THE ROLE OF ANAEROBIC BACTERIAL METABOLISM IN THE NUTRITION OF CRICKETS (ORTHOPTERA: GRYLLIDAE)

by

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ABSTRACT

THE ROLE OF ANAEROBIC BACTERIAL METABOLISM IN THE NUTRITION OF CRICKETS (ORTHOPTERA; GRYLLIDAE)

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The relationship between members of the family Gryllidae and the bacterial community in their anterior hindgut was investigated from two perspectives. In one approach, *A. domesticus* larvae were reared with (conventional) and without (germfree) microorganisms under different conditions of diet quality. In general, growth rates and measures of adult fitness were affected more by diet quality than by the presence of bacteria. In all cases, however, conventional larvae digested more, consumed less, and converted a greater portion of available food into biomass than germfree counterparts. Based on measures of excretion rates of uric acid and total N, the mechanism of increased food utilization efficiency was not related to nitrogen metabolism by gut bacteria. Digestibility values of major nutrient classes indicated that the gut bacteria bacteria acted principally on soluble carbohydrates. Enzymatic profiles also revealed that the bacterial community enhanced carbohydrase activity in general and produced at least one enzyme class not found in germfree crickets.

In the second approach, the overall bacterial community metabolism was investigated with a focus on carbon-based fermentation products. Volatile fatty acids (VFA) were found to be produced in quantities estimated to account for 75% of the observed

food utilization advantage observed in conventional crickets (ca 400 nmol/ cricket/ hr.). VFAs were consistently found in the anterior hindguts of a number of gryllid species, and over a range of diets and rearing conditions. The compounds were produced far in excess of their excretion rates, and were shown to readily transverse the hindgut wall. Acetate was the dominant acid formed and evidence indicated that a substantial portion of acetogenesis (ca 30%) could be derived from CO_2 reduction with H_2. The bacterial community/cricket symbiosis, therefore, appears to be based upon carbon conservation mechanisms via anaerobic bacterial metabolism. Gut microbes increase the metabolic diversity of crickets and allow a more efficient use of dietary carbohydrate resources.

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INTRODUCTION

Symbioses in insects.

Insect-microbe symbioses[1] are widespread and almost as diverse as the Class Insecta itself. These associations have been cataloged since the late 1800's, and several books and literature reviews attest to the multitude of insect species that serve as "hosts" to various types of microorganisms (Buchner 1965, Koch 1967, Richards and Brooks 1958, Breznak 1982, Houk and Griffiths 1980). In spite of nearly a century of active research in the area, and the detailed work with several particular groups of insects, most of our present knowledge of insect-microbe associations is descriptive. Given the estimate that less than 20% of insect species are thought to be known to man, and that only a fraction of these have been examined for specific microbial symbionts, it is safe to conclude that our ignorance in the area far exceeds our understanding. Investigations of these symbioses are fascinating and valuable from the pure science perspective, and have been instrumental in addressing questions of nutritional ecology, co-evolution, and microbial ecology. With the advent of pesticides derived directly from microorganisms and the need for non-chemical control agents,

[1] Within the context of this discussion, symbiosis and subcategories will follow the broad definitions of Starr (1971) and Buchner (1965) referring to the close physical association of two different species of organisms throughout all or part of their life cycles.

microbial symbionts of insects present a potential opportunity for manipulations leading to control of particular insect populations; an idea that has intrigued insect pathologists for decades (Lysenko 1985). At the very least, our knowledge of insect physiology and ecology - an invaluable prerequisite to any applied control measures - must include an awareness of the potential contributions of microorganisms.

The types of insect-microbe symbioses range from tightly-linked mutualisms and parasitisms; where the relationship between microorganism and insect is species-specific and obligate, to facultative associations in which neither member demonstrably benefits nor suffers from the relationship. In most cases the association has not yet been classified in terms of its degree of interdependence. Microorganisms can be categorized as ectosymbionts (living outside the body) or endosymbionts (living within the body of the animal). Endosymbionts can be further subdivided into extracellular or intracellular forms. The type of association is thought to indicate the degree of specialization/interdependence of the symbioses; with intracellular forms representing the extreme of mutualistic specialization and presumably a more highly co-evolved situation (Buchner 1965, Koch 1967). The evolution of intracellular endosymbionts is generally thought to have progressed from a condition of extracellular microbes living in the digestive tract to microbes becoming an integral component of specialized cells or groups of cells (mycetocytes). Microbes housed in specialized regions of the gut or contained in specialized organs within the hemoceol (mycetomes)

represent intermediate forms. Microorganisms housed in mycetocytes are transferred from generation to generation of insect via elaborate mechanisms, and in many ways must be considered inseparable components of the insect tissue. Examples include those found in several families of Homoptera(Houk and Griffiths 1980), Coleoptera (Buchner 1965), and Orthoptera (Brooks 1970, Richards and Brooks 1958). Although intracellular endosymbionts clearly represent a high degree of specialization, several other types of associations suggesting highly evolved symbioses occur in those relationships classified as ectosymbioses or extracellular endosymbioses. The relationship between bark beetles (Scotylidae) and certain fungi instrumental in the beetles' utilization of wood is one example (Graham 1967), and recent studies have demonstrated the importance of acquired enzymes from particular fungal ectosymbionts in the nutritional ecology of several insect groups (Martin 1984). Some extracellular endosymbioses are also thought to be highly specific relationships; an example being the association of unique protozoans with lower termites (Breznak 1982).

Although the insect-microbe associations which attract the most attention are those in which some specific interdependence or pathology is clearly suggested, the vast majority of associations cannot yet be classified in terms of any functional interaction. Even among the specific intracellular endosymbioses there is little empirical evidence in many instances that demonstrates the "role" of the microorganism. There is also some controversy regarding the validity of evidence reported by researchers investigating the functional roles of microbial symbionts. Buchner's (1965) treatise on

endosymbionts points out several sources of misinformation and erroneous interpretations from earlier work. More recently, Brooks (1970) has questioned much of the work with bacteroid symbionts in cockroaches, and Campbell and Nes (1983) have indicated that certain functional roles of aphid endosymbionts in cholesterol metabolism may have been misrepresented. Hill *et al.* (1976) have presented evidence that does not support the reported role of *Rhodnius* symbionts in B-vitamin production. Additionally, several assumed mutualistic associations in dipterans have been shown to be less obligatory than previously thought (Schneider *et. al.*, 1983, Howard *et al* .1985). This is not to dismiss the aforementioned examples as inconsequential symbioses; clearly further research is required to examine additional hypotheses about the relationship. It is rather to underscore the point that our understanding of even the best-known associations is fragmentary and sometimes based upon untested assumptions.

Microorganisms are ubiquitously found as residents of insect digestive tracts. It is currently unknown how many insect-gut microbe associations represent any type of symbiosis within the definitions employed here, but in general they are more apt to be facultative and non-vital associations. Many gut microbes are merely transients associated with ingested material; eventually ending up as nutritional sources themselves or being passed out with undigested material. In several insect groups, however, microbes proliferate in the gut tract and become permanent or semi-permanent symbionts. Ironically, gut microbes in insects are probably both the best and least-understood of non-pathological

insect/microbe associations. Research on termites has elucidated a story of nutritional interdependence that has become a classic example of mutualism. Termites and their intestinal protozoa and bacteria represent a highly-specialized, extracellular endosymbiosis. However, in most other insects that possess a permanent gut flora, mutualism has yet to be conclusively demonstrated. Further, in most other insect groups, the constancy and function of alimentary tract microorganisms is completely unknown.

Bignell (1984) discusses the insect digestive tract as an environment for microorganisms and points out that the highest densities of microbes and the more permanent populations are usually found in the hindgut region. The hindgut in insects is well-suited to microbes in many cases since it presents a relatively stable environment, presents a source of nutrients, and is osmotically favorable to most groups. The region is often differentiated morphologically and houses dense, multispecies communities of microbes. These hindgut modifications are variously termed fermentation chambers, diverticuli, paunches, or cecae (McBee 1977, Buchner 1965). The relative size and extent of development of hindgut modifications in insects is strongly correlated with diet (Buchner 1965). Known examples of insects with modified hindgut regions include all families of Isoptera, Scaraboidea, and the Tipulidae, Blattidae, and Gryllidae.

One of the principal roles which has been demonstrated and often postulated for gut microbes in insects is the degradation of refractory polymers that are components of the insects' diet. The digestion of native (crystalline) cellulose is a complex process

involving the action of at least three enzymes, and, with the possible exception of silverfish (Lasker and Giese 1956), it has not been conclusively demonstrated that any insect possesses the capability to completely digest the compound without the aid of one or more microbial enzymes (Martin 1983). In the case of lower termites and woodroaches, digestion of native cellulose in their wood-based diets is accomplished principally by flagellated protozoans inhabiting the hindgut (Breznak 1982, O'Brien and Slaytor 1982, Hogan *et al.* 1988). The lower temite, *Coptotermes lacteus*, produces enzymes active against soluble forms of cellulose in addition to those supplied by the gut protozoa (Hogan *et al.* 1988). Higher termite groups, which lack the protozoan component in their hindgut microbial community, are thought to synthesize some cellulose-degrading enzymes in their own tissue, and the activities of hindgut bacteria do not appear to be involved (O'Brien and Slaytor 1982). Bacteria in the hindguts of cockroaches (*Periplaneta americana*) are thought to be responsible for the utilization of carboxymethylcellulose by the insect (Bignell 1977, Cruden and Markovetz 1979). Cellulase activity was not specifically demonstrated in *Oryctes nasicornis* (Scarabaeidae) larvae by Bayon (1980); however, she indicates that hindgut microbial fermentation activity in the larvae is unaffected when ∂-cellulose is supplied as the only diet constituent. Hindgut bacteria in the field cricket, *Gryllus bimaculatus* , and house cricket, *Acheta domesticus* , have also been implicated in the degradation of cellulose (Martoja 1966, McFarlane and Distler 1984).

Although cellulose metabolism is the most widely-investigated of gut microbe activity, the roles of bacteria in the hindguts of insect

groups may include the utilization of other polymers. Hemicelluloses are apparently degraded by hindgut bacteria in termites (Breznak 1982) and by bacterial isolates from the grass grub, *Costelytra zealandica* (Coleoptera: Scarabaeidae) (Bauchop and Clarke 1975), but little is known about the importance of these activities *in vivo*. Chitin can be utilized by some isolates from cockroach hindguts (Cruden and Markovetz 1987), but there is no evidence to suggest that such a capability is quantitatively important in the insect, since roaches produce their own chitinase (Bignell 1981, House 1974). Less-resistant polysaccharides such as starch, and most oligosaccharides, fall within the metabolic capabilities of many gut bacteria isolated from insects, and these substrates are also normally degraded by insect enzymes. In general, gut microbes in insects are likely to contribute to the diversity of carbohydrases present (House 1974 and references therein), but research thus far has been limited in its focus.

Microorganisms in insect hindguts also are known to contribute to the nitrogen economy of the host. Again, the termite literature provides much of the evidence. N_2-fixation, an activity restricted to prokaryotic organisms, has been demonstrated in several species of termites from both higher and lower families (Prestwich *et al.* 1980, Breznak 1982, O'Brien and Slaytor 1982). The capability to fix atmospheric nitrogen in these insects is clearly related to the low nitrogen content of their wood diet and the activity can be reduced by supplementing the diet with additional nitrogen sources (Breznak 1982). The resulting available nitrogen could account for substantial portions of termite growth requirements, depending upon the

species, caste, and diet involved (Breznak 1982 Bentley 1984). N_2-fixing bacteria have been isolated from cockroaches (Cruden and Markovetz 1987), however its significance to the host is doubtful.

Recycling of uric acid nitrogen via gut microbes has also been demonstrated in termites by Potrikus and Breznak (1981). The insect is itself incapable of degrading the compound and hindgut bacteria are responsible for making a portion of the carbon and nitrogen from uric acid available to the termite in the form of acetate and ammonia (Potrikus and Breznak 1981). A similar mechanism has been postulated for cockroaches which, like termites, store uric acid internally (Cochran 1985), however mycetocyte symbionts are usually invoked as the main uricolytic sites in the insect. Uricolytic bacteria have been isolated from cockroach hindguts (Cruden and Markovetz 1987) and house cricket iliea (Ulrich *et. al.* 1981), but their *in situ* importance remains to be demonstrated.

The metabolism of other nitrogenous compounds by hindgut bacteria in insects is even less understood. The synthesis of some amino acids and the composition of termite protein and free amino acid pools is affected by the presence of gut microbes (O'Brien and Slaytor 1982)). It has also been demonstrated that gut microbes from cockroaches and crickets are capable of converting cysteine to methionine (Bignell 1981, Martoja 1966); a conversion not thought to generally occur in insect tissue (Dadd 1985). It has also been frequently postulated that gut bacteria supply B-vitamins to insects (House 1974, Wigglesworth 1972, Dadd 1985). However, evidence had come mainly from studies of *Rhodnius* and its gut symbionts (Baines 1956, Lake and Friend 1968), and the role of gut microbes in

this insect has been subsequently questioned by Hill *et al.* (1976). Although it seems quite clear that hindgut microorganisms could offer several classes of nitrogenous compounds to the host, it has rarely been shown that they would be available to the insect. Studies with mammals indicate that nitrogenous compounds from hindgut microbes are normally unavailable to the animal without coprophagy or some other means of harvesting gut microbe cellular components (McBee 1977, Reddy *et al* 1968).

Other possible nutritional roles of gut microbes are largely speculative. Incorporation of sulfate into the amino acids methionine and cysteine by gut bacteria has been demonstrated in a few insects (Martoja 1966, Dadd 1985), but the quantitative significance is unknown and the presence and availability of sulfate in normal diets has yet to be shown. Clayton (1960) has indicated that gut bacteria in roaches supply sterols, however, no bacteria are known to synthesis this class of lipids. Bacterial metabolism of other lipids in insect digestive tracts has not been investigated, nor has their possible influence on mineral metabolism, osmolarity maintenance, and excretion. The latter are a potentially important considerations in view of the hindgut/Malpighian tubule interface and the normal function of most hindgut tissue in resorption of water and soluble material (Bradley 1985, Phillips *et al* 1986).

Hindgut bacteria in insects may also interact beneficially in ways not directly related to nutrient metabolism. The production of aggregating pheromones by bark beetles (Scotlylidae) (Borden 1982), locusts (Nolte 1977), cockroaches (Mcfarlane and Alli 1986), and house crickets (McFarlane *et al.* 1983) has been attributed to

metabolites produced by hindgut bacteria. However, Charnley *et al.*, (1985) have recently presented evidence to contradict this contention in the case of locusts. It has also been suggested that gut microbes may act to detoxify ingested compounds (Jones 1984, Guthrie and Apple 1961). This is an interesting hypothesis that deserves investigation, however it currently remains untested. Among other possible non-nutritional roles, the inhibition of pathogenic microoganisms by endemic gut microbes has been of interest to invertebrate pathologists, but little effort has been specifically directed to this question. Dillon and Charnley (1986) have shown the bacterial community in locust hindguts inhibits the germination and viability of the fungus *Metarhizium anisopliae* . Proliferation of certain pathogenic bacteria is inhibited by members of the "normal flora" (Goodwin 1967, Lysenko 1985). Yet as Lysenko (1985) notes, studies of the normal flora of insects have "received surprisingly little attention" .

The roles of gut microorganisms in vertebrate groups have been studied more intensively, due in large part to the interest of agricultural (livestock) scientists and the medical research community. The subject is beyond the scope of this discussion and has been reviewed elsewhere (Luckey 1969, Gordon and Pesti 1971, Savage 1977, 1984, Hungate 1966, 1975, Wolin 1979). All of the same gut microbe metabolic characters discussed above for insect systems have been documented in vertebrate systems. Further, more detailed and specific examples of metabolic interactions with the host have been shown. Indeed, many hypotheses concerning the activities of hindgut microbes in insects originated from studies of

vertebrates. Research on vertebrate gut systems have also placed a much greater emphasis on interaction between members of the gut microbial community; particularly as it relates to pathogen resistance (Savage 1977, Lee 1985). In spite of the relatively intense research in the area, understanding of gut microbe/vertebrate associations, like similar research with insects, suffers from a lack of breadth in the type of organisms examined. Information has been heavily skewed toward ruminants and laboratory rodents. There are no doubt many parallels between vertebrate and invertebrate gut microbe systems, however the basis of comparison is currently restricted to a few examples.

The preceding discussion has emphasized the diversity of insect microbe associations, the potential metabolic capabilities of gut microbial communities, and the paucity of information available on all but a few systems. Within insect groups in particular, research in the area has focused upon obligate microbial associations in species feeding upon incomplete diets. Even the excellent and detailed microbiological characterization of the cockroach hindgut (Cruden and Markovetz 1987) has not clarified the relationship between bacteria and this omnivorous species. An intriguing association of bacteria with the anterior hindgut of crickets (Gryllidae, Gryllotalpidae) has been investigated thus far in only a descriptive manner. This insect hindgut/bacteria system is provocative and deserving of investigation because the association is non-obligate (Martoja 1966) and the insect is not restricted to particular, incomplete diets. Additionally, unlike roaches, nutritional relationships between gut microbes and insect would not be clouded

by the presence of intracellular symbionts as no cricket species are known to harbor such symbionts.

The cricket hindgut system.

Crickets are members of the order Orthoptera and as such represent an ancient group of hemimetabolous insects. There are over 2400 species in the family Gryllidae distributed worldwide in temperate and tropical regions (Alexander 1968). The most familiar groups are the subfamilies gryllinae, nemobiinae, and gryllotalpinae (house and field, ground, and mole crickets, resp.). Most gryllid species are associated with grassland and field soils, and mole crickets are almost exclusively subterranean. House crickets (*Acheta domesticus*), an introduced species in the United States, inhabit human dwellings and refuse dumps in Europe (Bates 1969,1971). As a group, crickets are omnivorous but generally consume plant material (Tennis 1983, VanHook 1971, Gangwere 1961, Walker and Masaki in press). The list of potential diet items is long and varied, and very little is known about the normal feeding and foraging behaviour of these insects. Mole crickets are also omnivorous, with degrees of herbivory and carnivory varying with species (Matheny 1981). Mole crickets (*Scapteriscus* spp.) are important pests of turf in parts of the southern United States (Walker and Nickle 1981, Walker and Ngo 1982) while field crickets (*Teleogryllus commodus*) are a serious pastureland pest in New Zealand (Blank and Olson 1981, Blank and Bell 1982). House crickets are the basis of a multi-million dollar industry which supplies bait shops, pet shops, and zoos in N. America.

In addition to their economic importance, crickets have served as models in several areas of research. Several gryllid species have been studied to address questions of speciation (Alexander 1968, Harrison 1979), mating behaviour (e.g. Cade1981), and physiology (e.g. Woodring *et al.* 1977, 1979). As such, there is a considerable information base, particularly in the area of physiology and nutrition, for these insects. A recent publication (see references in Walker and Masaki in press) reviews areas of research employing gryllids. There is limited information, however, concerning the microbial community associated with the digestive tract of these insects.

The digestive system of all gryllids is similar and is illustrated in Figure I.1. The digestive tract is largely unmodified from the presumed primitive insect gut (Chapman 1985) and reflects the ancient lineage of the group. Unique to crickets is a section of the hindgut located anterior to the insertion of the Malpighian tubules (ileum) and containing setaceous structures with a dense bacterial community. All gryllid subfamilies except for the myrmecophilinae, and all gryllotalpids examined thus far have a similar anatomy and association with bacteria (Martoja 1966, Nation 1983). In most members of the Gryllidae, the bacteria occupy the space between the peritrophic membrane and the gut wall (Fig. I.2, I.3): termed the ectoperitrophic space. The peritrophic membrane usually separates the food bolus from the bacterial community and is disrupted by muscular activity at the junction of the anterior hindgut and the remainder of the proctodeum. This membrane is absent in gryllotalpinae (Thomas and Nation 1984a).

