of the food bolus as it passes through this section.

Enzymatic work about amylase activity has been carried out in some pests of stored grains in order to find a potential natural control. Most of the work done was the isolation, purification and partial characterisation of the amylases present in the digestive system of different insects, which were then confronted with inhibitors (Krishna, 1955; Krishna and Saxena, 1962; Applebaum, 1964; Baker, 1983; Baker, 1987; Campos, et al., 1989; Lemos, et al., 1990; Baker, 1991; Sandoval-Cardoso, 1991). However, with the data available it is difficult to compare among the amylase activity of the different insects, mainly because researches worked either with crude extracts of guts or purified enzymes.
or partially purified. For instance, Baker (1983) working with crude extracts of larval midguts of *Sitophilus* spp., found one amylase at 5.0 pH for *S. oryzae* and another at 4.75 pH for *S. zeamais*. The same author in 1987, using purified enzymes, demonstrated the presence of two amylases at 4.5 and 5.0 pH in *S. oryzae*. Sandoval-Cardoso (1991) working with partially purified enzymes reported one amylase at 4.5 pH in *S. zeamais* (Table 3.2). Then, it seems that both species *S. oryzae* and *S. zeamais* have one amylolytic isoenzyme at 4.5 pH.

The enzymatic study of the levels of α-amylase activity per gut (Figure 3.3) in the digestive system of *P. truncatus* showed that this activity is about three times larger in the adult stage than that of the larval instar and this was true for all three insect strains studied, although the strain from Togo had 20% less activity than the other two. The high level of activity found in the adult gut gives an idea of its higher capacity to digest starch.

The α-amylase activity shown by using zymograms (Figures 3.5A and 3.6A), demonstrated that there are similarities among the three strains. The α-amylase activity for adult and larva present two bands of isoenzymes. The isoenzyme bands of the adult stage are similar in intensity, while in the larval instar the upper isoenzyme was less intense than the lower band. These zymograms also demonstrate that there is more α-amylase activity in the adult stage than in the larval instar.

Enzymatic work about the proteinase activity of some pests of stored grains has also been carried out following the same scheme as the amylase activity (Krishna, 1955; Birk, et al., 1962; Krishna and Saxena, 1962; Baker, 1982; Murdok et al., 1984; Gatehouse, 1985; Kitch et al., 1986; Wieman et al., 1988; Campos et al., 1989; Lemos et al., 1990; Liang et al., 1991; Sandoval-Cardoso, 1991; Silva et al., 1991; Houseman et al., 1993; Blanco-Labra, 1996). But again, it is difficult to compare among the insects
reported. Nissen et al. (1991) compared populations of *P. truncatus* from Africa, Central America and Mexico separating their isoenzymes by iso-electric focusing, and concluded that populations were different, although there was some affinity between the populations from Africa and Mexico.

The levels of proteolytic activity measured as trypsin activity per gut in the digestive system of *P. truncatus* were different among the adult and larval stages (Figure 3.4). There were also differences among the three strains studied (Table 3.9). The Tanzanian strain had less proteolytic activity for the adult and larva than strains from Mexico and Togo. These differences among both stages show the high capacity of the adult insect to digest proteins.

The proteolytic activity reveal by zymograms (Figures 3.7A and 3.8A) also showed differences among the three insect strains. The Mexican strain had most proteolytic activity, presenting four bands of isoenzymes, whereas the strain from Tanzania had least proteolytic activity, showing two isoenzymes. These differences among the three strains from different geographic sources suggest that the strains from Tanzania (East Africa) and Togo (West Africa) may have different origins. Neither of the two African strains was very similar to the Mexican strain in proteolytic activity.

