ALIMENTARY TRACT MICROBIOTA OF AQUATIC INVERTEBRATES

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ABSTRACT

ALIMENTARY TRACT MICROBIOTA

OF AQUATIC INVERTEBRATES

By

Amanda Kay Meitz

Microscopical examination of twenty-six species of aquatic invertebrates, primarily insect larvae, revealed that a gut microbiota is widespread. Microbiota was present in the midguts and, more frequently, in the hindguts of the larvae. Morphologically diverse microbiota was observed in the gut lumen and firmly adhering to the gut wall of a number of the larvae examined. Rods were the most frequently observed bacterial morphologies, however, prosthecate and filamentous bacteria and members of an obscure class of fungi, trichomycetes, were also noted. With some exceptions, the presence or absence of a gut microbiota was found to be correlated with the food habits of the insect. Detritivorous insects were observed to possess a dense midgut or hindgut biota, and occassionally both. Invertebrates living on more nutritious substrates such as algae or insect prey possessed a sparse microbial population in their alimentary tracts.

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INTRODUCTION

It is generally concluded that first to third order woodland stream communities depend upon inputs from surrounding terrestrial areas for the majority of their energy (Nelson and Scott, 1962; Hynes, 1963; Egglishaw, 1964; Minshall, 1967; Triska, 1970; Fisher, 1971; Hall, 1971; Fisher and Likens, 1972, 1973; Cummins et al, 1972, 1973). Particulate organic matter, primarily in the form of senescent leaf material, makes up a large percentage of this input with estimates of these inputs ranging from 0.97 to 5.0 $g/m^2/day$ (Peterson and Cummins, 1974). In temperate regions the initial stages of processing of these inputs of allochthonous organic matter occurs throughout the fall and winter via several mechanisms (Hynes, 1970; Kaushik and Hynes, 1971; Cummins, 1974). Coarse particulate organic matter (CPOM) such as leaves, twigs, branches, bark, needles, nuts, and fruits undergo abiotic leaching upon entering the stream and the majority of the soluble components are released within twenty-four hours (Nykvist, 1963; Kaushik and Hynes, 1971; Cummins, 1974, Petersen and Cummins, 1974). Other abiotic losses due to mechanical and physical processing of the CPOM as a result of the rigors of lotic conditions is estimated to account for approximately 5% of the total processing of this material (Cummins et al, 1973). In a scheme suggested by Cummins (1973) CPOM is material greater than 1 mm and includes leaf litter and fragments of plants and animals. Fine particulate organic matter (FPOM) is less

than 1 mm and includes plant and animal fragments, fecal material, free microorganisms and floculated or precipitated dissolved organic matter (DOM).

Concurrent with the abiotic processes the CPOM is colonized by fungi, protozoans, and bacteria. Colonization of CPOM by fungi (aquatic hyphomycetes) and bacteria occurs normally within two weeks in streams in the temperate zone (Suberkropp and Klug, Kellogg Biological Station, pers. comm.) This colonization leads to increases in the nitrogen content of the detritus (Kaushik and Hynes, 1971; Iverson, 1973; Suberkropp et al, 1975). The major processing of detrital materials occurs through the actions of the microorganisms and invertebrates as summarized in the trophic relations scheme in Figure 1 (from Cummins, 1973).

The two categories of organic particles (CPOM and FPOM) are processed in the stream by different categories of microorganisms and invertebrates. Fungi are credited with a major role in CPOM decomposition (Suberkropp and Klug, 1975) while bacteria are the primary decomposers of FPOM (Klug, pers. comm.) Aquatic animals, primarily aquatic insect larvae, have been divided into four categories according to their feeding behavior (Cummins, 1973): 1)shredders--animals consuming CPOM, 2)collectors--animals utilizing FPOM, 3)grazers--ingesters of periphyton, primarily diatoms, and 4)predators which utilize members of the other three feeding groups. Animals in each of the groups have morphological and behavioral adaptations which equip them for their roles of grazing algae, capturing insect prey or shredding or collecting detrital material. In Figure 1 Pteronarcys, Tipula, and Pycnopsyche are shown as typical shredders, Stenonema and Simulium as collectors, Glossosoma as a grazer and Nigronia and the fish Cottus and Salmo as predators. Litter microbes are characterized by

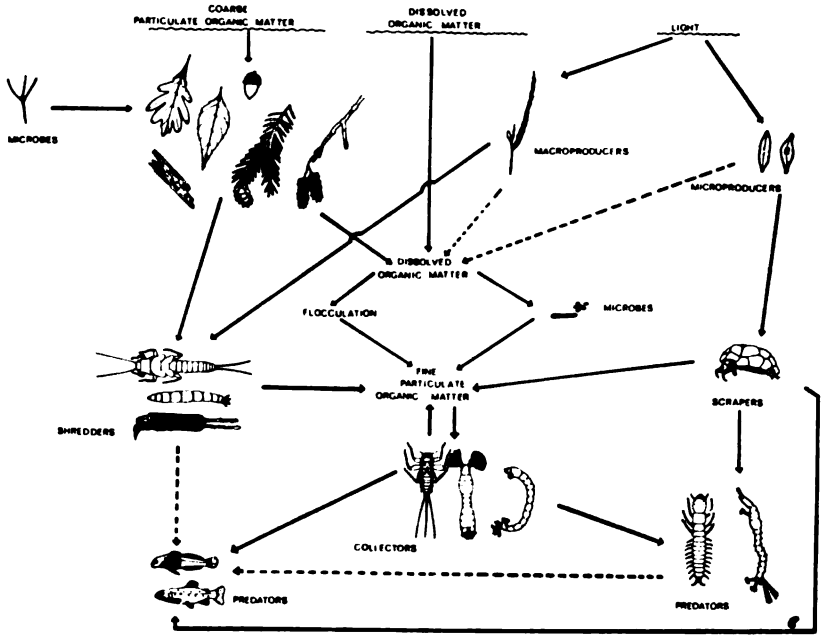


Figure 1. A simplified view of trophic relationships in a woodland stream community. Dashed arrows indicate less frequent exchange.

fungi (aquatic hyphomycetes) and fine particle microbes by bacteria. Deciduous leaves and photosynthesis by diatoms are utilized, together with soil runoff, as representative of energy inputs to the system.

An apparent nutritional dependence by some of the insects on the microbial flora associated with the ingested leaf material, rather than the leaf material itself, has been noted (Kaushik, 1969; Wallace et al, 1970; Kostalos, 1971; Liston, 1972; MacKay and Kalff, 1973; Iverson, 1973; Bärlocher and Kendrick, 1973a, 1973b, 1975). The insects presumably ingest the detrital material only to gain access to the colonizing microbes and fulfill their dietary needs at the expense of the microbes, while deriving little nutrition from the comparatively recalcitrant detrital material. A "peanut butter and cracker" analogy has been suggested by Cummins (1974) with the "peanut butter" being the microorganisms and the "cracker" the less nutritious leaf material. Alternatively, the insects may find the partially decomposed material more palatable (less tough) than non-colonized leaves.

