

INTERACTIONS OF ENTERIC BACTERIA WITH AMERICAN COCKROACHES
(*Periplaneta americana*) AND PHARAOH ANTS (*Monomorium pharaonis*)

By

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by

Deanna D. Branscome

This dissertation is dedicated to my Guardian angels: my Mother, Kathleen Duvall and my Grandmother, Florence Galek. I would also like to dedicate this work to Mischa, my vocal Russian Blue, Rosie, my wonderful canine companion and to Princess, the wonderdog whom I knew for too short a time.

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Microscopic examination of cross sectioned American cockroach pronota revealed the following structures from top to bottom: an external wax layer (~1 to 3 μm), an inner exocuticle (~2 μm), a lamellate sponge-like endocuticle (~24 μm), a gap (~2 μm) and an inner/ventral layer of transparent cuticle (~6 μm). Additionally, globular material between the dorsal and ventral cuticle was revealed, between which were two brown ovoids, identified as attachment points for the prothoracic legs.

Interaction of cuticle with enteric bacteria was also investigated. Pronota of American cockroaches were inoculated with 6.0×10^5 cells and incubated for 24, 48 and 72 h. No *Salmonella enterica* were recovered from pronota processed in deionized water. However, viable cells were recovered from the stock *S. enterica* solution at all time periods.

Scanning electron microscopy of pronota and tarsi fixed with osmium tetroxide vapors revealed few (<60) bacteria on pronota and none on tarsi. However, on one

pronotum, bacteria were observed underneath the wax layer. Another set of specimens was fixed with glutaraldehyde, removing significant amounts of wax from cuticular surfaces. However, thousands of *S. enterica* cells remained on epicuticle surfaces and within remaining wax.

Comparative attachment of *S. enterica*, *Escherichia coli* (*E. coli*) O157:H7 and non-pathogenic (NP) *E. coli* on pronota of American cockroaches was investigated, as was recovery of viable *S. enterica* from adult male American cockroaches and Pharaoh ant colonies over time. At an inoculum of 2.5×10^5 , *S. enterica* attached to epicuticle more effectively than either of the *E. coli*, with *E. coli* O157:H7 attaching more effectively than NP *E. coli*. At an inoculum of 1.0×10^5 , no bacteria were observed on any pronota.

Recovery of viable *S. enterica* cells from American cockroach pronota was dependant on inoculum dose, with greatest recovery across all time periods (24 to 120 h) occurring at an inoculum of 9.0×10^5 , with half the number of viable *S. enterica* recovered from cockroaches inoculated with 4.5×10^5 cells. *Salmonella enterica* recovery from Pharaoh ant workers and brood occurred across all time periods (3, 10 and 17 d) without significant decline in viability.

CHAPTER 1 INTRODUCTION AND LITERATURE REVIEW

American cockroaches, *Periplaneta americana* (L.), and Pharaoh ants, *Monomorium pharaonis* (L.), are often found in close association with humans, living in homes, businesses and health care facilities. The intimate association of American cockroaches and Pharaoh ants with humans has resulted in their relegation to pest status. Additionally, cockroaches and ants are perceived to be aesthetically offensive, with cockroaches especially perceived to be loathsome creatures (Rau 1924).

Although capable of flight, American cockroaches are primarily crawling insects, whereas Pharaoh ants are motile exclusively via crawling or by inadvertent distribution by humans (Edwards 1986). Because of their association with human environments, both American cockroaches and Pharaoh ants may incidentally contact and acquire human pathogens. Pathogens may then subsequently be mechanically transmitted to humans or to susceptible artifacts or surfaces which may then function as fomites or vehicles for indirect transmission.

It is because of their potential to mechanically transmit disease that the pathogens associated with American cockroaches and Pharaoh ants have been investigated. In particular, American cockroaches have been numerous linked to enteric bacteria such as the *Salmonellae* (Kopanic et al. 1994, Roth and Willis 1957, 1960), with fewer investigations revealing associations of Pharaoh ants with *Salmonella dublin* (Beatson 1972) and other bacteria (Granovsky and Howell 1983, Hughes et al. 1989). In general, the variety and types of bacteria isolated from the cuticle of crawling insects will depend

on the foraging and movement patterns of the insects themselves, as well as the types of bacteria that are present in the environment.

Insect cuticle exposed to the environment is a physical barrier against microbes and “presents an inhospitable environment for microbial development” (Boucias and Pendland 1998, p. 8). The dominant hydrocarbons of the cuticle are alkanes that do not provide suitable nutrition for microorganisms, and in some insect species, the cuticular lipids may act as antimicrobials (Boucias and Penland 1998). However, because human enteric pathogens have been cultured from the cuticle of American cockroaches and Pharaoh ants, the environment of the cuticle may not be immediately detrimental to their survival. Enteric bacteria are notoriously hardy and *Escherichia coli* can remain viable “for 2 months on a dry stainless steel work surface, and 2 to 5 months in natural bodies of water” (Black 1999, p. 645). Therefore, study of the American cockroach cuticle and the interaction of the cuticle with enteric bacteria could help elucidate the efficiency and effectiveness of their role as mechanical vectors of disease.

Salmonella cells are approximately 1-5 μm in size and would be prevented from passing through the alimentary canal of Pharaoh ant workers by their buccal tube filter (Petti 1998). Therefore, transmission of enteric bacteria by Pharaoh ant workers would be considered strictly mechanical via the cuticle, being transferred from contaminated cuticle surfaces rather than in contaminated feces. However, once transferred to the colony by infected foragers, enteric bacteria could be maintained within harborage areas and amongst brood and queens, creating a possible infection reservoir. Therefore, study of the prevalence and persistence of *Salmonella* within Pharaoh ant colonies could assist understanding of their potential role in disease transmission.

American Cockroaches

Origin and Distribution

American cockroaches are reported to have originated in the tropics of Africa from where they have been distributed throughout the world (Bell and Adiyodi 1981). The spread of American cockroaches was originally facilitated by international ship transport, possibly by European sailors (Kevan 1979) or as an artifact of human slave trade (Rehn 1945). On ships, American cockroaches have found suitable living conditions from which they have been distributed with cargo to new habitats.

In the United States, American cockroaches are intimately associated with human sewage and sewer facilities from which they will enter bathrooms and basements (Brenner et al. 1987). They also occur in “restaurants, grocery stores, bakeries, meat-packing plants and other places where food is prepared or stored” (Bell and Adiyodi 1981, p. 3).

Biology

American cockroaches are a relatively long-lived species. From 1st instar to adult, individuals may live for well over a year. After the molt to adulthood, females will mate within a period of one week, and will produce a sclerotized ootheca containing ~16 young (Bell and Adiyodi 1981). When fully developed, the oothecae will often be glued to a suitable substrate utilizing the saliva of the mother mixed with particles removed from the substrate to both secure the ootheca and provide camouflage. However, ootheca may also be deposited directly onto a surface, without the benefit of protective camouflage (Cornwell 1976, Fig. 79). After hatching, nymphs will undergo between 6 to 14 molts (Bell and Adiyodi 1981) over a period of several months (Barcay 2004) depending on environmental conditions.

Medical Importance

There are two primary reasons that cockroaches are cited as being detrimental to human health. First, cockroaches are a major source of allergens which cause asthma. Sensitivity to cockroach antigens was determined by exposing asthmatic adults and children to respiratory or dermal contact with cockroach feces or cuticle particulates (Bernton and Brown 1967, 1969, Bernton et al. 1972, Kang 1976, Kang and Chang 1985, Mendoza and Snyder 1970, Picone et al. 1975, Schulaner 1970). It was found that specific cockroach antigens were responsible for the dermal and/or respiratory reactions in people sensitized to cockroaches. Cockroach antigens were categorized according to their molecular weight, and it was determined that the heavier the antigen, the more definitive the allergic reaction (Chang et al. 1983, Twarog et al. 1977).

A comprehensive study of the role of cockroaches in asthma and allergies evaluated 476 children from inner-city areas diagnosed as asthmatics. The researchers challenged the children with cockroach allergens and determined that 36.8 percent were allergic to cockroaches (Rosenstreich et al. 1997), specifically the German cockroach allergens, Bla g 1 and Bla g 2, which continue to pose a health threat in low-income housing (Arbes et al. 2004).

Another major reason that cockroaches, primarily American cockroaches, are cited as detrimental to human health is their often close proximity to human fecal material (Eads et al. 1954, Schoof and Siverly 1954) and associated pathogens. Two references frequently cited as evidence that American cockroaches captured from the wild are carriers of a multitude of pathogens are Roth and Willis (1957, 1960). Roth and Willis (1957, p. 7) state that “except for parasites for which cockroaches are intermediate

hosts, the disease organisms are transmitted mechanically in the insect's feces, in its vomitus, and on its legs and body.”

There have been several important laboratory studies which have specifically demonstrated how *Salmonella* spp. may be potentially vectored via feces of infected cockroaches and/or by mechanical transmission via contaminated cuticle:

1) American cockroaches were fed human feces infected with four serogroups from two species of *Salmonella*: *S. typhimurium* and *S. montevideo*. It was found that recovery of *S. typhimurium* strain 5609 from the alimentary canal of cockroaches occurred consistently at both 2 and 7 d after ingestion. Whereas, recovery of *S. montevideo* occurred only at the highest ingested dose (3.0×10^5) for the B-33 strain (Jung and Shaffer 1952).

2) Olson and Rueger (1950) reported that *S. oranienburg* remained viable in the feces of an American cockroach for 85 and 199 d at humidities of 52 to 56 and 31% respectively, and on experimentally inoculated pronota for up to 78 d after inoculation. Additionally, when infected feces of American cockroaches were inoculated onto dried food products and glass surfaces, “survival time at room temperature was as follows: on corn flakes, 3 yrs 3 mo; on crackers, more than 4 yrs 3 mo; and on glass slides, more than 3 yrs 8 mo” (Rueger and Olson 1969, p. 538).

3) To demonstrate actual transmission, Kopanic et al. (1994) allowed American cockroaches to feed on a *S. typhimurium* infected food source. The cockroaches were then placed with other non-infected individuals. Cockroaches, food and water were then tested and were positive for *S. typhimurium* at 24, 48, 72 and 96 h. Methods of transmission were speculated to have been either through ingestion of infected feces or

food, contact with contaminated cuticle or ingestion of the contaminated water supply. Other experiments demonstrated that infected cockroaches could also contaminate eggs during a 24 h exposure period.

From these studies, it is clear that when relatively large amounts of inoculum are ingested, *Salmonella* can remain viable in the alimentary canal of American cockroaches for up to 7 d. If excreted, *Salmonella* may then remain viable in feces for up to 199 d, and for at least 4 yr when present in American cockroach feces on a dried cracker. However, although it was reported that *Salmonella* may be recovered from an experimentally inoculated American cockroach pronotum(a) for up to 78 d, the quantity of viable bacteria (colony forming units) was not reported, nor was the number of cockroaches that remained infected.

Overall, there is a dearth of information on how *Salmonella* spp. interacts with the cuticle. It is generally hypothesized that *Salmonella* is easily dislodged from cuticle which would therefore, not function as a long term reservoir. In order to test this hypothesis, it is important to understand the morphology, histology and function of the cuticle in mechanical transmission. However, “very little is known about any of the constituent layers [of the cuticle] except the wax” (Locke 1974, p. 143) especially for American cockroaches. To further complicate matters, there are conflicting reports on the exact components of the cuticle, and in particular, the epicuticle.

Cuticle

Cornwell (1968) reports that the overall thickness of the American cockroach cuticle is ~40 μm and composed of an inner, layered and pliable endocuticle, ~20-30

μm , an exocuticle, ($\sim 10\text{-}20 \mu\text{m}$) which is lamellate and melaninized. The outermost layer, the epicuticle, is hydrophobic and $\sim 2 \mu\text{m}$ thick.

Bell and Adiyodi (1981), writing in the book ‘The American Cockroach’, report that there are four distinct layers of the epicuticle: the inner and outer epicuticle and the wax and cement layers. However, much of their information was derived from investigations conducted on *Rhodnius prolixus* (Stal) (Hemiptera: Reduviidae: Triatominae). Dennell and Malek (1955) utilized chemical analysis and reported that the epicuticle of American cockroaches is composed of four distinct layers, the inner cuticulin (inner epicuticle), paraffin (outer epicuticle), wax and cement layers. However, Richards and Anderson (1942), utilizing transmission electron microscopy, report two layers of the epicuticle on the American cockroach pronotum, reportedly chemically distinct, but not corresponding to either a wax or cement layer.

It is widely accepted that the outer layer of the American cockroach cuticle is cement. The cement layer is reported to be a shellac-like substance covering a fluid and dynamic wax layer that is continuously sloughed and replenished, and which has been described as a “Vaseline like substance” (Beament 1955, p. 515). If the wax is indeed covered by a continuous, shellac-like substance, then bacteria contacting the surface would readily be dislodged, resulting in rapid mechanical transmission.

Pharaoh Ants

Origin and Distribution

As with many other pest insect species, Pharaoh ants are not native to the United States. The country of origin of Pharaoh ants is not definitively known. However, the

type specimen was described by Linnaeus from an individual collected in Egypt in 1758 (Klotz 2004).

Pharaoh ants have derived their pest status by living in close association with humans and within sensitive areas worldwide. Ebeling (1975, p. 267) reported that Pharaoh ants were widespread throughout the United States and Canada and that colonies have been found “in hotels, large apartment houses, grocery stores, and other places where food is commercially handled . . . indoors in wall voids, subfloor areas, attics, cracks, crevices, and behind wainscoting, baseboards, plaster, or mantles, under hearthstones, between flooring or in furniture.” Additionally, Pharaoh ants have been a problem in hospitals where they have been found feeding on humans, residing in clean linens and invading medical equipment (Beatson 1972, 1973). In general, Pharaoh ants prefer warm and inaccessible locations for their nests (Ebeling 1975)

Biology

Pharaoh ant colonies include queens, males, monomorphic workers and brood, to include the eggs, various staged larvae, prepupae and pupae. The complete life cycle of egg to adult has been estimated at ~38 d for workers and ~42 d for reproductives (Peacock et al. 1950), with eclosed workers living approximately 9-10 weeks and queens living much longer at ~39 weeks (Peacock and Baxter 1950). Colony size varies and may contain up to thousands of individuals (Peacock et al. 1955). Colonies breed year round, are polygynous and do not require mating flights for reproduction (Ebeling 1975).

Medical Importance

The primary medical significance of Pharaoh ants is their presence in hospitals (Beatson 1972) where they could potentially acquire and mechanically vector pathogens. Additionally, because Pharaoh ants prefer relatively warm nesting sites, ~27°C (Peacock

and Baxter 1949), acquired pathogens may survive within a colony for an indefinite period of time. Pharaoh ants have been found on intravenous fluid lines, underneath patient bandages and in tracheotomy tubes (Beatson 1973) and could therefore mechanically vector acquired pathogens.

Enteric Bacteria

Of the human bacterial pathogens, American cockroaches are most often associated with bacteria in the family Enterobacteriaceae, primarily those in the genus *Salmonella*. Of the enteric bacteria, there are two which are of contemporary medical concern: *Salmonella* spp. and *E. coli* O157:H7. Because species of *Salmonella* are very closely related to *E. coli* O157:H7 and non-pathogenic *E. coli* (NP *E. coli*), the route of acquisition and subsequent transmission by American cockroaches and Pharaoh ants would probably be similar. Therefore, it is useful to discuss the three groups of bacteria together.

Both *Salmonella* spp. and *E. coli* strains (to include *E. coli* O157:H7) are gram negative, facultatively anaerobic bacteria that are usually motile by means of peritrichous flagella. Both are also chemoorganotrophic, capable of either respiratory or fermentative metabolism, with an optimal reported growth temperature of 37°C (Holt et al. 1994).

Salmonella

There are >2,500 serovars of *Salmonella* that have been identified and are grouped into two (Holt et al. 1994) or three (Black 1999) species. Typhoid fever is a highly virulent enteric infection caused by *S. typhi*. However, most cases of Salmonellosis are caused by serovars of *S. enteritidis* and are characterized by intestinal pain, fever and diarrhea (Black 1999).

Normally Non-Pathogenic *Escherichia coli*

Whereas *E. coli* is usually non-pathogenic and a normal resident of human intestines, it can become a pathogen when introduced into a susceptible area of the body. For example, *E. coli* “is the causative agent in 80 percent of UTIs [urinary tract infections] . . . Poor hygiene . . . especially in females can introduce fecal organisms into the urethra” resulting in disease (Black 1999, p. 570). Other types of pathogenesis include infection of “the gallbladder, meninges, surgical wounds, skin lesions and lungs, especially in debilitated and immunodeficient patients” (Black 1999, p. 645).

Pathogenic *Escherichia coli*

The following information regarding the pathogenic *E. coli* has been derived primarily from the review article by Kaper et al. (2004). There are five primary categories for the intestinal disease causing *E. coli*: enteropathogenic *E. coli* (EPEC) with attaching and effacing histopathology, enterotoxigenic *E. coli* (ETEC) expressing heat-labile or heat-stable enterotoxins, enteroinvasive *E. coli* (EIEC) with expression of the type III secretion system, diffusely adherent *E. coli* (DAEC) “defined by the presence of a characteristic, diffuse pattern of adherence to HEp-2 cell monolayers” (p. 131) and enterohaemorrhagic *E. coli* (EHEC) resulting in either bloody or non-bloody diarrhea. The later category contains the strain *E. coli* O157:H7, first identified in 1982.