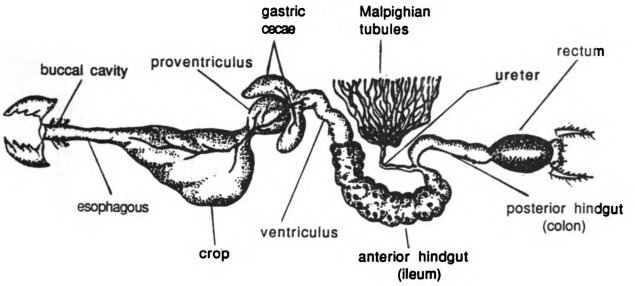


Figure I.1 Gryllid digestive tract. From Martoja (1966)

The anterior hindgut (ileum) in insects is generally thought to function mainly in the export of undigested material and products of metabolism excreted via the Malpighian tubules to the rectum. Mechanisms of water and solute resorption are associated with the posterior hindgut (rectum) (Bradley 1985). It is now known that the anterior hindgut is more than a passive channel to the rectum (Phillips *et al.* 1986), and in the case of crickets, plays a considerable role in digestion and absorption (Thomas and Nation 1984a, b).

The cricket hindgut as an environment for bacteria is likely to be influenced by substrates passing from the midgut, excretory products and activities of the surrounding tissues, and by the activities of the bacteria themselves. The presence of a peritrophic membrane will restrict the availability of large compounds (> 20 nM) to the bacterial community (Richards and Richards 1970, Chapman 1985). Contributions from the Malpighian tubules are likely to

Figure I.2 Scanning electron micrographs of the luminal side of the anterior hindgut wall of *A. domesticus*. (A) Conventional adult 3 weeks after molt; 200X. (B) Germfree adult 3 weeks after molt; 200X. (C) Close -up of setacaceous papillae in conventional adult; low mag 540X, inset 4500X. (D) Corresponding view of (C) in germfree version; low mag 540X, inset 3000X. (E) Close-up of wall between papillae in conventional adult; low mag 540X, inset 2700X. (F) Corresponding view of (E) in germfree version; low mag 180X, inset 2000X.

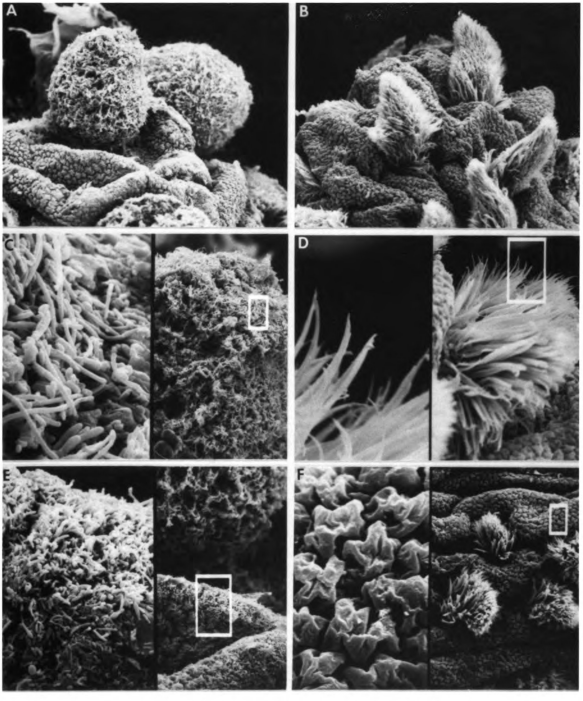
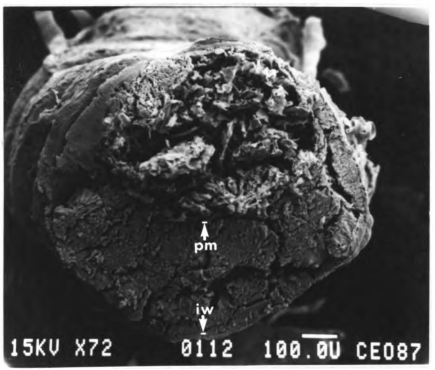
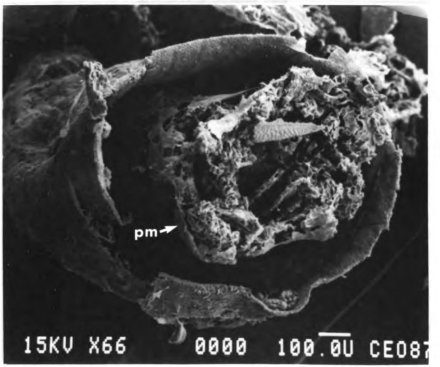

A
B
C
D
E
F

Figure I.3 Transverse section through anterior hindgut of germfree and conventional *A. domesticus* adults (SEM, freeze-fracture prep.). Material between peritrophic membrane (pm) and ileum wall (il) in conventional animal is bacteria and associated extracellular matrix. Bar = 100μM.

Conventional



Germ-free



include a pool of soluble materials (inorganic ions, amino acids, soluble carbohydrates) that are a primary filtrate of the insect's hemolymph (Bradley 1985). Additionally, the tubules concentrate uric acid for excretion as the insect's major nitrogenous waste product. It is unclear how much if any of the Malpighian tubule fluid contents are available to the bacteria in the cricket anterior hindgut, since the community is located "upstream" of the ureter's insertion point (Figure I.1). However, anterior movement of ectoperitrophic space fluid is known to occur in insect midguts (Dow 1986) and such a flow might carry Malpighian tubule effluent to cricket hindgut microbes in this case also.

The physiochemical environment of the gryllid hindgut is incompletely known. pH conditions within the digestive tracts of most gryllids is circumneutral; with the anterior hindgut reported to range from 6.2 - 7.8 (Teo and Woodring 1985, Thomas and Nation 1984b, Srivastava and Baigal 1983). Oxygen concentrations and eH values for the region have not been reported, but Ulrich *et al.* (1981) reported the isolation of strict anaerobes from the anterior hindgut of *A. domesticus* . Maintenence of anaerobic conditions is presumably a consequence of the metabolism of the underlying epithelium and the facultative bacteria present (Veivers *et al* 1980).

The bacterial community is found in the hindgut of very young instars and is present throughout the life of the insect (Martoja 1966). However, the community is periodically disrupted during the molting process since the hindgut cuticle is derived from ectoderm and must be shed for growth. This event occurs from 6 to 12 times during development of *A. domesticus* (Patton 1978) and necessitates

the redevelopment of the community. Martoja (1966) has observed the molting process and reports that the hindgut cuticle is shed separately and subsequent to the exoskeleton. He states that the bacterial community is reestablished by the fraction of the populations remaining after the old hindgut cuticle is defecated and that the recolonization is quite rapid (< 2 days). The original inocula for hatchlings is not known for certain, but clearly is initiated through the ingestion route. There is no evidence of transovariole (within the egg) transmission of microorganisms in crickets. Martoja (1966) concluded that the egg surface is contaminated with gut microbes as it is laid (some microbes possibly supplied by the male parent through spermatophore transfer) and that these provide the inoculum for hatchlings. There is, however, no demonstrated evidence of this event.

The composition of the bacterial community in gryllids has been examined in a descriptive manner in only one species, *A. domesticus* (Ulrich *et al* 1981). Facultative anaerobes such as *Citrobacter* spp., *Klebsiella* spp., *Yersinia* spp., and unidentified strains of Enterobacteriaceae dominated the isolates obtained. Strict anaerobes were less numerous and included *Bacteroides* spp. and *Fusobacterium* spp. Overall, the isolates and facultative nature of the community suggest a similarity with the bacteria isolated from termites (Schultz and Breznak 1978), however the isolates obtained from general media are likely to represent only a small percentage of forms present in vivo. Other gut systems such as the rumen, vertebrate hindguts, and the cockroach hindgut contain a higher

porportion of strict anaerobes (Hungate 1975, McBee 1977, Cruden and Markovetz 1987).

The metabolic capabilities of isolates obtained by Ulrich *et al* (1980) were characterized with API system techniques and were not remarkable in degradative capabilities. Isolates did not grow on ball-milled filter paper based media, but a high percentage were able to degrade uric acid. Fermentation products included acetate, propionate, and butyrate. However, *in vivo* capabilities were not assessed.

Although bacterial isolates from *A. domesticus* display no particularly unusual degradative capabilities, it is unclear whether members of the hindgut community supplement the cricket digestive system by acting on substrates for which no host enzymes are produced. Teo and Woodring (1985) found that only raffinase activity is likely to be restricted to the hindgut of house crickets. Interestingly, a *Fusobacterium* sp. isolated from crickets shows activity against the plant trisaccharide raffinose (Ulrich *et al.* 1981). It also seems likely that at least part of the microbial activity in the anterior hindgut could augment the cricket's own digestive capabilities by duplicating the enzymatic processes of the host; essentially providing an additional attack on solubles that escape digestion/absorption in the midgut. Teo and Woodring (1985) and Thomas and Nation (1984a) have shown that degradation of substrates in the anterior hindgut segment of crickets is reflective of enzymatic activity in the gastric cecae and midgut segments. Presumably, this activity results from the continued action of insect-produced enzymes. However, since activity was assayed as

disappearance rates of substrates, evidence of enzyme action in the anterior hindgut would also include microbial attack on the compounds.

Statement of purpose.

It is clear that basic information about the relationship between crickets and their hindgut microbes is fragmentary. The same can be said of omnivore/gut microbe associations in general. This study examines the functional relationship between gryllids and the microbial community in their digestive tract. In a broader sense, it addresses the question of interaction between host and gut microbe in omnivorous animals; where the association is non- obligate and inconspicuous from the perspective of nutritional interdependency. The approach taken here is bipartite and attempts to: a) determine the effect of the microbial community's presence on growth and food utilization by the insect over a range of diet conditions and b) to characterize the microbial community's metabolic activities most likely providing the interface between insect and microbe.

The first portion of the investigation addresses the question "Does the hindgut microbial community in *A. domesticus* contribute beneficially to the insect across conditions of varying diet quality?" It is hypothesized that any microbe-mediated nutritional benefit would be more pronounced as diet conditions deteriorate, and conversely, that microbial contributions to nutrition would be inconsequential when an abundant and complete diet is available.

The second part of the study is directed toward describing the bacterial community's fermentative metabolism in a range of cricket

species, identifying and quantifying metabolites potentially available to the insect, and determining the constancy of these metabolites across diets and in a range of gryllid species. Research is focused on terminal processes of anaerobic metabolism in order to gain an understanding of important interactions within the bacterial community and because these end products represent the primary pool of compounds available to the insect.

CHAPTER 1

Growth and food utilization parameters of germfree *Acheta domesticus* (Orthoptera: Gryllidae)

Introduction

The presence of symbiotic microorganisms in the digestive tracts of insects is usually correlated with refractory and/or incomplete diets. Most familiar are the obligate microbial associations in xylophagous insect species (Breznak 1982, Buchner 1965). The anterior hindgut of crickets (Gryllidae) is also characterized by high densities of microorganisms (Martoja 1966, Ulrich *et al.* 1981); however, potential nutritional roles of this bacterial community are obscured by the fact that crickets are omnivorous, opportunistic feeders and not restricted to particular, incomplete diets. Additionally, the associaton of gut microbes with crickets appears to be nonobligatory to the insect (Martoja 1966). It has been shown that considerable digestion and transport of nutritionally valuable compounds occurs in the gryllid anterior hindgut (Thomas and Nation 1984 a,b), but the contribution of the bacterial community to these processes is not yet understood.

In omnivorous cockroaches, hindgut microbes are thought to contribute to carbon and nitrogen metabolism in the insect through the degradation of cellulose (Bignell 1981) and uric acid (Cochran 1985). There are conflicting reports regarding utilization of cellulose by crickets. Martoja (1966) and McFarlane and Distler (1982) reported digestion of the polymer in *Gryllus bimaculatus* and *Acheta domesticus* , respectively. However, Teo and Woodring (1985) could find no evidence of a cellulase in *A. domesticus* and Ulrich *et al.* (1981) could not isolate cellulolytic bacteria from *A. domesticus*

digestive tracts. A potential for nitrogen recycling in crickets was suggested by the finding that a large percentage of microbes isolated from *A. domesticus* hindguts were able to degrade uric acid (Ulrich *et al.* 1981), but it remains to be seen if this activity is important *in vivo*.

The following report details initial investigations into the nutritional interactions between *Acheta domesticus* (Orthoptera: Gryllidae) and the bacterial community inhabiting its anterior hindgut. The objective was to determine the overall effects of the bacterial community's presence on the development of *A. domesticus* reared under dietary conditions varying in plant carbohydrate and nitrogen content.

Materials and Methods

Insects: *Acheta domesticus* individuals were obtained from stock cultures maintained in the laboratory for several years. The stock cultures were kept at 30°C, 60% R.H., and on a 12:12 L:D cycle. Crickets were fed a commercial diet (described below).

Germfree techniques: The terminology employed here to describe animals reared with and without microbes is discussed by Coates and Gustafsson (1984) . "Germfree" (=axenic) will refer to conditions of no detectable bacteria and fungi, while "conventional" will apply to insects with a "normal", undefined compliment of microorganisms.

Germfree cricket hatchlings were obtained in the following manner. Stock culture females oviposited into a dish of moist,

autoclaved sand for a 24 hr. period. Eggs were incubated in the oviposition dish for five to seven days and separated from the sand by washing with distilled water. Broken and discolored eggs were discarded, and the remainder rinsed several times with sterile distilled water. The eggs were then immersed in a 0.3% solution of benzalkonium chloride and gently agitated for 10 minutes. Following several rinses with sterile distilled water, eggs were transferred individually to brain heart infusion (BHI Difco Co., Detroit, MI) plates using aseptic techniques. After 24 hrs. incubation at 30°C, eggs showing no sign of contamination were aseptically transferred to fresh BHI plates and incubated for an additional 24 hrs at 30°C. Groups of 20-25 eggs which showed no evidence of microbial growth were transferred to sterile plastic petri plates (100 x 80 x 25 mm) containing moistened filter paper and incubated at 30° until hatching. All BHI plates used were also incubated for an additional 4-7 days to assay for slower growing contaminants. In practice, the majority of contamination manifested itself in the first 24 hrs. and subsequent prolonged incubations rarely indicated new growth. Subsamples (2-3) of young larvae (5-7 days after hatch) from each group were assayed for contaminants by crushing them with forceps to expose and open the digestive tract, and then inoculating media with the entire carcass. Several media were initially employed as screens; including BHI agar and broth, blood agar, PYG (peptone-yeast extract-glucose) plates, nutrient agar, and 2% malt extract broth. Preliminary work indicated that BHI plates and broth were equal to or better than other aerobic media in detection efficacy, and utilized anaerobic BHI or PYG broth (Holdeman and Moore 1971)

after transferring larvae to an anaerobic glove bag (Coy Manufact. Ann Arbor, MI). Though contaminant growth was rarely seen in malt extract broth, the media was used as a screen for yeasts in all initial and final tests.

All manipulations of surface sterilized eggs and subsequent transfers of germfree crickets were done at a laminar flow work station. Plates and growth chambers were kept in microisolater units (Lab Products Inc., Haywood, N.J.) which provided an effective secondary barrier during incubations in environmental chambers.

Groups of hatchlings deemed germfree with the above tests were subdivided into smaller groups as they matured by transferring the insects to fresh petri plates. Sterile food and water were aseptically placed in the 100 x 80 x 25 mm plastic petri plates (henceforth referred to as "growth chambers") prior to the introduction of insects. All food (diets described below) was stream-sterilized (121°C) for 30 min and dried. Water was introduced in 3 dram vials with cotton rolls as wicks. The vials, placed horizontally in the growth chambers, were equipped with stainless steel wire collars to reduce rolling movements. Crickets were transferred to new growth chambers at approximately weekly intervals. Transferring the insects was facilitated by chilling them (5°C, approx. 30 min.) prior to handling. Routine assay for contaminants was accomplished by plating fecal material and moist cotton swabbings of used chambers on aerobic BHI. Critical assays for contamination performed at the end of an experiment included aerobic and anaerobic incubation of fecal material and swabs of each chamber, phase microscopic examination of fecal material, placing whole, crushed insects in aerobic and

anaerobic broth, and examination of the anterior-hindgut intima of several animals with Scanning Electron Microscopy (SEM) (Ulrich *et al.* 1981). Efficacy of aerobic BHI plates for routine containment checks was confirmed by failure to detect microorganisms with any of the above methods when BHI plates showed no sign of growth.

Successive generations of germfree *A. domesticus* were obtained by transferring mated females to larger chambers (Pyrex Corning #3250 storage jars) containing oviposition dishes.

Diets: A "natural" diet for crickets and other opportunistic omnivores is unknown and variable enough to be essentially undefinable. A commercially available "cricket feed" (Country Mark Inc., Columbus, OH) was used as a basic diet for the maintenance of stock cultures and as an "optimal" reference diet (Diet one) for growth studies because it contained mixtures of unrefined plant and animal material that would be similar to the general classes of material encountered by "wild" crickets. The commercial diet consisted largely of a mixture of wheat middlings and soy bean meal, with animal protein and fat, vitamins, and minerals added as supplements. The diet was similar to that employed by Woodring *et al.* (1977) in studies of growth and food utilization by *A. domesticus.* Though ill-defined, cricket feed supported good growth of *A. domesticus* , contained no antibiotics, and remained palatable to the insects after autoclaving.

Diets two and three consisted of 50:50 (wt.:wt.) dilutions of Diet one with alfalfa hay and cellulose (ball-milled filter paper), respectively. Diet two increased the level of plant structural carbohydrates in the diet while only marginally decreasing total

nitrogen levels. Diet three increased structural carbohydrates and halved nitrogen content of ingested material. Diets two and three also presumably diluted fat and vitamin pools. Basic component percentages for each diet are presented with the results (Table 4). All diets were ball milled for 48 hrs., sieved (#40 mesh), and stored under desiccation. Diets were autoclaved (121°C) for 30 min. and dried prior to use in growth studies for both germfree and conventional animals.

Growth and food utilization experiments: Ten groups consisting of three larval crickets of each type (conventional and germfree) were reared from hatching to 10 days beyond the adult molt on each of the three diets. Germfree animals were third generation under axenic conditions and conventional larvae were taken from a parallel stock culture originating from the same batch of eggs. Conventional animals were reared in chambers identical to those described for germfree larvae except that the petri dish lids were perforated. Humidity levels within the conventional chambers were consequently 10-15% below levels within the germfree chambers. The humidity reduction was necessary to control occasional mold growth on uneaten food over the course of the experiment and did not appear to affect growth rates in preliminary experiments.

The food utilization parameters: approximate digestibility (AD), efficiency of conversion of ingested matter to biomass (ECI), efficiency of conversion of digested matter to biomass (ECD), and relative consumption rates (RCR) were calculated following Waldbauer (1968) and were determined during four consecutive periods encompassing the most linear portion of the larval growth

curve. Fecal material weight was corrected for uric acid content in estimates of digestibility (Waldbauer 1968). The growth periods were arbitrary divisions, but most larvae had reached the last instar by the start of period #4 on all diets. Larvae were weighed after each period and transferred to fresh chambers. Remaining food and fecal material was dried (50°C, 72 hrs), sorted, and weighed. Fecal material was then ground and stored under desiccation for later chemical analysis. Larval fresh weights were converted to dry mass with linear regression equations derived from wet-to-dry measurements of larvae taken from parallel cultures of germfree and conventional insects. Larvae were separated prior to the final molt and were weighed 5 days after reaching the adult stage. Adult females were also weighed and sacrificed 10 days after the final molt to determine the number of eggs produced.

Chemical analyses: Uric acid (UA) and nitrogen (N) content of fecal material was determined for each replicate group of crickets at each growth period. For UA determination, duplicate 10 mg subsamples of material were extracted for 30 min at 60°C in a Li_2CO_3/boric acid buffer (Cox *et al.* 1976), centrifuged, and the supernatant analyzed with a modified version of an HPLC method devised by Marquardt *et al.* (1983). A Regis ODS C18 column (25 x 4.6 mm) was used for separation of the compound eluted with a mobile phase of 50 mM Na PO_4 acidified to pH2 with phosphoric acid. The flow rate was 1.0/ml/min. at 40°C. U.V. dectection at 305 nm was used in the assay.

Total N content was measured for triplicate subsamples of each fecal sample using a Carlo Erba Elemental Analyzer (Model 1102).

Nitrogen content was also determined for six subsamples of each autoclaved diet using the above methods. Nitrogen in whole body tissue was determined for six larvae of each type during the final growth period. Larvae were obtained from parallel cultures of germfree and conventional crickets and were prepared as follows. The crop and contents were removed and the carcass was then dried and subsequently ground in a micro-Wiley mill (#40 mesh). Triplicate subsamples of each larvae were then analyzed as above for total N content.