Aside from the insects' preference for colonized vs. non-colonized leaves microbial-insect interactions are thought to occur among aquatic insect larvae comparable to those found in other insects. Microbes have been observed to occur within the insect body, either in mycetomes (specialized cells harboring bacteria or yeast) or in the alimentary tract of the insect. Microbes within the mycetomes, termed endosymbionts, have been observed in a number of insects including hemipterans, heteropterans, coleopterans, and orthopterans (Buchner, 1965). Buchner noted a correlation between nutritional categories of insects and endosymbionts. Insect predators which live on a complete diet do not have endosymbionts, while insects whose diets are incomplete possess endosymbionts. The American cockroach benefits from growth factors synthesized by mycetomal

bacteria, which are lacking from the normal cockroach diet. Aposymbiotic cockroaches (individuals in which the bacteria have been eliminated via antibiotics or gnotobiotic rearing) require a rich diet and are raised with difficulty in the laboratory (Brooks and Richards, 1955). Mittler (1971) has shown that individual omission of the ten essential amino acids from diets of aphids deprived of their endosymbionts substantially reduced the growth of these aphids compared to aphids possessing symbionts. For those aphids possessing endosymbionts the only "essential" amino acids were histidine, lysine, isoleucine and methionine.

A well documented example of a mutualistic symbiosis (favorable and obligatory for both symbionts) between an insect and the microbiota of its alimentary tract occurs in termites. Protozoa in the termite paunch digest cellulose to organic acids (primarily acetate) which are utilized by the termite for energy (Honigberg, 1970). Further, Breznak et al (1973) and Beneman (1973) have demonstrated nitrogen fixation in termites which has been attributed to the gut microbiota. This source of fixed nitrogen is presumed to be vital to the termite since a diet of wood is deficient in combined nitrogen as evidenced by a high C/N ratio.

Alimentary tract insect-microbe interactions are thought to also occur in larval stages of aquatic insects as evidenced by a dense, morphologically diverse bacteria population associated with the hindgut of larvae of the aquatic cranefly Tipula abdominalis (Klug and Kotarski, 1974). This microbiota was observed in the lumen and firmly adhering to the gut wall. The nutrient-poor characteristics of the detrital material ingested by the cranefly and other shredders and collectors suggest a possible insect-microbe gut symbiosis analogous to Buchner's observation for insects possessing mycetomes, i.e. a correlation between feeding

behavior of an insect and alimentary tract microbiota similiar to the correlation between feeding behavior and presence or absence of a mycetome.

The importance of the gut microbiota to the insect and to the processing of detritus in the stream may be minimal or vital depending upon the nature of the ingested food and the enzymatic capabilities of the host. A range of symbioses including mutualistic, protocooperative (favorable to both symbionts, but not obligatory), commensalistic (favorable to the commensal and the host not affected), or parasitic relationships could occur between an insect and members of the gut microbiota. For example, if the insect produced a wide range of polymer degrading enzymes, or possessed the behavioral and morphological adaptations so that a complete diet was available to a somewhat limited set of enzymes produced by the insect, a gut microbiota would be superfluous. Conversely, an insect consuming fiberous material low in nutrition and lacking the enzymes necessary to digest the various polymers would benefit from a microbial population with the capacity to hydrolyze the complex dietary constitutents. Other possibilities between these extremes are that the insect gut absorbs growth factors, essential amino acids, vitamins, or other small moleucles such as fatty acids, produced by microbial symbionts. The last set of possibilities is probably more frequent among insects than the two extremes of the continuum and certainly more difficult to characterize.

If the gut microbiota is superfluous or only marginally beneficial to the harboring insect the microbiota may be playing a role in the further conditioning and preparing of the detritus for the next trophic level. The biota of a shredder, for example, may not benefit the shredder, but may be a vital conditioning requirement for ingestion of shredder fecal material by the collectors; thus playing a relatively more

important role in processing allochthonous input to the stream than in providing nutrients to the host insect. Shredders and collectors consume 0.6% to 130% of their body dry weight/day (Cummins et al, 1973). Welch (1968) reported that for detritus feeders as much as 80% of the ingested food is execreted as feces. The ingested food is presumably modified as it passes through the gut with the less resistant compounds being assimilated by the insect. The material execreted as feces is thought to be more recalcitrant and less nutritious than that ingested. However, due to the associated bacteria the feces may be considerably more palatable and nutritious to the collectors than if the bacteria were absent. The role of the bacteria associated with this FPOM may be analogous to that of the hyphomycetes associated with the CPOM, acting as the "peanut butter" to induce collectors to ingest the FPOM. Although particles in feces have attached bacteria, this possibility is not as attractive as it might seem because feces of several aquatic insects have been observed to disperse immediately upon defecation, releasing free bacteria and detrital particles.

The integration of these considerations concerning the role of gut microbiota into the scheme of trophic relations in a woodland stream is not possible, without more information concerning the presence or absence of a gut microbiota in aquatic insects aside from the cranefly. To provide this information a survey of aquatic insect larvae was undertaken to assess whether a gut microbiota is widespread among aquatic insect larvae and if the presence of a microbiota is correlated with the feeding behavior of the insects examined. The basic hypothesis being that a gut microbiota would frequently be observed among animals consuming detritus (shredders and collectors) and absent in animals

utilizing more nutritious substrates (predators and grazers). To test this hypothesis selected members of the various feeding categories were dissected and their alimentary tracts examined with phase microscopy. Gut morphology, presence or absence of significant numbers of bacteria, their location in the guts, and whether the population was lumen or gut wall associated were noted. An estimate of the numbers present was determined and light and electron photomicrographs of typical examples of the microbiota obtained.

MATERIALS AND METHODS

Collection and Maintenance of Insects

Most of the insect larvae examined were collected from Augusta Creek, a small woodland stream in Kalamazoo and Barry counties, Michigan. Larvae of insects were maintained in containers of water and detritus, stones colonized with algae, or insect prey depending on the feeding category of the insect to be examined. In this way the larvae had available a fairly normal diet prior to death and dissection. The chambers were aerated via compressed air forced through an aquarium bubbling stone. Temperature was held at 5-10° C for periods of several days for most larvae to several months for the larval craneflies. Four species from streams in the Cascade Mountains were kindly provided by members of Dr. James Sedell's laboratory at Oregon State University. Selection of insects was based upon availability of information concerning their feeding habits, ease of collection and potential interest as an unusual or typical insect. A crustacean, Gammarus, and a mollusk, Goniobassis, were also included in the survey because of their frequent occurrence in Augusta Creek and their similiar feeding behavior to some insects.

Light Microscopy and Enumeration of Biota

Larvae were killed by immersion in boiling water for approximately five seconds or by decapitation and examined using a variety of microscopic techniques. After dissection in 0.1 M phosphate buffer, pH 7,

gut morphology was traced using a camera lucida attachment on the dissecting microscope. Wet mount preparations of lumen contents and washed gut wall were made by excising portions of the gut (foregut, midgut, pylorus, and rectum) and slicing the resulting cylindrical tissue longitudinally to obtain a flat preparation. The lumen contents removed during this process were collected in a drop of buffer on a microscope slide. The gut wall was vortexed vigorously for 10-20 seconds in 0.1 M phosphate buffer, placed on a slide, and the surrounding muscle teased away. The resulting tissue was examined for adhering microorganisms with phase microscopy.

Numbers of bacteria in the wet mounts of lumen contents were determined subjectively. More than fifty bacteria/field at 1000X was categorized as +2, 1-50 bacteria/field as +1, and less than one bacterium/field as 0. Subsequently the Petroff-Hauser counter was used on one-half of the larvae initially examined to provide further documentation of the subjective observations. For enumeration of gut microflora larvae were dissected and midgut and hindgut dimensions were recorded. Midguts and hindguts were macerated in a tissue grinder in phosphate buffered 4% formalin solution. With the larger larvae it was possible to macerate individual midguts and hindguts. For analysis of the smaller larvae several midguts and hindguts were pooled. Pieces of gut wall and detrital particles in the gut were observed to settle within a minute after maceration and were not disturbed when samples of suspension were placed in the Petroff-Hauser chamber and counted at 640X. The suspension was agitated and large particles allowed to resettle between successive samples. Three to six samples of the suspension were counted, each count including three to twenty-five fields (twenty-five fields equals volume of Petroff-Hauser counter) depending upon bacterial density. The gut

was presumed to be a cylinder and volume of the gut was calculated from the gut measurements. Average numbers of bacteria per midgut and hindgut were calculated from the Petroff-Hauser counts and numbers/ml of gut were calculated.