Escherichia coli O157:H7 differs from non-pathogenic *E. coli* in that it contains specific somatic (O) and flagellar (H) antigens that confer pathogenicity. The results of pathogenic gene expression in *E. coli* O157:H7 can cause hemolytic uremic syndrome (HUS) and/or thrombotic thrombocytopenic purpura (TTP) which may lead to renal failure and death. Infection with *E. coli* O157:H7 is especially dangerous for young children and the elderly.

In summary, American cockroaches and Pharaoh ants are crawling insect species that are ubiquitous within the urban environment. Also ubiquitous within the urban environment are pathogenic and non-pathogenic enteric bacteria. Contact of enteric bacteria with American cockroaches or Pharaoh ants would likely occur as a result of the insects' traversing a source of infection. Therefore, even if ingested from an infected food source or via grooming, the cuticle would be the initial point of contact for an enteric pathogen. After contact, the bacteria could subsequently be mechanically transmitted to humans.

Statement of Purpose

The goals of this research were to first, examine the cuticle of American cockroaches with scanning and transmission electron microscopy (SEM and TEM) to develop integrated visual documentation of its structure in relation to pathogen acquisition and transmission. Secondly, to document the presence, prevalence and distribution of *S. enterica*, *E. coli* and *E. coli* O157:H7 cells on the cuticle of American cockroaches via SEM. Third, to quantify the amount of *S. enterica* on the pronota of American cockroaches over time after experimental inoculation. Fourth, to determine the viability and percent recovery of *S. enterica* from Pharaoh ant colony workers and brood over time.

CHAPTER 2
EXAMINATION OF THE CUTICLE OF AMERICAN COCKROACHES BY
ELECTRON MICROSCOPY

Introduction

American cockroaches are ubiquitous throughout the United States and are often found living in close association with humans. Preferred habitats of American cockroaches are basements, sewer systems and commercial sewage facilities (Roth and Willis 1960). It is because of their association with sewage that American cockroaches are strongly implicated as mechanical vectors of enteric bacteria, primarily those in the family Enterbacteriaceae. Of the enteric bacteria, *Salmonella* spp. are those most frequently isolated from wild cockroaches (Roth and Willis 1957, 1960).

Mechanical transmission by cockroaches is indirect, being defined as when a disease organism is acquired from a contaminated surface and is then transferred without “development or multiplication” (Foil and Gorham 2000, p. 461) to a susceptible individual, food or food contact area. A major route of mechanical transmission involves the external surface of the cockroach cuticle. Therefore, increasing our understanding of the American cockroach cuticle may increase our understanding of their role as mechanical vectors.

The cuticle, to include sternites, tergites and pronota of American cockroaches has been studied by histological and chemical means (Binnington and Retnakaran 1991, Locke 1974, Nation 2001). Additionally, there have been studies of American cockroach cuticle with transmission (Richards and Anderson 1942) and scanning (Scheie et al.

1968) electron microscopy. However, a unified visual account of the superstructure of the cockroach cuticle has not been resolved.

A diagrammatic representation of a generalized insect cuticle is given by Noble-Nesbitt (1991, p. 256), composed from inside to outside of a basement membrane, epidermis, endocuticle, mesocuticle, exocuticle and epicuticle. The epicuticle is then further divided into an inner and outer epicuticle, wax layer and cement layer with wax blooms.

Similarly, the American cockroach cuticle has also been represented diagrammatically (Bell and Adiyodi 1981) as consisting of an innermost cellular epidermis, an endocuticle, exocuticle and an outermost epicuticle composed of four sections. According to Dennell and Malek (1955), the epicuticle is, from inside out, composed of an inner epicuticle or cuticulin layer, an outer epicuticle known as the paraffin containing layer, a wax layer and a cement layer. The protective wax and cement layers (external layers), due to their exposure to environment, are directly involved in interaction with pathogens. However, of the cuticular layers, the external layers have been particularly difficult to study by other than chemical means.

The objectives of this study were three-fold. My first objective was to determine a method of preparation which would preserve the wax and cement layers for scanning electron microscopy. My second objective was to examine the cuticle with SEM to visually document cuticular layers. My third objective was to examine the cuticular layers with transmission electron microscopy for comparative analysis with SEM visualization and previously reported examinations.

Materials and Methods

Cockroaches

All cockroaches were housed at the Urban Entomology laboratory, University of Florida, Gainesville, FL and reared in cloth covered glass jars (4 liter, 22.2 cm diam x 25.4 cm deep) at $\sim 26^{\circ}\text{C} \pm 2^{\circ}\text{C}$ and $\sim 55\%$ RH, with a photoperiod of 12:12 h (L:D).

Cockroaches were provided a diet of laboratory rodent diet (PMI Nutrition International, Inc, St. Louis, MO) and water in plastic vials (59 ml) stoppered with cotton balls. Adult male American cockroaches of various age were used for all experiments.

Scanning Electron Microscopy

Three techniques were attempted to prepare cuticular specimens for examination via scanning electron microscopy (SEM):

1) Fixation with osmium tetroxide vapors. Fully sclerotized American cockroach cuticle does not require stabilization via fixation to preserve morphological characteristics, structure or volume. However, freshly removed or dissected cuticle is flexible, and will not produce clean breaks necessary for SEM observation. In previous examinations of cockroach cuticle, I observed that specimens exposed to osmium tetroxide vapors (OsO_4) became desiccated. Therefore, in order to prepare specimens for breakage, desiccation of dissected cuticle with osmium vapors was attempted.

Cockroach pronota ($n=2$) were placed into a laminar flow hood within a glass Petri dish (Pyrex®, 100 x 10 mm, Fisher Scientific, Pittsburgh, PA) with a glass well containing ~ 0.5 ml of osmium tetroxide. The lid was then placed on the Petri dish and the specimens were exposed to 4% osmium vapors (Osmium tetroxide, ultrapure, TEM grade, Tousimis Research Corp., Rockville, MD) for 15 min, at which time the lid was removed. After fixation, specimens were allowed to dry for 2 h, at which time they were

removed from the hood. Breakage of specimens was attempted by placing a pair of forceps on the anterior and posterior edge of each pronotum and bending each edge downward.

2) Desiccation. The second preparation method was via desiccation in Drierite[®] (Drierite[®], anhydrous, Fisher Scientific, Pittsburgh, PA). Pronota (n=3) and sternites (n=5) were removed from live adult male cockroaches. All specimens were placed within a nonvacuum desiccator, (Fisherbrand[®] heavy glass desiccators with porcelain plate, I.D. 200 mm, Fisher Scientific, Pittsburgh, PA) with Drierite[®] and allowed to dry for 9 d. After desiccation, cuticle specimens were broken into transverse sections. After breaking, specimens were mounted on aluminum stubs with adhesive (Graphite, conductive adhesive 154, Summers optical, Ft. Washington, PA) which was allowed to dry until fully hardened. After drying, specimens were placed into a DESK II vacuum (Denton Vacuum, Moorestown, NJ) and sputter coated with gold (D. Williams, personal communication).

3) Freezing. Pronota (n=3) were removed from live cockroaches and placed into an ultra-low temperature freezer at $\sim(-27^{\circ}\text{C}) \pm 2^{\circ}\text{C}$ for ~ 5 min. While still in the freezer, specimens were broken by placing a pair of forceps on the anterior and posterior edge of each pronotum and bending downward, creating a transverse break. After breaking, specimens were mounted on aluminum stubs with adhesive and allowed to dry until fully hardened. After drying, specimens were coated with gold as described previously.

All specimens were individually examined with an Hitachi S-450 scanning electron microscope at an accelerating voltage of 20 kV. Photographs were taken with a Pentax

ZX-50 camera with T-MAX 100, Kodak 35 mm black and white film. Negatives were developed and scanned. Final images were resolved with PhotoShop 7.0.

Transmission Electron Microscopy

Two different techniques were attempted to prepare cuticular specimens for examination via transmission electron microscopy:

1) Embedding in LR White or Epon embedding resin. To preserve external layers, pronota were not fixed prior to embedding. Pronota were removed from live cockroaches and cut into strips (~3 by 15 mm) and placed into either LR White or Epon embedding media. Samples were incubated at $60^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 48 h prior to sectioning.

2) Fixation. Pronota were removed from living cockroaches and cut into strips (~3 by 15 mm). Cut pronota were then fixed in a 2% glutaraldehyde and 2% formaldehyde (~0.25 ml 10% glutaraldehyde, 0.13 ml formaldehyde, 0.6 ml 0.1 M cacodylate buffer, pH 7.2) solution for 3 h. After fixation with aldehydes, pronota were triple washed with 0.1 M cacodylate buffer (15 min each wash), and then further processed in a 50:50 4% osmium tetroxide, 0.2 M cacodylate buffer solution for 1.5 h, at which time they were triple rinsed, 15 min each rinse, in deionized water. After rinsing, specimens were dehydrated with a graded ethanol series, with final dehydration in acetone. After drying, specimens were gradually infiltrated with Spurr's, sectioned and examined with a Zeiss EM-10-CA transmission electron microscope (D. Williams, personal communication).

Results and Discussion

Scanning Electron Microscopy

1) Fixation with osmium tetroxide vapors. Fixation with osmium vapors did not result in complete desiccation of cuticle. Rather, cuticle remained pliable and did not

break. Therefore, osmium fixation was discarded as a viable method for specimen preparation.

2) Desiccation. Desiccation with Drierite[®] caused specimens to become brittle and break easily. However, the desiccation process also removed the wax and cement layers (external layers), revealing the bare epicuticle. Therefore, investigation of the external layers could not be performed. However, as remaining cuticular layers were well defined and easily observed with SEM, pronota were examined.

Desiccated, broken transverse sections of pronota contained two distinct layers, presumably the exocuticle and endocuticle. Underneath the endocuticle was a gap and then an inner layer of cuticle. A basement membrane was not evident at 2.50 k. The endocuticle was observed to be $\sim 24 \mu\text{m}$ and composed of loosely packed laminae, conferring a spongy appearance. The exocuticle was compressed, measuring $\sim 2 \mu\text{m}$ with lamella not visible. Examination of the pronota also revealed a $\sim 2 \mu\text{m}$ gap, under which lay another $\sim 6 \mu\text{m}$ layer of undifferentiated material, presumably another inner layer of cuticle (Fig. 2-1).

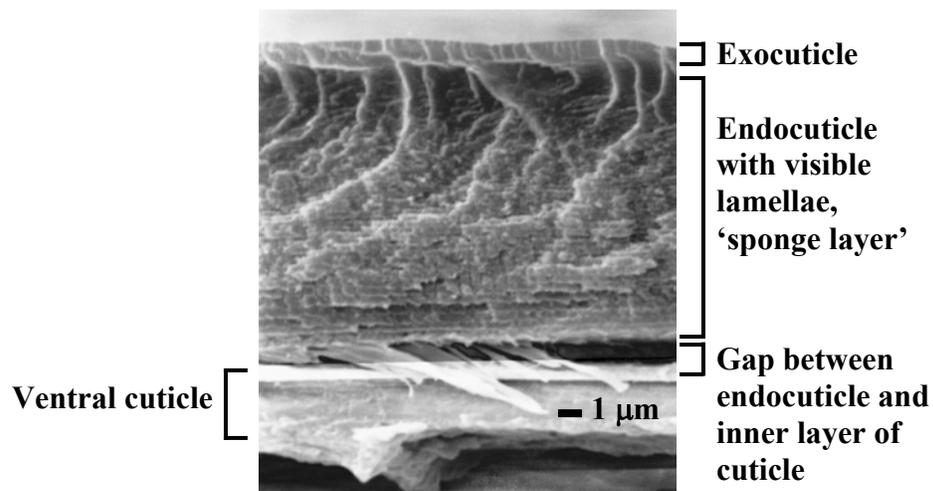


Figure 2-1. Desiccated, fractured transverse section of adult American cockroach pronotum with external layers removed.

Micrographs taken at an angle of approximately 15° revealed the denuded surface of the epicuticle and the presence of numerous dermal pores (Fig. 2-2), the ‘perforations’ reported by Sheie et al. (1968). The function of the dermal pores in American cockroaches is believed to be for the secretion of cement. Production of the cement layer has been reported to occur during adult ecdysis, after which, the dermal glands atrophy (Kramer and Wigglesworth 1950).

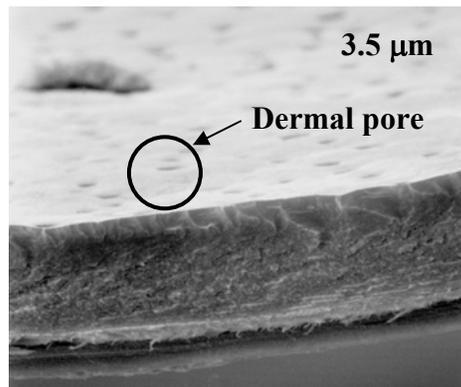


Figure 2-2. Surface of epicuticle without external layers, revealing numerous dermal pores.

3) Freezing. Microscopic examination of an adult male American cockroach pronotum revealed that the external layers remained intact, but did not reveal differentiation between wax and cement layers or between an inner and outer epicuticle. Measurements of the external layers ranged from the thinnest area of ~1 μm to 3 μm for a convex area extruding from the surface, which agree with the 1 to 2 μm previously reported for the wax layer (Cornwell 1968). As was seen with desiccated pronota, a gap existed, under which there appeared to be an another, inner layer of cuticle (Fig. 2-3). In one examination, the external layers could clearly be seen, folded over the edge of the procuticle (Fig. 2-4). When viewed horizontally, the presence of numerous spines were observed emerging from the ventral surface of the pronotum (Fig. 2-5). When the

pronotum is viewed with the ventral surface facing up, the spines may be seen extending from the edge of the pronotum, inward for a distance of ~ 0.75 mm (Fig. 2-6). Further examination of the ventral surface of a fractured pronotum revealed that in an area where the ventral cuticle had broken away, there were numerous tonofibrillae ~ 20 μ m in length, spaced ~ 10 μ m apart (Figs. 2-7, 2-8) extending from the dorsal cuticle to the surface of the ventral cuticle, except where the ventral cuticle had been removed.

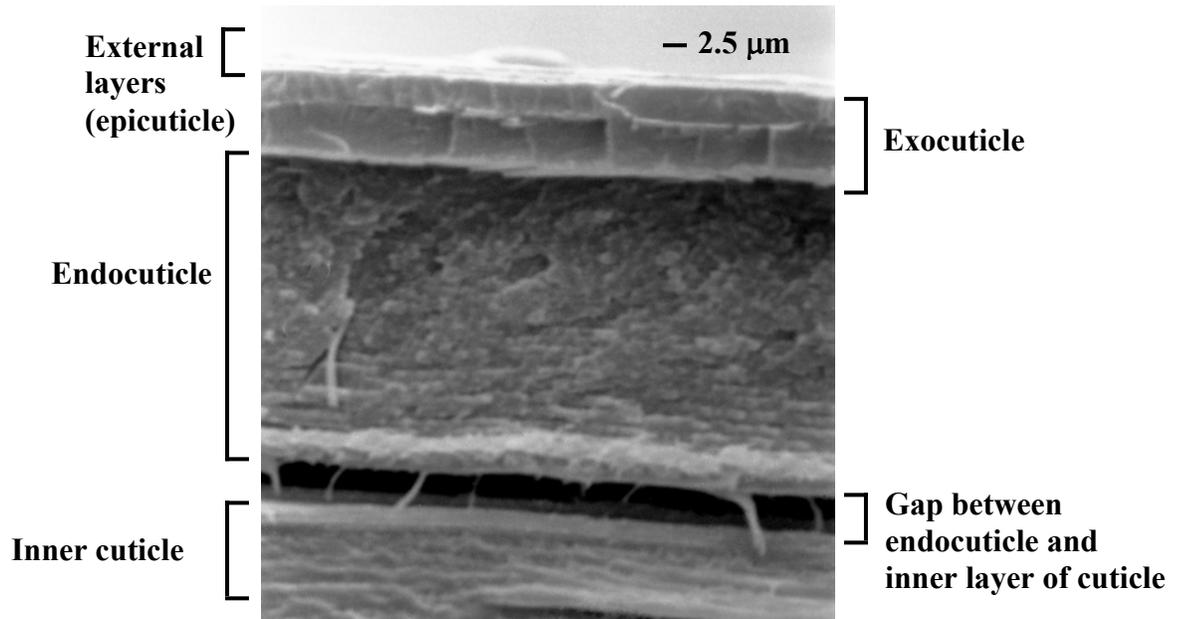


Figure 2-3. Frozen, fractured American cockroach pronotum with external layers intact.