The microbiological study showed for the first time the presence of cellulolytic bacteria in the digestive system of *P. truncatus* adult and larval guts. These cellulolytic bacteria as well as methanogens were found only in the alimentary system of both adult and larva of the strain from Tanzania. Bacteria found in the digestive system of the other insect strains are also different. This study also showed that the bacterial fauna is located in the ileum of the LGB hindgut (Figures 4.11 to 4.15) and that these bacteria are not free in the digestive system but they are embedded in a microbial biofilm, which protects them from internal or external factors.
Cellulolytic activity in *P. truncatus* is a characteristic shared between Anobiidae and Bostrichidae families, which are phylogenetically similar. In *P. truncatus* the activity is associated with gut symbionts. Insects from the strain collected in Tanzania were alone in having methanogens, usually associated with decomposition of cellulose under anaerobic conditions.

The presence of cellulolytic bacteria in the gut of *P. truncatus* could explain the presence of this insect in fields where are cultivated crops different to maize, and even on other material away from any maize field. Also, these cellulolytic bacteria should be taken into account when predicting the future of *P. truncatus* as a potential pest of any crop.

The presence of different bacteria in the three strains studied and the conditions where they were found, supports the idea that these three strains are from different geographic zones, and that they have different origin. Thus, the introduction of *P. truncatus* to East and West Africa may have different sources.

There are several areas of this study that suggest where future work should be done. In order to determine if the whole bacterial fauna in the digestive system of the three strains is different, it would be necessary to use a nutrient medium instead of the restricted one here used. It is important to know how much of the enzymatic activity detected in the digestive system of *P. truncatus* is from the bacteria present in the hindgut. Because the proteinase activity was different among the three geographical insects, it would be interesting to determine whether this activity is affected by the presence of bacteria in the hindgut and how they are involved in the digestive process of LGB.

If the difference in bacteria and protease activity in the Tanzanian strain is important in determining host range, there could be implications for the spread of and the damage done by *P. truncatus*. More geographical work should be therefore done in order to assess whether the differences found here between bacteria and the protease activity in
the three strains differ consistently between different populations of the same geographical region. For instance, an exhaustive study could be done in Mexico to examine variation in bacterial fauna and protease activity within the whole range of conditions where *P. truncatus* is found in Mexico, as well as in maize stores.

Some of the differences in enzyme activity may represent polymorphism in the insects, and the basis of the differences found in the three strains of LGB needs to be established. For example, the PCR (Polymerase Chain Reaction) technique could be used to characterise the DNA profiles of the three strains studied, and in other strains.

This study has established that there are differences between the three geographical strains of *P. truncatus*. Whether these differences are inherited or acquired (e.g. gut fauna), and whether they represent “random” differences between strains reared in the laboratory or differences that are consistent between geographical populations, can only be established by further studies.

Finally, the answers found during this study to the questions posed in the section 1.4 are:

1. There is no evidence from the small number of insect strains studied here that *P. truncatus* was introduced into Africa from Mexico. This negative conclusion is based on the differences found in the proteolytic activity and the bacteria present in the digestive system of LGB from the three different strains studied.

2. It also seems that populations from Togo and Tanzania may have different origins because of the above differences.

3. It was found that *P. truncatus* from Tanzania has the capacity to digest both starch and cellulose, but this capacity was not found in strains from Mexico and Togo.
4. The presence of cellulolytic bacteria and methanogens in the Tanzanian strain suggests that cellulolytic digestion in *P. truncatus* may be carried out with the collaboration of symbionts.

5. The cellulolytic capacity to digest cellulose is shared by both Anobiidae and Bostrichidae families.

These answers should not be considered as conclusive because this study was carried out with only three geographical strains of *P. truncatus*, but they are consistent with the results obtained by other researchers.
APPENDIX 1: Preparation of Insect Material

Insects

_P. truncatus_ adults were maintained in 2 litre containers with maize (cacahuazintle race) at room temperature (approximately 27°C and 65% r. h.). Three strains of _P. truncatus_ were used, which came from Mexico, Tanzania, and Togo. The strains from Tanzania and Togo came originally from the Natural Resources Institute (NRI), Chathan, UK. The Mexican colony was captured in the surroundings of the Center for Research and Advances Studies (CINVESTAV), Irapuato, Mexico. _P. truncatus_ larvae were developed using 20 adult insects in 250 ml containers with a mixture of 20 g of maize (cacahuazintle race), and 10 g of cacahuazintle flour at the above conditions (Howard, 1983).