The wet mount procedure was used for all invertebrates examined (Tables 1 and 4) and Petroff-Hauser counts on invertebrates listed in Tables 2 and 3. Since bacteria populations were, to some extent, similiar among insects possessing biota and due to time constraints phase, light, and electron micrographs presented in the "Results" are of microbiota from fourth instar larvae of the cranefly Tipula. This was chosen for photography since it was large and easier to manipulate than smaller larvae, possessed greater morphological diversity of bacteria than some of the smaller larvae, and was readily available.

For phase photography of lumen populations contents from Tipula gut were suspended in phosphate buffer or 0.75% sodium chloride solution and drops of the suspension placed on agar slides. Agar slides were made by dripping a sterile 1.5% agar solution on microscope slides, allowing it to spread out, wiping off excess agar, and allowing the remainder to solidify on the slide.

Sections 1 μm thick were cut on an LKB microtome from epon-embedded alimentary tract tissue (described below) and placed in drops of 10% ethanol on microscope slides. Excess ethanol was blotted off and the remainder evaporated over a hot plate causing the sections to adhere to the slide. Several drops of 1% toluidine blue and 1% sodium borate (1:8) were added with the slide still on the hotplate. The hotplate was allowed to heat until the edges of the staining solution were dry. The staining solution was washed off with distilled water, excess water blotted and

remaining water near the sections allowed to air dry. The thick sections were photographed using light microscopy. All photographs were taken with Kodak Panatomic or Plus-X-Pan film on a Zeiss Universal Microscope equipped with a 35 mm camera.

Electron Microscopy

Preparations of lumen bacteria for electron microscopy were made by dissecting larvae of the cranefly Tipula in 0.75% saline. Portions of the hindgut were placed in test tubes, sliced longitudinally, and vortexed in 1% glutaraldehyde in 0.1 M phosphate buffer for thirty minutes at room temperature. The cells were washed in distilled water to remove the glutaraldehyde and placed on 200 mesh formvar coated grids. The grids were shadowed with platinum-palladium (80:20) in a Kinney Vacuum Evaporator prior to viewing in the electron microscope.

Larvae for embedding in epon were decapitated and dissected in 3% cold glutaraldehyde in 0.1 M phosphate buffer. Guts were perfused with 6% purified buffered glutaraldehyde (Electron Microscopy Sciences) to insure immediate contact with the fixative. The tissue was fixed overnight at 4^{o} C, washed in phosphate buffer, post-fixed in 1% osmium tetroxide in S-collidine buffer and embedded in epon according to the procedures of Luft (1961). Silver or gold sections were cut with glass knives and placed on grids. Sections were stained with aqueous 5% uranyl acetate and lead citrate according to Reynolds (1963). A Siemens Elmiskop 1a at 80 KV was used to examine the grids.

RESULTS

Gut Morphology

The alimentary canal in insects is divided into three main regions: the foregut, derived from the embryonic ectoderm, the midgut which is endodermal in origin and the ectodermally-derived hindgut. Depending upon the morphological complexity of the insect these regions may be further divided: the foregut into the oesophagus, crop and proventriculus; the midgut is comprised of the gastric cecae and ventriculus; and the hindgut includes the pylorus, ileum, and rectum (Chapman, 1971; Wigglesworth, 1974). In the insects examined boundaries of the foregut, midgut, and hindgut were usually fairly distinct; the subdivisions of these regions were often less discernible or absent. A tissue change and often a color change was observable where the foregut and midgut connected. The hindgut was separated from the midgut at the point where the Malpighian tubules intersect the alimentary tract.

The majority of the insect guts examined exhibited morphological similarity as shown in Table 1. The simple gut possessed no gastric cecae, proventriculus, convolutions or enlarged fermentation chamber. Small variations of the simple gut occurred and correlated with the phylogeny of the insect; for example the three mayflies were more similar to each other than to any of the others in this group. The simple gut shown (Figure 2) is a tracing of Hydatophylax hesperus and is characteristic of the limnephilids and filipalpians. The remaining

trichopterans and elmid beetle had a longer, narrower, and slightly curved ileum. Ephemeropterans possessed a wider ileum and the rectum was surrounded by a prominent layer of muscles.

A few insects exhibited more complex morphology including a proventriculus in the foregut, gastric cecae in the midgut, or a convolution or fermentation chamber in the hindgut (Table 1). The diagram of one of the more complex guts is that of the megalopteran Nigronia. Two other guts exhibited an intermediate complexity between the simple gut and the more complex megalopteran gut shown. The pylorus and ileum of the blackfly Prosimulium were not well defined, forming a loop that lead to an enlarged rectum. The caddisfly Hydropsyche possessed a straight tube gut with the addition of a proventriculus. The cranefly Tipula was the only insect with a truly enlarged fermentation chamber in the hindgut (Figure 2). In the majority of insects the midgut was the most voluminous and prominent of the three regions, frequently larger than the hindgut by a factor of ten. In the cranefly and megalopterans these proportions were reversed, though in Tipula the hindgut was only two to three times larger than the midgut (Table 2).

Hindguts of all of the insects examined were fairly durable tissues and could be manipulated for observation as described previously. The midgut of the majority of insects was fragile and could not be handled in this manner. It was possible to make squash mounts of midgut and observe fragments of wall, but not to make a clean preparation of wall. The four exceptions--the chironomids and simulid, possessed a midgut wall that consisted of a tough, transparent membrane (Figure 7). Due to the small size of these guts slicing the cylindrical midgut longitudinally to aquire a flat tissue was not achieved. Instead, the contents could

Table 1. Gut morphologies and taxonomy of invertebrates

SIMPLE GUT
Hexagenia limbata (Ephemeroptera, Ephemeridae)
Stenonema spp. (Ephemeroptera, Heptageneidae)
Leptophlebia nebulosa (Ephemeroptera, Leptophlebiidae)

Pteronarcys pictetii (Plecoptera, Filipalpia, Pteronarcidae)
Pteronarcys sp. (Plecoptera, Filipalpia, Pteronarcidae)*
Taeniopteryx parvula (Plecoptera, Filipalpia, Taeniopterygidae)

Lara sp. (Coleoptera, Elmidae)*

Brillia flavifrons (Diptera, Chironomidae, Orthocladiinae)
Brillia sp. (Diptera, Chironomidae, Orthocladiinae)
Stictochironomus annulicrus (Diptera, Chironomidae, Chironominae)

Platycentropus radiata (Trichoptera, Limnephilidae)
Pycnopsyche guttifer (Trichoptera, Limnephilidae)
Hydatophylax argus (Trichoptera, Limnephilidae)
Hydatophylax hesperus (Trichoptera, Limnephilidae)*
Lepidostoma costalis (Trichoptera, Lepidostomatidae)
Heteroplectron sp. (Trichoptera, Calamoceratidae)*
Brachycentrus occidentalis (Trichoptera, Brachycentridae)
Glossosoma nigrior (Trichoptera, Glossosomatidae)

CONVOLUTION
Prosimulium sp. (Diptera, Simuliidae)

PROVENTRICULUS
Hydropsyche bronta (Trichoptera, Hydropsychiidae)

PROVENTRICULUS + GASTRIC CECAE + CONVOLUTION
Paragnetina sp. (Plecoptera, Setipalpia, Perlidae)

Corydalus sp. (Megaloptera, Corydalidae)
Nigronia serricornis (Megaloptera, Corydalidae)

GASTRIC CECAE + FERMENTATION CHAMBER
Tipula abdominalis (Diptera, Tipulidae)

NON-DIFFERENTIATED GUTS
Goniobassis sp. (Mollusca, Gastropoda)

Gammarus pseudolimnaeus (Crustacea, Amphipoda)

* species from Oregon

Figure 2. Examples of gut morphologies observed

Left: Hydatophylax hesperus gut, characteristic of the simple gut morphology.