Total lipid, soluble carbohydrate, insoluble carbohydrate, protein and ash content were estimated for food and fecal material collected during growth period 4. Food material analyses were done on six subsamples of each autoclaved diet, while analysis of fecal material was done on material from individual larvae replicates. Total lipids were estimated by the methods of Bligh and Dyer (1959). Soluble carbohydrates were determined as those sugars released after extraction with 0.1N H_2SO_4 and quantified with the phenol-sulfuric method (Dubois *et al.* 1956). Woodring *et al.*. (1979) reported that virtually all carbohydrate available to the normal *A. domesticus* digestive system is released upon extraction with 0.1 N H_2SO_4, and the soluble carbohydrate refered to here is equivalent to their "digestible" carbohydrate. Total residual (=insoluble) carbohydrate was estimated by successive extraction of the material with hot water (100°C, 30 min) and hot 1.0N H_2SO_4 (100°C, 30 min), followed by chloroform/methanol (25°C, 30 min) to remove lipids and serve as a final wash. The ash-free dry weight of the material remaining

remaining after these extractions was taken to be a relative estimate of refractory structural carbohydrate content.

Protein content was estimated from total nitrogen values (x 6.25) after adjustment for uric acid, and percent ash was determined by standard methods. Approximate digestibility (AD) values for each component were calculated as described above for total dry weight (Waldbauer 1968).

Enzyme comparison: Comparisons of germ free and conventional enzyme activity were made using API ZYM system (API Analytical Products, Sherwood Medical, Plainview, NY). Digestive tracts were excised from eight insects of each type and subdivided into two regions. The midgut region included gastric cecae and the ventriculus, and the hindgut segment consisted of the bacteria-rich ileum. Gut segments from each category were pooled and homogenized in 2 ml of deionized water with a tissue grinder. Eight each of germ free and conventional adult *A. domesticus* midgut sections were pooled for the analyses, and eight germ free and four conventional hindgut segments were used. Fewer conventional hindgut segments were used in order to keep the weight of homogenized material approximately equivalent to the germ free material. A subsample of each homogenate was inactivated by heating at 95°C for 15 minutes and used as a blank. Subsamples of the homogenate were added to substrate containers as per API instructions, and incubated for 5 hrs at 30°C. Semi-quantitative measures of enzyme activity levels were then determined by comparisons of color development in active vs blank homogenate treatments for each gut segment category.

^{14}C-cellulose (ICN) was purified by methods adapted from Papson (1963). and added as an aqueous suspension to excised digestive tracts of conventional and germfree *A. domesticus*, and the field cricket, *G. pennsylvanicus*, in 2.5 ml Venoject® tubes. The suspension and gut material were homogenized with a glass rod, after which the tubes were flushed with N_2 and incubated for 24 hrs. at 30°C. The suspension was then diluted with 1.0 ml deionized water, clarified by centrifugation, and filtered (0.22 μM). Filtrates were assayed for radioactivity in aqueous solution with liquid scintillation techniques.

Statistics: Parameters were compared as their log transformed values with standard ANOVA and regression techniques (Sokal and Rohlf 1969, Kleinbaum and Kripper 1978). Experiments were initially analyzed in a three-way factorial design. When significant interaction was found, further analyses were performed with separate ANOVA of parameters within each diet or growth period. Unless otherwise indicated, probability levels less than 0.05 were considered significant.

Results

Growth parameters: Growth rates (Figure 1.1), size at maturity, maturation time, and fecundity estimates (Table 1.1) were generally very similar for germfree and conventional insects. Although conventional animals tended to be slightly larger than germfree

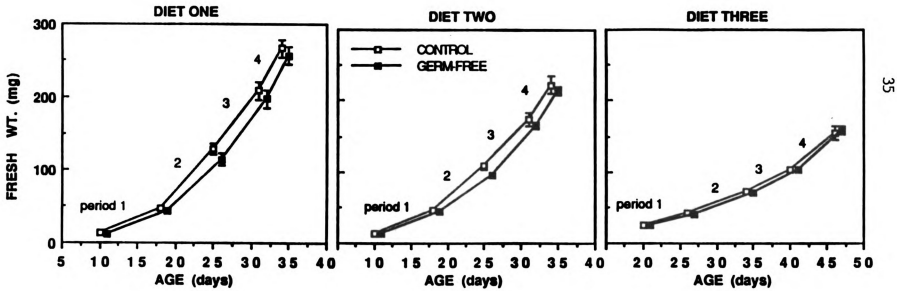


Figure 1.1 Growth of germfree and conventional *A. domesticus* larvae on three diets. Bars are S.E. n = 25-30.

Table1.1 Adult size, maturation time, mortality, and fecundity values for germfree and conventional *A. domesticus* on three diets. Adult weights were taken 5 days after the final molt. Values are mean + S. E. (n).

		Diet One	Diet Two	Diet Three
Male wt. (mg)	Conventional	369.9 ± 8.9 (14)	283.4 ± 7.9 (15)	215.0 ± 6.8 (11)
	Germfree	327.5 ± 8.5 (15)	274.6 ± 7.7 (16)	250.6 ± 5.4 (14)
Female wt. (mg)	Conventional	432.6 ± 11.7 (16)	370.4 ± 10.1 (15)	257.2 ±7.3 (14)
	Germfree	408.3 ± 7.3 (12)	339.6 ± 8.5 (14)	254.2 ± 8.9 (11)
Maturation time (days)	Conventional	38 ± 0.4 (30)	39 ± 0.6 (30)	51 ± 1.3 (25)
	Germfree	39 ± 0.6 (27)	39 ± 0.4 (30)	51 ± 0.6 (25)
# eggs per female	Conventional	444 ± 13.9 (16)	331 ± 10.3 (15)	137 ± 14.9 (14)
	Germfree	441 ± 13.1 (12)	324 ± 11.8 (14)	207 ± 9.6 (11)
Total % mortality	Conventional	3.3	3.3	16.7
	Germfree	3.3	3.3	16.7

counterparts on diets one and two, the only statistically significant difference in any of the growth parameters was found between fecundity estimates for crickets reared on diet three; conventional crickets produced fewer eggs during the first 10 days of maturity. Adult male size of conventional insects reared on diet three was also reduced, but not significantly.

Food utilization: Differences between germfree and conventional animals were manifested clearly in the insects' abilities to digest food. Conventional crickets digested a greater percentage of each diet (AD, Figure 1.2) and converted food into biomass more efficiently (ECI, Figure 1.2). The AD differences were significant across diets and time periods, and ECI differences were significant. Overall, crickets with hindgut microbes digested 34-50% more of ingestia than germfree crickets and gained 21-39% more biomass per unit of food ingested on average over the four time periods (Table 1.2). Germfree crickets tended to convert digested food into biomass more efficiently (ECD, Figure 1.2), but the difference was significant only within periods 1 and 3 of the Diet two treatments and period 2 of the Diet three treatment. Though conventional animals were more efficient in converting food to biomass, growth rates were similar to those in germfree animals because conventional crickets consumed significantly less food (RCR, Figure 1.2) across all diets and time periods.

Food utilization parameters changed with diet treatments for both germfree and conventional crickets. Efficiencies decreased from diets one to three, while consumption rates generally increased with the exception of period 4. Changes in food utilization parameters

Figure 1.2 Digestibility, food utilization efficiencies, and relative consumption rates for germfree and conventional *A. domesticus* larvae on three diets. Periods are growth intervals illustrated in Figure 1.1. Bars are S. E., n = 6-10.

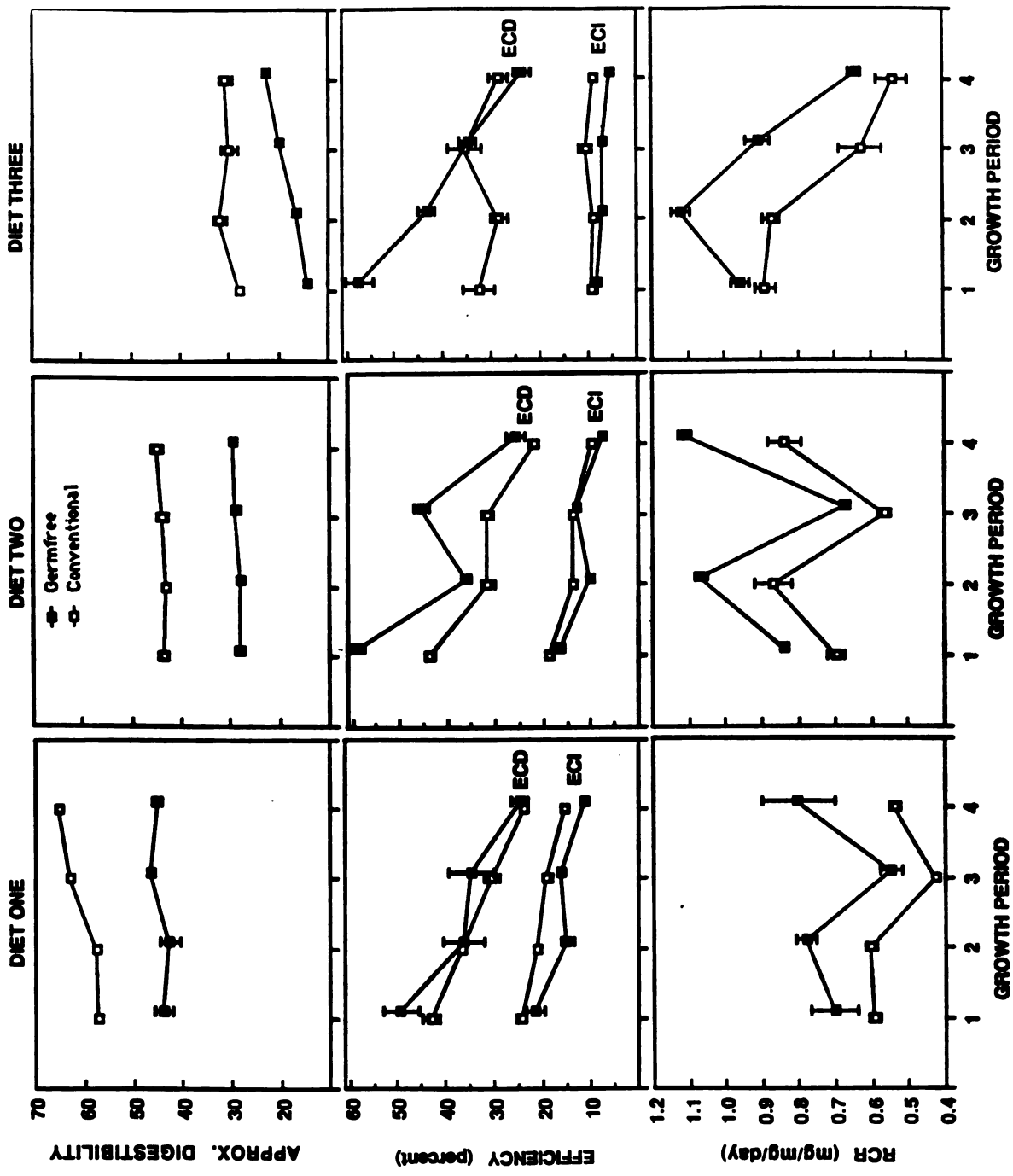

DIET ONE
DIET TWO
DIET THREE
Germfree
Conventional
ECD
ECI
APPROX. DIGESTIBILITY
EFFICIENCY (percent)
RCR (mg/mg/day)
GROWTH PERIOD

Table 1.2 Overall digestibility and biomass gain per 100 mg of food consumed by germfree and conventional *A. domesticus* during the larval stage. Values are combined estimates from all growth periods. All values are mg dry weight.

	Diet one		Diet two		Diet Three	
	Digested	Biomass gain	Digested	Biomass gain	Digested	Biomass gain
Conventional	**63**	**19**	**44**	**13**	**30**	**9**
Germfree	**47**	**15**	**30**	**10**	**20**	**7**

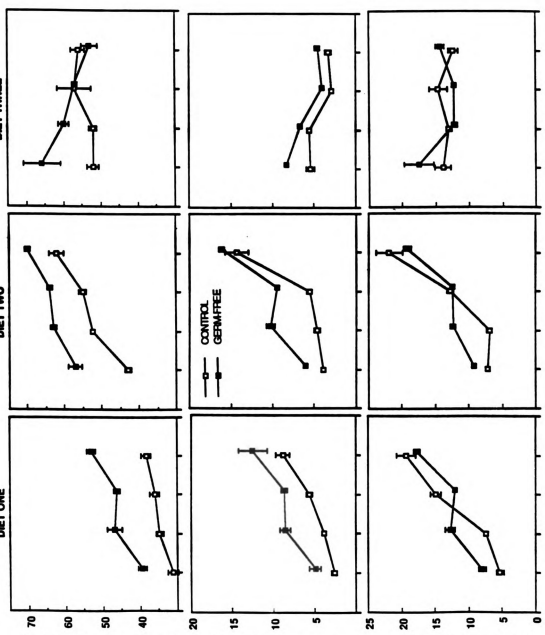
across diets were the same for germfree and conventional animals; i.e. differences between germfree and conventional animals in these parameters remained nearly constant as diet quality was decreased.

Nitrogen excretion (Figure 1.3) paralleled overall food utilization efficiency patterns on diets one and two; conventional larvae excreted a smaller portion of consumed N and apparently conserved the element. However, older instars of both larval types on diet three showed no difference in overall N balance. Total N percentages of combined body tissues did not differ significantly between types on any diet treatment, but larvae reared on diet three tended to contain a smaller percentage of N (Table 1.3). Although conventional animals excreted less uric acid in total than did germfree versions (Figure 1.3), the two groups produced similar amounts of the compound per unit N consumed (Figure 1.3). On diets one and two, however, younger instars (growth periods 1 and 2) of conventional crickets excreted less UA and older instars excreted more than germfree larvae. On diet three, uric acid excretion did not increase with age and no difference was found between germfree and conventional larvae during any period.

Table 1.3 Nitrogen content of combined body tissues of last instar *A. domesticus* larvae. Values are mean %N (S.E.), n = 6.

	Diet one	Diet two	Diet three
conventional	10.4 (0.3)	9.8 (0.1)	9.0 (0.4)
germfree	9.9 (0.4)	9.8 (0.1)	9.4 (0.3)

Figure 1.3 Total nitrogen (N) and uric acid (UA) excreted by germfree and conventional *A. domesticus* larvae on three diets. Periods are growth intervals illustrated in Figure 1.1. Bars are S. E., n = 6-10.


DIET ONE
DIET TWO
DIET THREE
CONTROL
GERM-FREE
N EXCRETED (% N consumed)
UA EXCRETED (µg/mg/day)
UA EXCRETED (% N consumed)
GROWTH PERIOD
GROWTH PERIOD
GROWTH PERIOD

The cricket/microbe complex was more efficient at removing water-soluble and acid-soluble carbohydrates from food material. Fecal material from germfree animals contained a high proportion of soluble carbohydrate, while material from conventional crickets was correspondingly higher in residual carbohydrates (Table 1.4) on all diets. Differences in calculated digestibility of the acid-soluble pool were significant between groups, while digestibility of the remaining insoluble carbohydrate was virtually identical in germfree and conventional animals. Lipid and ash digestibility estimates did not differ significantly between the two groups of crickets, but conventional animals digested protein (N) more efficiently than germfree animals on diets one and two.

Enzyme activity levels of gut sections from germ free and conventional *A. domesticus* indicated that no major differences existed between groups in midgut preparations (Figure 1.4). Additionally, midgut preparations in general showed higher activity levels than hindgut preparations. Conventional hindgut extracts, however, showed higher activity levels toward most carbohydrates and apparently could degrade a-galactoside bonds while germ free insects could not. Some a-galactosidase activity was also noted in the midguts of conventional crickets. Purified cellulose was not solubilized above blank values in the isolated guts of any cricket examined (Table 1.5).

Table 1.4 Composition of crude nutrient fractions in diets and fecal material, and approximate digestibility estimates for each fraction in germfree and conventional *A. domesticus* during growth period 4. Values are mean + S. E., n = 6.

			CONVENTIONAL		GERMFREE	
DIET	COMPONENT	FOOD % dry wt.	FECES % dry wt.	AD	FECES % dry wt.	AD
ONE	Residual carbohydrate	26.1 + 0.8	39.0 + 0.9	45	26.0 + 1.2	47
	Soluble carbohydrate	47.1 + 0.9	20.2 + 0.3	85	39.0 + 1.0	58
	Lipid	3.8 + 0.3	1.5 + 0.1	85	1.4 + 0.2	81
	Protein	18.1 + 0.6	9.6 + 0.6	80	11.3 + 0.4	66
	Ash	9.0 + 0.2	13.8 + 0.7	43	9.8 + 0.4	42
	Uric acid	--	3.4 + 0.2	--	1.9 + .04	--
TWO	Residual carbohydrate	37.8 + 1.0	45.9 + 0.6	33	36.4 + 1.0	33
	Soluble carbohydrate	32.6 + 0.8	12.9 + 0.3	78	21.6 + 0.8	54
	Lipid	3.1 + 0.1	2.4 + 0.1	57	1.7 + 0.1	62
	Protein	16.9 + 0.6	11.6 +0.3	63	11.5 + 0.2	52
	Ash	10.3 + 0.2	13.7 + 1.2	26	10.6 + 0.9	28
	Uric acid	--	1.7 + 0.7	--	1.3 + .02	--

Table 1.4 (cont.)

			CONVENTIONAL		GERMFREE	
DIET	COMPONENT	FOOD % dry wt.	FECES % dry wt.	AD	FECES % dry	AD
	Residual carbohydrate	57.4 + 0.7	72.0 + 1.0	12	64 + 1.3	11
THREE	Soluble carbohydrate	22.3 + 0.6	10.3 + 0.3	69	14.0 + 0.6	50
	Lipid	1.9 + 0.2	1.3 + 0.1	52	1.1 + 0.1	54
	Protein	8.8 + 0.7	5.5 + 0.2	56	4.9 + 0.1	55
	Ash	5.7 + 0.1	5.8 + 0.4	17	5.0 + 0.2	18
	Uric acid	--	0.7 + 0.1	--	0.6 + .02	--

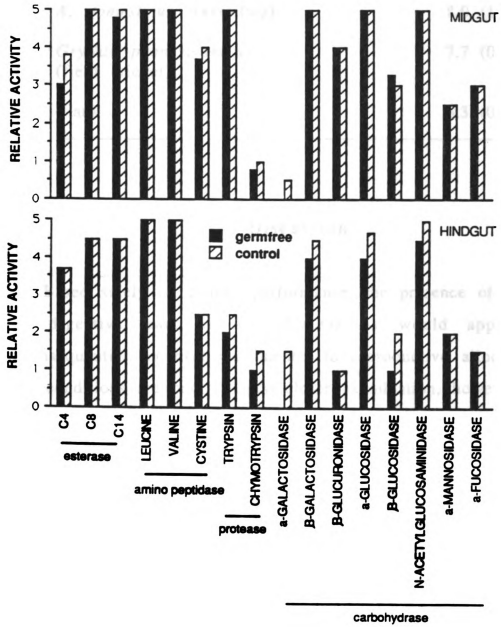


Figure 1.4 Activity levels of major enzyme classes in the digestive tract of germfree and conventional *A. domesticus* .

Table 1.5. Percent solubilization of ^{14}C-labelled cellulose in excised cricket digestive tracts. Mean (S.E.) n = 3 for all cases.

A. domesticus (conventional)	8.3 (1.2)
A. domesticus (germfree)	8.0 (1.2)
Gryllus pennsylvanicus (field cricket)	7.7 (0.7)
Blank	6.3 (0.3)

Discussion

Based solely on growth performance, the presence of bacteria in the digestive tract of *A. domesticus* would appear to be inconsequential, or even detrimental to reproductive allocation when diet conditions are poor. Upon closer examination, however, the gut symbionts enhance the extraction of soluble nutrients from the food bolus; reducing the amount of ingested material necessary for optimal growth across a range of diets.