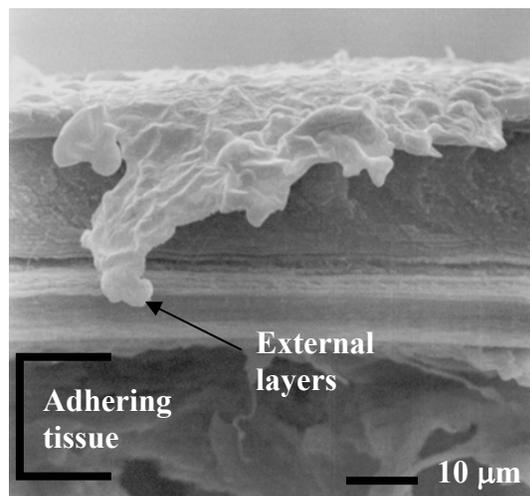


Figure 2-4. Transverse section of a frozen, fractured American cockroach pronotum with external layers draped over the epicuticle and procuticle.

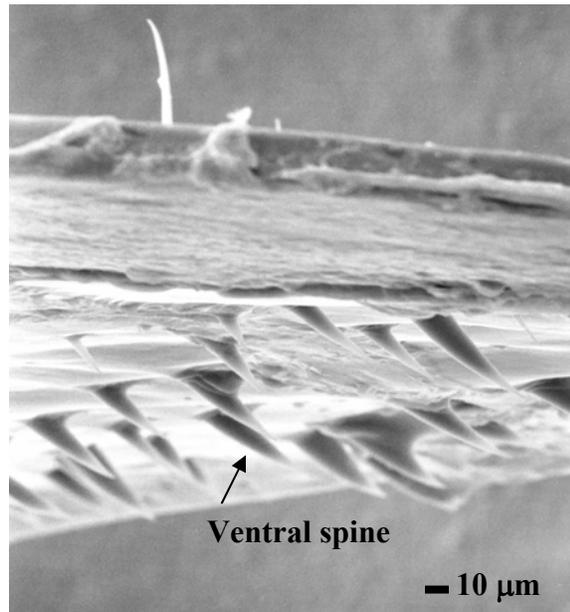


Figure 2-5. American cockroach pronotum in cross-section after slicing with a dissection knife. Spine-like structures on ventral surface.

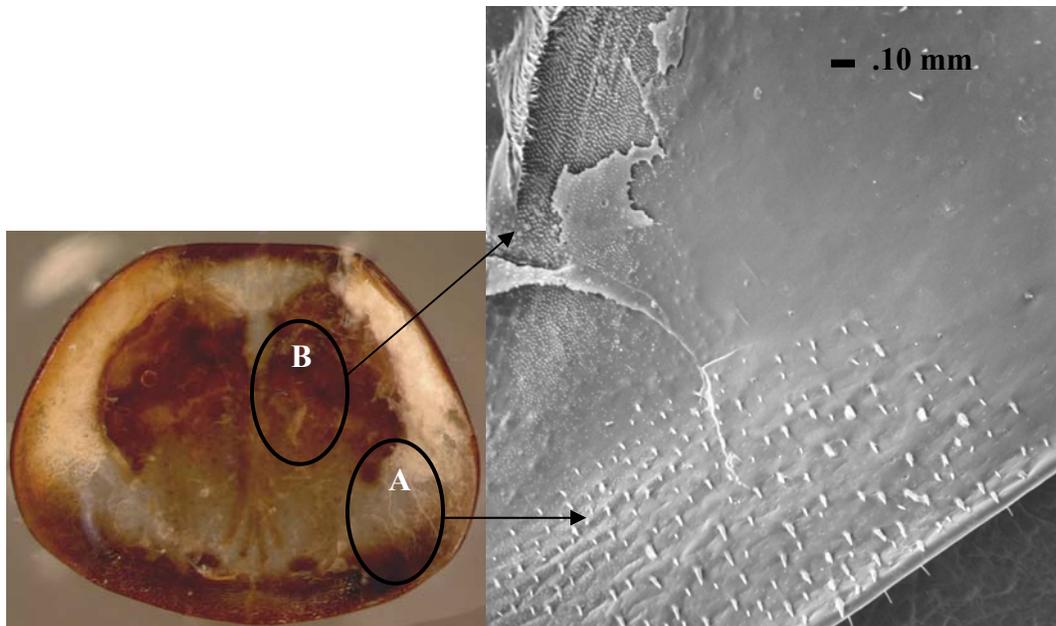


Figure 2-6. Left: Photograph of the ventral surface of a detached American cockroach pronotum. Right: SEM of the ventral surface of a detached American cockroach pronotum after fixation with osmium tetroxide vapors. A) spine-like structures present on the ventral surface of an American cockroach

pronotum. B) tonofibrillae in area where prothoracic legs were attached prior to removal of pronotum from living cockroach

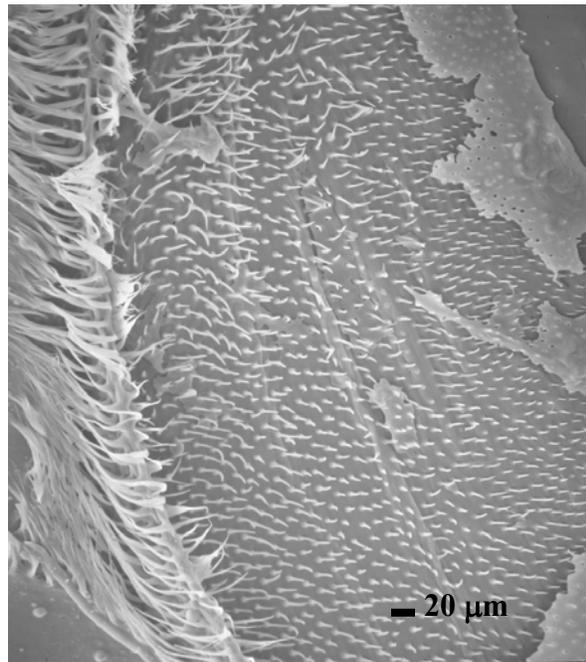


Figure 2-7. Tonofibrillae extending from the dorsal cuticle layer of a broken American cockroach pronotum.

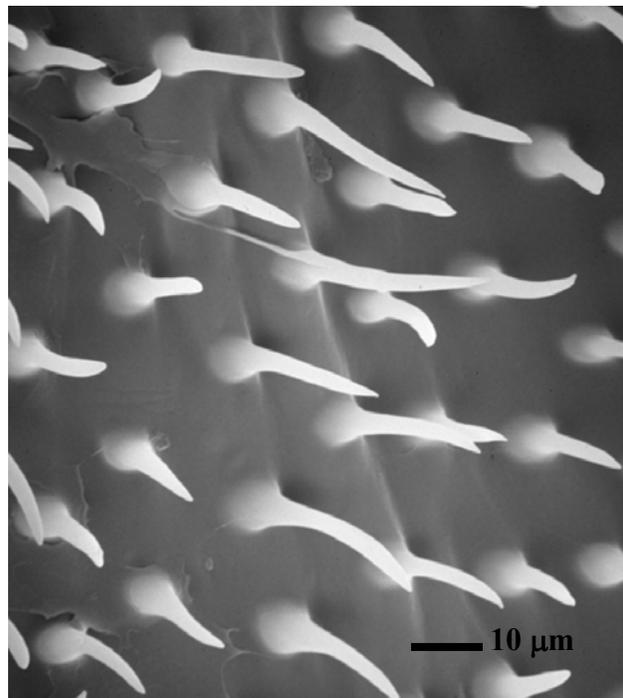


Figure 2-8. Detailed view of tonofibrillae extending from the dorsal cuticle layer into the body of a broken American cockroach pronotum.

Pronota. Examination of the dorsal surface of a pronotum revealed a globular substance extruding from the edges of a fractured area (Fig. 2-9). Dissections of pronota removed from living cockroaches further revealed that the ventral surface of pronota is covered by a transparent layer of cuticle, except in the distinctive brown twin eyespot pattern of the central pronota. The brown eyespots were discovered to be the point of attachment for the prothoracic leg muscles. The ventral surface of a pronota with the transparent cuticle removed on the right side is shown in Figure 2-10. The removed section of transparent cuticle is shown in Figure 2-11. Cuticle with removed globular substance is shown in Figure 2-12, with a magnified view of the globular substance shown in Figure 2-13.

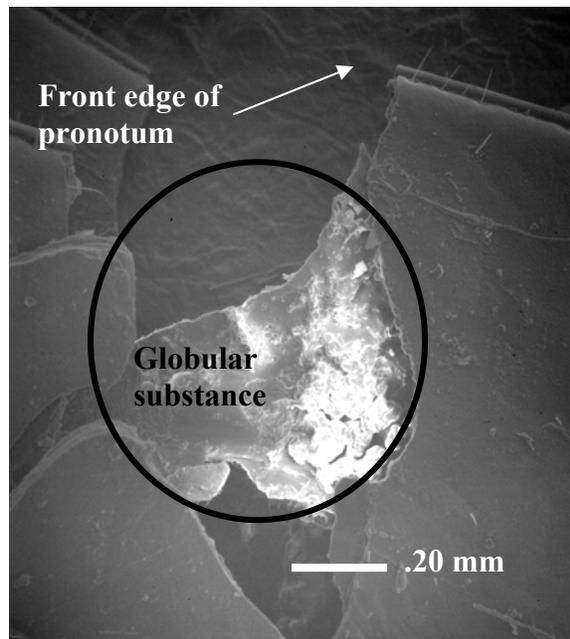


Figure 2-9. Fractured dorsal surface of an adult male American cockroach pronotum with extruded globular substance.

Sternites. Examination of the dorsal surface of the sternites revealed an absence of external layers on the external cuticle surface which were probably removed by handling. The sternites were composed of longitudinal striations and were pitted with dermal pores

(Fig. 2-14). The pattern on the surface of the sternites closely matches that found on the surface of tergites (Kramer and Wigglesworth 1950).

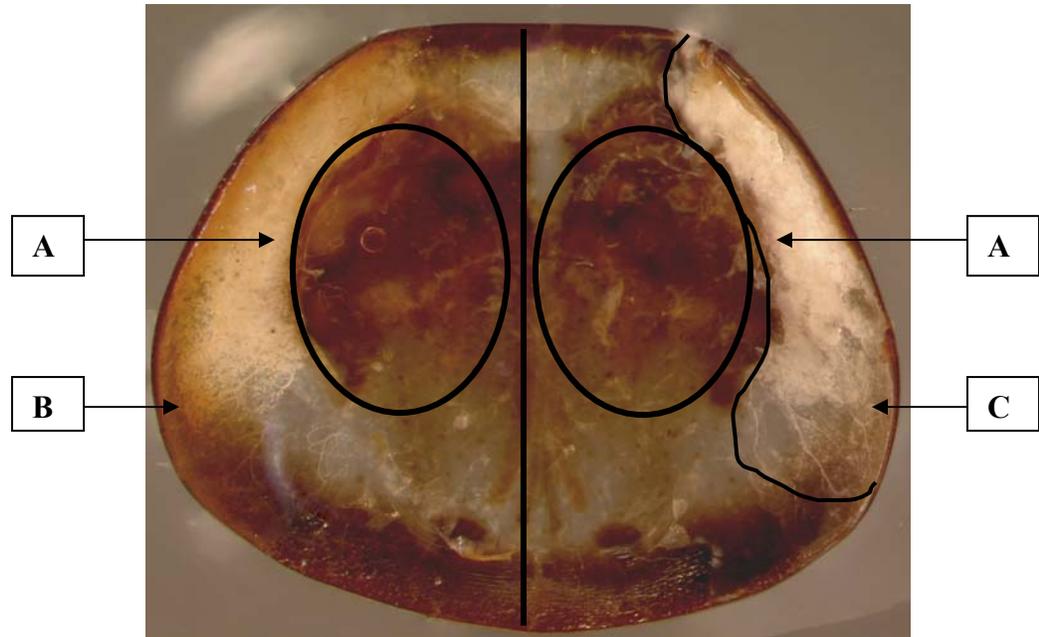


Figure 2-10. Ventral surface of pronotum: A) point of prothoracic leg attachment, B) intact transparent section of cuticle with underlying globular substance, and C) globular substance with transparent cuticle removed.



Figure 2-11. Ventral section of transparent cuticle dissected from pronotum.

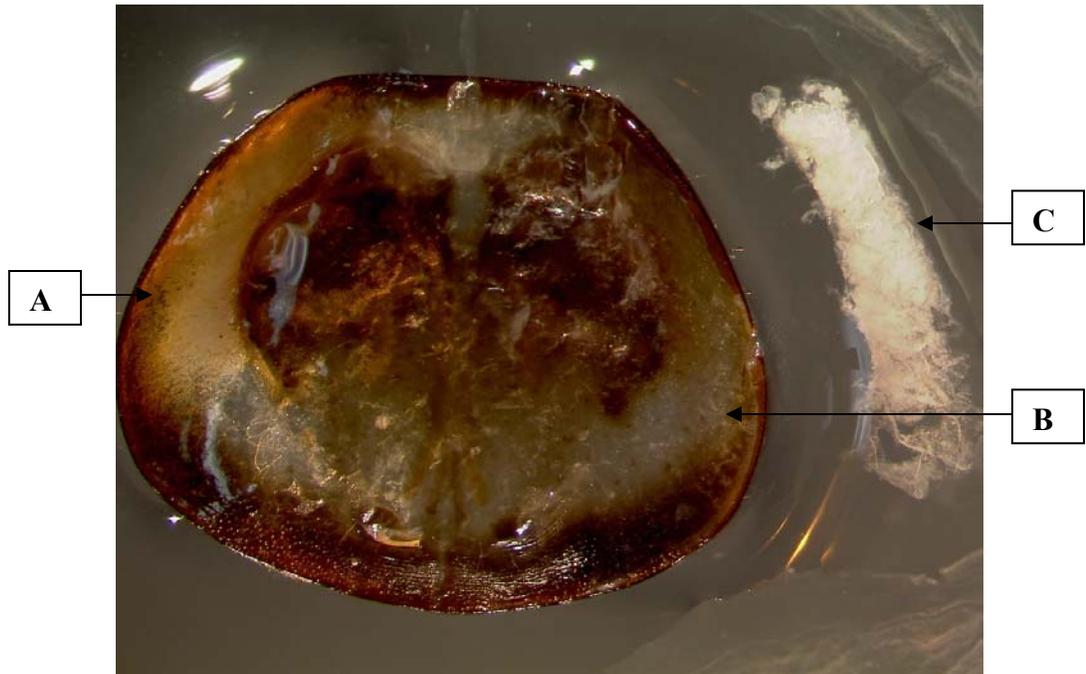


Figure 2-12. Ventral surface of pronotum: A) left side of pronotum with ventral transparent cuticle intact and globular substance in place, B) right side of pronotum with ventral transparent cuticle and globular substance removed and C) globular substance removed from right side of pronotum.



Figure 2-13. Magnified view of globular substance removed from pronotum.

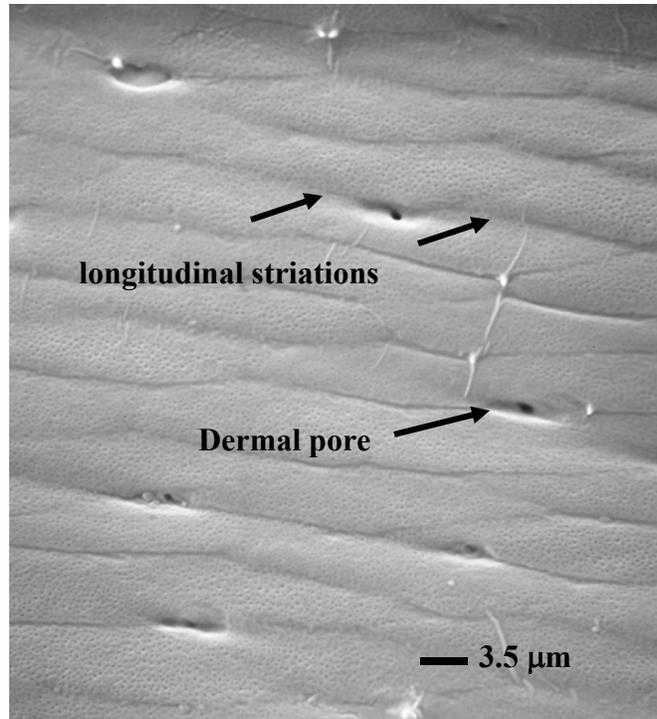


Figure 2-14. Outer surface of American cockroach sternite with external layers removed.

Examination of the surface of the sternites and pronota also revealed numerous small openings, smaller than the dermal pores, $\sim 0.15 \mu\text{m}$ in diameter covering the entire surface. The presence of small openings on the surface of the epicuticle has not been previously recorded. On both sternites and pronota, the openings are irregular in shape, round to ovoid and spaced ~ 0.25 to $0.50 \mu\text{m}$ apart (Figs. 2-15, 2-16). It is possible that the holes correspond to the wax channels reported by Locke (1960) or the pore canals of Richards and Anderson (1942) and Kramer and Wigglesworth (1950) which have been implicated in wax transfer (Bell and Adiyodi 1981), and would therefore allow deposition of wax over the cuticle surface.

However, it has alternately been reported that “the pore canals can be eliminated as a means of [wax] transfer since they do not penetrate the surface” (Cornwell 1968, p. 108). If the pore canals do penetrate to the surface, then the presence of the openings

discovered in this study would satisfactorily explain how the wax would then be deposited onto the cuticle surface.