Center: Nigronia serricornis gut, a more complex gut morphology.

Right: Tipula abdominalis, a complex gut with a fermentation chamber.

Foreguts and hindguts are outlined with a heavy line to denote chitinization. Malpighian tubules attach at the line where the midgut and hindgut meet.

g--gastric cecae
i--ileum
pr--proventriculus
py--pylorus
r--rectum
rl--rectal lobe
rs--rectal sac

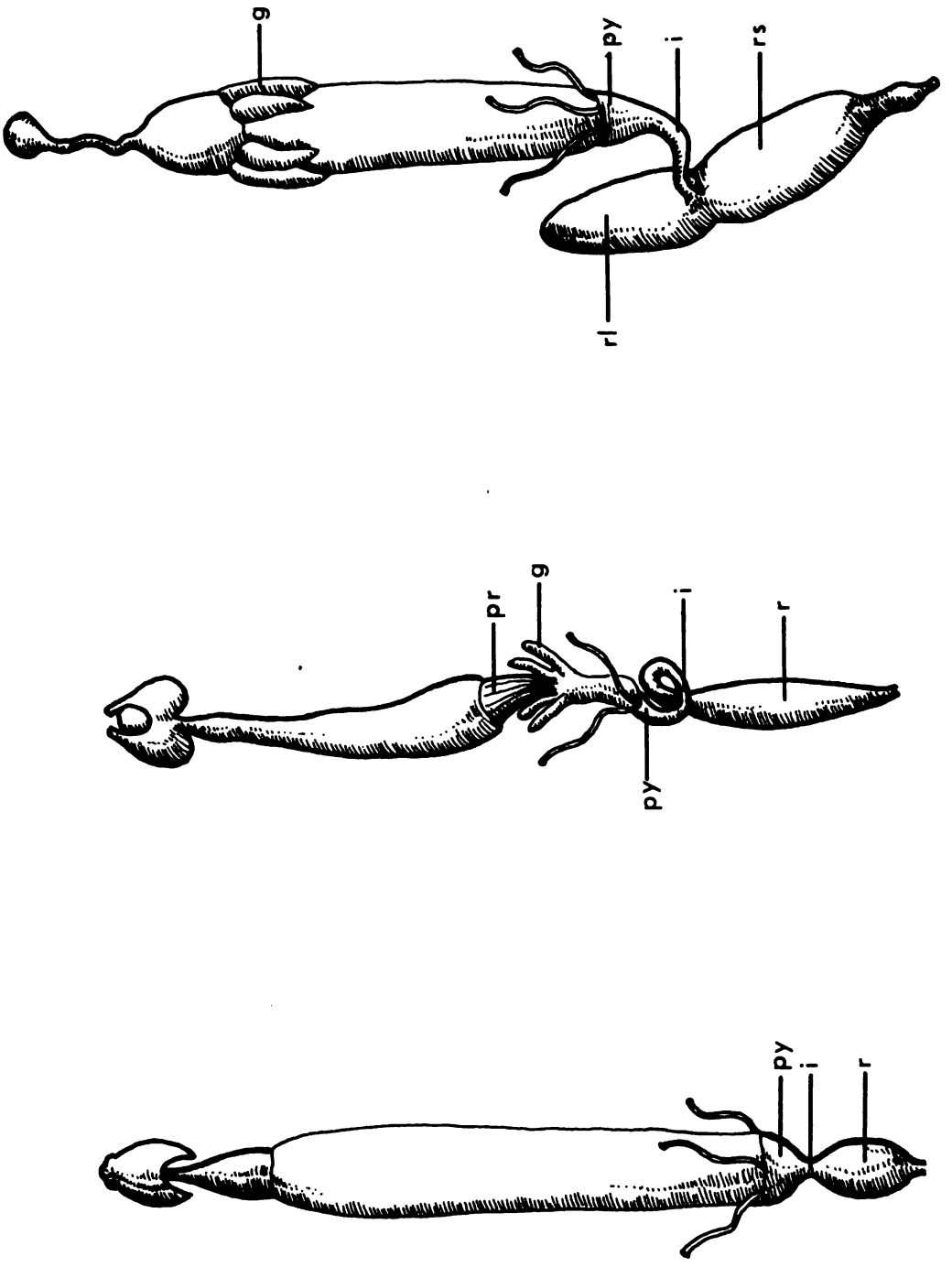


Figure 2.

be extruded from the tubular gut by applying pressure to the exterior of the gut with tweezers, thus obtaining preparations of contents and gut wall (Figure 7).

Neither the amphipod Gammarus or the snail Goniobassis possessed the foregut, midgut, and hindgut divisions of the alimentary tract that is characteristic of insects. Alimentary tracts of both were undifferentiated, consisting of fairly fragile membranes surrounding the ingested food.

Gut Microbiota

Two populations of organisms were distinguished in the insects examined--lumen and wall-associated organisms. Lumen organisms were defined to be those organisms removed when the gut wall was sliced longitudinally and vortexed in buffer. (The majority of the lumen organisms were removed when the contents were collected by longitudinally slicing the gut wall and allowing the contents to fall in a drop of buffer on a glass slide.) The wall organisms remained attached to the tissue in spite of the vigorous washing. For the Petroff-Hauser counts the lumen population was assumed to consist of those organisms that were suspended as a result of the tissue grinding procedure. Only rarely were fragments of typical wall-attached organisms observed in the lumen preparations.

The predominent lumen bacteria in all guts were flagellated rods ranging in length from 1 μm to 30 μm with the majority 5 μm long or smaller. In regions of guts with 10^5-10^7 cells/ml only small rods, approximately 1-3 μm long were observed. Guts with greater numbers possessed greater diversity including larger sporulating and non-sporulating rods, spiral-shaped organisms, and more unusual morphologies.

Morphological diversity of the lumen biota varied slightly from insect species to species and between individual members of the same species as shown in the percentages of bacterial morphological types in Table 3. Morphological diversity and flagellation among members of the lumen population of the cranefly Tipula are shown in Figure 3. Similar or somewhat less diversity was observed in wet mount preparations of hindgut lumen contents of the other obligate shredders, collectors, and predators that possessed a dense microbiota.

Thick sections of non-washed epon-embedded gut wall from the cranefly Tipula (Figure 4) revealed dense populations close to the wall and somewhat less dense populations and detrital particles in the lumen. The gut is surrounded by two layers of muscles. A total of three nuclei of gut wall cells can be seen in the two photomicrographs. Thick sections of the caddisflies Pycnopsyche and Hydropsyche revealed a similiar situation to that shown in the cranefly. Thin sections of washed cranefly gut wall revealed bacteria surrounded by amorphous material in close proximity to the wall (Figure 4). The wall consists of a cuticle adjacent to the bacteria. Parallel infoldings of the cell membrane with mitochondria between occupy approximately one-half of the cell. The nucleus and nuclear membrane are prominent above an electron-transparent area and the basement membrane. Preliminary electron microscopy of thin sections of the caddisfly Pycnopsyche hindgut revealed a similiar association.