Comparisons of axenic and conventional versions of insects in nutritional studies have usually focused on differential survival, growth, or fecundity in the presence or absence of symbionts. Relatively few insects which normally possess large and permanent gut microbe populations have been examined with this approach, and none have quantified food utilization efficiency parameters. Charnley *et al.* (1985) found little distinction in growth and development between germfree and conventional locusts free of gregarine

parasites. Bracke *et al.* (1978) reported that cockroaches developed poorly when a large portion of the normal gut bacterial community was eliminated with antibiotics. Although not quantified, Martoja (1966) notes that *Gryllus bimaculatus* seemed to develop normally when reared without gut bacteria. Studies of vertebrate animals are much more extensive (reviews in Savage1984, Gordon and Pesti 1971, Luckey 1969) and indicate that germfree animals generally show better growth than conventional individuals if the diet is "complete". Cecal microorganisms did, however, enhance the digestibility of dry matter in rodents and rabbits (Gordon and Pesti 1971, Yoshida *et al.* 1968), but the degradation of crude fiber was the principle mechansim. Findings here suggest the cricket hindgut microbes interact with the host in a manner which may be analogous to cecal microorganisms, but that the mechanism is not as dependent upon structural carbohydrate degradation.

The mechanisms of increased food utilization efficiency by conventional crickets are presently unknown, but would not seem to involve substantial degradation of cellulose. Calculated digestibility for the residual carbohydrate fractions do not differ with the presence and absence of gut microbes (Table 1.4) and growth of conventional crickets is not enhanced as this fraction increases as a dietary constituent. It is also apparent that purified cellulose is not degraded to any extent (Table 1.5). Additionally, ADs for both types of crickets are almost exactly halved as the basic diet is diluted by 50% with cellulose (Figure 1.2). Previous reports of cellulose digestion in gryllids (Martoja 1966, McFarlane and Distler 1982) may reflect impurities in the cellulose used, or perhaps a difference in the

microbial communities found in the cricket populations assayed. There are several additional lines of evidence to suggest that substantial degradation of plant polymers would be an improbable occurrence in the cricket digestive tract. First, gut retention times are relatively short (ca 3 hrs) for both field and house crickets (Chauvin 1947, Turcek 1967). Secondly, the bulk of the microbial community in gryllids lies within the ectoperitrophic space in the anterior hindgut (Martoja 1966). This would greatly restrict contact of all but the smallest of cellulose fragments with the bacteria and would necessitate a profusion of extracellular enzymes. Although extracellular release of enzymes is not unknown in bacteria, degradation of plant polymers in the rumen usually require close physical association with the substrate (Hobson and Wallace 1982). Finally, Ulrich *et al.* (1981) and Teo and Woodring (1985) could find no evidence of cellulolytic bacteria or cellulase in *A. domesticus* digestive tracts. None of these observations absolutely and independently prove that cellulolytic activity does not occur in the hindguts of crickets; some species may harbor capable microbes and further research is warranted. The observations also do not preclude the possibility of gut microbe attack on various soluble forms of cellulose (e.g. cellobiose, carboxymethylcellulose). The soluble carbohydrate fraction assayed in this study could include a wide range of compounds including sugars, hemicelluloses, gums, and fragments thereof. Therefore it is possible that microbial activity includes attack of soluble fiber. A further partitioning of extracted carbohydrates will be necessary to address the question.

Enzyme activity levels in germfree and conventional hindguts (Figure 1.4) demonstrate that gut microbes enhance the ability of the insect to attack carbohydrates , and to some degree proteins. It should be pointed out that activity levels illustrated were measured for equal weights of gut material, but that on a per cricket basis, the conventional levels would increase by a factor of two. The results are completely consistent with the calculated digestibilty values (Table 1.4) which reveal that carbohydrate utilization accounts for much of the gut microbe activity. The results also suggest that gut microbes in crickets do not substantially inactivate host enzymes. In some vertebrate systems, host enzyme levels drop in the hindgut of conventional animals; presumably because gut microbes use the enzymes as protein sources for growth (Yoshida *et al.*. 1968, Coates 1984). Charnley *et al.*. (1985) showed, however, that activity of a-glucosidase was increased in the locust hindgut in the presence of gut microbes in locusts. They suggest that the finding reflects an enzyme of microbial origin and not an enhancement of the activity of host enzyme present.

Since the results only show activity levels of broad classifications of enzymes, it cannot be determined how much of the microbial activity is a duplication of insect degradative capabilities. It is likely that the bacteria provide different isozymes within a broad substrate category, and that this allows more efficient attack of carbohydrate classes. It is also possible that insect enzyme activity levels are enhanced due to microbial maintenance of more "optimal" physiochemical conditions. Midgut pH and anterior hindgut pH are similar in normal *A. domesticus* (Teo and Woodring 1985); implying

that insect enzymes would operate with similar efficiency throughout most of the digestive tract in this species. Hindgut environmental condition differences in germfree and conventional crickets have not been examined; however, pH and osmolarity in rat cecae are considerably altered by germfree conditions (Gordon and Bruckner 1984, Gordon 1968).

The finding here that microbes are responsible for a-galactosidase activity confirms the suspicion of Teo and Woodring (1985) that the raffinase activity found in the anterior hindgut of *A. domesticus* was produced by microbes. Raffinose is a plant trisaccharide composed of galactose and sucrose in an alpha linkage. It is likely to be only a minor component of most plant tissues. However, the presence of raffinase activity demonstrates that cricket gut microbes not only raise enzyme activity levels in general, but they also increase the enzymatic diversity of the insect. It is likely that other (e.g. *Gryllus* spp.) species of crickets harbor a more diverse bacterial community than lab-reared *A. domesticus* , and the potential for increased metabolic diversity in the hindgut is even greater in field species..

Whether gut microbes in crickets act chiefly on common substrates or on those that are normally unavailable to host enzymes, it is clear that the bacteria process "leftovers" and make a portion of the energy available to the insect. The fact that fecal material from germfree insects contains a substantial portion of soluble carbohydrate indicates that the house cricket digestive system is relatively inefficient to begin with, and that microorganisms are not competing with the animal under normal

conditions. It remains to be seen how microbial trapping of "leftover" energy is transferred to the insect.

There are two major routes of transfer possible. The first would involve a utilization of material by the bacteria and the subsequent release of metabolites which are absorbed by the insect. This scenario is thought to characterize the transfer of energy in carbohydrates in most gut/microbe systems studied to date (Oldeson and Breznak, 1983, Hungate 1975). Anaerobic production of organic acids like acetate provides useful compounds to the host with a minimal loss of potential ATP. Such compounds are rapidly and preferentially transported by the hindgut epithelium (Phillips *et al.* 1986). The hindgut community in gryllids is anaerobic and produces several short-chain organic acids (Martoja 1966, Chapter 2), and the quantitative significance of these metabolites is currently being investigated. Additional compounds such as vitamins and cell exudates are also conceivably made available to the insect. Vitamin synthesis is thought to be one function of hindgut microbes in vertebrates but there is conflicting evidence regarding the availability of the compounds to the host animal and in some cases microbes compete with the host for essential vitamins (Coates 1984). The issue has not been critically examined in insects. Although conventional crickets seemed to benefit little from microbes when reared on the vitamin-diluted diets two and three in this study, the question of vitamin nutrition was not specifically addressed in the experiment.

A second possible method of converting excess digestia to cricket biomass would involve the harvesting of microbial cells. Microbial

biomass is generally discounted as a possible nutritive source in animals with hindgut microbes, except in cases where coprophagy is employed (McBee 1977). There is no evidence to indicate that crickets are coprophagic under the rearing conditions employed (excess food), but natural populations have not been observed. Reasons that microbial biomass harvesting have been dismissed as a significant mechanism in hindgut systems center around the fact that microbes are concentrated posterior to the major sites of digestion and adsorption in the animal (McBee 1977). The unusual anatomy of the cricket anterior hindgut, however, may allow an exception to these generalities. The presence of a peritrophic membrane in the hindgut presents the potential for anterior movement of fluid in the ectperitrophic space, as has been demonstrated in the midguts of several insect species (Dow 1986). It is not known if a similar mechanism occurs in crickets, but bacterial cells in the anterior hindgut conceivably could move anteriorly to digestive/absorptive sites in the midgut. The evidence of some a-galactosidase activity in the midgut preparations of conventional crickets hints that a portion of the microbes or their extracellular products may move forward in the digestive tract.

Nitrogenous excretion patterns do not clarify the issue, as they suggest microbes aid in N conservation when dietary N is in excess, but when dietary N becomes less available they appear to compete with the host. This is implied because overall nitrogen economy is not enhanced by microbes in late instar larvae on diet three (Figure 1.3), and egg production; a highly N-dependent process (Chen 1985), was reduced in conventional animals on the same diet. A

scavenging/recycling of nitrogen as it becomes scarce in the diet is often postulated as a role for gut microbes (Salter 1984, Cochran 1985), but the evidence from the cricket system suggests otherwise. If microbial protein is being harvested, it may be highly dependent upon the population dynamics and metabolic activities of the bacterial community. Conceivably, population turnover rates were greatly reduced in the gut bacterial community as low nutrient intake by the larvae on diet three became critical. In ruminant systems, microbial biomass production is reduced as diet quality deteriorates (Hungate 1975). When nutrient levels were adequate (diets one and two), population turnover rates and biomass production would be greater; potentially making more microbial N available to the insect. The possibility of hindgut bacterial protein being made available to the host is supported by the findings of Martoja (1966) who reported incorporation of gut bacteria amino acids into the tissue of *G. bimaculatus*.

The low levels of soluble carbohydrates in diet three may also have changed the composition of the bacterial community and/or caused a shift in its metabolism. With fewer readily available carbohydrates, the bacteria may have switched to utilizing nitrogenous compounds (amino acids, peptides) that would have been uncontested under conditions of excess. There is evidence to suggest that gut microbes in other systems act mainly on endogenous, soluble pools of nitrogenous compounds (Salter 1984) and hindgut fluid may contain significant quantities of amino acids from normal excretory processes (Bradley 1985). If hindgut bacteria in crickets shift to utilizing these compounds as other sources are

depleted, then nitrogen reclamation routes normally employed by the insect could be disrupted. The tendency for a decreased nitrogen utilization efficiency with age in conventional crickets reared on diet three suggests that the microbial use of nitrogen changes or that the insect's own nitrogen metabolism is disrupted only gradually by microbial competition for substrates.

The role of microbes in the nitrogen metabolism of crickets remains unclear, but there is little evidence from this study to suggest that uric acid degradation by gut bacteria constitutes a meaningful nitrogen conservation mechanism. The performance of conventional animals on the low nitrogen diet indicates that if uric acid is degraded, then the nitrogen is unavailable to the insect or is balanced by a loss of exported microbial nitrogen. A peculiarity in the localization of the hindgut microbes - they are concentrated anterior to the insertion of the Malpighian tubules - may preclude opportunities to degrade significant quantities of the compound in any case. Most of the urate would enter the digestive tract "downstream" of the microbial community, and be passed out of the system in an insoluble form (Bradley 1985). The uric acid-degrading capabilities of the hindgut microbes in crickets may be more important to the survival of those cells which are subsequently exported to a different environment (e.g. soil) with egested material.

Gut bacteria have been shown to recycle uric acid in termites (Potrikus and Breznak 1981) and have been implicated in performing a similar function in cockroaches (Cochran 1985) and birds (Salter 1984, Mortensen 1984). Termites and roaches are known to store uric acid internally for mobilization under conditions

of N stress (Mullins and Cochran 1975a,b). Such a system of "storage excretion" (Cochran 1985) is not evident in house crickets (Cochran 1976, Ulrich *et al.* 1981, Nation and Patton 1961), however, and strategies for nitrogen conservation are apparently different or less important in this insect.

Interestingly, efficiency of food utilization in conventional larvae exceeded that of germfree larvae even when overall nitrogen digestion and excretion patterns were identical in late growth periods of the diet three treatment. This would imply that the mechanism of enhanced efficiency is not principally channeled through nitrogenous compounds. It is not known how any weight gain was partitioned between carbohydrate, lipid, and protein during this study. However, it has been established that larval growth in *A. domesticus,* particularly during late instars, is proportionally greater in the lipid component (Lipitz and McFarlane 1971, Woodring *et al.* 1979). Microbes do not appear to affect the overall digestibility of lipids, but they do salvage carbohydrates which may serve as lipid precursors.

Crickets were apparently unable to totally compensate for nutrient dilution with increased consumption as has been demonstrated for locusts (McGinnis and Kastins 1967) and cockroaches (Gordon 1972). In general, food was consumed at a higher rate by young cricket larvae and individuals reared on diet three. Patterns of consumption appear to be different in larvae reared on diet three, but the high RCRs of larvae during growth period 4 on diets one and two (Fig. 2) is most likely an artifact of the growth period duration. The fourth growth period of diets one and

two treatments was shortened to 3 days because food became depleted in some germfree chambers. At this time, most larvae were in the middle of the last instar and consumption rates decline rapidly after that point (Woodring *et al.* 1979). Therefore, if the period had been extended to 7 days (length of period 4 in diet three treatment), little additional food would have been consumed, but the calculated RCR (mg/mg/day) would have dropped to approximately 1/2 of the rate illustrated in Figure 1.2; effectively making the patterns of consumption with age similar across the diet treatments.

It is difficult to reconcile the facts that crickets with microbes used food more efficiently, but failed to translate the benefit into better growth. The reduction in consumption of food by conventional animals, which accounts for the discrepancy, may be related to a change in physiology due to microbial activity or the physical presence of the bacterial community: e.g. the mass of microbes in the anterior hindgut acts on stretch receptors that signal a slowing of feeding because the region is perceived to be "full". There is evidence to suggest that food movement and feeding behavior can be partially controlled by such feedback sensors in various regions of insect digestive tracts, including the hindgut (Bernays 1985, Simpson 1983). Gut retention times were measured using a chromic oxide marker in house crickets and no discernable differences between germfree and conventional animals were found (Kaufman unpub.). The rates were between 2 and 3 hrs., but it was impossible with the method to estimate rates with a precision of greater than $\pm$ 15 minutes. In any case, the regulation of feeding behavior is certainly more complex than merely controlling the passage of ingestia.

Internal physiological status (e.g. hemolymph titres of sugars and amino acids) may play a role in determining how frequently and how much an insect consumes, and osmolarity of the hemolymph has been shown to affect feeding behavior of locusts (Bernays 1985). It is possible that microbial activity interacts at that level by releasing metabolites and products of digestion to the hemolymph, which subsequently act to inhibit ingestion.

Growth rates and food utilization parameters reported here for conventional animals reared on diet one are in close agreement with values measured for other lab populations of *A. domesticus* given a similar diet (Clifford *et al.* 1977, Patton 1978, Woodring *et al.* 1977, Crossley and Van Hook 1970). ECI values from this study are also comparable to those reported for ultimate instar *A. domesticus* reared on a totally synthetic diet (McFarlane and Distler 1982). Growth and food utilization for both germfree and conventional crickets generally decreased with diet quality and most likely varied because of diet nitrogen (available protein) content, as was determined by *Gryllus bimaculatus* (Merkle 1977). Dietary nitrogen was not controlled specifically and variation in performance could be related to several factors. A more defined diet manipulation would be necessary to resolve the question; however, in preliminary experiments using only conventional larvae, the addition of cholesterol, B-vitamins, and vitamins E and K to diet three did not increase growth performance (Kaufman, unpub.).

Although conventional crickets digested a greater percentage of ingested material on all diets, conversion of digested material into body mass (ECD) was in general, less efficient than in germfree

insects. This also supports the idea that the flow of nutrients and energy in the excess digested material goes through the bacterial community prior to being incorporated into insect tissue. Some of the potential energy and biosynthetic precursors would be lost to bacterial metabolism and export of bacterial cell matter with the feces. Approximately 1 x 10^7 bacterial cells are excreted per mg of fecal material by crickets from stock cultures (Kaufman, unpub.).

It is somewhat perplexing that an increased efficiency was not translated into a growth advantage by conventional crickets. The tendency toward larger body size in conventional larvae and adults, though not statistically significant, would seem to indicate a beneficial interaction when sufficient nitrogen is present. Results from several preliminary growth studies employing diets one and two failed to demonstrate any difference in growth parameters between conventional and germfree crickets and the combined data merely support the observations of this study (Appendix I.). Conditions of the experiment, however, may have influenced the growth performance. As indicated, relative humidity levels in conventional animal growth chambers were necessarily lower to inhibit fungal growth, allowing quantitation of food and fecal material. Though our preliminary work and that of Bates (1971) indicated no substantial difference in growth rates within a humidity range of 60-85%, and that conventional and germfree crickets contained identical water as percent body weight on all diets, there may have been a subtle interaction between humidity and diet. As Clifford *et al.* (1977) have pointed out, crickets will not drink to repletion from wick sources, and conventional crickets in slightly

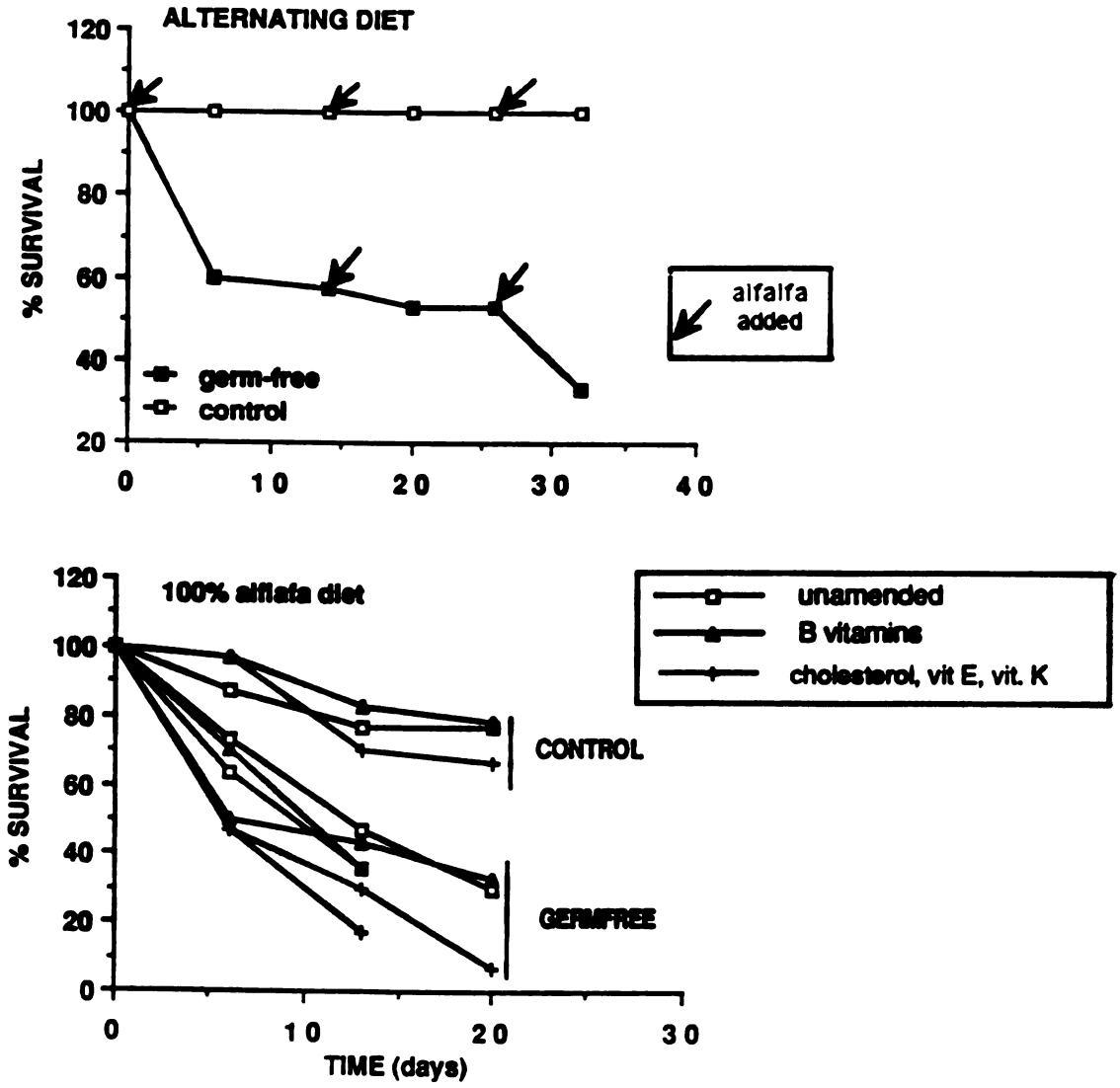
drier chambers may have paid higher metabolic costs to maintain a water balance. The poorer egg production of conventional animals on the low quality diet may, in particular be related to relative humidity levels, since nutrition in many insects is a synergistic function of nitrogen and water content of food material (Mattson 1980)

The experimental conditions also precluded selective exclusion of parasitic or pathogenic organisms from conventional animals. Zuk (1987) showed that parasitism by gregarine sporozoans did not adversely affect host field crickets unless diet conditions were stressful, and there is substantial documentation that host susceptibility is related to nutrient status in many other insects (Vago 1963). Though *A. domesticus* cultures were free of protozoan parasites - typical orthopteran gregarine parasites do not seem to colonize house crickets - the prevalence of other pathogens has not been examined.