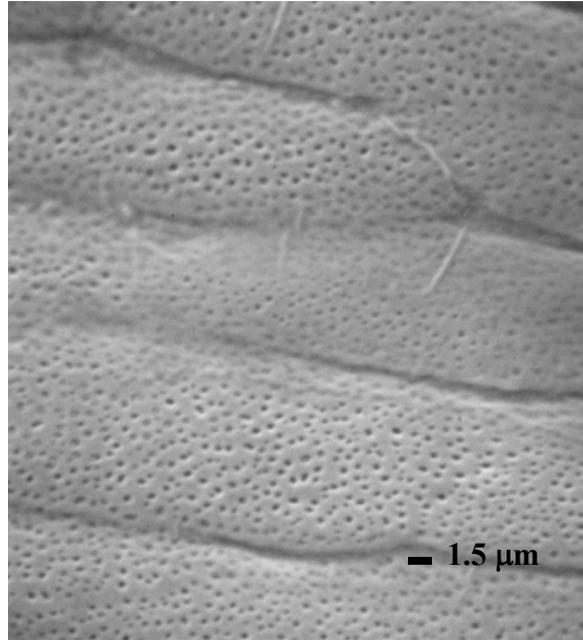


Figure 2-15. Outer surface of an American cockroach sternite with numerous small dermal openings.

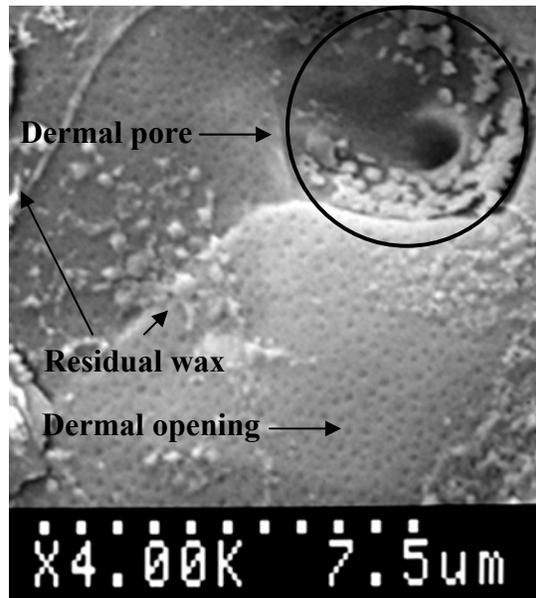


Figure 2-16. Outer surface of an American cockroach pronotum with numerous small dermal opening. Dermal pore for size comparison.

Transmission Electron Microscopy

1) Embedding in LR White and Epon. Due to water present in cuticular specimens and the presence of the external wax and cement layers, embedding attempts were unsuccessful, resulting in separation of cuticle from the embedding media, with the additional complication of the cuticle disintegrating during sectioning. However, after repeated sectioning, one usable sample was obtained. As with SEM, the cuticle appeared as a multi-lamellate structure (Fig. 2-17). Within the lamellae were observed numerous pore canals as described by Richards and Anderson (1942). However, due to compression during sectioning, the spongy appearance seen with SEM was not observed.

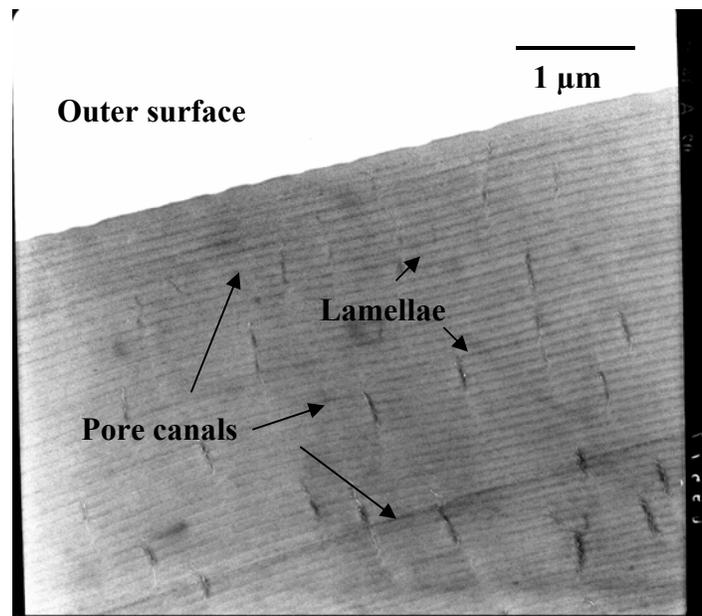


Figure 2-17. Transmission electron micrograph (16k) of adult male American cockroach cuticle incompletely embedded in LR White.

2) Fixation. With fixation, embedding of cuticle pieces was more efficient than embedding alone although some separation of plastic from the cuticle did occur with a thin layer of cuticle adhering to the plastic. However, it was possible to obtain complete sections of cuticle for examination.

Lamellae were observed on the dorsal half to be compressed more tightly than ventrally (Figs. 2-18 to 2-20). Pore canals were again evident and did not terminate in wax filaments as depicted by Chapman (1969). The canals become more tightly compressed dorsally with penetration of all observable cuticular layers, agreeing with results of silver-staining observed by Kramer and Wigglesworth (1950). Improved microscopy techniques have allowed verification that pore canals do penetrate all cuticular layers. Additionally, the openings observed on the surface of the cuticle could allow deposition of wax onto the surface of the exocuticle.

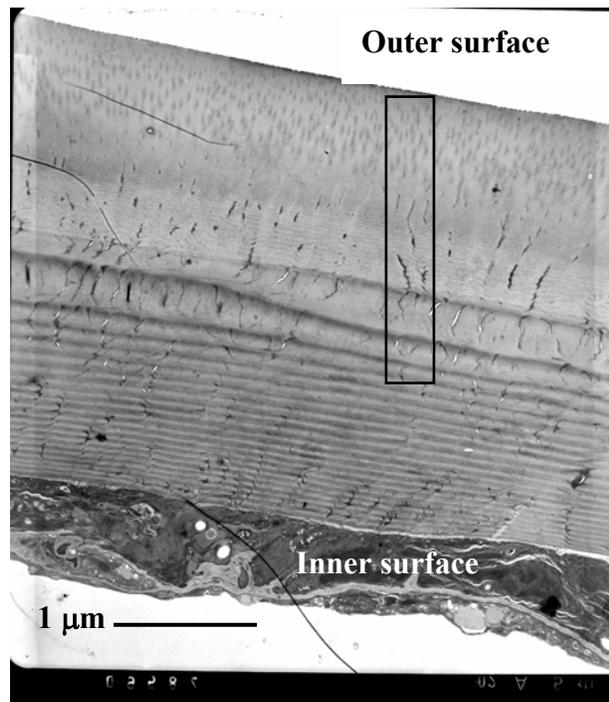


Figure 2-18. Cross section of pronotum (20k) of fixed adult male American cockroach cuticle. Rectangle around pore canals.

Preparation of American cockroach cuticle for observation via electron microscopy is complicated by the hydrophobic nature of the cuticle and the instability of the external wax and cement layers. In this examination, it was determined that for observation of the cuticle with SEM, freezing of specimens at -27°C and then fracturing resulted in

preservation of all cuticular layers. For examinations which do not require preservation of the external layers, dehydration of the cuticle with Drierite[®] and then fracturing results in clean, easily examined specimens.

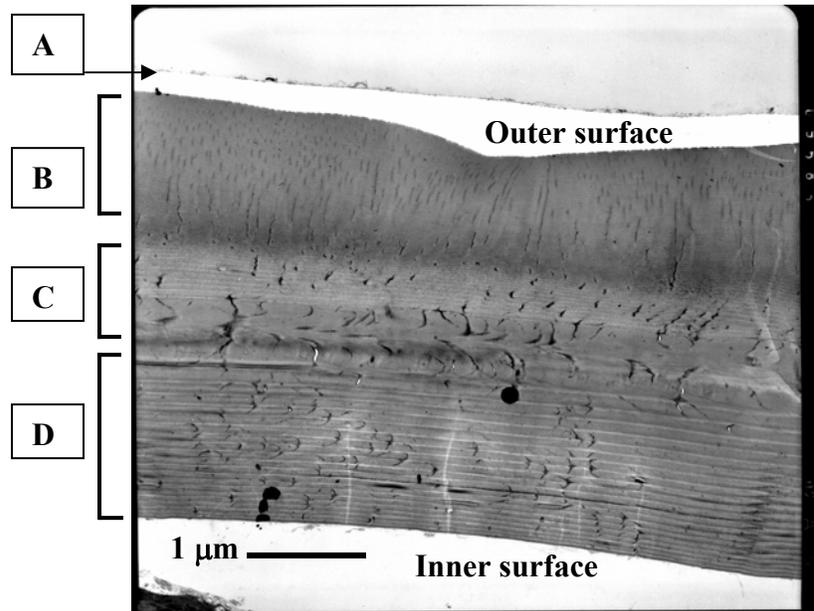


Figure 2-19. Cross section of pronotum (20k): A) epicuticle attached to separated embedding media, B) lamellae of exocuticle, C) severely compressed endocuticle (sponge layer observed with scanning electron microscopy) and D) lamellae of the ventral or inner layer of (transparent) cuticle.

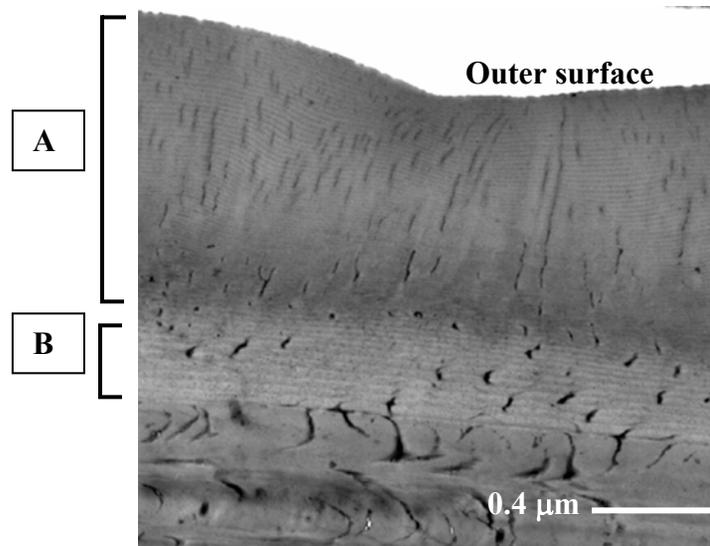


Figure 2-20. Magnified view of: A) exo- and B) endocuticle with greater detail of lamellae and pore canals.

When the pronotum of adult male American cockroach is observed with SEM, two major cuticle layers are obvious: the exo- and endocuticle, agreeing with the diagrammatic representation of tergites provided by Kramer and Wigglesworth (1950). However, the basement membrane could not be discerned and four distinctly different layers of the epicuticle discernable by chemical analysis (Dennell and Malek 1955) were not visually discernable. This examination did verify the presence of a morphologically variable wax (possibly wax/cement) layer, although inner and outer epicuticle layers were not observed.

Further, SEM examination of pronota revealed a hereto unreported globular substance within pronota. Subsequent dissections and microscopic examination revealed another unreported morphological characteristic, the presence of an inner, transparent section of cuticle. Additionally, the presence of tonofibrillae at the point of leg muscle attachment is reported as is the presence of spines on areas of the ventral side of pronota.

Processing for TEM examination of cuticle results in artificial compression. Additionally, fixation of specimens for TEM examination removes external layers. Therefore, images of cuticle should be interpreted cautiously by researchers utilizing TEM. In this study, TEM examination of the cuticle revealed a very thin layer of epicuticle. The exocuticle sponge layer observed with SEM was also present and composed of tightly packed lamellae. Endocuticle also appeared as lamellae of thicker sections (~3X) than that found in exocuticle. Because of compression, the gap between the endocuticle and inner cuticle layer observed with SEM was not present. However, the transparent, inner section of cuticle observed was composed of lamellae not obvious with SEM examination. Richards and Anderson (1942, p. 166) reported that the

endocuticle of a pronotum observed with TEM is “divided into two similar layers by a line of material that reflects light differently.” I suggest that the two layers of Richards and Anderson’s (1942) endocuticle are the endocuticle and inner cuticle of my TEM examination.

Interwoven within lamellae were pore canals, presumably the helical pore canals of Richards and Anderson (1942). The pore canals penetrated all layers present in TEM examinations. Also, when the outer surface of both sternites and pronota were examined, dermal openings were observed. I suggest that the pore canals do correspond to the dermal openings and are responsible for wax secretion onto the exterior cuticle surface.

In summary, it was determined that for SEM examinations, freezing and fracturing of American cockroach cuticle provides clean breakage of specimens without removal of external layers. However, no differentiation between a wax and cement layer is observed. Additionally, distinct inner and outer epicuticle layers were not observed. For TEM, due to the hydrophobic nature of the American cockroach cuticle, specimens must undergo fixation, removing external cuticle layers.

The results of the current study also suggest that the diagrammatic representation of American cockroach cuticle presented in entomology texts does not accurately reflect its composition and morphology. Cuticle morphology differs widely between insect species (Binnington and Retnakaran 1991). However, because of the importance of American cockroaches as both a laboratory insect (Bell and Adiyodi 1981) and as a potential mechanical vector of disease, accurate representation of the cuticle will assist researchers in obtaining a more complete understanding of this insect pest and how pathogens may interact with the cuticle in the process of mechanical transmission. The current research

on the American cockroach has described hereto unreported data on the histology and morphology of the cuticle. Therefore, based on the results of this study, a proposed diagram of the adult male American cockroach cuticle is presented in Figure 2-21.

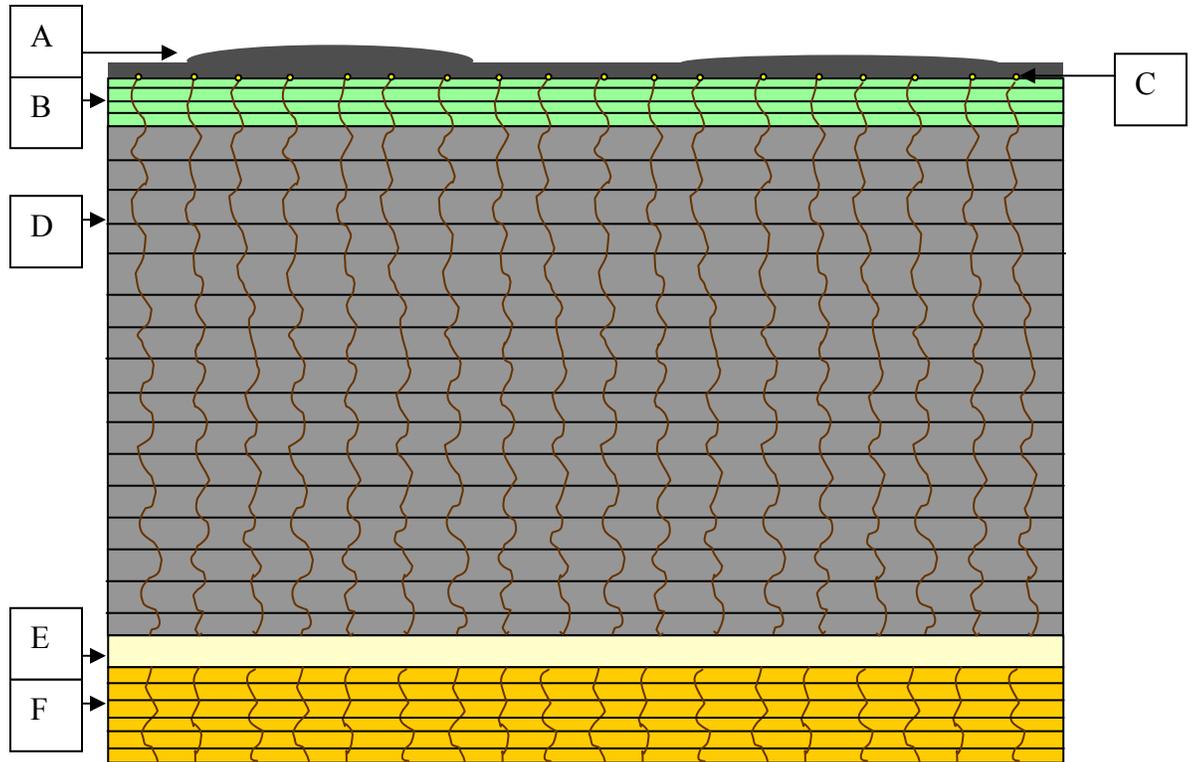


Figure 2-21. Diagrammatic representation of the cross section of an adult male American cockroach pronotum derived from scanning and transmission microscopy: A) wax layer of epicuticle B) lamellate exocuticle penetrated by pore canals, terminating in dermal openings, C) pore canal terminus D) lamellate endocuticle penetrated by pore canals, E) gap between endocuticle and inner transparent cuticle containing globular substance and F) transparent inner section of cuticle penetrated by pore canals.

CHAPTER 3
DIFFERENTIAL RECOVERY OF *Salmonella enterica* FROM THE CUTICLE OF
AMERICAN COCKROACHES

Introduction

American cockroaches are one of the main pest cockroach species in the United States. In urban areas, American cockroaches are primarily found in basements and sewer systems, as well as in commercial sewage treatment facilities (Roth and Willis 1960), bringing them into contact with a wide variety of potentially pathogenic bacteria. American cockroaches are also very mobile and often invade human dwellings through sewer drains (Brenner 2002).