The most distinctive members of the wall populations were filamentous bacteria. Phase and electron microscopy of these organisms (Figure 5) revealed an end-on attachment to the wall and extension several hundred μm into the lumen. At the proximal end of the filaments few septa were observed. At the distal end of the filaments numerous septa and

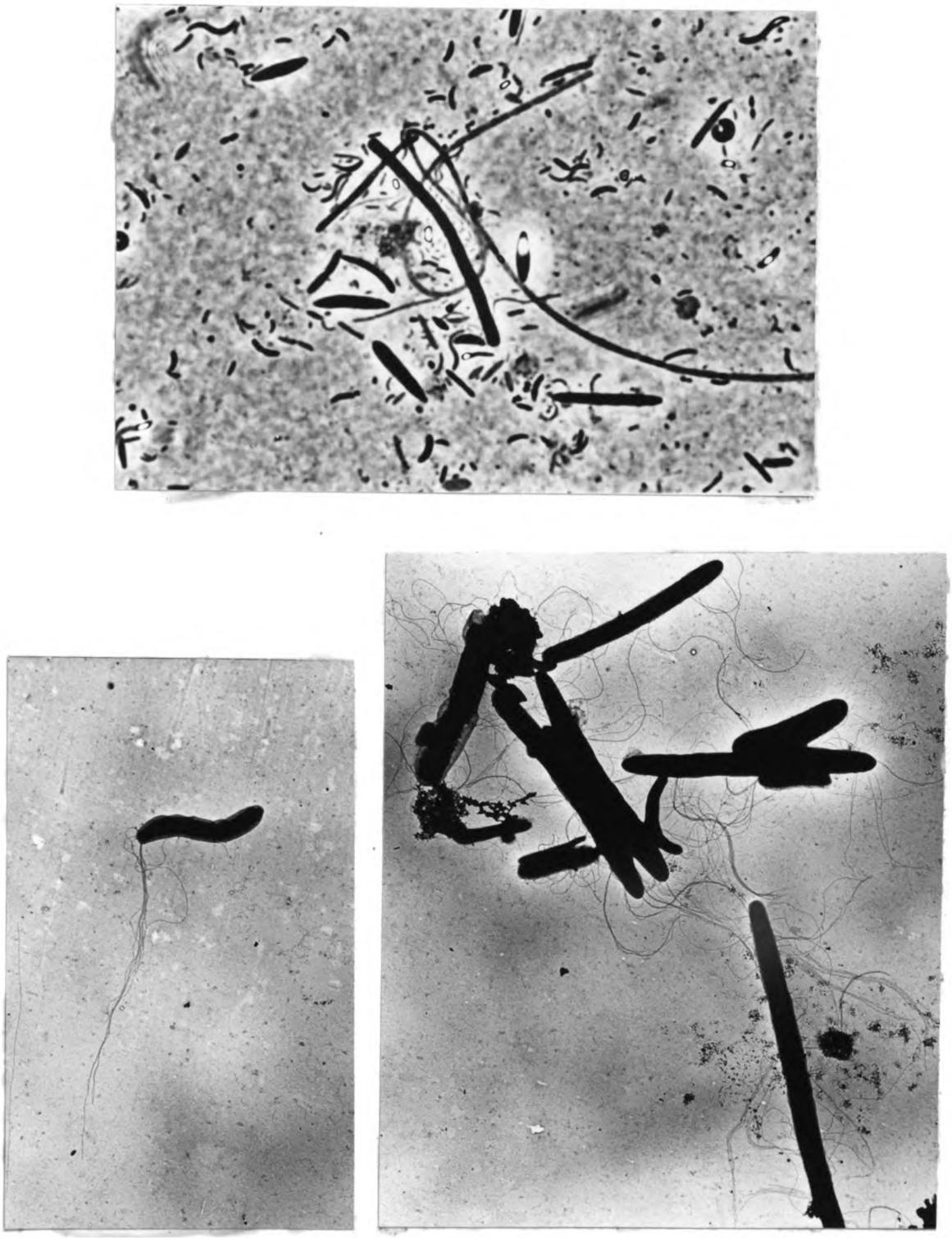


Figure 3. Lumen bacteria from Tipula.

Upper: Agar slide photomicrograph. 1300X

Lower: Shadowed electronmicrographs. Left: 5280X

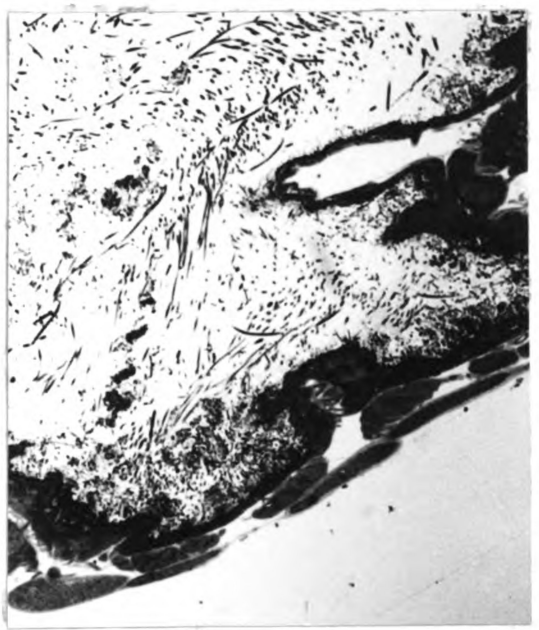
Right: 5320X

Figure 4. Tipula rectal sac and bacteria.

Upper: Epon-embedded thick sections of non-washed rectal rectal sac and lumen contents. Left: 310X