Dissection of _P. truncatus_ adult gut

The adult gut of _P. truncatus_ was dissected following the next steps:

1) Adult insect was put on a dissection glass with a drop of insect Ringer solution (Appendix 2).

2) Elytra and wings were separated with insect pins.

3) Head was fastened with tweezers and hindgut was exposed from the last abdominal segment with insect pins.

4) Head was cut off with insect pins and the entire gut was carefully pulled from the abdominal end with tweezers.

5) Gut extraction was facilitated when the abdomen was separated from the rest of the body once head was cut off.
Dissection of *P. truncatus* larval gut

The larval gut of *P. truncatus* was dissected following the next steps:

1) Larva was put on a dissection glass with a drop of insect Ringer solution (Appendix 2).

2) Head was pinned and the hindgut was exposed from the last abdominal segment with insect pins.

3) Head was cut off with insect pins and the entire gut was carefully pulled from the abdominal end with tweezers.

4) Gut extraction was facilitated when the abdomen was gently pressed from the head to the last segments once head was cut off.
APPENDIX 2: Preparation of Chemical Solutions and Gels

1% acetic acid

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>1 g</td>
</tr>
<tr>
<td>Deionized water</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

Acidic I-KI

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-KI</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>1M HCl</td>
<td>12.5 ml</td>
</tr>
<tr>
<td>Deionized water</td>
<td>250 ml</td>
</tr>
</tbody>
</table>

0.5% Agarose

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agarose</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Deionized water</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

0.1% AgNO₃

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>AgNO₃</td>
<td>0.1 g</td>
</tr>
<tr>
<td>Deionized water</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

2% casein

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>2 g</td>
</tr>
<tr>
<td>0.04 M succinic acid buffer, pH 6.5</td>
<td>100 ml*</td>
</tr>
</tbody>
</table>

* Casein was dissolved in 10 ml of boiling 0.04 M succinic acid buffer, pH 6.5 and then, after cooling, adjusted to 100 ml with the same buffer.

Dubos solution, pH 6.0

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>K₂HPO₄</td>
<td>1.0 g</td>
</tr>
<tr>
<td>NaNO₃</td>
<td>0.5 g</td>
</tr>
<tr>
<td>MgSO₄·7 H₂O</td>
<td>0.5 g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.5 g</td>
</tr>
</tbody>
</table>
**F eS 0 F H2O**  0.01 g
**Deionized water**  1,000 ml

**Dye reagent concentrate**
- **Coomassie Brilliant Blue G-250**  100 mg
- **Ethanol 95 %**  50 ml
- **Phosphoric acid 85 %**  100 ml

**Electrode buffer, pH 8.3**
- **Tris**  15 g
- **Glicina**  72 g
- **SDS**  5 g
- **Deionized water**  1000 ml

**Electrophoretic 10 % resolving gel**
- **Deionized water**  4.02 ml
- **1.5 M Tris-HCl, pH 8.8**  2.5 ml
- **10 % sodium dodecyl sulfate (SDS)**  100 μl
- **30 % acrylamide-bis**  3.33 ml
- **10 % ammonium persulphate**  50 μl
- **0.1 % N,N,N',N'-tetramethylethylenediamine (TEMED)**  5 μl