Bacteria isolated from the exterior surfaces of American cockroaches are frequently those belonging to the family Enterobacteriaceae, especially those in the genus *Salmonella* (Roth and Willis 1957). Therefore, American cockroaches are considered potential mechanical vectors of *Salmonella* spp. bacteria via contaminated cuticle. Mechanical cuticular transmission occurs when pathogens are acquired and then directly transferred to a susceptible food, food contact area or individual, usually without replication (Reisen 2002).

For cockroaches, mechanical cuticular transmission occurs in the context of the epicuticle which is composed of four layers: the inner epicuticle, the outer epicuticle, the wax layer and the outermost cement layer. The generalized model of the American cockroach cement layer is as either a discrete, continuous layer (Bell and Adiyodi 1981)

or as a discontinuous substance intermingled with the wax layer (Locke 1965). The function of the cement layer is to protect and provide stability for the wax layer.

The cement layer is important in the currently accepted theory of mechanical transmission of bacteria. Bacteria are believed to adhere to the rigid, 'shellac-like' (Beament 1955) surface of the cement layer, readily available for transfer to the environment. Therefore, when attempting to isolate *Salmonella* or other pathogenic organisms from cockroaches, the external cuticle may either be washed (Cloarec et al. 1992, Fathpour et al. 2003), or parts of the insect cuticle swabbed against a nutrient media (Frishman and Alcamo 1977, Kopanic et al. 1994).

However, underneath or enmeshed within the cement layer, the cuticle of American cockroaches is also covered by a fluid and dynamic wax layer that is continuously sloughed and replenished and has been described as a "Vaseline like substance" (Beament 1955, p. 515). Therefore, it is not known if *Salmonella* cells are actually available on the surface of the cuticle, or if they may somehow become embedded within the wax layer of the cuticle, leaving them unavailable for detection and potentially unavailable for transmission.

In addition to the dynamics and mobility of the wax layer, *Salmonella* are living organisms, individually capable of movement by means of peritrichous flagella, and which may, when grown on appropriate media, exhibit active swarming behavior in groups (Harshey 1994). Therefore, the interaction of *Salmonella* with the cuticle of American cockroaches may be important in attempts to recover and isolate the bacteria as well as whether or not the bacteria may be mechanically transmitted.

The objectives for this study were to: 1) Document the presence of *Salmonella* cells on the cuticle of American cockroaches via scanning electron microscopy. 2) Quantify the amount of bacteria remaining on the pronota of American cockroaches 24 h after inoculation and without amplification via enrichment media. 3) Document bacterial distribution on the surface of the pronotum and within external cuticular layers. 4) Investigate the interaction of *S. enterica* with the cuticle via videography.

Materials and Methods

American Cockroaches

All cockroaches were housed at the Urban Entomology laboratory, University of Florida, Gainesville, FL and reared in cloth covered glass jars (4 liter, 22.2 cm diam x 25.4 cm deep) at $\sim 26^{\circ}\text{C} \pm 1^{\circ}\text{C}$ and $\sim 55\%$ RH, with a photoperiod of 12:12 h (L:D). Cockroaches were provided a diet of laboratory rodent diet (PMI Nutrition International, Inc, St. Louis, MO) and water in plastic vials (59 ml) stoppered with cotton balls. Adult male American cockroaches of various age were used for all experiments. Cockroaches, rearing tubs, water sources and food were tested to ensure that the colony was free from existing *Salmonella* contamination.

Bacteria

Salmonella enterica subspecies *enterica* JAVA was obtained from the University of Florida, School of Veterinary Medicine and maintained at $4^{\circ}\text{C} \pm 2^{\circ}\text{C}$ until use. Prior to use, bacteria were incubated in Selenite broth (Remel, Apogent Company, Lenexa, KS) at $38^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for 18-24 h. After incubation, a loop of the Selenite suspension was plated onto Hektoen Enteric (HE) agar plates (Remel, Apogent Company, Lenexa, KS) and incubated at $38^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for 18-24 h. Cells were removed from HE plates with a loop and suspended in deionized water until the desired cell concentration for inoculation

(6.0×10^5 - 2.4×10^6) was achieved. Prior to inoculations, count plates were inoculated to determine the number of viable colony forming units (cfu) in each inoculum.

Cells were negatively stained with 1% uranyl acetate and examined via transmission electron microscopy to document the presence of flagella (Fig. 3-1). Additionally, to verify motility of cells, a 0.5 ml aliquot of a 10 ml *S. enterica*/deionized (DI) water suspension was placed onto a glass slide, covered with a cover slip and examined under a light microscope.

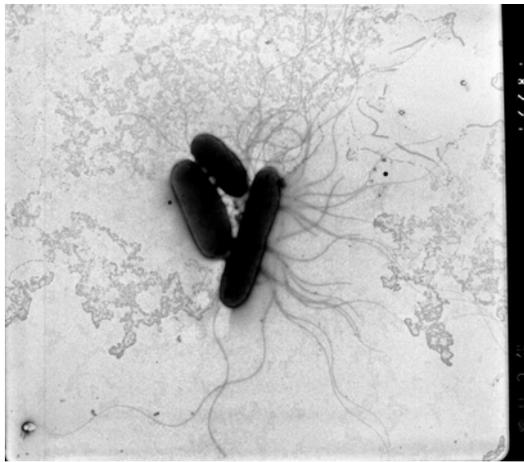


Figure 3-1. *S. enterica* with flagella.

Cockroach inoculation for Scanning Electron Microscopy (SEM)

Cockroaches (N=72: 36 for osmium tetroxide fixation, 36 for glutaraldehyde fixation) were either inoculated on their pronotum or a single tarsus. For pronotal inoculation, cockroaches (n=36) were immobilized in an upright position by securing their wings with a wooden clothespin with a 2.5 cm strip of Velcro[®] attached to the exterior edge of one clothes pin grip. The grip with Velcro[®] was then attached to a complementary strip of Velcro[®] on a length of board. Cockroaches were spaced so that their antennae were not able to come in contact with adjacent individuals. Cockroaches were thus held in place for inoculation and subsequent incubation. After immobilization,

a 2 μ l droplet of a suspension containing 5.0×10^5 *S. enterica* cells was inoculated onto the central portion of each pronotum (n=18). Controls (n=18) were inoculated with 2 μ l of deionized (DI) water.

Cockroaches to be inoculated on their tarsi (n=36) were placed onto sticky traps (Catchmaster insect trap and monitor, Atlantic Paste & Glue Co., Inc., Brooklyn, NY) on their dorsal side, securing the legs with rubber bands. Tarsal claws/puvilli (n=18) were inoculated with 0.5 μ l of a suspension containing 1.25×10^5 cells. Control cockroaches (n=18) were inoculated with 0.5 μ l DI water.

After inoculation, cockroaches still attached to their board with clothes pins were incubated at $\sim 27^\circ\text{C} \pm 1^\circ\text{C}$ for 24 h, at which time the pronotum of each cockroach was removed to be processed for SEM. To facilitate handling of tarsal specimens, the leg of each inoculated cockroach was removed at the mid-point of the tibia. All samples were then processed for SEM.

Scanning Electron Microscopy

Due to the rigidity of the cuticle, fixation was required only for bacteria present on pronota or tarsal specimens. Fixation of specimens was accomplished with either 4% osmium tetroxide vapors or with 2% glutaraldehyde. Specimens to be fixed with osmium vapors (Osmium tetroxide, ultrapure, TEM grade, Tousimis Research Corp., Rockville, MD) were placed into a laminar flow hood within a glass Petri dish (Pyrex®, dish, 100x10 mm, Fisher Scientific, Pittsburgh, PA) with a glass well containing ~ 0.5 ml of osmium tetroxide. The lid was then placed on the Petri dish and the specimens were exposed to the osmium vapors for 15 min, at which time the lid was removed. Specimens were allowed to dry for 2 h in the hood prior to sputter coating.

Specimens to be fixed with glutaraldehyde were placed into separate microcentrifuge tubes (Fisherbrand® Tube, Micro centrifuge, 1.5 ml, Fisher Scientific, Pittsburgh, PA). A 6 ml stock solution containing 1.2 ml of 10% glutaraldehyde (Glutaraldehyde, 10% ultrapure, TEM grade, Tousimis Research Corp., Rockville, MD), 3 ml of 0.2 M cacodylate buffer (Cacodylic acid, sodium salt, molecular biology grade, Fischer Biotech, BP325-50, Fairlawn, NJ), and 1.8 ml of deionized water was prepared and pipetted into each tube until the specimen was completely covered. At 15 min, the glutaraldehyde solution was removed and the specimens were washed with 0.1 M cacodylate buffer for 15 min, twice each. Specimens were dehydrated with a graded ethanol series at concentrations of 25, 50, 75, 95 and 100% for 10 min, with two washes at 100%. After dehydration, specimens were washed with hexamethyldisilazane (HMDS) for 2 x 10 min, and allowed to dry for 2.5 h.

After drying, specimens were affixed to a metal stub and placed into a DESK II vacuum (Denton Vacuum, Moorestown, NJ), and sputter coated with gold. Specimens were then individually examined with a Hitachi S-450 scanning electron microscope at an accelerating voltage of 20 kV. Photographs were taken with a Pentax ZX-50 camera with T-MAX 100, Kodak 35 mm black and white film. Negatives were developed and scanned. Final images were resolved with PhotoShop 7.0.

Cockroach Inoculation for Microbial Recovery

Cockroaches (N=9) were immobilized with clothes pins as previously described. Pronota were inoculated with 1 μ l *S. enterica* suspension containing 6.0×10^5 cells and incubated at $27^\circ\text{C} \pm 1^\circ\text{C}$. At 24, 48 and 72 h, 3 cockroaches were removed from clothes pins and completely submerged into 10 ml DI water, where the pronotum of each

cockroach was gently scrubbed with a sterile swab (Fisherbrand[®], sterile swabs, calcium alginate, fiber tipped, wood applicator swab, Fisher Scientific, Pittsburgh, PA) to dislodge *S. enterica* cells. The cockroach was then removed and the solution was agitated gently to distribute cells throughout the water. Additionally, at 24, 48 and 72 h, 1 ml of the original *S. enterica* stock solution was diluted into 9 ml DI water. From the cockroach/*S. enterica* solution and from the diluted stock solution, 1 μ l droplets were removed and serially diluted to 10^{-6} . For each dilution, three, 1 μ l droplets were removed, pipetted onto HE agar and incubated at $38^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for 18-24 h, at which time, cfu for each sample were determined.

Bacterial Distribution on Pronotal Surface and Within Lipid Layer

Cockroaches (N=24) were immobilized by clothes pins as previously described. After immobilization, the pronotum of each cockroach was inoculated with 2 μ l of suspension containing 4.5×10^5 *S. enterica* cells. Inoculated cockroaches were incubated for 24 h at $\sim 27^{\circ}\text{C} \pm 1^{\circ}\text{C}$. At 24 h, each pronotal surface was gently swabbed with a sterile swab moistened with Selenite broth. The swab was then placed into a vial with 9 ml of Selenite broth.

After swabbing, pronota were removed and placed into separate vials of Selenite broth. All Selenite vials containing surface swabs and pronota were incubated for 48 h at $38^{\circ}\text{C} \pm 1^{\circ}\text{C}$. After incubation, two, 1 μ l droplets were removed from each tube and serially diluted to a concentration of 10^{-6} . For each dilution, three, 1 μ l droplets were plated on HE agar and incubated for 24 h at $38^{\circ}\text{C} \pm 1^{\circ}\text{C}$. Therefore, for each swab and pronotum, paired data for a total of six cfu counts was obtained. Number of cfu was

recorded for each sample. The numbers of cfu from surface swabs and pronota were compared with a paired *t*-test (SAS Institute 2000).

For each sample, a single cfu was removed from the HE agar and streaked onto urea agar (Urea agar, Remel Inc., Lenexa, KS) slants, along with positive and negative controls. Slants were incubated for 24 h and examined. Additionally, RapID One (RapID One, Remel Inc., Lenexa, KS) analysis was conducted for biochemical confirmation that suspect isolates were *Salmonella*.

Videography of *S. enterica* on Cockroach Pronotum

The pronotum of a living American cockroach was removed, placed onto a glass slide, and inoculated with 4 μ l of a *S. enterica* suspension containing 2.4×10^6 cells/ μ l. After inoculation, a cover slip was placed over the pronotum. The inoculated pronotum was then mounted on a light microscope stage modified for videography with a video camera to microscope coupler (HRP042-NIK, 0.42X F-Mount HRP Coupler less clamp, Sterling Heights, MI). A camera (Canon XLI-S, Mini DV, 3CCD digital video camcorder, Canon, U.S.A., Inc.) was mounted into the coupler and the activity of the *S. enterica* cells on the surface of the pronotum was recorded at a magnification of 600X. Digital video analysis was performed on an Apple Macintosh G4/933 Computer (Apple Computer, Eupertino, CA).

Results and Discussion

Scanning Electron Microscopy: Osmium Tetroxide Fixation

Cuticle morphology. For pronota and tarsal specimens fixed with osmium vapors, it was observed that the wax and cement (external layers) layers remained intact. On living specimens, external layers are transparent with the epicuticle clearly visible. On

osmium fixed specimens examined with SEM, the external layers appear white to gray in contrast to the epicuticle, which appears dark gray to black.

Examination of pronota revealed that the morphology of the external layers was highly variable on all the 18 specimens and across all pronotal regions (9 treated, 9 control). On some samples, the external layers contained pits, cracks and waxy globules of variable size, ranging from one to several microns in width (Fig. 3-2). On one sample, the external layers were more or less uniform in appearance (Fig. 3-3), when compared to other samples where external layers were formed into globules (Fig. 3-4) or pits (Fig. 3-5) of variable size. External layers were variable in thickness. It was not possible to measure the depth of the external layers as all observations were made from a dorsal view. However, it has been estimated that the wax or lipid layer is 1-2 μm thick (Cornwell 1968).

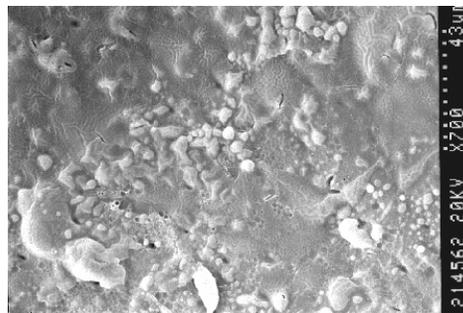


Figure 3-2. Irregular pronotal surface with pits and wax globules.

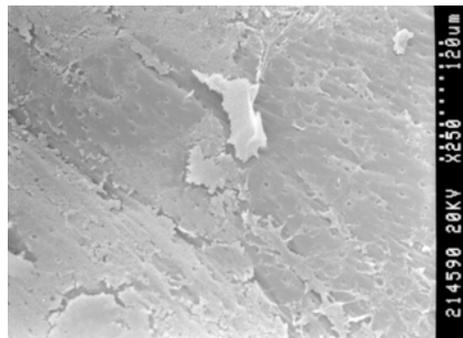


Figure 3-3. Relatively smooth surface of pronotum.

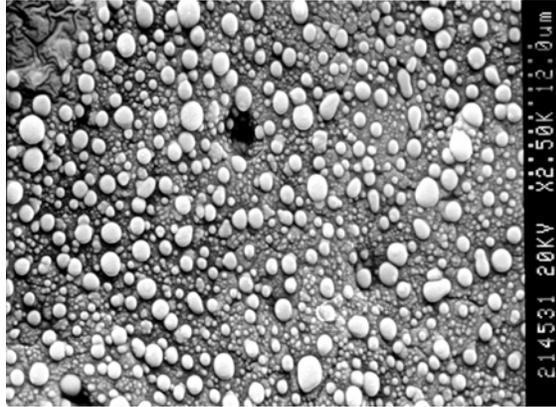


Figure 3-4. Pronotal surface with “bubbles” of wax.

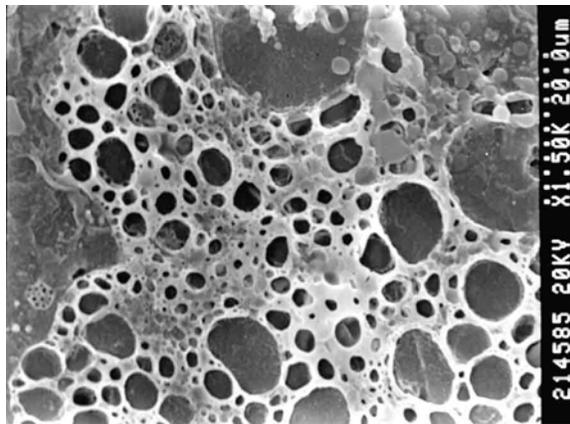


Figure 3-5. Pronotal surface with craters.

For tarsi fixed with osmium vapors, the intact external layers covering the pupilli formed a smooth, crenulated surface (Fig. 3-6). Surface layers of the tarsal claws and tarsus resembled the appearance of those observed on pronota (Fig. 3-7).