Right: 780X

Lower: Electronmicrograph of washed rectal sac. 11,550X



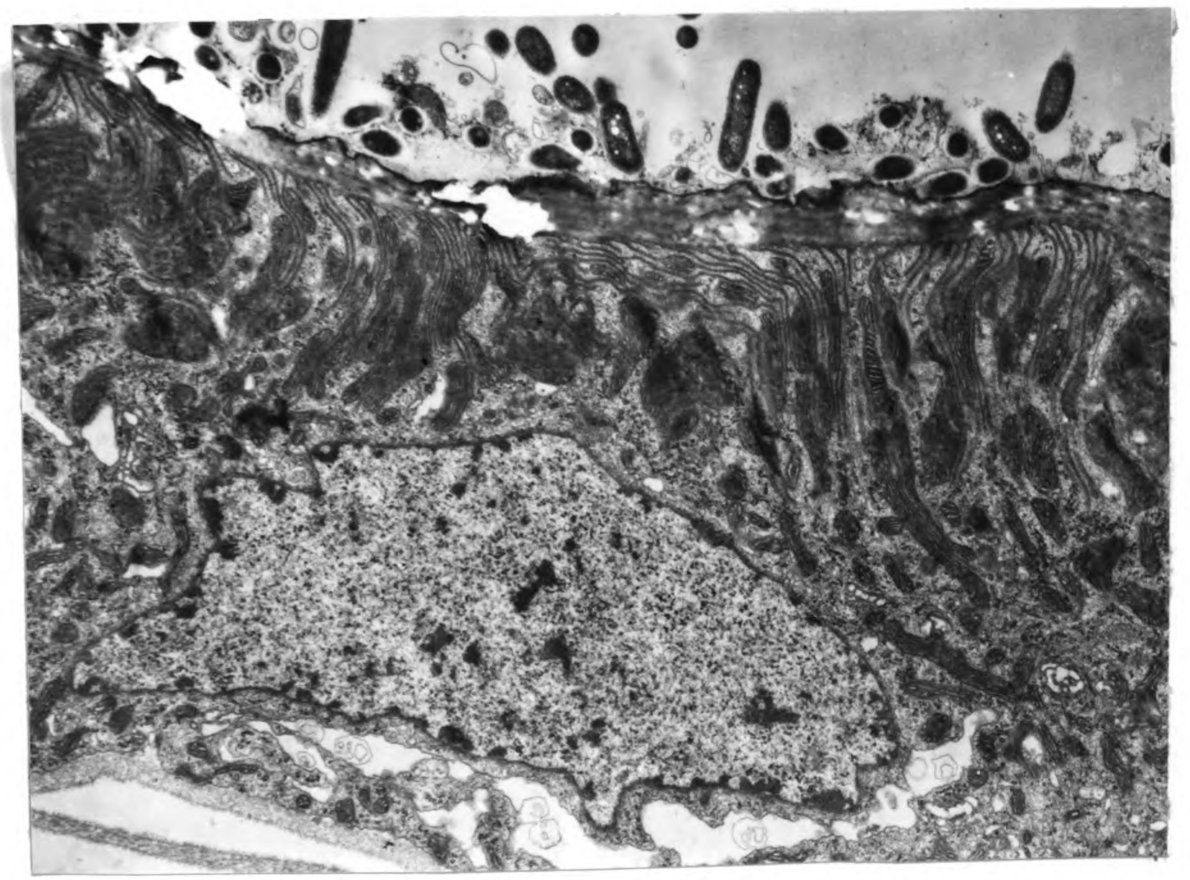


Figure 4.

Figure 5. Bacterial filaments from Tipula.

Upper left: Wet mount of sporulating filamentous bacteria and gut wall near juncture of the ileum, rectal sac, and rectal lobe. Masses of shorter rods are against the wall. 800X

Upper right: Agar slide preparation of the distal ends of the filamentous bacteria with spores and in the presporulation stage following septum formation. 1300X

Lower: Electronmicrograph of the filaments and cuticle of gut wall in the region of the ileum, sac, and lobe juncture. 11,550X

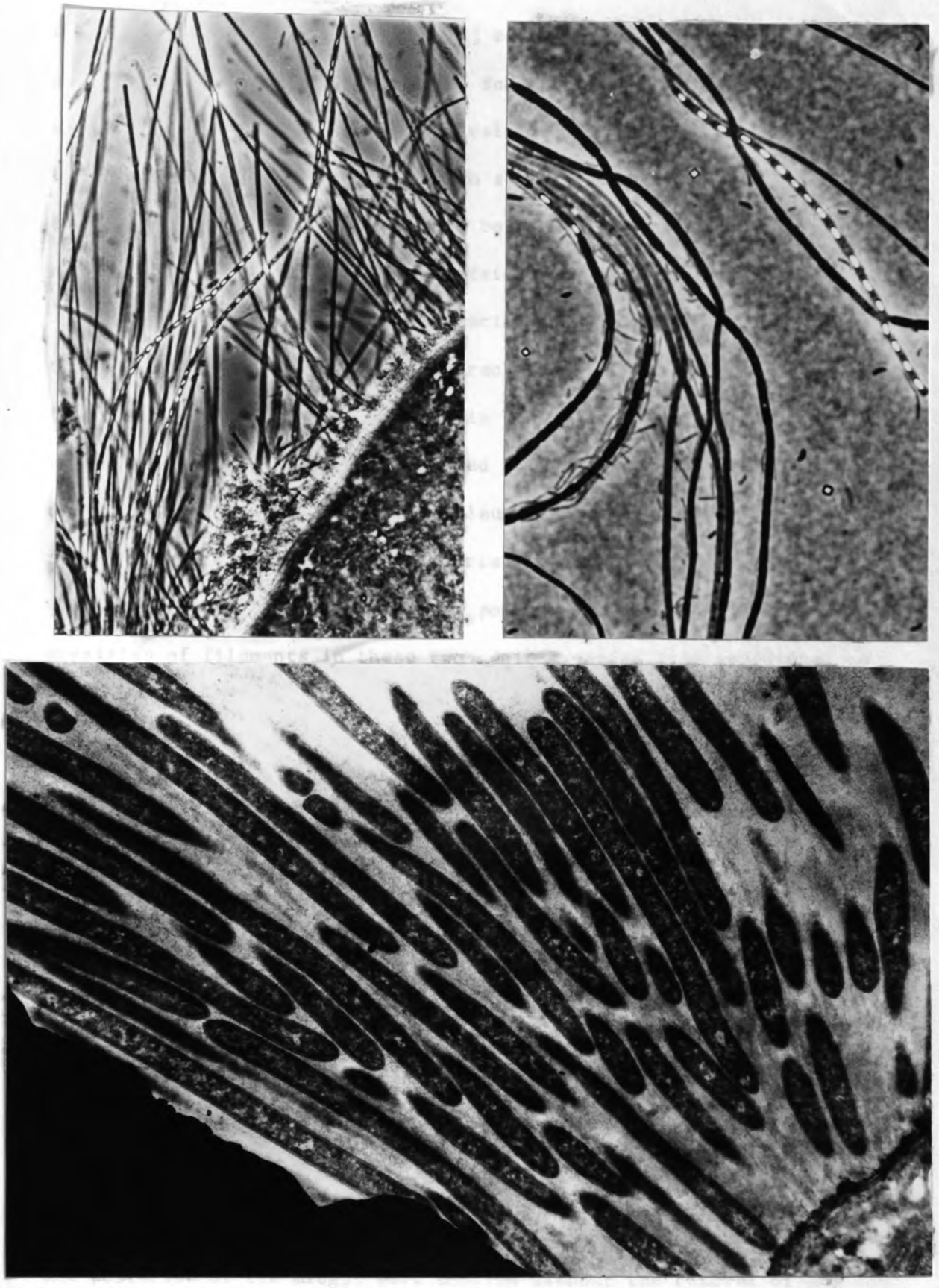


Figure 5.

inclusion bodies, presumably spores, occur. During the sporulation process the cells appear to shorten somewhat as evidenced in the agar slide preparation (Figure 5) where cells without spores are slightly longer and not as wide as those with spores. Distal portions of filamentous bacteria in the lumen can also be observed in Figure 4 with the attachment site presumably being outside the sectioned area.

In the cranefly Tipula the bacterial filaments were most dense at the juncture of the ileum with the rectal sac and rectal lobe. However, this localization was not observed in every individual. In some individuals these organisms were observed to colonize the rectal sac in densities similiar to that at the ileum-sac-lobe juncture. In the trichopterans the filamentous bacteria were localized in the posterior half of the pylorus or the anterior portion of the rectum. Relative densities of filaments in these two regions varied from individual to individual. Location of the mass of filaments could frequently be observed immediately after opening the body cavity and exposing the alimentary tract. The mass of filaments would impart a yellowish tinge while the rest of the gut was brown due to the detrital food material. Under the dissecting microscope (120X or 250X) the filaments appeared as fine wispy strands protruding from the gut wall.

Localization of bacteria in a portion of the gut was particularly evident in the wood boring midge Brillia. A very prominent clump of sporulating rods (approximately 8 X 1 μm) occurred just anterior to the juncture of the Malpighian tubes with the alimentary tract. This mass of rods was observable as a light brown clump through the body of the insect at 120X. Individuals were observed to defecate and the hindgut and posterior of the midgut were emptied without the removal of the

mass. Phase microscopy of the feces revealed woody particles and less than one bacterium/field. Apparently, the mass of rods inscribes the midgut wall and is firmly attached. Clumping of the rods was observed and difficulties encountered when attempts were made to spread the bacteria for observation on agar slides. That the bacteria were not removed with the feces together with the clumping implies that the rods form a "donut" inside the midgut and adhere while allowing the feces to pass through the "hole" of the "donut."