Although growth performance of house crickets was not enhanced by the presence of microorganisms, a general increase in food utilization efficiency across a range of diets might be of substantial benefit to crickets in natural environments. Gryllids are omnivores which feed sporadically; food resources are likely to be limited in quantity and variable in quality. House crickets occupy a variety of habitats indirectly created by humans (Bates 1969,1971) and are probably restricted in their foraging by predation and other factors. An ability to efficiently extract nutrients from an unpredictable resource would certainly be adaptive.

In an attempt to simulate fluctuating diet conditions, a preliminary experiment was performed using alternating diets (Figure 1.5). Germfree and conventional larvae were reared from hatching to an age of 15 days on diet one, and then given a diet of 100% alfalfa hay for one week. Diets (diet one and 100% alfalfa) were then alternated weekly until the adult molt and insects were kept on 100% alfalfa for the duration. Results showed that germfree larvae suffered substantial mortality when diets were switched to alfalfa, while conventional larval survival was 100%. Further investigation with the alfalfa diet showed that germfree crickets repeatedly showed higher mortalty rates which were not reduced by the addition of cholesterol, fat-soluble vitamins, or water soluble vitamins. Conventional larvae also showed increased mortality independent of vitamins and cholesterol when kept exclusively on the alfalfa diet. Growth rates were also reduced; however, the presence of microbes allowed most conventional larvae to reach the adult stage, while no germfree crickets survived to that stage. Digestibilty of the hay diet was enhanced by microbes following the patterns seen in the study discussed above. Mean AD (approximate digestibilty) was 26.6 $\pm$ 1.3 (n =7) for conventional larvae, and 8.7 $\pm$ 2.5 (n = 6) for germfree larvae. It seems likely that this added digestive capability provided the minimum nutrition necessary for survival over the short term and may aid in growth over long term exposure to poor diets. These results support the evidence gathered from studies with the diluted diets. With sufficient nitrogen levels in the diet, the microbial community's extraction of carbohydrates allowed subsistence and minimal growth. The gut microbial activity

Figure 1.5 Percent survival of germfree and conventional *A. domesticus* reared on diets with alfalfa hay. Top - survivorship when hay is alternated with basal diet (diet one). Bottom - Survivorship on 100% alfalfa with and without growth supplements.


ALTERNATING DIET
% SURVIVAL
120
100
80
60
40
20
0
10
20
30
40
germ-free
control
alfalfa added
100% alfalfa diet
unamended
B vitamins
cholesterol, vit E, vit. K
CONTROL
GERMFREE
TIME (days)

enabled the insect to utilize a food source that was apparently inadequate and unavailable to the insect's endogenous systems.

An important concern in viewing the any of the results presented here is that laboratory populations of insects were utilized. *Acheta domesticus* is cosmopolitan in its native Europe and Asia (Bates 1971), but it is an introduced species in the U.S.; reared commercially in large concentrated populations (cricket "farms") or maintained in laboratories as a tool for insect physiologists. It remains to be seen if lab populations of crickets contain a "normal" bacterial community and some previous work would suggest that rearing conditions and diet do affect gut microbe community composition in other insects (Brooks 1963, Hunt and Charnley, 1981, Carpentier et al 1978). Other authors, however, have emphasized the stability in gut microbe community composition over a range of conditions (Savage 1977, Lee 1985). Work with various gryllid species (Chapter 2) indicates that the bacterial community in crickets is functionally similar between lab and field-collected individuals. Furthermore, because fermentation metabolites of microbes in the anterior hindgut of *A. domesticus* are identical to those seen in native populations of field crickets (*Gryllus* spp.), it appears that the Gryllidae as a group is likely to posses a functionally similar microbial community.

Chapter 2

The production of volatile fatty acids by the bacterial community in crickets (Orthoptera: Gryllidae): Potential significance to the insect.

Introduction

Crickets are omnivorous insects which possess a densely-populated bacterial community in a modified region of the anterior hindgut (Ulrich *et al.*. 1981, Martoja 1966). In the house cricket, *Acheta domesticus* , the bacteria enhance food utilization efficiency by larvae and appear to conserve soluble carbohydrates that escape digestion by the insect (Chapter 1). Little is known about the metabolic activities of the bacterial community in gryllids, but the mechanism of increased efficiency is likely to be expressed through these activities.

In almost all other animal/hindgut microbe systems studied to date, fermentation metabolites in the form of volatile fatty acids (VFA) represent the principle pool of microbially-produced compounds which are available to the host. The importance of these compounds to the host is thought to vary considerably with the species of animal and its feeding habits (McBee 1977). Among invertebrates, VFA produced by hindgut microbes in termites could support almost all of the insects basal metabolism energy requirements (Odelson and Breznak 1983). Although fermentation metabolites have been measured in the gut systems of other insects (Bracke *et al.* 1980, Bayon 1980, Bauchop and Clarke 1975), their significance is unknown.

The bacterial community in gryllids is thought to function under anaerobic conditions (Ulrich *et al.* 1981) and utilizes soluble carbohydrates (Chapter 1). These observations strongly suggest that fermentation metabolites in the form of VFA are produced; potentially providing a link between microbial activity and food utilization efficiency by the insect. It is the intent of this study to examine and characterize the major end products of the fermentation in gryllid hindguts, and to evaluate their potential contribution to the insect. Effort has been focused on the production and distribution of VFA over a range of cricket species and diets.

Materials and Methods

Insects: *A. domesticus* were obtained from stock cultures and maintained as reported previously (Chapter 1). Field specimens of *Gryllus rubens* and *Scaptericus vicinus* (gryllotalpinae) were supplied by Dr. T. Walker, Univ. Fla., Gainesville and were collected from fields adjacent to the campus. Dr. Walker also provided lab-reared specimens of *G. assimilis*. Field specimens of *G. pennsylvanicus* were collected from grassland fields near the Kellogg Biological Station, Hickory Corners, MI. All field-collected specimens were either analyzed immediately or were maintained for short periods (1-3 days) in covered aquaria and fed autoclaved cricket feed (see Chapter 1) prior to analysis. A stock culture of *G. rubens* was maintained in the laboratory under the same conditions as *A.*

domesticus colonies. Germfree *A. domesticus* were obtained with methods reported in Chapter 1.

Diets: Diets for *A. domesticus* have been described previously (Chapter 1). They consisted of a commercial cricket feed (Country Mark, Inc., Columbus, OH) as the basic diet, with dried alfalfa hay and cellulose (ball-milled filter paper) as constituents added to decrease protein and soluble carbohydrate content.

VFA concentration: Anterior hindgut segments were quickly excised from live crickets and placed individually in 300 µl of deionized water. 100 µl of 1 mM 3-methyl pentanoic acid in NaOH was added as an internal standard. The gut was then homogenized with a stainless steel rod and the suspension was clarified by centrifugation. Supernatants were collected and stored at -70°C. VFAs were quantified after distillation following methods of (Lovley and Klug 1982). VFA pool size of fluid in the ectoperitrophic space was assessed by exposing the anterior hindgut segment of living *A. domesticus* adults and collecting fluid in capillary pipettes. The ventral portion of the segment was first pierced with an insect pin and fluid was collected as it flowed from the opening. Samples from several insects were pooled until approximately 20 µl were obtained. Five separate, pooled samples were each placed in 300 µl water with 100 µl internal standard, and samples were treated as above. Cell material content of the fluid was determined by collecting subsamples of the fluid in capillary pipettes, sealing the opening with paraffin, and centrifuging the pipettes for 30 min. at 10,000 rpm (Odelson and Breznak 1983). Percent cell material content was taken

to be the volume of the pellet as a percentage of the total volume collected.

VFA production: Estimates of VFA production rates were obtained following the general methods of Odelson and Breznak (1983) using a time-zero approach. Individual anterior hindgut segments were excised and placed in 2.5 ml Venoject® flushed with an atmosphere of 95% N_2, 5% CO_2. These were then incubated from 0, 2, and 4 hrs at 30°C. Freezing at -70°C terminated the incubations and samples were subsequently assayed for VFA content as described above.

Estimates of VFA excretion were obtained by homogenizing 10 mg of fresh (< 1hr.) fecal material collected from terminal instar larvae in 0.5 deionized water and subsequent analysis of the extract as described above. Excretion rates were then estimated by the product of VFA / mg feces and total hourly fecal production during the last larval growth period (Chapter 1).

Non-volatile fatty acids: *A. domesticus* anterior hindguts were prepared as above for VFA production up to the point of termination of the incubation. Samples were then prepared for HPLC as follows. Guts were homogenized in 400 µl purified water and the homogenate clarified by centrifugation, followed by acidification of the supernatant with 2.5 N H_2SO_4 (10% acid: extract, v:v). Equipment and column conditions were as reported by Lawson and Klug (in press).

H_2 and CH_4 evolution: Isolated anterior hindgut segments were incubated for four hours at 30°C in 2.5 ml Venoject® tubes flushed with N_2. Headspace gas was sampled and analyzed by gas chromatography (3 M Porapak N column, nitrogen carrier). Living

crickets were examined for H_2 and CH_4 gas production by placing them in 36 ml serum vials containing soda lime to remove CO_2. O_2 was added hourly in 0.5 ml quantities to compensate for respiratory consumption (see Appendix II.). Headspace samples were taken after incubation at 30°C for 4 hrs. and analyzed by gas chromatography (Dacey and Klug 1982).

CO_2 fixation/acetogenesis: The potential for VFA production through reduction of CO_2 with H_2 was examined in *A. domesticus* anterior hindguts following the methods of Breznak and Switzer (1986). This method will subsequently be referred to as the "suspension" method to distinguish it from a separate experiment. Ten gut segments were homogenized in 2.0 ml BSS (Breznak and Switzer 1986) in an anaerobic glove box (atmosphere 5% H_2, 10% CO_2, 85% N_2). 200 µl of the suspension was added to each of nine 2.5 ml Venoject® tubes which were then removed from the glovebox and flushed with H_2. Chloroform, which blocks the methylation of formate to acetate in the acetogenic reaction (Prins and Langhurst 1977), was added to each of six tubes in a final concentration of 10, 50, and 100 µM (two replicates per concentration). The remaining three tubes served as controls and received deionized water in volumes equal to those added to the $CHCl_3$ block treatments. 5 µl of 36mM $Na^{14}CO_3$ (8.3 µCi/ µM) was then injected into the tubes as an aqueous solution (pH = 9.5). The tubes were then incubated with agitation at 30°C for 3 hrs. The incubation was terminated by the addition of 50 µl of 1.0 N HCL and the tubes were flushed under CO_2 for 10 min. 60 µl of 1.0 N NaOH was added to neutralize the suspension, followed by 180 µl of deionized water to bring the

volume up to 500 µl. Following centrifugation, 50 µl subsamples were counted by liquid scintillation to determine the total counts fixed in the soluble fraction. The remaining supernatant was prepared for HPLC as described above for nonvolatile organic acids. Fractionation of the HPLC eluent was accomplished by methods reported in Lawson and Klug (in press). Under conditions of the analysis, formate and fumarate co-eluted and fractions reported here as formate are those collected from the single formate/fumarate peak. As a consequence, concentrations of formate could not be determined.

The acetogenic reaction was also examined in a slightly different manner; henceforth referred to as the "whole gut" method. Two gut segments were placed in 2.5 ml Venoject® tubes under a flow of N_2 or H_2. One µl of $Na^{14}CO_3$ (aqueous, pH = 9.5) was injected through the wall of each segment and the guts were then macerated with a stainless steel rod to form a more homogenous slurry and to distribute the label more uniformly. It was thought that this procedure would be an effective compromise between the disruption of the spatial distribution of the bacterial community likely to occur with the suspension method, and the problems associated with an uneven distribution of label as it is introduced by injection into the intact gut system. The tubes were incubated for 3 hours at 30°C without agitation. Incubations were terminated and homogenates analyzed as described above for the suspension method.

Permeability studies: The permeability of the hindgut cuticle and epithelium to major VFAs was assessed by injecting ^{14}C-labelled acetate, propionate, and butyrate into isolated hindgut segments from *A. domesticus*. guts were removed from living insects and

quickly immersed in a saline solution isosmotic with cricket hemolymph (J. P. Woodring, Louisiana State University, person. comm.). The composition of the saline was as follows: NaCl-160mM, KCL-6mM, $CaCl_2.2H_2O$-8mM, Na-citrate-5mM, $NaCO_3$-5mM, glycine-10mM. The gut segments were ligated at the hindgut/colon junction and label was introduced by inserting a 30 gauge needle with blunted tip through the lumen of the midgut and into the anterior hindgut segment. The label (1 µl) was slowly injected as the needle was withdrawn and a ligature was tightened at the midgut/hindgut junction immediately after the needle was withdrawn. The isolated gut was then rinsed in fresh saline, blotted, and placed in 100 µl of saline. Subsamples of the saline bath were taken after 10 minutes and at hourly intervals for two hours. Subsample volume (50 µl) was replaced with fresh saline at each time point. Gut integrity was assessed by inclusion of ^{3}H-labelled polyethylene glycol (PEG, 4000 mw, 0.1 µCi per µl solution) in the injected solution as described by Bracke *et al.* (1980). Each acid was added at concentrations within the range of those found in the ectoperitrophic space fluid of conventional insects and were as follows: acetate, 100 mM (5.4 µCi); propionate, 10 mM (0.5 µCi); butyrate, 5 mM (0.13 µCi).

Statistics: Data were compared as their log transformed values with standard ANOVA and regression techniques (Sokal and Rohlf 1969, Kleinbaum and Kripper 1978). Probability level for significance was 0.05 unless otherwise indicated.

Results

VFAs were found in the anterior hindgut segments of all cricket species examined (Figure 2.1). Acetate dominated the pool in all species, but propionate was prominent and characteristically accounted for 15 to 20% of the total acids. Other VFAs generally consisted solely of butyrate, with trace quantities of isobutyrate, isovalerate, and valerate found inconsistently. Germfree *A. domesticus* hindguts contained significantly less total VFA, with acetate comprising over 98% of the pool. Lab-reared species and field collected species showed strikingly similar patterns. Only *S. vicinus*, the mole cricket representative, seemed to differ; however, neither total VFA concentration nor % composition differed between conventional species.

Production rates of VFA and the percent composition of acids produced during the incubations (Figure 2.2) were also very similar between field-collected *G. rubens* and *G. pennsylvanicus*, and lab-reared *A. domesticus*. Estimated rates (slopes from linear regression) were not significantly different, nor were acid distributions. Germfree *A. domesticus* hindgut incubations (not illustrated) failed to demonstrate any VFA production. In a direct comparison of lab-reared and field-collected *G. rubens* (Figure 2.3), production rates and percent composition did not differ significantly, although field crickets' rates tended to be slightly lower and the VFA pattern indicated slightly elevated levels of propionate.

Fluid in the ectoperitrophic space of *A. domesticus* (Figure 2.4) contained similar proportions of acids to those measured for entire

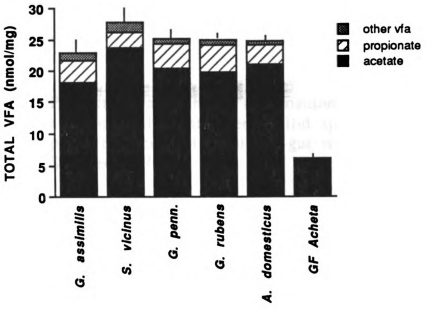
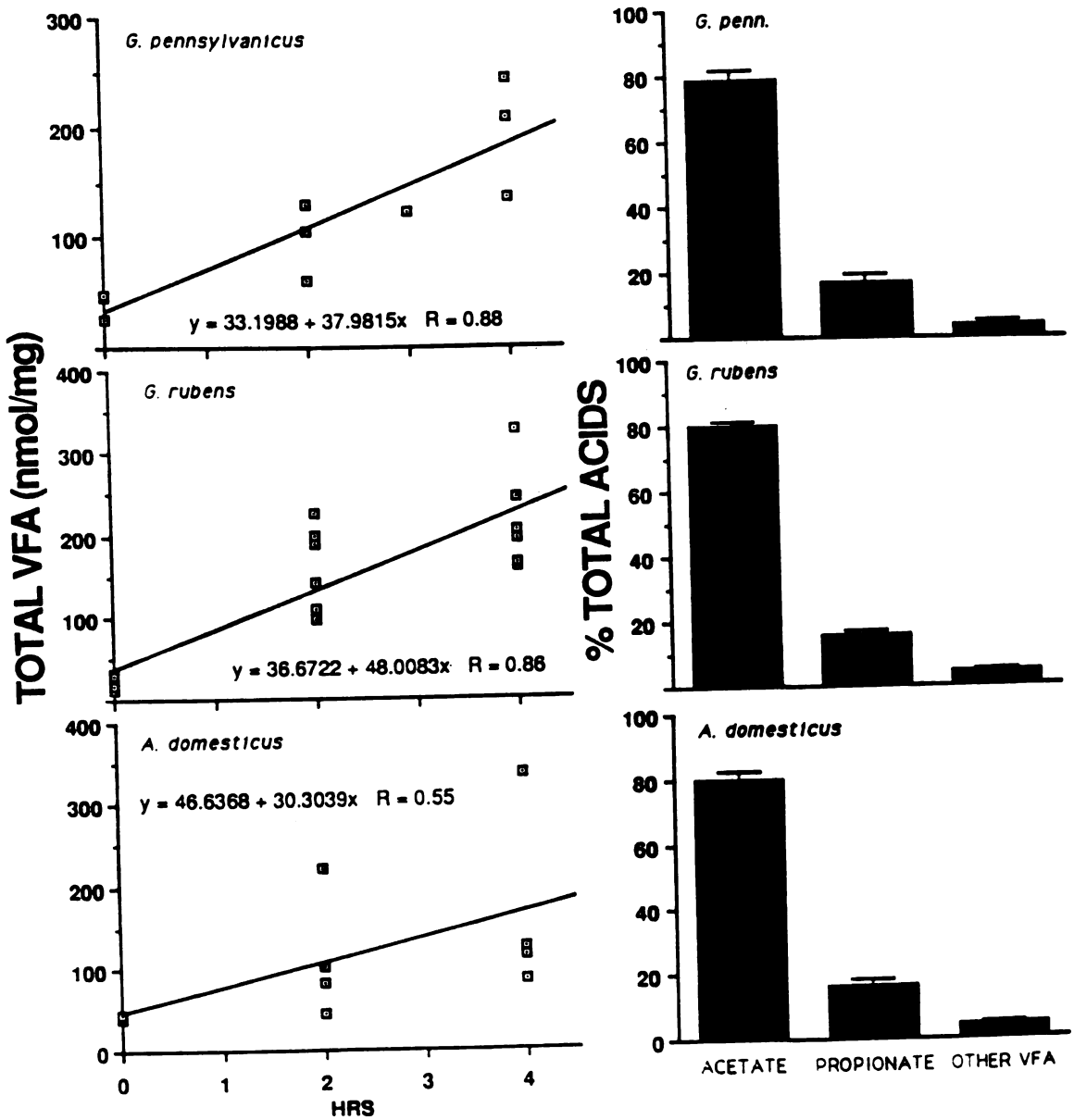


Figure 2.1 VFA concentration in the anterior hindgut of various gryllid species. Bars are S. E., n = 6-14.

Figure 2.2 Production rates and % composition of VFA in the anterior hindgut of three gryllid species. Each point in the production curve is a single gut replicate. Error bars are S.E., n = 7-14.