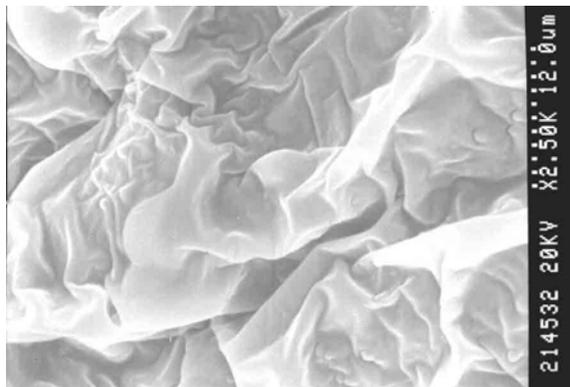


Figure 3-6. Osmium fixed, close-up of arolium showing smooth crenulations of external layers.



Figure 3-7. Osmium fixed tarsi.

Bacterial attachment. Of the nine treated pronota fixed with osmium vapors, on one were 5 visible cells whereas on another were <60 discernable cells (Fig. 3-8).

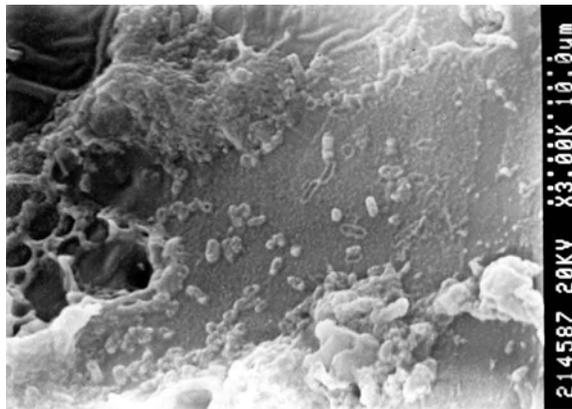


Figure 3-8. Osmium fixed pronota with bacteria on surface and with bacteria-shaped indentations in wax.

On the same specimen with the <60 cells, bacteria could be seen underneath the wax, resulting in bacteria shaped protrusions (Fig. 3-9) The remaining seven specimens had no evidence of bacterial attachment. No bacterial cells were seen on any of the osmium vapor fixed tarsi, or on control pronota and tarsi (Table 3-1).

Scans of the entire surface of all specimens revealed minimal numbers of bacteria on only two pronota. However, due to the large number of *S. enterica* cells in the

inoculum (6.0×10^5 cells/ μ l), it was expected that bacteria would be evident on both pronota and tarsi. Therefore, recovery of bacteria was attempted with microbiological techniques on another set of inoculated cockroaches.



Figure 3-9. Osmium fixed pronotum with bacterial cells under external layers.

Cockroach Inoculation for Microbiological Recovery

No *S. enterica* bacteria were recovered from any of the inoculated cockroaches at 24, 48 and 72 h after inoculation. Whereas, recovery of *S. enterica* from the original stock solution at 24, 48 and 72 h was 3.3×10^5 , 4.3×10^3 and 4.7×10^3 respectively. It has been reported that when cockroaches carry bacteria, they may readily be dislodged into the environment (Brenner et al. 1987). However, I was unable to recover any *S. enterica* from water washes of inoculated cockroaches, although recovery of cells from the unenriched stock solution revealed that *S. enterica* cells were still viable. A similar recovery method by Cloarec et al. (1992) was able to recover various bacterial pathogens from German cockroaches collected from apartment buildings in France. However, 5 cockroaches were used for each sample and were washed in an unknown quantity of sterile water with the addition of 0.01 ml of Triton X-100, a nonionic surfactant to assist in bacterial recovery, which may have also removed cuticular lipids.

The results of this study substantiate the results of SEM examination of osmium fixed pronota that bacteria does not remain on the surface of external layers, 24 h after inoculation. Therefore, it was considered that the *S. enterica* cells might have become embedded within cuticular lipids where they could not be seen with SEM examination, and would also not easily be dislodged into the DI water. Therefore, SEM examination of specimens was again attempted, utilizing an alternate fixation technique, glutaraldehyde fixation.

Scanning Electron Microscopy: Glutaraldehyde Fixation

Cuticle morphology. Glutaraldehyde fixation removes the wax layer from cockroach cuticle. Additionally, with a series of 12 washes, the fixation procedure would theoretically remove a significant amount, if not all, bacteria from pronota and tarsal specimens. However, since SEM examination of osmium fixed specimens reveal <50 bacterial cells, the glutaraldehyde fixation method was attempted.

For specimens fixed with glutaraldehyde, the majority of the external layers were removed, leaving behind waxy patches. With the majority of the wax and cement removed, the surface of the bare epicuticle was clearly visible (Fig. 3-10). Across the surface of the epicuticle were numerous pores consisting of an outer depression containing the pore opening, that averaged ~3 microns in size (Fig. 3-11).

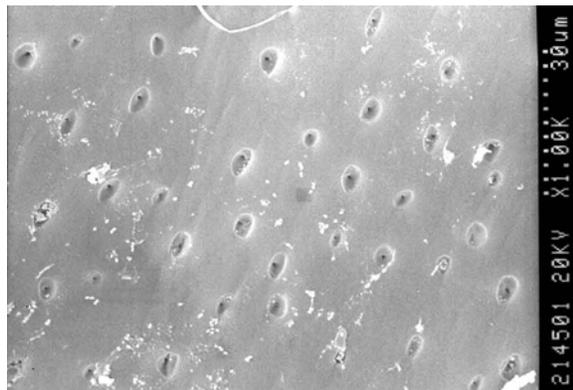


Figure 3-10. Glutaraldehyde fixed pronotum with bare epicuticle visible.

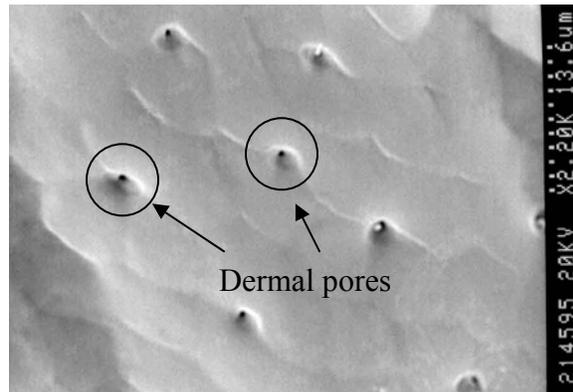


Figure 3-11. Glutaraldehyde fixed pronotum with dermal pores clearly evident.

Epicuticular pores labeled as “perforations” identified as openings of the dermal ducts, have also been reported by Scheie et al. (1968, p. 1346) on “unwashed” specimens viewed with SEM. Numerous hairs of varying lengths and widths were also evident.

For tarsi fixed with glutaraldehyde, the majority of the surface layers were removed (Fig. 3-12), and as with pronota, some waxy patches remained. Additionally, a large opening between the pulvilli and distal tarsal segment was revealed, containing numerous rows of structures resembling inverted cones $\sim 3 \mu\text{m}$ in height by $\sim 3 \mu\text{m}$ in width (Fig. 3-13).

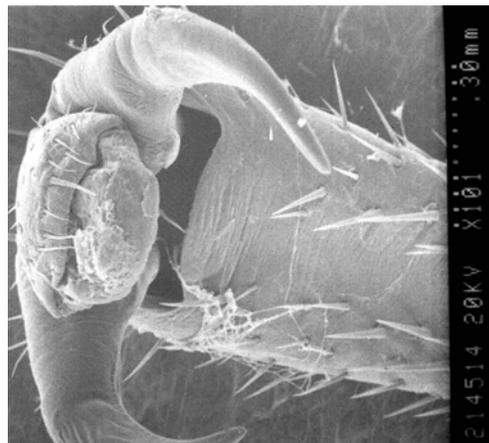


Figure 3-12. Glutaraldehyde fixed tarsus showing large opening under arolium.

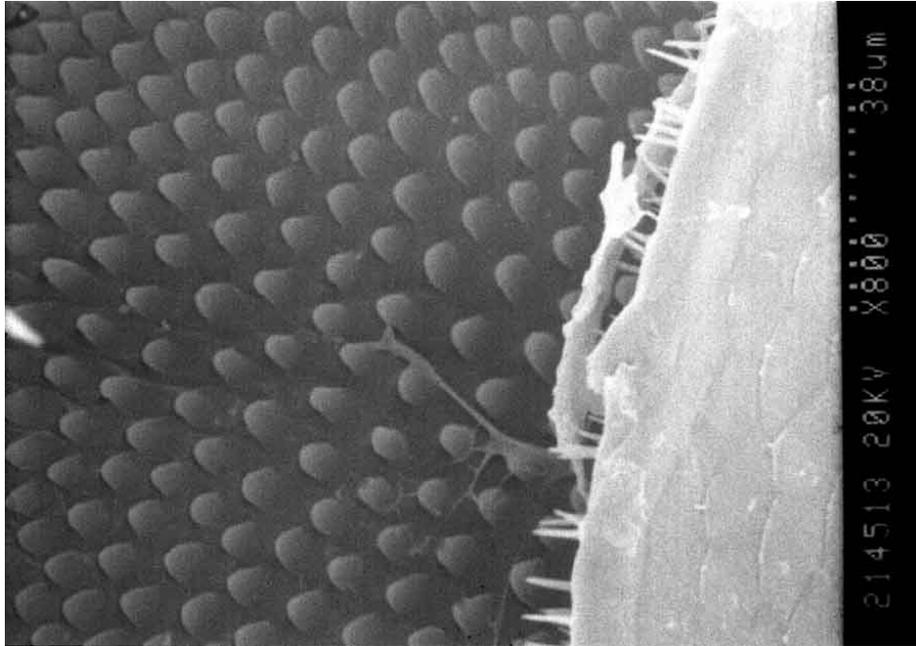


Figure 3-13. Glutaraldehyde fixed tarsus. Interior shot of opening under arolium.

For all treated pronotal and tarsal specimens fixed with glutaraldehyde, large numbers of *S. enterica* cells, too numerous to count, were randomly distributed in clumps and singly across the surface of inoculated areas. No bacteria were visible on controls (Table 3-2). On one inoculated pronotum, a sheet consisting of thousands of bacterial cells was present (Fig. 3-14). Cells also formed clumps (Fig. 3-15), or were scattered individually across the surface of the bare epicuticle (Fig. 3-16). In some areas where cell attachment occurred, it often appeared that the cells were enmeshed within a waxy residue (Fig. 3-17). The waxy residue may have been remnants of the external cuticular layers of the cockroach, or in some cases, an extracellular matrix of the bacterial cells, or a combination of both an extracellular matrix and remnants of external layers. An examination made by Gazivoda and Fish (1985) of wild German cockroaches demonstrated the presence of unknown bacteria, also enmeshed within a waxy residue after fixation with both glutaraldehyde and osmium.

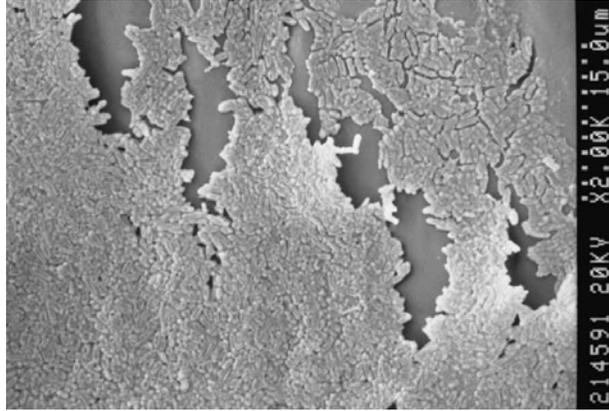


Figure 3-14. Glutaraldehyde fixed pronotum. Sheet of *S. enterica* on surface of bare epicuticle.

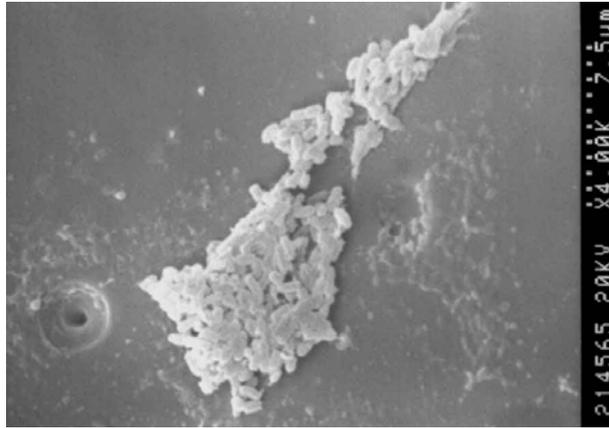


Figure 3-15. Glutaraldehyde fixed pronotum. Clump of *S. enterica* next to dermal pore.



Figure 3-16. Glutaraldehyde fixed pronotum. *S. enterica* scattered across surface of bare epicuticle.

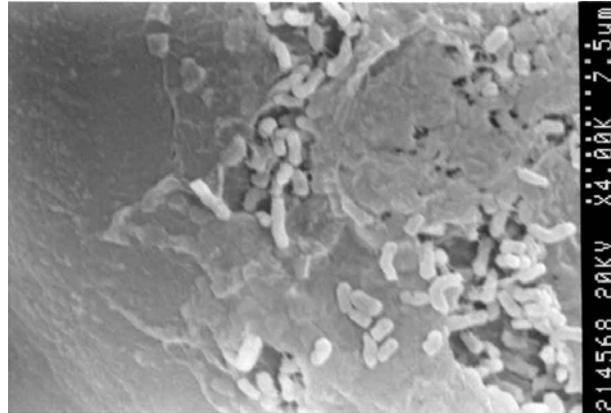


Figure 3-17. Glutaraldehyde fixed pronotum. *S. enterica* in residue of external layers.

Bacterial Distribution on Pronotal Surface and Lipid Layer

Of the 24 inoculated cockroaches with the pronota removed and incubated in enrichment media, *S. enterica* was recovered and confirmed with RapID One analysis, from 20 pronotal surface swabs (83.3%) and 16 pronota (66.7%). Of those cockroaches from which *S. enterica* was recovered, there were no significant differences in the mean number of cfu from swabs ($y = 1.2 \times 10^5$) or pronota ($y = 1.3 \times 10^5$) ($t = -0.37$, $df = 38$, $P = 0.7102$).

From one cockroach, there was no recovery of *S. enterica* from either surface swab or pronotum. From another cockroach, isolated bacteria were negative for *Salmonella* spp. For two cockroaches, cfu for *S. enterica* could not be obtained due to incompletely removed tissue adhering to detached pronota causing bacterial contamination on HE plates. Kopanic et al. (1994) found that recovery of *S. typhimurium* from cockroaches self-inoculated by an infected food source (2 ml @ $\sim 1.0 \times 10^{7-8}$ cfu/ml) resulted in 93% recovery at 24 h, similar to the surface swab recovery rate of the current study.

Videography

Immediately after inoculation of a detached American cockroach pronotum with 4 μ l of *S. enterica* suspension, the bacteria could be seen swimming within the water, with

some cells exhibiting Brownian movement. Due to capillary action, 2 min after initiation of filming, the DI water moved from under the cover slip, carrying with it the majority of the *S. enterica* cells. However, once the water was removed from the pronotum, several remaining cells could clearly be seen, exhibiting strong swimming/tumbling movement within the wax layer. The following contains video of *S. enterica* swimming in the cockroach wax layer:

Object 3-1: Surface of a detached American cockroach pronotum inoculated with *S. enterica* in deionized water. *S. enterica* cells swimming within the wax layer at 400X. Filmed under Leica light microscope (MPEG movie file. 5.0 MB, 3 min, 22 sec)

In summary, examination via SEM of inoculated pronota and tarsi fixed with osmium vapors revealed no to few (<60) *S. enterica* on the surface of the external layers of 17 samples. One pronotal sample did have evidence that bacteria had at some point been embedded and then removed. Because osmium fixation does result in drying and hardening of samples, it is possible that during subsequent processing, bacteria on the surface of samples could have been dislodged by handling or lost in the vacuum of either the sputter coater or the SEM. However, in this study, lack of a significant amount of *S. enterica* on the surface of pronota that were not subject to osmium fixation was verified by microbiological examination of DI water washes from inoculated cockroaches. Thus, the microbiological evidence indicates that bacteria initially inoculated onto the surface were probably not dislodged.

Further SEM investigation of inoculated cockroach pronota and tarsi fixed with glutaraldehyde revealed that *S. enterica* cells were visible within and under external cuticle layers. As was documented via videography, the motile *S. enterica* were able to swim through the external cuticular layers, and as was observed with SEM, became

embedded within wax and on the surface of the bare epicuticle. Further, those cells that were observed with SEM were attached firmly enough to avoid being dislodged by the several washes involved in glutaraldehyde fixation. However, because most of the external layer was removed, it is probable that additional *S. enterica* cells were lost in processing.

Recovery of *S. enterica* from pronotal surface swabs and from pronota did reveal that there were bacteria on the surface of and within the external cuticular layers. However, 48 h enrichment of surface swabs was required in order to detect the presence of *S. enterica*. Additionally, the surface swabs may actually have removed some of the external layers, increasing recovery of *S. enterica*. Because *S. enterica* cells were recovered with enrichment, it is suggested that DI water washes may dilute bacteria that are present on the surface to numbers too few to be counted, or if recovered, would represent only a portion of *S. enterica* cells also present within the wax layer. Additionally, recovery of bacteria from swabbing cockroach cuticle on nutrient media may also result in reduced recovery as bacteria residing within the wax may not be transferred to media.