Not only were specific members of the flora localized (the filaments of some shredders and collectors and the mass of rods in the wood borer), but frequently the entire flora was localized in the anterior portion of the rectum or in the pylorus. Due to the size of the washed Tipula gut wall the most notable example of localization of the entire gut flora to a region of the hindgut was observed by Klug and Kotarski (1974). They described a "line of demarcation" just anterior to the rectum, observable macroscopically on a washed gut wall. The wall had a fuzzy white appearance in colonized areas and was clear and transparent at the posterior end. The shredding caddisflies also possessed a distinct line between colonized and non-colonized regions.

Prosthecate bacteria, shown in Figure 6, possess appendages (prosthecae) approximately 0.2-0.7 μm X 0.05-0.12 μm and the longest dimension of the cell (excluding prosthecae) is approximately 0.6-1.3 μm across. Prosthecae of the cells are surrounded by the bacterial cell wall (lower electronmicrographs, Figure 6). Some morphological variability occurs among the prosthecate bacteria and many of them contain vesicles. They are embedded in a somewhat more electron-dense material than the other bacteria in the gut. Prosthecates were observed in thin sections of gut wall with bacteria in the rectal lobe, rectal sac, or ileum-sac-

lobe juncture in six different cranefly larvae. Occurrence was fairly rare, but when they were observed they were in groups very often within ten μm of the gut wall. Prosthecates have also been noted by Klug and Kotarski (1974) in scanning electron micrographs of Tipula gut wall.

Trichomycetes, a poorly understood class of fungi were observed in the midguts of three dipterans and the hindgut of a mayfly (Table 4). After removal of portions of detritus and bacteria from the midguts of the dipterans the trichomycetes could be observed through the intact transparent midgut wall (Figure 7). Observation of trichomycetes in the mayfly hindgut was by the usual method of slicing open the gut. Colonization of larvae by trichomycetes varied with time. The mayfly, for example, was well colonized in some collections and poorly colonized later in the spring. Degree of colonization also varied between individuals collected on the same day. Asexual reproductive structures, trichospores, were characteristic of Stachylina or Harpella, members of the Harpellales, the only order of trichomycetes known to conjugate. Occassionally conjugation of hyphae was observed in the blackfly Prosimulium and the midge Stictochironomos.

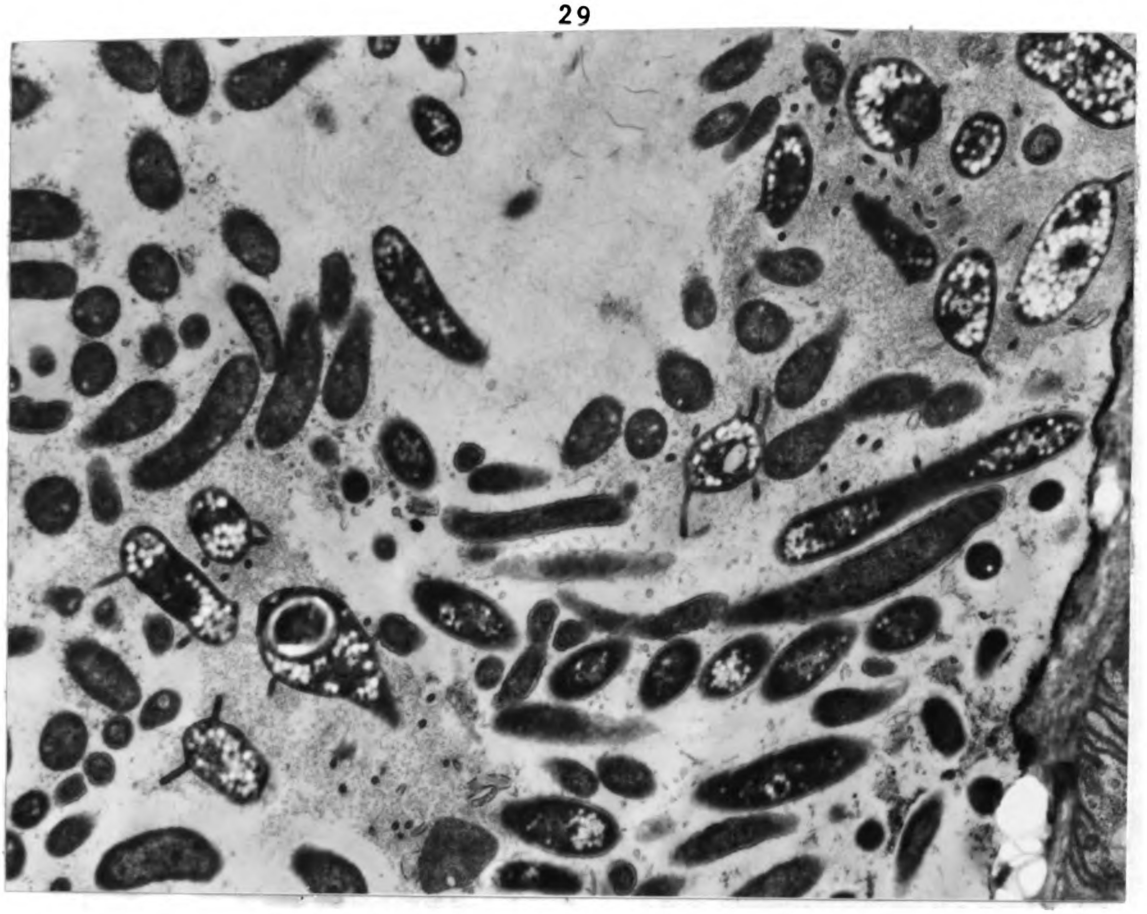
The subjectively determined categories of +2, +1, and 0 were found to correspond to 10^9-10^{10}, 10^8, and 10^5-10^7 bacteria/ml of gut respectively in the Petroff-Hauser counting procedure for the insects that were counted. Half of the insects were examined by both methods and half received subjective examination only. Due to the correlation noted above between the subjective and Petroff-Hauser examinations it is reasonable to extrapolate from the insects that were counted in the Petroff-Hauser chamber to those not counted as was done in Table 4. Tables 2 and 3 list

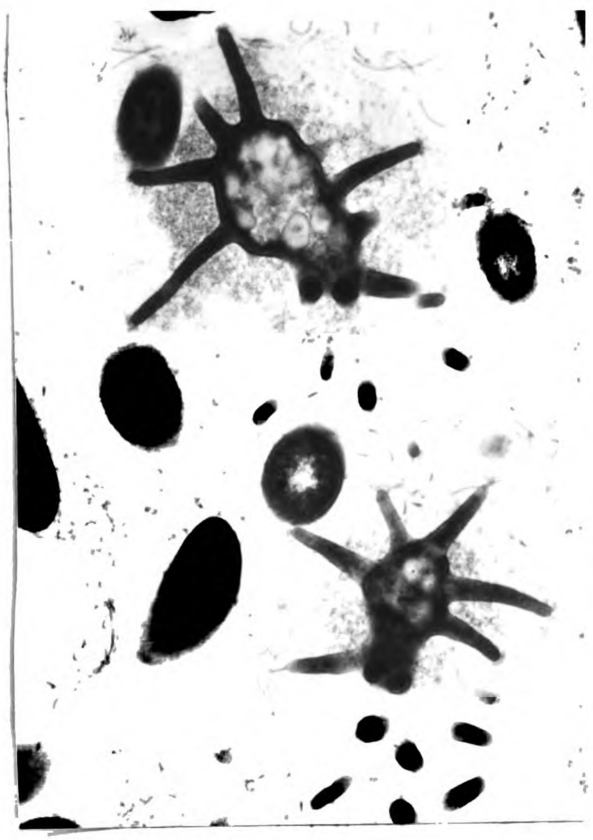
Figure 6. Prosthecate bacteria from Tipula.