**Electrophoretic 12 % resolving gel**
- **Deionized water**  3.35 ml
- **1.5 M Tris-HCl, pH 8.8**  2.5 ml
- **10 % sodium dodecyl sulfate (SDS)**  100 μl
- **30 % acrylamide-bis**  4.0 ml
- **10 % ammonium persulphate**  50 μl
- **0.1 % N,N,N',N'-tetramethylethylenediamine (TEMED)**  5 μl
**Electrophoretic 4 % stacking gel**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deionized water</td>
<td>6.1 ml</td>
</tr>
<tr>
<td>0.5 M Tris-HCl, pH 6.8</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>10 % SDS</td>
<td>100 µl</td>
</tr>
<tr>
<td>30 % acrylamide-bis</td>
<td>1.3 ml</td>
</tr>
<tr>
<td>10 % ammonium persulphate</td>
<td>50 µl</td>
</tr>
<tr>
<td>0.1 % TEMED</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

**10% ethanol**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>10 ml</td>
</tr>
<tr>
<td>Deionized water</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

**Ethanol-acetic acid**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>30 ml</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>10 ml</td>
</tr>
<tr>
<td>Deionized water</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

**0.02% formaldehyde (37%)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formaldehyde (37%)</td>
<td>0.02 ml</td>
</tr>
<tr>
<td>Deionized water</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

**I-KI**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>I&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.5 g</td>
</tr>
<tr>
<td>KI</td>
<td>5 g</td>
</tr>
<tr>
<td>Deionized water</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

**Insect Ringer solution, pH 7.0**

<table>
<thead>
<tr>
<th>Component</th>
<th>Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>2.288 g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.0503 g</td>
</tr>
<tr>
<td>CaCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.0502 g</td>
</tr>
<tr>
<td>Component</td>
<td>Amount</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>--------------</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>0.025 g</td>
</tr>
<tr>
<td>Deionized water</td>
<td>250 ml</td>
</tr>
<tr>
<td><strong>3% Na₂CO₃</strong></td>
<td></td>
</tr>
<tr>
<td>Na₂CO₃</td>
<td>3 g</td>
</tr>
<tr>
<td>Deionized water</td>
<td>250 ml</td>
</tr>
<tr>
<td><strong>Sample buffer</strong></td>
<td></td>
</tr>
<tr>
<td>Deionized water</td>
<td>4.0 ml</td>
</tr>
<tr>
<td>0.5 M Tris-HCl, pH 6.8</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.8 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>1.6 ml</td>
</tr>
<tr>
<td>2-β-mercaptoethanol</td>
<td>0.4 ml</td>
</tr>
<tr>
<td>0.05% bromophenol blue</td>
<td>0.2 ml</td>
</tr>
<tr>
<td><strong>0.125% starch</strong></td>
<td></td>
</tr>
<tr>
<td>Starch</td>
<td>0.125 g</td>
</tr>
<tr>
<td>Deionized water</td>
<td>100 ml*</td>
</tr>
<tr>
<td>* Starch was dissolved in 10 ml of boiling deionized water and then, after cooling, adjusted to 100 ml with 0.04 M succinic acid buffer, pH 6.5.</td>
<td></td>
</tr>
<tr>
<td><strong>0.5% starch</strong></td>
<td></td>
</tr>
<tr>
<td>Starch</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Deionized water</td>
<td>100 ml*</td>
</tr>
<tr>
<td>* Starch was dissolved in 10 ml of boiling deionized water and then, after cooling, adjusted to 100 ml deionized water.</td>
<td></td>
</tr>
<tr>
<td><strong>0.04 M succinic acid buffer, pH 6.5</strong></td>
<td></td>
</tr>
<tr>
<td>Succinic acid</td>
<td>1.180 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.876 g</td>
</tr>
<tr>
<td>Buffer Type</td>
<td>Components</td>
</tr>
<tr>
<td>-----------------------------------</td>
<td>--------------------------------------</td>
</tr>
<tr>
<td><strong>0.15 M Tris-(hydroxy-methyl)aminomethane (Tris) buffer, pH 8.1</strong></td>
<td>CaCl$_2$</td>
</tr>
<tr>
<td></td>
<td>Deionized water</td>
</tr>
<tr>
<td></td>
<td>Tris</td>
</tr>
<tr>
<td></td>
<td>CaCl$_2$.2H$_2$O</td>
</tr>
<tr>
<td></td>
<td>Deionized water</td>
</tr>
<tr>
<td><strong>0.5 M Tris-HCl, pH 6.8</strong></td>
<td>Tris</td>
</tr>
<tr>
<td></td>
<td>Deionized water</td>
</tr>
<tr>
<td>* 1N HCl was used to adjust pH.</td>
<td></td>
</tr>
<tr>
<td><strong>1.5 M Tris-HCl, pH 8.8</strong></td>
<td>Tris</td>
</tr>
<tr>
<td></td>
<td>Deionized water</td>
</tr>
<tr>
<td>* 1N HCl was used to adjust pH.</td>
<td></td>
</tr>
</tbody>
</table>
APPENDIX 3: Microbiological Techniques