In light of the findings presented here, I conclude that the current theory of mechanical transmission should be revised. Motile *S. enterica* cells are able to swim through the wax layer, indicating that the cement layer of adult male American cockroaches is not a continuous barrier as proposed by Kramer and Wigglesworth (1950). Also, if cement is intermingled with wax, as has been proposed by Locke (1965) it does not represent a significant barrier to penetration of the wax layer by motile bacteria.

Because *S. enterica* does become enmeshed within the wax layer, cells may be shed or rubbed off with the wax over time, resulting in continual transmission risk until cells are no longer viable or until the reservoir is depleted. While it is not known how long the potential for transmission may occur, it has been reported that *S. enterica* can be recovered from American cockroaches for up to 78 d after inoculation (Olson and Rueger 1950). Therefore, future research into mechanical transmission of *S. enterica* by cockroaches should consider the dynamics of the interaction of *S. enterica* with the external layers of the cockroach cuticle.

Table 3-1. Visible *Salmonella enterica* cells (24 h) on pronota and tarsi of American cockroaches inoculated with 6.0×10^5 cells, fixed with osmium tetroxide vapors and viewed with scanning electron microscopy.

Cockroach number	Pronota		Tarsi	
	Treated	Control ^a	Treated	Control
1	-	-	-	-
2	-	-	-	-
3	-	-	-	-
4	-	-	-	-
5	+ (<50)	-	-	-
6	-	-	-	-
7	+ (5 ^b)	-	-	-
8	-	-	-	-
9	-	-	-	-

^aControl pronota and tarsi for both osmium and glutaraldehyde fixed specimens.

^bCells also observed under external layers.

Table 3-2. Visible *Salmonella enterica* cells (24 h) on pronota and tarsi of American cockroaches inoculated with 6.0×10^5 cells, fixed with glutaraldehyde and viewed with scanning electron microscopy.

Cockroach number	Pronota		Tarsi	
	Treated	Control ^a	Treated	Control
1	++++ ^b	-	++++	-
2	++++	-	++++	-
3	++++	-	++++	-
4	++++	-	++++	-
5	++++	-	++++	-
6	++++	-	++++	-
7	++++	-	++++	-
8	++++	-	++++	-
9	++++	-	++++	-

^aControl pronota and tarsi for both osmium and glutaraldehyde fixed specimens.

^b++++ = Too numerous to count.

CHAPTER 4
COMPARITIVE EXAMINATION OF *Salmonella enterica*, *Escherichia coli* AND *E. coli* O157:H7 ON THE CUTICLE OF AMERICAN COCKROACHES, WITH RECOVERY OF *S. enterica* FROM AMERICAN COCKROACHES AND PHARAOH ANTS OVER TIME

Introduction

Of the various insect species associated with disease transmission, biting insects have received the most attention in their role as direct or horizontal transmitters of human pathogens. Secondarily, insects such as the house fly, *Musca domestica* (L.), have been heavily scrutinized in their role as mechanical vectors. Of the crawling insects, American cockroaches, *Periplaneta americana* (L.), and pharaoh ants, *Monomorium pharaonis* (L.), are frequently associated with human structures and both have been implicated as potential mechanical vectors of disease (Eldridge and Edman 2000).

Mechanical transmission is not a direct transference of a disease organism to a host, but occurs as a result of contaminative contact with a susceptible individual, food or food contact area, usually without multiplication or change of the etiological agent. Because of the indirect transmission, it has been notoriously difficult to create a direct association between pathogens carried by a crawling insect and the occurrence of human disease.

However, the potential for mechanical disease transmission by crawling insects does exist. In urban areas, American cockroaches are primarily found in basements and sewer systems, as well as in commercial sewage treatment facilities (Roth and Willis 1960), bringing them into contact with a wide variety of potentially pathogenic bacteria. American cockroaches are also very mobile and often invade human dwellings through

sewer drains (Brenner 2002), or by flying toward lights in populated areas, entering houses and other residential dwellings. Pharaoh ants, although found outdoors in warmer climates, derive their pest status from their close association with humans. Pharaoh ants may be found in offices, restaurants and most importantly, within hospitals (Cornwell 1979) where they may inhabit sensitive areas such as operating rooms, heated food carts, vending machines and clean linens (Beatson 1972). Because of their presence in hospitals, and their willingness to feed on human body fluids and food products (Burrus 2004), the potential for pharaoh ants to cause hospital acquired infections is substantive.

In addition to the vector capacity of an insect, the physiology of a pathogen also plays a determinative role in transmission potential. Obligate intracellular parasites such as viruses, rickettsias and protozoa are unlikely to be mechanically transmitted.

However, enteric bacteria such as *Salmonella enterica* (*S. enterica*), *Escherichia coli* O157:H7 (*E. coli* O157:H7) and non-pathogenic *E. coli* (NP *E. coli*) can survive outside their hosts where they may be acquired by crawling insects. Although genetically similar, *S. enterica*, pathogenic *E. coli* (various) and NP *E. coli* possess different mechanisms for attachment to and/or effacement of human epithelial cells (Goosney et al. 1999) which may also affect how effectively they can survive on an arthropod host as well as how efficiently they may be mechanically vectored.

The objectives for this study were three-fold. My first objective was to conduct a visual comparison of the attachment efficacy of *S. enterica*, *E. coli* O157:H7 and NP *E. coli* to the pronota of adult male American cockroaches utilizing SEM. My second objective was to determine the viability of *S. enterica* over time on experimentally inoculated adult male American cockroach pronota via microbiological recovery. My

third objective was to determine the viability and percent recovery of *S. enterica* from Pharaoh ant colony workers and brood over time.

Materials and Methods

Insects

All insects were housed at the urban entomology laboratory, University of Florida, Gainesville, FL. American cockroaches were housed at the Urban Entomology laboratory, University of Florida, Gainesville, FL and reared in cloth covered glass jars (4 liter, 22.2 cm diam x 25.4 cm deep) at $\sim 26^{\circ}\text{C} \pm 1^{\circ}\text{C}$ and $\sim 55\%$ RH, with a photoperiod of 12:12 h (L:D). Cockroaches were provided a diet of laboratory rodent diet (PMI Nutrition International, Inc, St. Louis, MO) and water in plastic vials (59 ml) stoppered with cotton balls. Third instars and adult males of varying age were used. Pharaoh ants were reared as described by Burrus (2004) at a temperature of $\sim 26^{\circ}\text{C} \pm \sim 7^{\circ}\text{C}$ and a mean of 37% relative humidity. Ants were fed dead cockroaches and/or termites and a 10% sugar water solutions. Cockroaches, ants, rearing containers, water sources and food were tested to ensure that colonies were free from *Salmonella* or *Escherichia coli* O157:H7 contamination.

Bacteria

Salmonella enterica. Frozen glass seed beads coated with *Salmonella enterica* subspecies enterica JAVA were obtained from the University of Florida, School of Veterinary Medicine. Beads were maintained at $4^{\circ}\text{C} \pm 1^{\circ}\text{C}$ until use, at which time an individual bead was placed into Selenite broth (Remel, Apogent Company, Lenexa, KS) and incubated at $38^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for 18-24 h. After incubation, a loop of the Selenite suspension was plated onto Hektoen Enteric (HE) agar plates (Remel, Apogent Company, Lenexa, KS) and incubated at $38^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for 18-24 h. Cells were gently

removed from HE plates with a loop and suspended in deionized (DI) water until the desired cell concentration of inoculation (1.0×10^5 - $2.4 \times 10^6/\mu\text{l}$) was achieved.

Escherichia coli. Dehydrated NP *E. coli* (ATCC# 25922, culti-loops, Chrisope technologies, Remel Inc., Lenexa, KS) and *E. coli* O157:H7 (ATCC# 35150, Remel Inc., Lenexa, KS) were rehydrated in tryptic soy broth for 24 h and inoculated onto MacConkey agar (Remel, Apogent Company, Lenexa, KS) and MacConkey agar w/sorbitol (Remel, Apogent Company, Lenexa, KS) plates respectively. Bacteria were then incubated for 24 h, at which time, suspensions of the bacteria were made as was previously described for *S. enterica*. Prior to all bacterial inoculations, count plates were inoculated to determine the number of viable colony forming units (cfu) in each inoculum.

Scanning Electron Microscopy

Cockroaches (N=21) were immobilized in an upright position by placing them onto strips of corrugated cardboard (14 x 27 cm) and securing them with Scotch[®] tape (Gloss finish transparent tape, 3M corporation, St. Paul, MN). Cockroaches were spaced so that there was no contact between individuals, and were thus held in place for inoculation and subsequent incubation. After immobilization, the central portion of each pronotum was inoculated with 2 μl of a suspension containing either 1.0×10^5 or 2.5×10^5 *S. enterica* cells (n=6), *E. coli* O157:H7 (n=3) or NP *E. coli* (n=6). Controls (n=6) were inoculated with 2 μl of DI water. After inoculation, cockroaches were incubated at $27^\circ\text{C} \pm 1^\circ\text{C}$ for 24 h, at which time the pronotum of each cockroach was removed to be processed for SEM.

For fixation, specimens were placed into separate microcentrifuge tubes (Fisherbrand® Tube, Micro centrifuge, 1.5 ml, Fisher Scientific, Pittsburgh, PA). A 6 ml stock solution containing 1.2 ml of 10% glutaraldehyde (glutaraldehyde, 10% ultrapure, TEM grade, Tousimis Research Corp., Rockville, MD), 3 ml of 0.2 M cacodylate buffer (Cacodylic acid, sodium salt, molecular biology grade, Fischer Biotech, BP325-50, Fairlawn, NJ), and 1.8 ml of DI water was prepared and pipetted into each tube until the specimen was completely covered. At 15 min, the glutaraldehyde solution was removed and the specimens were washed with 0.1 M cacodylate buffer for 15 min, twice each. Specimens were dehydrated with a graded ethanol series at concentrations of 25, 50, 75, 95 and 100% for 10 min, with two washes at 100%. After dehydration, specimens were washed with hexamethyldisilazane (HMDS) for 2 x 10 min, after which they were allowed to dry for 2.5 h.

After drying, specimens were affixed to a metal stub and placed into a DESK II vacuum (Denton Vacuum, Moorestown, NJ), and sputter coated with gold. Specimens were then individually examined with a Hitachi S-450 scanning electron microscope at an accelerating voltage of 20 kV. Photographs were taken with a Pentax ZX-50 camera with T-MAX 100, Kodak 35 mm black and white film. Negatives were developed and scanned. Final images were resolved with PhotoShop 7.0.

***Salmonella enterica* Recovery from American Cockroaches Over Time**

Cockroaches (N=50) were immobilized in an upright position by placing them onto the inverted half of a Petri dish (Petri dish, 60 x 15 mm, disposable polystyrene, Becton Dickenson labware, Becton Dickenson and Company, Lincoln Park, NJ) and securing them with Scotch® tape. Cockroaches were spaced so that there was no contact between individuals. After immobilization, the central portion of each pronotum was inoculated

with 2 μ l of a suspension containing either 4.5×10^5 (n=25) or 9.0×10^5 (n=25) *S. enterica* cells. Controls (n=15) were inoculated with DI water. Cockroaches were provided with a food pellet (Laboratory rodent diet PMI Nutrition International, Inc, St. Louis, MO) and water in microcentrifuge tubes (Fisherbrand® Tube, Micro centrifuge, 1.5 ml, Fisher Scientific, Pittsburgh, PA) stoppered with cotton. After inoculation, cockroaches were incubated at $27^\circ\text{C} \pm 1^\circ\text{C}$. Cockroaches (n=5) were randomly selected at 24, 28, 72, 96 and 120 h after inoculation to be processed for recovery of *S. enterica*.

To quantify recovery of *S. enterica*, pronota from all cockroaches were removed and placed into separate vials of Selenite broth. Additionally, 2 ml of the original inoculum was pipetted into a vial of Selenite broth. All vials were incubated at 38°C for 24 h. To obtain paired data for each sample, 2, 2 μ l samples were removed from each vial as well as from the original, unenriched stock solution. All samples were serially diluted to 10^{-6} . For each dilution, three, 1 μ l droplets were removed, pipetted onto HE agar and incubated at $38^\circ\text{C} \pm 1^\circ\text{C}$ for 18-24 h to determine mean CFU for each sample.

Recovery of *S. enterica* from Pharaoh Ants

For each of the three replications, there was one *S. enterica* treated and one DI water (control) arena tested at 3, 7 and 17 d in a completely randomized design, for a total of 18 experimental arenas. For each replication, ~300 adult workers and ~150 mixed brood, to include last instar larvae, were removed from rearing containers and equally distributed into arenas (Glad®, 733 ml, 14 wide x 5 cm deep) coated on the entirety of the inner rim with Fluon® (Polytetrafluoroethylene, grade AD1, AG Fluoropolymers, Asahi glass fluoropolymers USA Inc., Chadds Ford, PA) to prevent ant escape. Each arena contained a moistened harborage cell (Burrus 2004), DI water and

10% sugar water solution provided in vials (Fisherbrand[®], disposable culture tubes, borosilicate glass, 13x100 mm, Fisher Scientific, Pittsburgh, PA) stoppered with cotton balls. Vials containing water were secured in each arena with hot glue. All ants were starved for protein for 5 d.

At 5 d of protein starvation, each arena was provided with a cockroach inoculated with *S. enterica* (treated) or DI water (control). Third instar cockroaches were removed from rearing containers and frozen at $-37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for 20 min. After freezing, cockroaches were allowed to thaw at room temperature. After thawing, each treated (n=9) cockroach was inoculated with 5 μl of a DI water/*S. enterica* suspension containing 1.5×10^5 cells between the prothoracic legs. Control cockroaches (n=9) were inoculated with 5 μl of DI water. Each cockroach was placed into a weigh boat and randomly assigned to an arena.

At 3, 7 and 17 d after introduction of cockroaches, one treated and one control arena was broken down and surveyed for distribution of *S. enterica*. For each arena, each individual worker ant, larva or pupa was removed and placed into separate vials of Selenite broth. All Selenite vials were then incubated for 48 h. At 48 h, a swab was placed into each Selenite vial until moistened and then streaked across an HE plate. Plates were incubated for 24 h at which time the presence of bacterial growth was observed. Pure isolates of all suspect *S. enterica* samples were obtained and streaked onto urea slants (Remel, Inc. Apogent Company, Lenexa, KS). For isolates negative for urea hydrolysis, 10% were further tested via RapID One (RapID One, Remel Inc., Lenexa, KS) biochemical analysis.

Statistical Analysis

For analysis of recovery of *S. enterica* over time from American cockroaches, one-way analyses of variance (ANOVA) were conducted with treatment (unenriched stock, enriched stock or cockroaches) as the main factor with a different set of cockroaches at each time interval. Means were separated with Tukey's Studentized range test ($P = 0.05$; SAS Institute 2001).

For analysis of recovery of *S. enterica* over time from Pharaoh ants, one-way ANOVA were performed for main factors: number of workers or brood recovered and number positive for *S. enterica*. Means were separated with Tukey's studentized range test ($P = 0.05$; Minitab 2004).

Results and Discussion

Scanning Electron Microscopy

At the 2.5×10^5 inoculum level, thousands of bacteria were attached to all cockroach pronota ($n=3$) inoculated with *S. enterica*. For pronota inoculated with *E. coli* O157:H7, there were minimal cells (14) attached to one pronota, with large clumps of bacteria attached to the remaining specimens ($n=2$) to equal ~30 to 50% the number of *S. enterica* cells observed. However, examination of pronota inoculated with NP *E. coli* ($n=3$) revealed no to few (<110) bacteria. At the 1.0×10^5 inoculum level, no bacteria were observed on pronota inoculated with either *S. enterica* ($n=3$) or NP *E. coli* ($n=3$), and no bacteria were observed on any of the DI water control cockroach pronota (Table 4-1).

Differential attachment of *S. enterica*, *E. coli* O157:H7 and NP *E. coli* has also been reported to occur on alfalfa sprouts, with serovars of *S. enterica* attaching and growing significantly more effectively than serovars of *E. coli* O157:H7 or NP *E. coli*

(Barak et al. 2002, Charkowski et al. 2002). Additionally, significant correlations between the amount of *Salmonella* in an inoculum and the number of cells adhering to produce has been reported for both tomatoes (Montserrat et al. 2003) and green pepper slices (Liao and Cooke 2001) with reduced numbers in the inoculum leading to reduced bacterial attachment.

Cockroach cuticle is normally covered with a large amount of wax (Dennell and Malek 1956), the majority of which is removed with glutaraldehyde fixation prior to SEM examination. However, observations of pronota revealed that after fixation, ~50% of the observable bacteria were enmeshed within the wax residue not removed by fixation (Figs. 4-1, 4-2). Bacteria that were not enmeshed in wax were attached to the relatively smooth surface of the epicuticle (Fig. 4-3).