G. pennsylvanicus
y = 33.1988 + 37.9815x R = 0.88
G. rubens
y = 36.6722 + 48.0083x R = 0.86
A. domesticus
y = 46.6368 + 30.3039x R = 0.55
TOTAL VFA (nmol/mg)
HRS
G. penn.
G. rubens
A. domesticus
% TOTAL ACIDS
ACETATE
PROPIONATE
OTHER VFA

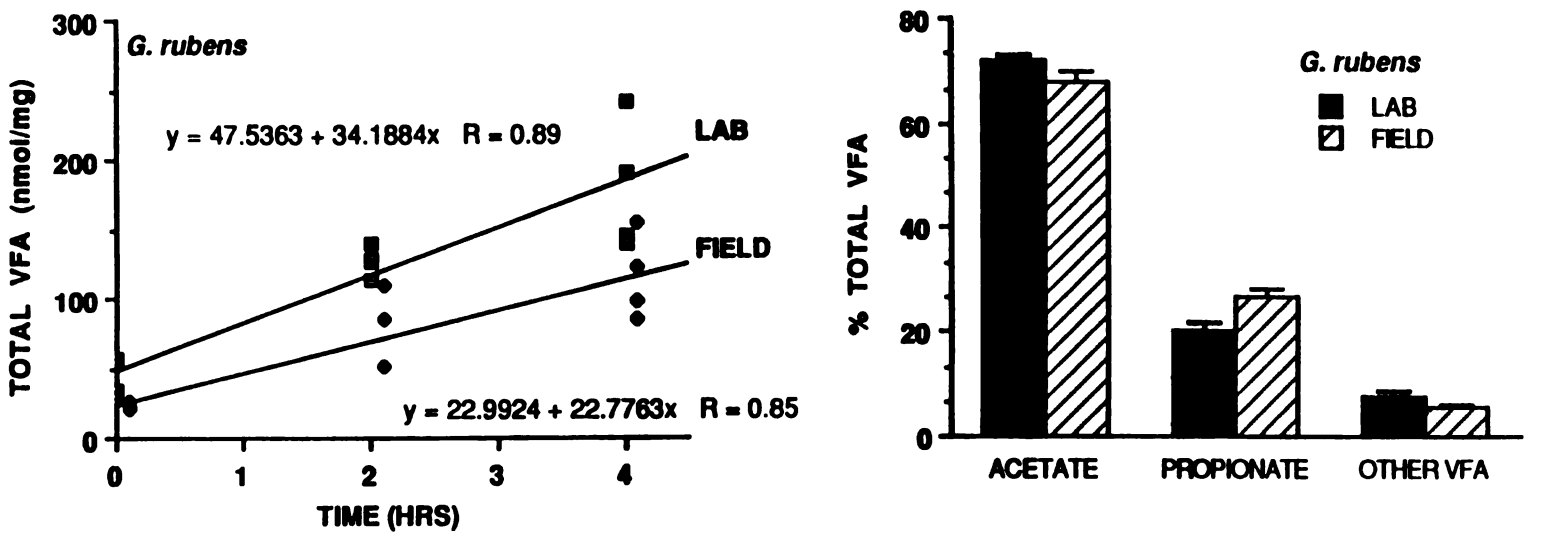


Figure 2.3 Comparison of VFA production rates and composition in anterior hindguts of lab-reared and field-collected *G. rubens*. Each point in the production curve is a single gut replicate. Error bars are S. E., n = 7-8.

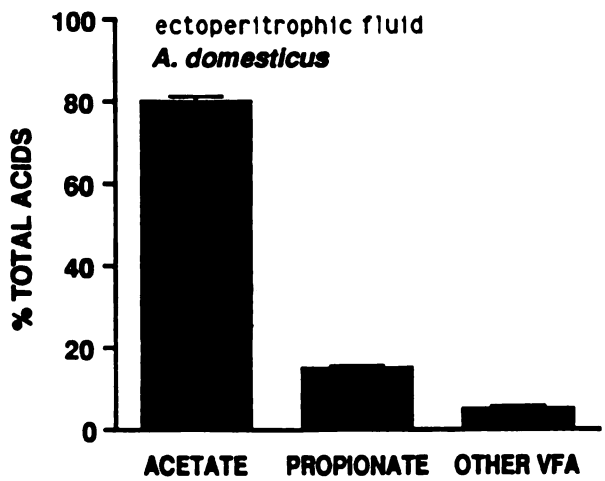


Figure 2.4 Percent composition of VFA in the ectoperitrophic fluid of the anterior hindgut of *A. domesticus*.. Error bars are S.E., n = 5.

guts. Cell material volume of the fluid was calculated to be 48% (S.E.=1.4, n=5) and mean concentrations for acetate, propionate, and total other VFA were 80 (±9.7) mM., 13 mM (±1.9), and 5 (±0.7) mM, resp.

The rates and acid composition of hindguts from young (20 -25 days old) *A. domesticus* larvae (Figure 2.5) were indistinguishable from those of late instar and adult house crickets .

Long term exposure to varying diets did not appreciably affect estimated production rates of VFA in *A. domesticus*, however, the distribution of acids was changed significantly (Figure 2.6). Dilutions of the basic diet with alfalfa hay or cellulose tended to increase concentrations of propionate and butyrate in incubated hindgut segments. Maintenance on a diet of alfalfa hay alone reduced propionate concentrations, but elevated butyrate. Other VFAs in all cases remained at trace levels.

Non-volatile organic acid production was not evident from incubations of isolated hindguts and subsequent HPLC analysis. There was some indication of lactate production; but results were inconsistent in repeated trials. Acetate and propionate did consistently increase with incubation time in the same analyses (results not illustrated);however, indicating that the method was able to detect production of fermentation metabolites.

Based upon VFA production estimates of 200-400 nmol/ last instar cricket/ hr., excretion rates of VFA (Table 2.1) would account for less than one percent of production.

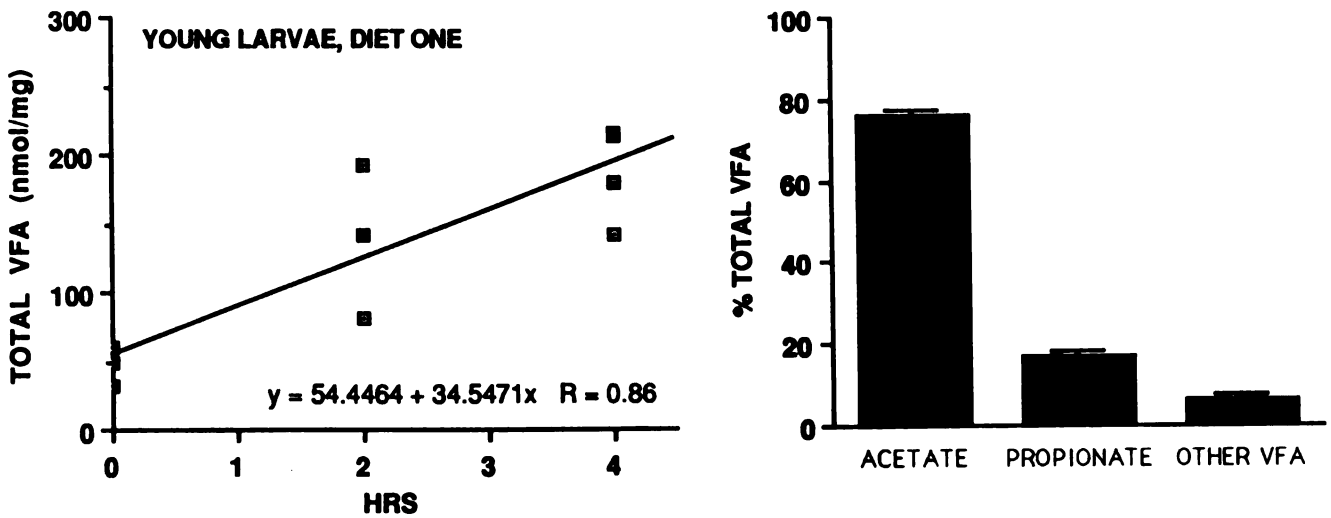


Figure 2.5 Production rates and composition of VFA in the anterior hindgut of young (20-25 days) *A. domesticus* larvae. Each point in the production curve is a single gut replicate. Error bars are S. E., n = 8.

Figure 2.6 Production rates and composition of VFA in the anterior hindgut of last instar larvae of *A. domesticus* reared on four diets. Each point in production curve represents a single gut replicate. Error bars are S. E., n = 4-20.

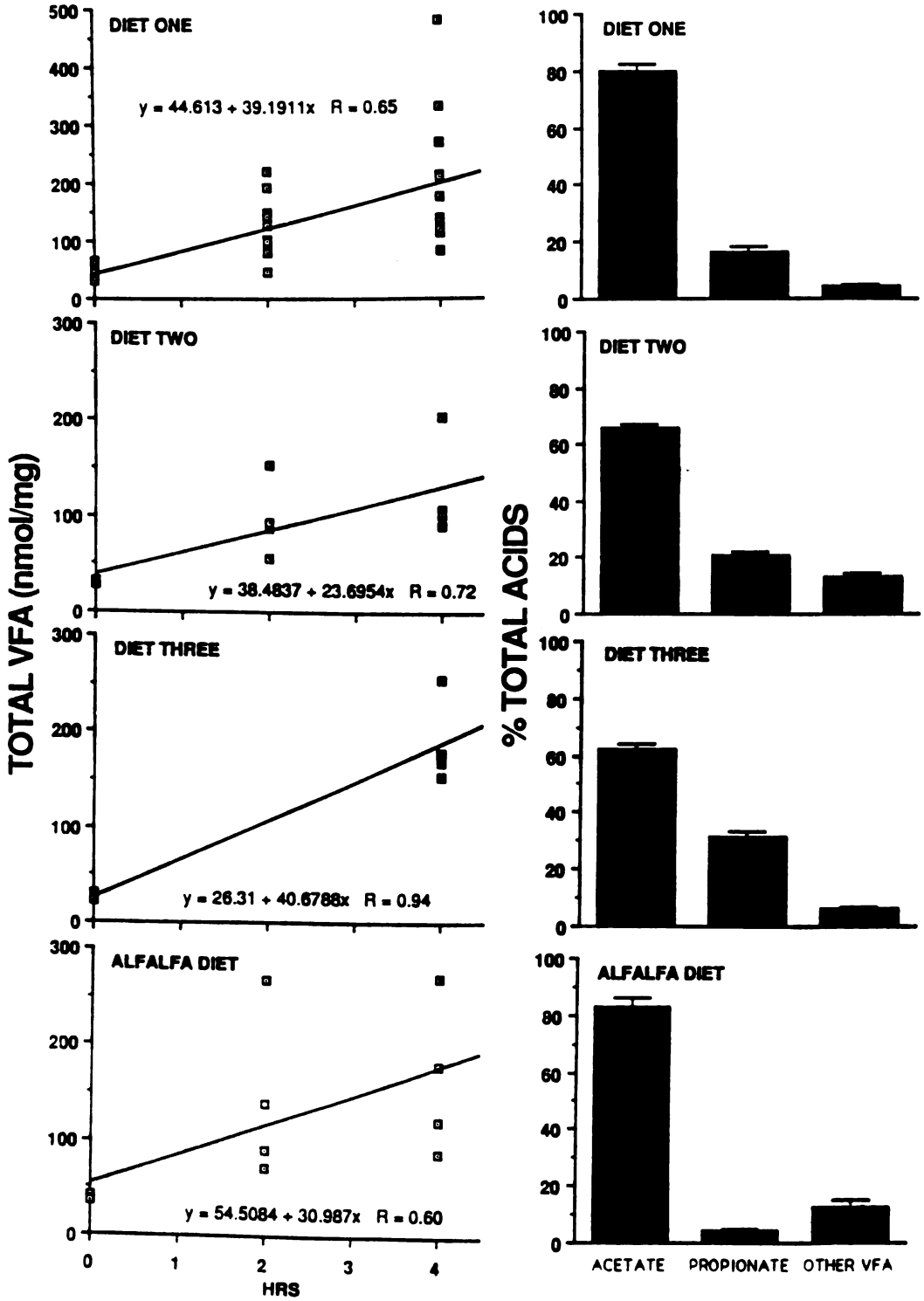

DIET ONE
y = 44.613 + 39.1911x R = 0.65
DIET TWO
y = 38.4837 + 23.6954x R = 0.72
DIET THREE
y = 26.31 + 40.6788x R = 0.94
ALFALFA DIET
y = 54.5084 + 30.987x R = 0.60
TOTAL VFA (nmol/mg)
HRS
% TOTAL ACIDS
ACETATE
PROPIONATE
OTHER VFA

Table 2.1. VFA excretion rates in last instar larvae of conventional *A. domesticus* .

Diet	Total VFA in feces (nmol/mg)	Feces produced (mg/cricket/hr.)	Estimated excretion (nmol/cricket/hr.)
1	5.7 (0.9)	0.3 (0.1)	1.7
2	2.9 (0.7)	0.5 (0.2)	1.4
3	2.3 (0.6)	0.6 (0.2)	1.4

Analysis of gas evolution from isolated hindgut segments of *G. rubens* (field collected), *G. pennsylvanicus* (field collected), and *A. domesticus* indicated that hydrogen was produced, but no evidence of methane was found in any of the crickets examined (Table 2.2). Additionally, H_2 gas could be detected in the headspace of vials containing live *A. domesticus*, but levels were low, not consistently detected from individual crickets, and were therefore not quantified.

$^{14}CO2$ was fixed by anterior hindgut microbes in a homogenized suspension and in whole gut preparations (Table 2.3). Label was not fixed when added to germfree hindguts (not illustrated) . The percentage of added label fixed in was enhanced by the presence of hydrogen and by the more thorough homogenization (suspension method) of the hindgut material (Figures 2.7, 2.8). Label appeared principally in acetate. However, propionate and butyrate fractions contained considerable counts; particularly in the slurry preparation. Label distribution varied with the incubation method: acetate specific activity increased disproportionately with the suspension method.

Table 2.2 Hydrogen and methane in headspace of gut incubation vials after 4 hrs. Values are mean ± S.E. (n).

	H_2 (µl/ mg)	CH_4
A. domesticus	0.33 ± 0.03 (14)	n. d. (14)
G. rubens (lab)	0.32 ± 0.03 (8)	n. d. (8)
G. rubens (field)	0.25 ± 0.03 (8)	n. d. (8)
G. pennsylvanicus (field)	0.29 ± 0.07 (8)	n. d. (8)

n. d. = not detected.

Table 2.3 Percentage of added $^{14}CO_2$ fixed under various conditions of gut incubation. Mean (S.E.), n = 4-6.

Suspension		Whole gut	
H_2	$H_2 + CHCL_3$	H_2	N_2
77.5 (0.71)	47.2 (1.01)	44.5 (2.1)	25.8 (2.3)

Figure 2.7 Distribution of fixed $^{14}CO_2$, specific activity of partitioned label, and total VFA formed per gut during "suspension" method incubations with and without $CHCL_3$ block. H2 = hydrogen atmosphere, BLOCK = hydrogen atmosphere and $CHCL_3$ added to the suspension.

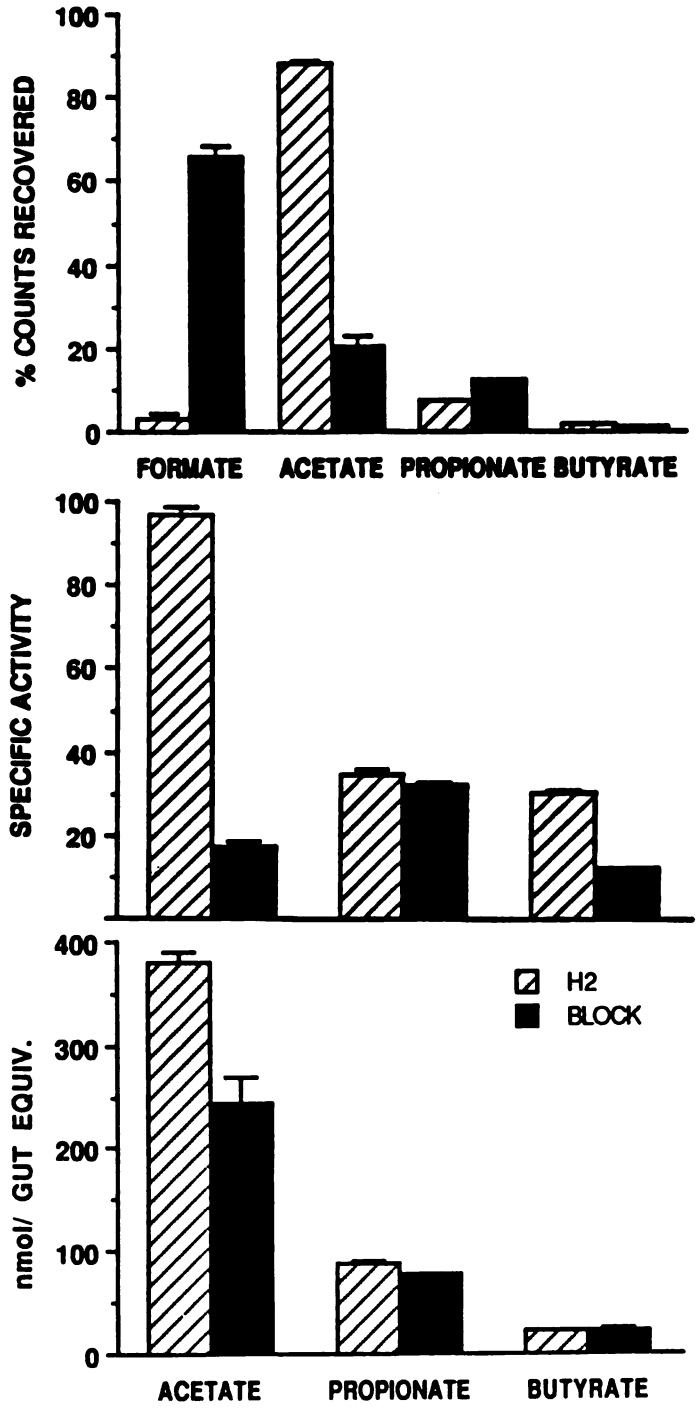
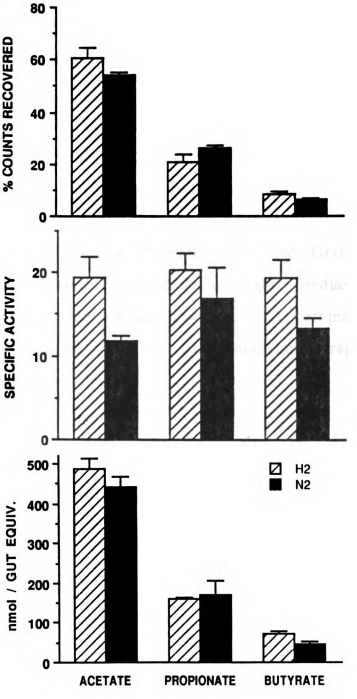

% COUNTS RECOVERED
100
80
60
40
20
0
FORMATE
ACETATE
PROPIONATE
BUTYRATE
SPECIFIC ACTIVITY
100
80
60
40
20
nmol/ GUT EQUIV.
400
300
200
100
0
H2
BLOCK
ACETATE
PROPIONATE
BUTYRATE

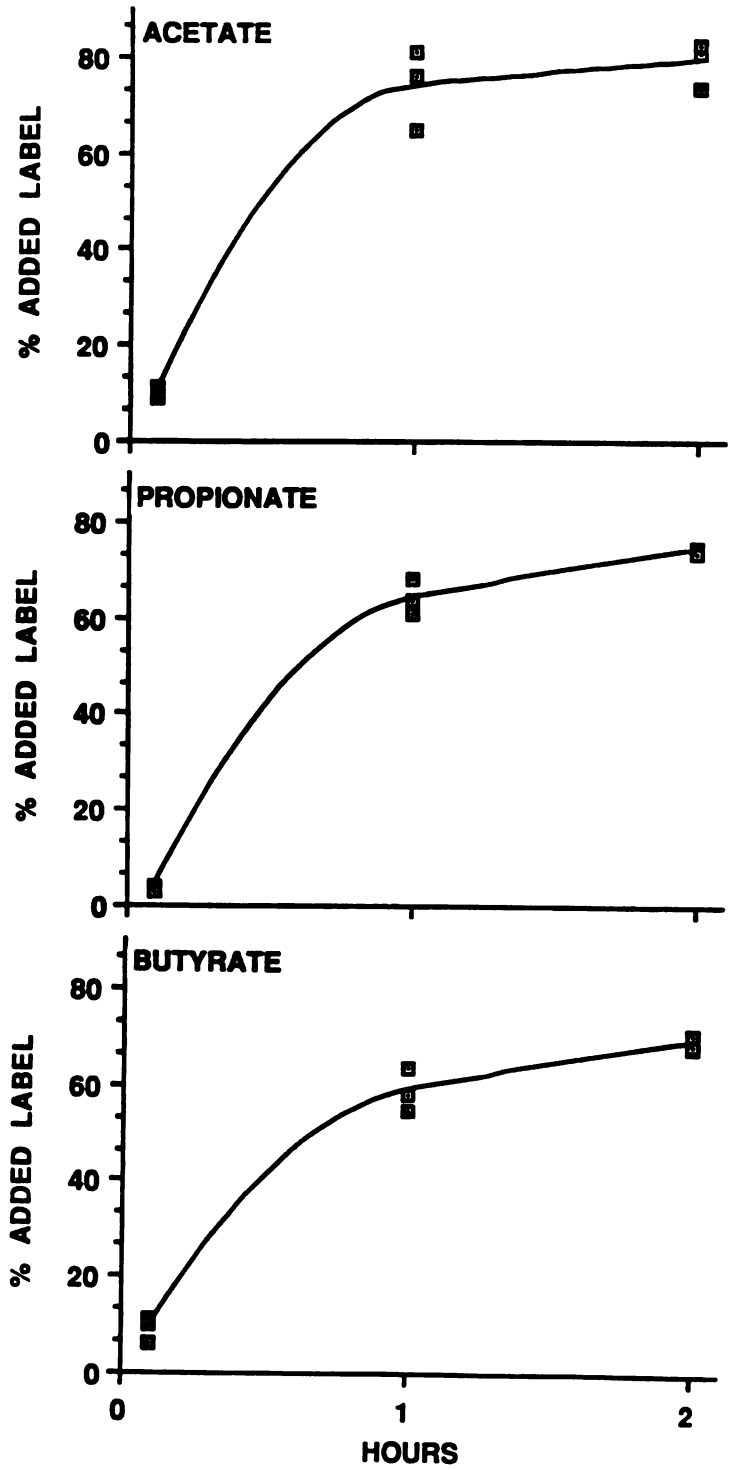
Figure 2.8 Distribution of fixed $^{14}CO_2$, specific activity, and total VFA produced per gut in "whole gut " incubations. N2 = nitrogen atmosphere, H2 = hydrogen atmosphere.