Gram Staining

A loopful from the pure cultures was spread in thin film over a slide, dried in air, and fixed by passing the slide through flame. The slide was flooded with crystal violet solution for 1 min and immediately flooded with iodine solution for 3 min, and then decolorized for about 20 sec with ethanol. Gram-positive cells are purple and Gram-negative cells are colourless. Finally, the slide was flooded with safranin solution for 1 to 2 min and then washed with tap water. Gram-positive cells remain purple and Gram-negative cells are red. A drop of oil was placed on the slide, which was examined by using a light microscope with 100x objective (Brock et al., 1994, pp. 43-88).

Streak Plate

Cultures in which growth has taken place can be transfered to the surface of agar plates, where strains develop from the growth and division of single cells. Picking and restreaking from the isolated colony is a major method of obtaining pure cultures. The method of making a streak plate to obtain pure cultures is as follows:

1. A loopful of inoculum is removed from test tube.
2. Streak is made over a sterile agar plate, spreading out the organisms as it is showed in Figure 1.
Spread Plate

The method for evenly spreading of bacterial cultures (Figure 2) used during the susceptibility to antibiotics is as follows:

1. Sample is pipetted onto surface of agar plate.
2. Sample is spread evenly over surface of agar using sterile glass spreader.
3. Incubation.
4. Typical spread-plate results.

Figure 1. Streaked plate before incubation.

Figure 2. Spread plate method.
**Antibiotic Inhibition.**

The distribution of antibiotics and inhibition haloes is shown in Figure 3.

![Inhibition haloes](image)

Figure 3. Sensitivity to some antibiotics indicated by zones of inhibition around discs.
REFERENCES


Bell, R. J. and F. L. Watters. (1982). Environmental factors influencing the development and rate of increase of *Prostephanus truncatus* (Horn) (Coleoptera: Bostrichidae) on stored maize. *Journal of Stored Products Research* **18**: 131-142.


*Biochemical Journal* **56**: 86-96


New York.


(Prostephanus truncatus (Horn)). Folleto Misceláneo No. 4, 426 pp. Secretaria de

Grossen Kornbohrer Prostephanus truncatus (Coleoptera: Bostrichidae).
Diplomarbeit, Freie Universität Berlin.

Investigation on the development capability of Prostephanus truncatus (Horn)
(Coleoptera: Bostrichidae) on different types of wood. In Fifth International

Nahrungssubstrate für den Großen Kornbohrer, Prostephanus truncatus (Horn)

on the Containment and Control of the Larger Grain Borer, Shulten, G. G. M. &


Dufour, L. (1834). Recherches anatomiques et cinsidérations entomologiques sur
quelques insectes Coléopteres. Annales des Sciences Naturelles. B. Zoologie 1: 56-
84.


Enzyme Nomenclature. (1978). Recommendations of the Nomenclature Committee of the
International Union of Biochemistry on the Nomenclature and Classification of