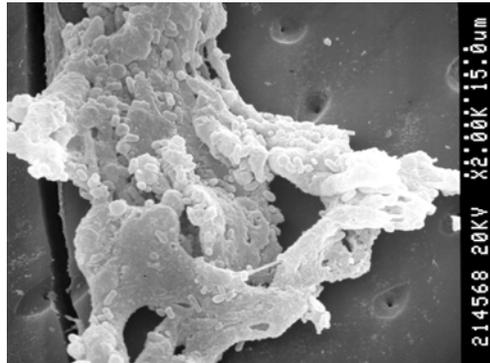


Figure 4-1. *Escherichia coli* O157:H7 enmeshed within the waxy residue after glutaraldehyde fixation on an adult male American cockroach pronotum.

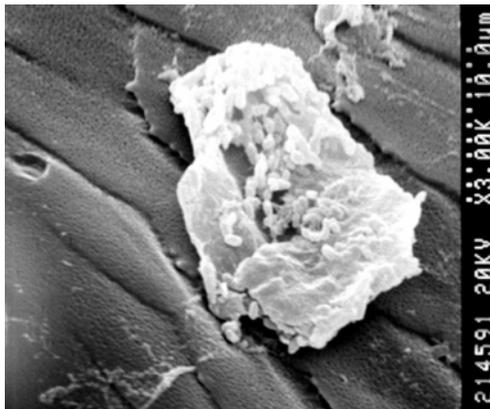


Figure 4-2. *S. enterica* enmeshed within waxy residue remaining after glutaraldehyde fixation on an adult male American cockroach pronotum.

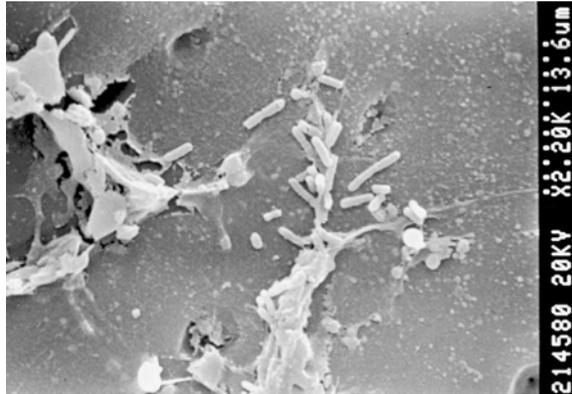


Figure 4-3. Non-pathogenic *Escherichia coli* attached to the surface of an adult male American cockroach pronotum.

Examinations of captured wild German cockroaches also reveal the presence of unknown bacteria enmeshed within a waxy residue after fixation with glutaraldehyde (Gazivoda and Fish 1985). However, although the authors proposed that the residue was possibly bacterial glycocalyx or slime, the bacteria formation they observed is similar to the bacteria/wax formations in my examinations.

Based on the results reported here, the attachment of bacteria to cockroach cuticle and subsequent observation via SEM is dependant on several factors: 1) First, the attachment efficacy of the bacterial strain, 2) number of bacterial cells in inocula, 3) numbers of bacteria enmeshed within wax, and 4) amount of wax that survives processing. Therefore, attempts to recover enteric bacteria from cockroaches should take into account the numerous factors which may influence results.

***Salmonella enterica* Recovery from American Cockroaches Over Time**

At the 4.5×10^5 inoculum level, there were negligible cfu recovered from the unenriched *S. enterica* stock solution (<0.0) for each day throughout the entire testing period. In contrast, significantly more *S. enterica* were recovered at 5 d than at 1 d for

the enriched stock solution ($F = 15.58$; $df = 4$; $P < 0.0001$). Recovery of *S. enterica* from cockroaches was higher at 1 d after inoculation than at all other days, with no recovery at 3 and 4 d.

At the 9.0×10^5 inoculation level, significantly less *S. enterica* was recovered from unenriched stock at 96 and 120 h than at either 24 or 48 h ($F = 18.86$; $df = 4$; $P < 0.0001$). For the enriched stock solution, there was no significant difference in recovery of *S. enterica* at 24 or 120 h. However, a significant drop in recovery occurred at 72 h ($F = 4.68$; $df = 4$; $P = 0.0058$). From cockroaches, there were no significant differences in recovery of *S. enterica* across the entire testing period ($F = 2.02$; $df = 4$; $P = 0.1303$).

At both inoculum doses, recovery of *S. enterica* from cockroaches was not significantly less at 120 h when compared to 24 h after inoculation. However, except at 24 h, although the smaller inoculum dose was half that of the larger, less than half comparative recovery occurred at all time periods (Table 4-2).

Quantitative recovery over time of *S. enterica* from cockroach cuticle inoculated with a known quantity of bacteria has not previously been reported. However, Kopanic et al. (1994) demonstrated that cockroaches exposed to an infected food source will acquire *S. typhimurium*, distributing it to other individuals and to susceptible foods. The authors were also able to recover *S. typhimurium* from cockroaches for up to and including 96 h, whereas Olsen and Rueger (1950) were able to recover *S. orientalis* from American cockroaches up to 78 d after inoculation.

It is evident that once an American cockroach becomes inoculated with *Salmonella*, the bacteria can remain viable for up to several days to several months. In the current study, it is shown that for at least 120 h after inoculation, there is no significant reduction

in the numbers of bacteria recoverable via microbiological methods. However, there is a correlation between the number of bacteria inoculated onto a cockroach and the amount of bacteria recovered, with greater recovery occurring at the higher (9.0×10^5) inoculum.

Recovery of *S. enterica* from Pharaoh Ants

For treated arenas, significantly more workers (67.0 ± 10.0) were recovered at 17 d than at 10 d (54.7 ± 4.0) ($F = 6.10$; $df = 2$; $P = 0.036$). However, there was no significant difference in recovery of workers between 17 d and 3 d, or with recovery of brood within the testing period ($F = 0.57$; $df = 2$, $P = 0.5912$). For control arenas, there were no significant differences in recovery of either workers ($F = 1.34$; $df = 2$; $P = 0.3310$) or brood ($F = 0.31$; $df = 2$; $P = 0.7414$).

The significant differences in recovery of workers from treated arenas at 17 d was probably due to emergence of adult workers from pupae toward the end of the testing period. In control arenas, although recovery of workers at 17 d was not significantly higher than at 3 and 10 d, there was an increased number of worker ants collected. Additionally, at 17 d for both treated and untreated arenas, recovery of workers included those that were not fully sclerotized, indicating that they had recently eclosed (Table 4-3).

Recovery of *S. enterica* from Pharaoh ant workers and brood occurred at 3, 10 and 17 d from all treated arenas, with recovery of *S. enterica* from workers at 3 d averaging a third (34%) of all workers recovered. At 10 d, recovery of *S. enterica* from workers increased to two-thirds (66%) and then significantly declined to 22% at 17 d ($F = 10.47$; $df = 3$; $P = 0.0111$). There were no significant differences in recovery of *S. enterica* from brood ($F=3.07$; $df = 2$; $P = 0.1206$), although recovery did decline considerably from 38.3% at 3 d to 2.7% at 17d. There was no recovery of *S. enterica* from any workers or brood from any of the control arenas (Table 4-3).

It has been well established that Pharaoh ants are capable of establishing colonies in residential structures (Hedges 1997, Klotz 2004). However, investigations on the association of *Salmonella* spp. with Pharaoh ants within structures is relatively scarce, although various human pathogens, to include *S. dublin*, have been recovered from Pharaoh ants foraging in hospitals (Beatson 1972).

Although *Salmonella* has been recovered from foraging workers, it has not previously been demonstrated how the pathogen will interact and survive within a colony. My examination has quantified the percentage recovery of *S. enterica* from both workers and brood of individual colonies after introduction of a known amount of inoculum. It has also been determined that after acquisition of *S. enterica* from an infected food source, the bacteria can be distributed throughout the colony and persist among both workers and brood for up to 17 d.

In summary, the results of my study demonstrate that when 2.5×10^5 bacteria were inoculated onto cockroach pronota, *S. enterica* attached and survived processing more effectively than either *E. coli* O157:H7 or NP *E. coli*. Additionally, *E. coli* O157:H7 attached and survived processing more effectively than NP *E. coli*, with visible attachment of NP *E. coli* to pronota $<0.04\%$ of the number of cells in the original inoculum. At an inoculum level of 1.0×10^5 , neither *S. enterica* nor NP *E. coli* were observed on any pronota.

Microbiological recovery of bacteria over time from inoculated adult male American cockroach pronota was also dependant on inoculum dose with greater recovery occurring across all time periods (24 – 120 h) from cockroaches inoculated with 9.0×10^5 cells compared to those given a 4.5×10^5 inoculum. Additionally, when Pharaoh ants

were fed cockroaches inoculated with 5 μ l of a suspension containing 1.5×10^5 cells of *S. enterica*, the bacteria were acquired and distributed throughout pharaoh ant colonies, and viable cells were recovered from both workers and brood for up to 17 d after exposure.

Based on the data presented here, it is suggested that the mechanical vector capacity of the American cockroach depends on several factors: 1) the ability of the pathogen to attach to the cuticle of the insect, with differential attachment occurring between strains, 2) the amount of the pathogen that is encountered by an insect and 3) the amount of time between pathogen acquisition and contact with a susceptible host, food or food contact area.

The same factors necessary for successful pathogen transmission by American cockroaches may also occur for pharaoh ants, but were not investigated in this study. However, it was determined that *S. enterica* can be acquired by foraging ants from an infected food source and then distributed throughout a colony. Additionally, the persistence of the pathogen within the colony can last for up to 17 d after initial exposure.

Table 4-1. Visible *Salmonella enterica*, *Escherichia coli* O157:H7, and non-pathogenic *Escherichia coli* cells on pronota of adult male American cockroaches inoculated with $n = 1.0$ or 2.5×10^5 cells, incubated (24 h), fixed with glutaraldehyde and viewed with scanning electron microscopy.

Cockroach number	1.0×10^5			2.5×10^5			
	<i>S. enterica</i>	NP <i>E. coli</i>	Control	<i>S. enterica</i>	<i>E. coli</i> O157:H7	NP <i>E. coli</i>	Control
1	-	-	-	++++ ^a	++++	++(<100) ^b	-
2	-	-	-	++++	+(14)	+(2)	-
3	-	-	-	++++	++++	-	-

^a++++ = Too numerous to count.

^bNumbers indicate the amount of countable bacterial cells.

Table 4-2. Recovery of *S. enterica* from American cockroaches and original inoculum of either 4.5×10^5 or 9.0×10^5 over time (5 d).

Mean colony forming units (CFUs) \pm SE at 10^{-4} dilution								
4.5×10^5				9.0×10^5				
Hour after inoculation	Stock (U) ^b	Stock (E) ^c	n	Cockroaches ^d	Stock (U)	Stock (E)	n	Cockroaches
24	0.0 \pm 0a	3.0 \pm 0.2a	5	5.4 \pm 1.9a	10.5 \pm 1.8a	23.3 \pm 2.1a	5	8.6 \pm 1.8a
48	0.0 \pm 0a	16.3 \pm 1.8b	5	0.8 \pm 0.8b	9.7 \pm 1.3a	22.3 \pm 2.7a	5	9.9 \pm 1.0a
72	0.0 \pm 0a	7.0 \pm 1.6ac	5	0.0 \pm 0b	3.0 \pm 1.1b	12.3 \pm 0.8b	5	2.9 \pm 2.9a
96	0.0 \pm 0a	11.7 \pm 0.7bc	5	0.0 \pm 0b	0.8 \pm 0.3b	18.7 \pm 2.2ab	5	5.0 \pm 2.4a
120	0.0 \pm 0a	11.2 \pm 1.4bc	5	1.2 \pm 1.2ab	0.3 \pm 0.2b	17.3 \pm 1.8ab	5	6.3 \pm 0.9a

Table 4-2. continued.

Means within a column followed by different letters are significantly different ($P < 0.05$, Tukey's studentized range test [SAS Institute 2001]).

^a No recovery of *S. enterica* from control (DI) water cockroaches.

^b Unenriched stock inoculum: serially diluted and plated directly onto HE media.

^c Enriched stock inoculum: incubated in nutrient broth prior to serial dilution and plating onto HE media.

^d Pronota of inoculated cockroaches removed and incubated in nutrient broth prior to serial dilution and plating onto HE media.

Table 4-3. Mean number (\pm SE) of Pharaoh ants recovered from arenas containing cockroaches inoculated with *S. enterica* or deionized water and percent (\pm SE) of ants positive for *S. enterica* at 3, 10 and 17 days after inoculation.

Day	n	Treated				Control			
		Workers		Brood		Workers		Brood	
		Recovered	% Positive	Recovered	% Positive	Recovered	% Positive	Recovered	% Positive
3	3	54.7 \pm 2.3ab	33.7 \pm 7.2a	20.7 \pm 7.4a	38.3 \pm 18.9a	56.7 \pm 7.3a	0.0 \pm 0.0a	22.7 \pm 4.6a	0.0 \pm 0.0a
10	3	47.3 \pm 3.4a	66.3 \pm 10.0b	29.7 \pm 3.0a	34.5 \pm 3.7a	58.7 \pm 5.2a	0.0 \pm 0.0a	23.7 \pm 7.5a	0.0 \pm 0.0a
17 ^a	3	67.3 \pm 5.8b	21.9 \pm 0.7a	22.0 \pm 7.8a	2.8 \pm 1.6a	71.0 \pm 7.4a	0.0 \pm 0.0a	18.0 \pm 3.0a	0.0 \pm 0.0a

^aAt 17 d, recovery of workers included those that were not fully sclerotized, indicating that they had recently eclosed. Means within a column followed by different letters are significantly different ($P < 0.05$), Tukey's Studentized range test [SAS Institute 2001]).

CHAPTER 5 SUMMARY AND CONCLUSIONS

This research has determined that the traditional model of the American cockroach as a mechanical vector of bacteria needs to be revised. It has been popularly described that bacteria are picked up by American cockroaches, adhering to an external cement layer from which they are readily dislodged into the environment. However, the interaction of enteric bacteria with the external surface of the cockroach cuticle is more complex than previously described, and depends upon a number of factors.

First, the morphology of the cuticle and specifically, the presence of the wax layer are important aspects of bacterial attachment. The current research verifies that the wax layer is a fluid (Beament 1955) and morphologically variable substance. However, although a shellac-like cement layer (1955) has been reported to cover the cuticular wax, the current investigation was not able to verify the presence of cement. Examination of American cockroach pronota via scanning electron (SEM), transmission electron (TEM) and light microscopy did verify the presence of the following cuticle components: an external wax layer (~1 to 3 μm), an inner exocuticle (~2 μm), a lamellate sponge-like endocuticle (~24 μm), a gap (~2 μm) containing a globular substance and an inner layer of transparent cuticle (~6 μm).

In addition to cuticle morphology, the ecology, physiology and behavior of bacteria are also determinative in the role of American cockroaches as potential mechanical vectors. Enteric bacteria such as the *Salmonellae* are often motile and able to swim in an aqueous medium. In the current investigation, *Salmonella enterica* were observed via

videography to readily swim within the wax layer of an American cockroach pronotum. Further examinations revealed that when American cockroach pronota are inoculated with *S. enterica* that, even when inoculated with large amounts of bacteria, no to few (<60) *S. enterica* cells remain on the surface of the wax layer. Rather, the bacteria penetrate the wax layer, becoming either enmeshed within the wax or attached to the surface of the epicuticle. Therefore, the wax, which is continuously sloughed off over time, may serve as a reservoir for *S. enterica* or other Salmonellae.

Finally, comparative examination of the attachment efficacy of three enteric bacteria was conducted: *S. enterica*, *Escherichia coli* O157:H7 and non-pathogenic (NP) *E. coli*. It was found that *S. enterica* attached to cockroach cuticle more effectively either of the *E. coli*, with *E. coli* O157:H7 attaching more effectively than NP *E. coli*. However, when the inoculum dose was reduced by 60%, no bacteria were visible within residual wax or attached to the epicuticle.

To determine recovery of *S. enterica* over time, two insect species were utilized in investigations, American cockroaches and Pharaoh ants. Recovery of *S. enterica* occurred at 24, 48, 72, 96 and 120 h after inoculation, with no significant reduction in recovery over time. However, when inoculated with 50% of the original number of *S. enterica* cells, recovery of *S. enterica* was reduced by more half, with no recovery of bacteria occurring at 72 and 96 h. For Pharaoh ants provided with an infected food source, *S. enterica* was recovered from both workers and brood for up to 17 d.

In summary, there are several factors which influence the ability of an insect to acquire and transmit a disease organism. The physiology of the cuticle will determine the adherence efficiency, as will the physiology of the pathogen itself. Adult male American

cockroaches have a fluid wax layer with no visible or functionally apparent cement barrier. Motile enteric bacteria are able to penetrate the wax and remain viable for a period of at least 5 days. Pharaoh ant workers may also acquire enteric bacteria, *S. enterica*, and transfer it throughout the colony where it becomes recoverable from other workers and from brood.

Based on the results presented here, it is suggested that further examination of the interactions between pathogens and insects traditionally considered mechanical vectors be continued. Specifically, the interactions between American cockroaches and Pharaoh ants with enteric bacteria should be further investigated. Our knowledge of how enteric bacteria interact with pest insects is minimal and increased understanding of those interactions can lead to more effective protections for humans against disease.

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BIOGRAPHICAL SKETCH

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