% COUNTS RECOVERED
80
60
40
20
0
SPECIFIC ACTIVITY
20
10
0
nmol / GUT EQUIV.
500
400
300
200
100
0
H2
N2
ACETATE
PROPIONATE
BUTYRATE

$CHCl_3$ effectively decreased total fixation of $^{14}CO_2$, and label to accumulated in formate (Figure 2.7). The total counts fixed and distribution of label in various fractions in the block treatments was independent of $CHCl_3$ concentration, and the illustrated results reflect the pooled results from 10, 50, and 100 μM $CHCl_3$ treatments. The $CHCl_3$ block reduced the specific activity of acetate and butyrate, but propionate remained unchanged. The block also significantly reduced acetate concentrations in the suspension, while propionate and butyrate were unaffected (Figure 2.7).

Labelled acetate, propionate, and butyrate easily transversed the hindgut cuticle and epithelium; most of the added label could be recovered in the saline bath after the first hour (Figure 2.9). Butyrate appeared to move at a slightly reduced rate compared to the other two compounds, however, high permeability is still evident and would indicate that all compounds could rapidly diffuse into the hemolymph.

Figure 2.9 Rate of movement of ^{14}C-labelled VFA across the anterior hindgut wall of *A. domesticus in vitro*. Each point represents the percentage of added label found in the saline incubation medium.


ACETATE
% ADDED LABEL
0
20
40
60
80
PROPIONATE
% ADDED LABEL
0
20
40
60
80
BUTYRATE
% ADDED LABEL
0
20
40
60
80
0
1
2
HOURS

Discussion

VFA pool sizes and production rates found in gryllid hindguts indicate an active fermentation that could explain a portion of the enhanced food utilization found to be provided by microorganisms in house crickets (Chapter 1). Excretion rates of VFA are almost negligible in relation to production estimates and the hindgut wall is readily permeable to the principle acids produced. Assuming produced VFA are absorbed and oxidized completely to CO_2, the acids could provide 4-5% of basal respiratory demand in late instar *A. domesticus* , based on an oxygen consumption figure of 0.5 ml O_2/ cricket / hr (see Appendix III). This figure is comparable to that estimated for pigs, but generally falls below estimates for other hindgut fermenters (McBee 1977). VFA production is calculated to provide nearly 100% of respiration needs in termites (Odelson and Breznak 1983 and between 5 and 16% of the demand in *Tipula abdominalis* (Lawson and Klug, in press).

The production rates in crickets are not trivial, however, since they represent a potential conservation of 0.6 mg glucose equivalents/ cricket/ day. Using a reported growth efficiency (dry weight gained/ dry weight food consumed) of 0.15 for late instar conventional insects and an efficiency of 0.11 for germ free versions (Chapter 1) the difference due to microbes is 0.04. Given a consumption rate of approximately 20 mg/cricket/day for conventional insects (Chapter 1, Woodring *et al.* 1977) the efficiency difference would account for 0.8 mg of dry weight gain per day: a figure remarkably similar to potential savings calculated from VFA

production estimates. Clearly, calculations from estimates include many possible sources of error, and conversion of VFA into biomass would not be 100% efficient. Nonetheless, the figures suggest that VFA production is well within the range necessary to account for a substantial portion of microbe-enhanced food utilization efficiency in *A. domesticus*.

VFA production rates from *in vitro* measurements may also be underestimates of *in situ* rates (Hume 1984) since removal of products by active gut tissue would be inhibited. There is evidence of fermentation product build up in the gut incubations reported here because longer chain volatile fatty acids tend to increase in proportion compared to time-zero points and ectoperitrophic fluid (Figure 2.4). Additionally, total VFA production in the entire cricket digestive tract was not quantified; only the anterior hindgut segment. Conceivably, microbial activity in the posterior hindgut and rectum contribute to the overall digestion and similar metabolites could be formed. This has not yet been investigated.

VFA contribution to food utilization may also be underestimated by simply calculating potential oxidation rates. VFA may enhance overall efficiency indirectly; by interacting with gut epithelial cells. There is evidence from vertebrate systems that VFAs stimulate development of gut cells (Sakata and Yajuma 1984), provide a large portion of gut cell energy requirements (Roediger 1980), and enhance fluid movement and transport activity (Demigne *et al.* 1980). Insect systems are not as well studied, but locust rectal tissue absorption of fluid and ions from the lumen is sustained by acetate transport, and acetate also supplies a portion of the tissue's

energy demands (Baumeister *et al.* 1981). Part of the overall digestive efficiency in crickets may include a similar mechanism.

The characteristics of fermentative metabolism in the gryllid hindgut suggest an unusual system compared to previously-reported hindgut fermentations. In insect systems investigated thus far, acetate dominates the pool of detected VFAs in termites, grass grubs, and craneflies (Table 2.4). The scarab, *Orcytes nasicornis*, however, has a digestive tract fermentation that produces a suite of VFA similar to crickets (Bayon 1980), but the standing pool of VFA in the proctodeal dilation of this beetle was reported (by the same author) to contain over 90% acetate (Bayon and Mathelin 1980). In vertebrates, fermentation products include a higher percentage of propionate and butyrate than those reported for insects; with changes in diet causing considerable variation. Odelson and Breznak (1983) suggest that the dominance of acetate in termites reflects both the insoluble nature of the substrate consumed and the high relative numbers of protozoa in the system. Aquatic detritivores consuming a diet of decomposing leaf material also have hindgut fermentations characterized by high levels of acetate and few longer chain VFA (Lawson and Klug in press), but the community is composed entirely of bacteria (Klug and Kotarski 1980). The pattern in crickets appears to be somewhat intermediate between the few insects studied and vertebrates. Propionate values consistently comprise 15-20% of VFA, while butyrate values are much lower than in vertebrate systems. In another anomaly, methane has not yet been detected in any cricket species, whereas its production has been measured in termites (Breznak 1982), scarab beetles (Bayon 1980)

Table 2.4 Comparison of gut VFA in various animal groups.

Animal	Diet	Production rate	% Composition Act.	Pro.	Other	Source
Insects (hindgut)						
Costelytra zealandica	roots	-	100	-	-	Bauchop and Clarke 1975
Orcytes nasicornis	dung	2.5 nmol/mg	81	17	2	Bayon and Mathelin 1980
" "	cellulose	1.2 nmol/mg	88	11	1.5	" "
Reticulitermes flavipes	wood (lab)	20 nmol/mg	95	3	2	Oldeson and Breznak 1983
"	wood (field)	40 nmol/mg	99	-	-	" "
Tipula abdominalis	detritus	14 nmol.mg	95	3	2	Lawson and Klug in press
Mammals						
Laboratory rat (cecum)	commercial feed	-	59	11	32	Høverstad and Midvedt 1986
"	feed + cellulose	-	66	19	13	Topping et al 1985
"	feed + gum arabic	-	61	19	20	" "
"	feed + gum arabic + cellulose		50	26	22	" "
"	feed + wheat starch	-	74	24	2	Demigne et al 1980
"	feed + pectin	-	76	18	6	" "

Table 2.4 (cont.)

Animal	Diet	Production rate	% Composition Act.	Pro.	Other	Source
Sheep (rumen)	roughage	-	65	17	13	Lu and Jorgensen 1987
"	grain conc.	-	59	18	19	" "
Cattle (rumen)	roughage	46 nmol/mg	65	20	15	Hungate 1966 Wolin 1979
Birds (cecum)						
Ptarmigan	buds, twigs	90 nmol/mg	44	32	25	McBee 1977
Grouse	"	-	60	13	17	"
Turkey	comm. feed	-	97	1	2	Sudo and Duke 1980

and numerous vertebrates (Prins 1977, McBee 1977). Finally, the microbial community in crickets does not appear to rely as much upon refractory structural polymers as substrates (Chapter 1), as is thought to drive much of the metabolism in other gut systems. Evidence presented thus far does not preclude the bacterial metabolism of soluble fiber (e.g. some hemicellulose, pectins, starches), however, and this may be of significance in certain dietary regimes.

The constancy of the fermentation pattern seen across species of crickets (Figures 2.1, 2.2), age (Figure 2.5), and rearing conditions (Figure 2.4) indicates an overall functional similarity in the bacterial communities that is maintained over a range of habitats. Hungate (1975) has noted that although the community composition of microbes in the rumen may vary across geographical region, the overall pattern of VFA remains remarkably consistent. It would in fact be surprising to find identical species of bacteria in different cricket groups, however, if metabolite production forms the basis of the relationship, the bacterial community composition may be irrelevant. The results also show that lab-rearing does not appear to alter the overall community metabolism in the hindgut. Again, nothing is known of the bacterial species present in crickets other than *A domesticus*, but a sufficiently "normal" compliment of microbes is retained even after several generations of artificial conditions. VFA represent only the final products of the fermentation, however, and the initial degradative pathways in hindguts of lab and field groups of crickets have not been critically compared. Lab-reared crickets may be deficient in certain members

of the bacterial community that have more relevance (in terms of substrate utilization capabilities) to wild cricket populations.

The shift in VFA patterns that is seen when *A. domesticus* is reared on different diets (Figure 2.6) suggests a change in substrates available to the bacteria and/or a shift in bacterial populations. As acid-soluble carbohydrate content drops, propionate and butyrate percentages increase. The addition of alfalfa hay to the diet raised butyrate levels in the fermentation, while dilution with cellulose raised levels of both propionate and butyrate. The shift to longer chain VFA is still evident when crickets are maintained on Diet three: a diet diluted with insoluble cellulose but containing no "new" carbohydrates compared to the basic diet. It may be that the insect's midgut scavenges a greater percentage of the pool of readily utilizable carbohydrates in the diluted diet; leaving a quantitatively different pool for the hindgut microbes. The leftovers reaching the hindgut from diet three would be poorer in easily-assimilable compounds, and the bacteria may have been forced to utilize other energy sources that were ignored in the richer diet. Increases in the relative proportions of propionate and butyrate in the rodent cecum are associated with increasing fiber content , however, the response is variable and dependent on the type of fiber available. In general, it appears from studies of vertebrate gut systems, that increasing cellulose and hemicellulose content in a mixed diet. favors more butyrate formation (Demigne *et al.* 1980, Sudo and Duke 1980, Hungate 1975). However, xylophagous insects utilizing strictly cellulose and hemicellulose have hindgut fermentations characterized by acetate proportions of 95% or greater (Odelson and Breznak

1983). The soluble pectins, guar gum and gum arabic, were also shown to elevate butyrate and propionate concentrations in the rat cecum (Tulung *et al.* 1987, Topping *et al.* 1985). Interestingly, a VFA pattern similar to that reported here for crickets was shown to develop in the rat cecum when the animal was fed wheat starch or pectin diets (Demigne *et al.* 1980). The basal diet employed here contains a high proportion of wheat middlings and the gut microbial community in crickets may be functioning in a similar manner to the rat cecum community. It is currently unknown if pectins, hemicelluloses, and soluble cellulose fragments are important fermentation substrates for cricket hindgut bacteria, but their increased utilization may explain the shifts in VFA patterns as diets changed.

A point that emerges from the preceding discussion is that the pattern of the hindgut microbial fermentation is not predictable when diet changes are considered independently of the particular gut system. The resulting change (or lack of) is also a response of the endemic microbial community present. It remains to be seen how much of the VFA pattern shift is due to metabolic changes or population shifts within the bacterial community; or both.

From the view point of the cricket, the changes in fermentation patterns with diet may be meaningful from a nutritional standpoint. If production rates for total VFA remain constant, a shift to longer chain acids represents an increase in available carbon. Assuming equal assimilation costs, propionate and butyrate would provide more oxidative/biosynthetic precursor per

mole than acetate. Details of this study are insufficient to quantify the answer to the question, but further investigation is warranted.

The production rates of VFA do not appear to differ markedly in response to diet, however, variation in values around time points is considerable, and critical comparisons of slopes would necessitate larger sample sizes in most cases. In the case of lower soluble carbohydrate diets, estimates of rates from 4 hour incubations may even be understated because VFA content in individual gut segments tends to begin to level off between 2 and 4 hrs and a linear model may not be the best fit of the data (Figure 2.10). Rates calculated between 0 and 2 hrs in that case would be higher, and perhaps more relevant, since transport of VFA may occur rapidly (discussed below). The tapering-off of VFA production in some incubations may indicate that substrates are being depleted. Production rates of acetate in termites vary with diet and conditions of the insect (Odelson and Breznak 1983) and VFA production rates in vertebrate gut systems are known to increase as more readily-digestible carbohydrates increase in the diet (Hungate 1975, Hume 1984, McBee 1977).

Although a characteristic feature of the cricket hindgut fermentation is the production of propionate, acetate clearly dominates the pool in all cases and represents the principle product available to the insect. Acetate can be derived directly through carbohydrate fermentation , but it can also be formed through the terminal process of CO_2-reduction with hydrogen (Ljungdahl 1986). The latter may be of particular significance in the cricket system since it represents a conservation of carbon and reducing equivalents

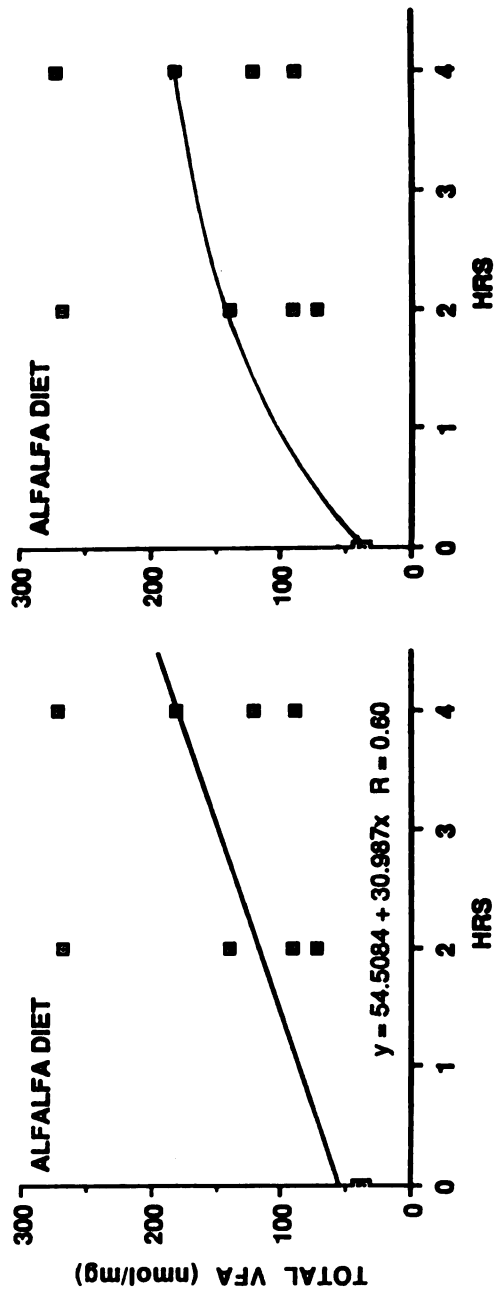


Figure 2.10 Comparison of linear best fit model with computer interpolation curve of VFA production in *A. domesticus* reared on a diet of 100% alfalfa hay.

The formation of acetate from CO_2 and H_2 is strongly suggested by the $^{14}CO_2$-fixation reported here. Conclusive evidence would necessitate the confirmation by NMR techniques of both carbon atoms of acetate being labeled . However, the results of the experiments here leave little doubt that the reaction occurs and is of considerable importance to the overall fermentation in *A. domesticus* hindguts. Based upon the reduction in acetate quantities that followed the addition of chloroform, H_2/CO_2 acetogenesis could account for approximately 35% of the acetate produced. This figure is nearly identical to that estimated for termite hindguts (Breznak and Switzer 1986). Although the incubation method (suspension) would limit extrapolation to intact gut systems, % composition of VFA produced in the suspension (not illustrated) are in agreement with values obtained for whole gut preparations. This would indicate that the overall fermentation was not greatly disrupted by the addition of $CHCL_3$ to the system.

Results from the "whole gut" incubations also demonstrated an active CO_2 fixation into VFA. The activity was enhanced under hydrogen, but considerable label was fixed under N_2; indicating that hydrogen evolution in the microbial community is sufficient to drive the reaction. Overall, less fixation of added label occurred in the whole gut method compared to the suspension incubations; indicating that distribution of the label was not adequate in the former. Even though label may not have been introduced uniformly in the whole gut method, its distribution in VFA after incubation is puzzling. Specific activities in acetate, propionate, and butyrate are

approximately equal in the whole gut incubatons, (Figure 2.8) while acetate specific activities in the suspension are disproportionately greater (Figure 2.7). The discrepancy requires further investigation, but may indicate that disruption of spatial relationships of bacteria in the community affects the transfer of fixed product between different groups.

The substantial incorporation of label into propionate raises questions about mechanisms involved in propionate formation. Of those known to involve CO_2 fixation are a *Bacteroides* -type pathway (Macy and Probst 1979) and a less well known reductive carboxylation of acetate (Schink 1984). CO_2 incorporation into propionate occurs via succinate in several species of *Bacteroides* (Macy and Probst 1979). Since this genus has been isolated from crickets (Ulrich *et al.* 1981) a potential explanation for the high specific activity found in propionate here may be due to *Bacteroides* metabolism of CO_2. There was little evidence of labeled succinate in any of the incubations, but turnover to propionate may have been too rapid for detection. Results from the chloroform block (Figure 2.7) support a *Bacteroides*-type fixation. $^{14}CO_3$ incorporation into propionate occurs independent of the reaction leading to acetate: specific activity of propionate does not change if acetate production from H_2 and CO_2 is inhibited (Figure 2.7). The evidence from the $CHCl_3$ treatment, then, is contrary to expectations, if propionate was formed from a reductive carboxylation of acetate. Incorporation of substantial amounts of $^{14}CO_3$ (up to 79%) into propionate have been reported by researchers primarily concerned with CO_2 fixation into acetate, but few have discussed the issue (Prins and Lankhorst 1977,

Goldberg and Cooney 1981, Schink 1984). The formation of C3 compounds from C2 compounds was called "enigmatic" by Schink (1984) and deserves more attention; particularly in systems where propionate is a significant end product. The high specific activity of propionate relative to acetate in the slurry incubations remains unexplained, but suggests that reductive carboxylation of acetate to propionate should not be dismissed as a possible pathway of CO_2 fixation in vivo.

The $CHCl_3$ block does indicate that incorporation of $^{14}CO_3$ into butyrate is dependent upon the acetogenic reaction. Condensation of two acetate molecules to form butyrate has been demonstrated previously (Kirby *et al.* 1983). The reaction may take on greater importance in crickets fed an alfalfa-based diet, as the butyrate proportion increases in the hindgut fermentation on that diet (Figure 2.6)

The significance of acetogenesis to terminal metabolism in anaerobic systems is only beginning to be understood. The process is generally thought to be less important than methanogenesis in most anaerobic systems, including the rumen (Breznak and Switzer 1986, Hungate 1975). Interest is currently focused on why methanogens seem to be more prevalent or active as terminal anaerobic metabolism. Since methanogenesis results in the net loss of carbon and reducing equivalents, the formation of acetate from H_2 and CO_2 would be advantageous in systems where fermentation products are linked to animal metabolism. In the case of termites, acetogenesis in the hindgut is relatively more important than methanogenesis, and represents a benefit to the insect (Breznak and Switzer 1986).

Although crickets are not as dependent upon their gut microbes, the presence of acetogenic bacteria as terminal process organisms fits in well with the apparent strategy of carbohydrate conservation in the insect. Methanogenesis apparently outcompetes acetogenesis in the hindgut of another omnivore; the American cockroach (*Periplaneta americana*) (Breznak and Switzer 1986). The reasons for dominance of acetate formation over methane formation in cricket and termite gut systems is an intriguing question: in the case of termites, acetogens appear to outcompete methanogens in the system. However in crickets, there is no evidence that methanogens ever establish in the system. Further research will hopefully clarify the issue.

The ability of injected VFA to move across the hindgut wall of crickets adds support to several other studies that reveal the insect hindgut permeable to a wide variety of small soluble organic compounds (Maddrell and Gardiner 1980, Hogan *et al.* 1985, Bracke *et al.* 1980, Phillips *et al.* 1986, Lawson and Klug, in press, Thomas and Nation 1984, Bayon and Mathelin 1980). As Phillips *et al.* (1986) point out, most studies involving ligated, *in vitro* gut incubations do not really measure transport rates. The research reported here for crickets is more accurately a measure of potential transport rates which would ultimately have to be determined *in vivo*. VFA movement across the cricket anterior hindgut wall was rapid. Rates were comparable to rates reported for termites (Hogan *et al.* 1985), but generally was higher than *in vivo* estimates for cockroaches (Bracke *et al.* 1980), cranefly larvae (Lawson and Klug, in press), and scarab beetles (Bayon 1980). The incubation temperature was

higher in this study (30°C) than in the aforementioned works, however, and may account partially for the high rates reported here. Additionally, the incubation bath used in this study did not contain VFA at physiological concentrations.

The pattern of label accumulation into the saline medium for all three acids suggests a passive transport of VFA (diffusion) in the anterior hindgut. Much of the absorption of compounds from insect midgut tissue is passive (Dow 1986), and the cricket anterior hindgut is in some ways a hybrid of midgut and proctodeum (Nation 1983). Thus, a passive transport is consistent with known mechanisms of absorption. Concentration gradients between the lumen and hemolymph would favor movement of VFA from the hindgut. Acetate concentrations in the hemolymph are approximately 10 mM (Kaufman unpub), while ectoperitrophic fluid concentrations are 80 mM (see above). Butyrate and propionate concentrations in hemolymph are below the mM range, and these acids would also have a favorable concentration gradient for leaving the hindgut.

It has been demonstrated previously that the anterior hindgut of crickets is permeable to products of digestion. Martoja (1966) found that ^{14}C from labelled cellulose in the diet could be found in the hemolymph of *G. bimaculatus* and that microorganisms were responsible for the activity. He did not identify the form of the transported label, but speculated that it likely was in the form of organic acid. Thomas and Nation (1984b) demonstrated that glucose, glycine, and palmitate could move across the gut wall of *G. rubens*, *G. assimilis* and *Scaptericus acletus*, but that palmitate tended to remain in the gut tissue, while glycine and glucose passed into the bathing

medium. These studies and the data presented here leave little doubt that the cricket anterior hindgut represents an absorptive site for products of microbial metabolism and possibly, cellular components released upon cell lysis (e.g. longer chain fatty acids). Although rates of movement in in vitro incubations are merely estimates of potential transport, they suggest that the hindgut can allow movement of produced VFA. Additionally, rectal tissue in some Orthoptera has been shown to actively transport acetate at rates that would greatly exceed production estimates reported here (Baumeister *et al.* 1981). Acetate transport mechanisms are reportedly unsaturable in the hindgut of *Panesthia cribrata* (Hogan *et al.* 1985). This has yet to be demonstrated in crickets, but similar salvaging of acetate and other VFA is implied by the low excretion rates of these compounds.

Since VFA are produced and absorbed in crickets, it is assumed they are utilized. Acetate is readily detectable in the hemolymph of *A. domesticus* in concentrations approaching 10mM (Kaufman, unpub.). Propionate and butyrate have also been found in hemolymph in trace amounts (< 100 μM), but detection limits were approached and no reliable quantitation is available. Acetate has been shown to be incorporated into lipids (Blomquist *et al.* 1982) in *A. domesticus* and serves as precursor to hormones, chitin and several other components of insect tissue. Additionally, there is little doubt it is oxidized as a source of energy in insect tissue (Odelson and Breznak 1983). Propionate and butyrate have received little attention, but presumably could serve as lipid precursors or oxidized substrates also. In vertebrates, propionate is particularly important

since it is one of the few lipids capable of being converted to carbohydrate via gluconeogenesis. Propionate is also incorporated into cuticular hydrocarbons in insects (Blomquist *et al.* 1979 and references therein). In view of the prominence of propionate in the cricket hindgut fermentation, and the role of gut microbes in food utilization efficiency (Chapter 1), the incorporation of propionate into insect carbohydrate deserves further research attention.

Although many aspects of the relationship between hindgut microbes and gryllids remain unclear, the results of this and previous studies (Chapter 1) strongly suggest a role in carbon conservation. Approximate digestibility of food components and enzymology comparisons between germ free and conventional *A. domesticus* reveal that soluble carbohydrates are the principle substrates of the microbiota. The hindgut bacteria add to the diversity of carbohydrases present and provide an increased degradative capability to the insect through a duplication of enzyme activity. VFA patterns also indicate that microbes ferment carbohydrates and that these compounds are made available to the insect in the form of products of anaerobic metabolism. The evidence for acetate formation from H_2 and CO_2 also suggests that microbial recycling of compounds that would normally escape the system is a significant piece of the carbon efficiency story. In many ways, the cricket hindgut/microbe system is unusual in comparison to the insect-microbe symbioses previously examined. Further research is needed to determine if similar mechanisms exist in other omnivorous species.

SYNOPSIS

The preceding chapters have examined the relationship between crickets and their hindgut symbionts from two slightly different perspectives. In one investigation, the effects of the microbial community on larval growth and digestive capabilities were examined. In another, the microbial fermentative metabolism was characterized and related to observed improvement of digestion efficiency in conventional animals. A synthesis of the two appears to explain the relationship between insect and microbe in *A. domesticus* and, possibly, other members of the Gryllidae.

The absence of a microbial community in the hindgut of *A. domesticus* had little obvious outward effect on growth, appearance, and behavior. The principle and consistent difference between conventional and germfree insects was the relative inefficiency of the latter in the utilization of food. It was apparent that acid-soluble carbohydrates were the microbes' principle substrates, and that carbohydrase activity in general was enhanced by the presence of hindgut bacteria. Nitrogenous compounds may also have been utilized more effectively by conventional crickets under some conditions. Unexpectedly, however, results from rearing the insects under low dietary N conditions did not indicate that a nitrogen conservation mechanism was operating, and in fact suggested that microbes interfere with nitrogen metabolism in adult females. The increased food utilization efficiency of conventional larvae was still maintained on a low nitrogen diet, however, and is likely explained

by the disproportionate increase in lipid content as cricket larvae mature.

The germfree/conventional comparison, therefore, strongly suggested a role for the hindgut bacteria in carbon metabolism and conservation. The cricket hindgut bacterial community was found to produce volatile fatty acids in quantities greatly exceeding excretion rates. The types of acids produced were entirely consistent with those expected of a carbohydrate fermentation, with acetate and propionate dominating the pool. In addition, a terminal aaerobic process and mechanism of carbon and reducing equivalent conservation was found to contribute substantially to the fermentation: acetogenesis from carbon dioxide and hydrogen. Since the bacterial metabolism is anaerobic and a mechanism for recycling CO_2 exists within the bacterial community, much of the energy of the carbohydrate precursors would still be available to the host. The potential savings amount to approximately 0.6 mg dry weight/cricket/day and agree well with estimated microbial enhancement of weight gain per unit of food consumed. These acids are highly permeable to the hindgut cuticle and epithelium, and have been shown elsewhere to be incorporated into lipid and carbohydrate components of insect tissue.

Through microorganisms, crickets increase their metabolic diversity. Although the study presented here uncovered only two specific examples (a-galactosidase activity and CO_2 fixation into acetate), there are undoubtedly many more. This would potentially allow the insects to utilize a wider range of diets, and the experimental evidence indicates that microbial-enhanced food

utilization is maintained across a range of diets. In natural situations, diet is likely to be even more variable.

Kinnear *et al.* (1983) have postulated that the chief benefit of gut microbes is to allow "niche expansion". They hypothesize that the success of ruminants and ruminant-like animals is not principally based upon the microbes abilities to degrade specific compounds (i.e. cellulose), but rather that microbes allow the utilization of a wider array of plant compounds which allows them to outcompete other herbivores in low nutrient situations. In other words, ruminants are not specialists on high fiber diets, but they are generalists with a large diet breadth. Certainly gryllids and other omnivores have not evolved as tight a dependence on microbes as ruminants, however, a portion of their success may also lie with expanded food utilization capabilities due to microbial metabolism. Jones (1984) has also forwarded several hypotheses concerning microbial associations with insect herbivores. Among others, he hypothesized that microbial mutualists should allow a utilization of marginal resources and function to "even out resource heterogeneity". His ideas are similar to those presented by Kinnear *et al.* (1983) as he emphasizes the increased metabolic diversity supplied by microbe mutualists and suggests that they are beneficial in low nutrient conditions, but detrimental in situations of better resources.. Buchner (1965) has postulated that microbial associations in general are more specific and pronounced as diets become specialized and deficient in one or more nutrients. How then, is the microbial symbiosis in crickets and other omnivores compatible with the above hypotheses?

The role of microbes in the nutrition of omnivores is likely to be more subtle and supplemental than in herbivores; but similarly not linked exclusively to particular diets. It may be a more general mechanism likely to be valuable throughout a range of fluctuating conditions. The idea is still compatible with the discussed hypotheses because increased food utilization efficiency in conventional crickets does become relatively more important as dietary carbohydrate is diluted. Conventional cricket food utilization efficiency was 40% greater than germfree values on the poorest diet, and 20% greater than germfree values on the highest quality diet (Table 1.2). In contrast to the predictions of Jones' (1984) hypotheses, however, the symbionts do not appear to be detrimental on diets of higher nutrient levels. Further, in terms of nitrogen metabolism, symbionts in crickets may be beneficial on high quality diets, and detrimental on nitrogen poor diets. The preceding study demonstrates that definitions of mutualism between microbes and animals must qualified in many cases because of interactions with nutrient type and availability, and growth stage of the animal. Diets employed in this study may not have been sufficiently different to test the above hypotheses, however, because the suite of available nutrients did not differ greatly in quality of compounds, but were more accurately a gradient of quantity. Support for the above hypotheses is provided by the differential survival of conventional and germfree crickets on the alfalfa diet (Figure 1.5) and indicate that microbes do indeed allow exploitation of marginal resources through increased metabolic diversity. Crickets as a group are diverse in habitat and feeding strategies. Examination of the

microbial symbioses in other species of crickets over a range of natural food items might provide a more rigorous test of the ideas.

Although microbial metabolites in the form of VFA may account for a substantial portion of the food utilization story in *A. domesticus*, there are several other tenable postulates concerning contributions from the bacteria. As discussed in previous chapters, a primary target for further investigation should be the question of cricket utilization of bacterial biomass and cellular constituents from the hindgut. Of most insect hindgut/bacteria systems studied thus far, the gryllid system has one of the highest potentials for such a mechanism independent of coprophagy because of its peculiar anatomy: the bulk of the bacterial biomass is not associated with the posterior moving food bolus. The unexpected observation that nitrogen may be conserved by conventional crickets on a high nitrogen diet, but that microbes may have a detrimental effect when nitrogen is scarce, is still unexplained. The examination of this will question require an understanding of the bacterial population dynamics within the hindgut under various nutrient conditions.

There also remain the possibilities of essential amino acid metabolism, supply of vitamins, and inhibition of pathogens as important roles. These questions were not directly addressed in this study, however, the germfree methodology employed will allow a means to investigate the possibilities. Additionally, the methods should allow an assessment of the microbe function in other species of crickets. The cricket hindgut/bacterial system remains a viable and intriguing opportunity for further research. The results presented in this dissertation represent only an initial, broad-based

look at the symbiosis, and it is hoped that future investigations will provide details.

APPENDICES

APPENDIX I

Early growth studies

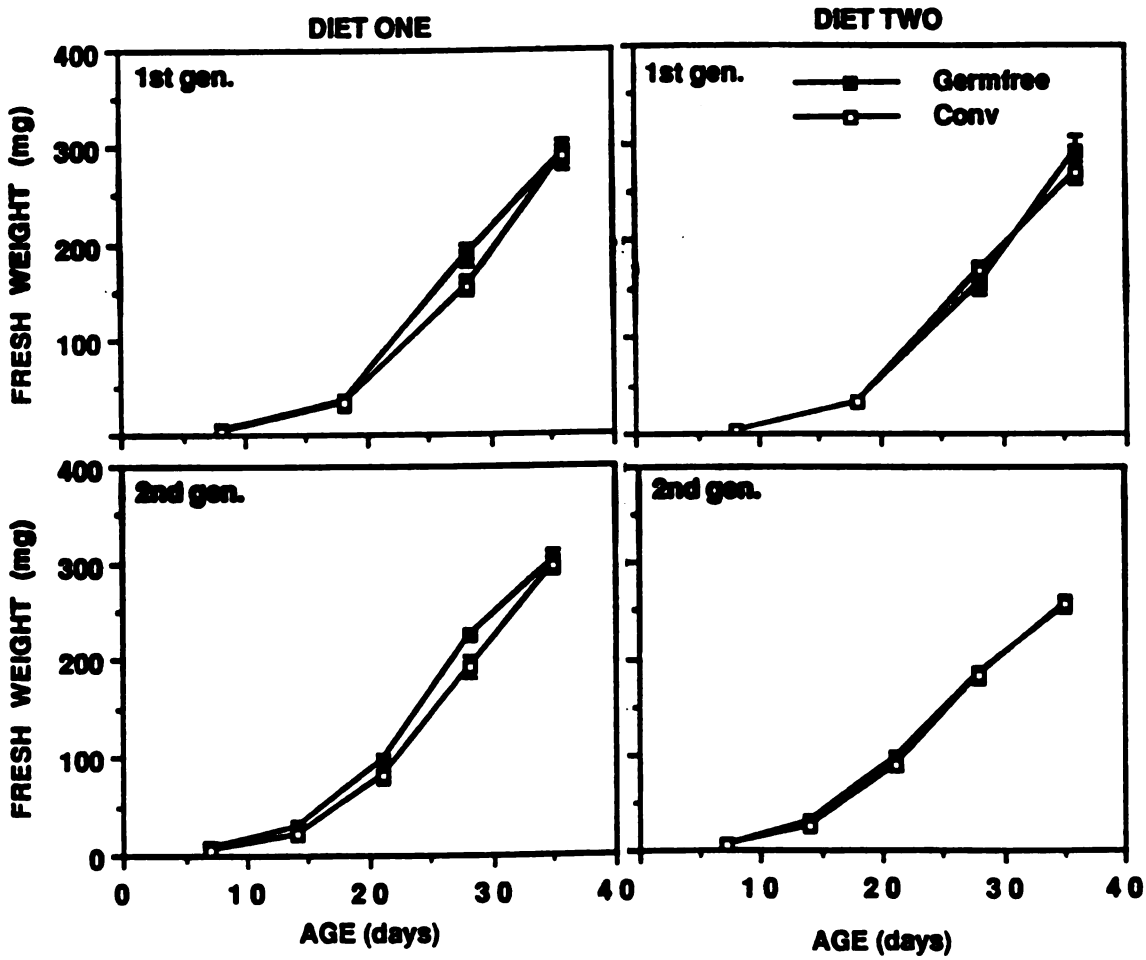


Figure AI.1 Larval growth curves of germfree and conventional *A. domesticus* reared on diets on and two, for two successive generations.

APPENDIX I. (cont.)

Table AI.1 Adult size (10 days beyond molt) and maturation times of first generation germfree and conventional *A. domesticus*. Values are mean ± S.E. (n).

		Diet one	Diet two
Males (mg)	Convent.	328.1 ± 4.4 (16)	296.9 ± 6.0(15)
	Germfree	337.6 ± 6.8 (14)	275.5 ± 10.4 (13)
Female (mg)	Convent.	415.3 ± 9.8 (12)	356.6 ± 7.5 (15)
	Germfree	411.1 ± 4.5 (13)	357.0 ± 7.6 (12)
Mat. time days	Convent.	39 ± 0.5 (28)	40 ± 0.6 (30)
	Germfree	38 ± 0.4 (27)	40 ± 0.6 (25)
Total % mortality	Convent.	0	6.7
	Germfree	0	10.0

Table AI.2 Adult size (10 days), fecundity, mortality, and maturation times of second generation germfree and conventional *A. domesticus*. Values are mean ± S.E.(n).

		Diet one	Diet two
Males (mg)	Convent.	344.2 ± 7.7(16)	307.8 ± 7.9 (15)
	Germfree	323.4 ± 6.5 (14)	276.9 ± 10.4 (13)
Females (mg)	Convent.	414.1 ± 7.8 (15)	349.3 ± 11.3 (15)
	Germfree	411.1 ± 8.4 (12)	339.8 ± 7.5 (15)
Mat. time (days)	Convent.	38 ± 0.4 (27)	39 ± 0.3 (29)
	Germfree	38 ± 0.4 (27)	39 ± 0.3 (30)
# eggs per female	Convent.	428 ± 11.2 (13)	318 ± 12.5 (14)
	Germfree	434 + 9.8 (12)	309 + 10.8 (15)
Total % mortality	Convent.	0	6.7
	Germfree	0	6.7

APPENDIX II

Whole Insect Respiration

O_2 consumption and CO_2 production of germ free and conventional *A. domesticus* was measured by placing insects in wire mesh tubes (6 x 30 mm) plugged with cotton at both ends to limit movement by the animal. The tubes were then placed in 36 ml serum vials and incubated at 30°C. For O_2 consumption, soda lime was placed in the serum vial prior to introduction of the insect in order to eliminate CO_2 accumulation during the incubation. Headspace was subsampled at 3 minute intervals for 2 hrs and analysed for O_2 and N_2 concentrations by GC (Dacey and Klug 1982). CO_2 production was assessed in a similar manner (soda lime was eliminated from the incubation vessel). O_2/N_2 and CO_2/N_2 ratios were used in place of absolute concentration values to correct for subsampling without replacement of headspace gas. Rates are reported as the slopes from linear regression analysis of computed ratios vs incubation time. R^2 values for all regressions were 0.98 or higher.

Table AII.1 Oxygen consumption and carbon dioxide production by last instar,germfree and conventional larvae of *A. domesticus*. Values are mean (S. E.), n = 5.

	O2 Consumed (ml/g /hr.)	**CO2 produced** (ml/g /hr)	**R. Q.**
Conv. female	1.5 (0.1	1.9 (0.1)	0.8 (0.1)
Conv. male	1.9 (0.2)	2.2 (0.3)	0.9 (0.1)
GF female	1.4 (0.1)	1.8 (0.1)	0.8 (0.0)
GF male	1.8 (0.1)	2.2 (0.2)	0.8 (0.1)

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