Upper: Prosthecates in close proximity to the gut wall. 16,800X

Lower left: 24,800X

Lower right: 42,750X





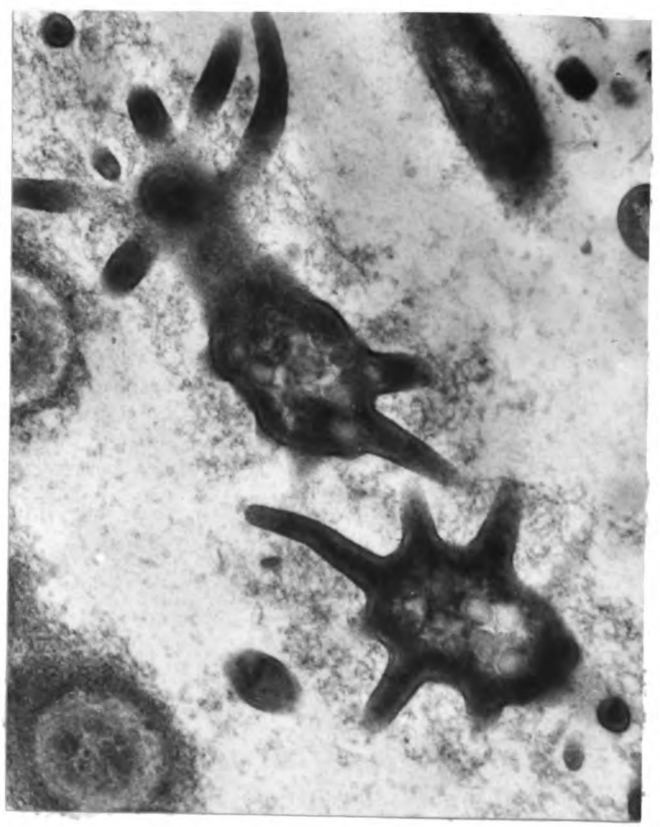
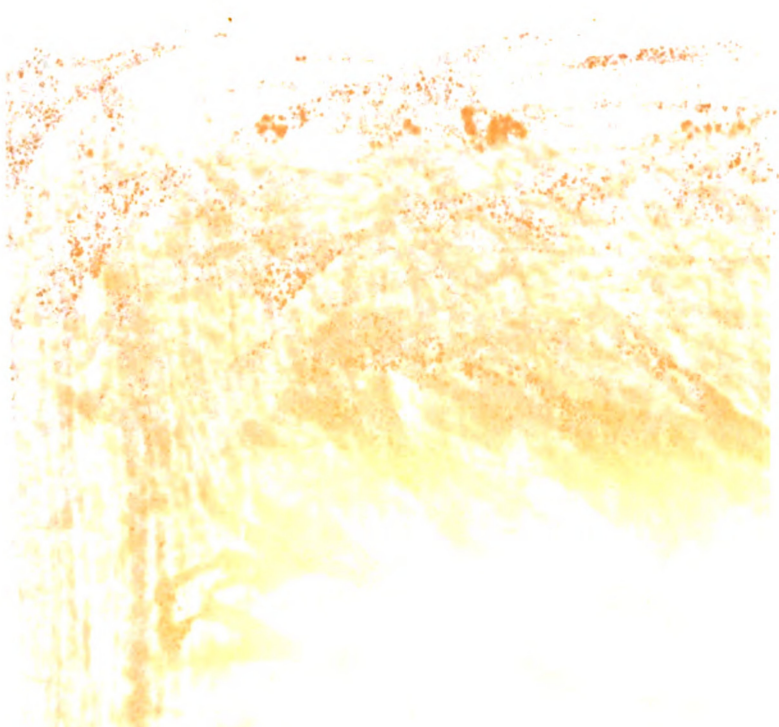


Figure 6.



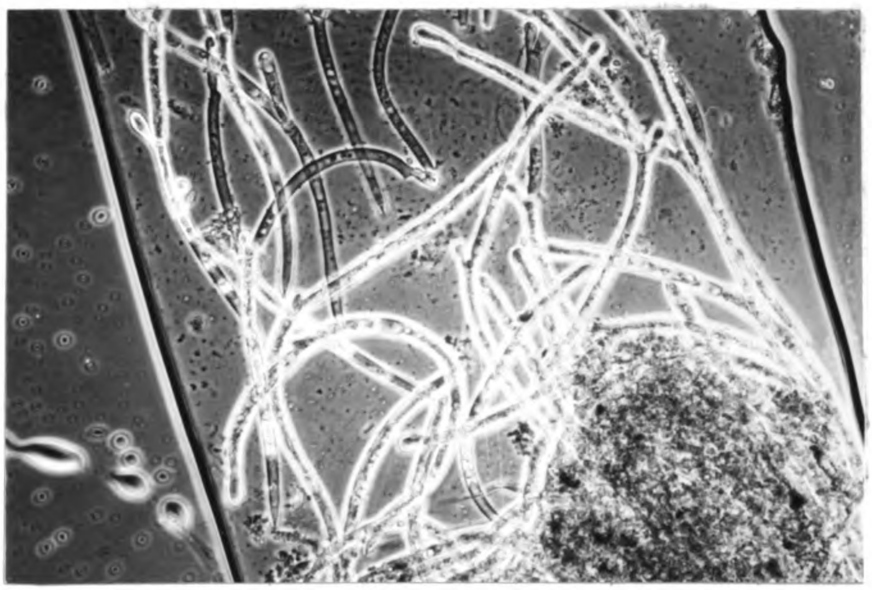


Figure 7. Trichomycete hyphae with immature spores, bacteria and detritus visible through the intact midgut wall of the blackfly Prosimulium.

Table 2. Gut dimensions and Petroff-Hauser counts of bacteria in selected invertebrates

Animal	Location	No. of Animals*	Gut Volume (ml)	No. Bacteria/ Gut	No. Bacteria/ ml of Gut Contents
Tipula abdominalis	ileum to anus	1	9.8×10^{-2}	1.7×10^{8}	1.7×10^{9}
	(posterior	1	1.0×10^{-1}	1.4×10^{8}	1.4×10^{9}
	hindgut)	1	4.0×10^{-1}	8.0×10^{9}	2.0×10^{10}
		1	1.9×10^{-1}	1.0×10^{8}	5.4×10^{9}
		1	3.5×10^{-1}	4.7×10^{9}	1.4×10^{10}
		1	3.4×10^{-1}	1.2×10^{9}	2.8×10^{10}
	pylorus	1	9.4×10^{-3}	3.5×10^{6}	3.7×10^{8}
		1	6.3×10^{-3}	1.5×10^{5}	2.4×10^{7}
	midgut	1	1.2×10^{-1}	1.7×10^{6}	1.4×10^{7}
		1	1.4×10^{-1}	4.5×10^{5}	3.2×10^{6}
Pycnopsyche guttifer	hindgut	1	8.2×10^{-3}	8.6×10^{7}	1.0×10^{10}
		1	1.2×10^{-2}	9.2×10^{7}	7.8×10^{9}
		1	1.6×10^{-2}	8.0×10^{7}	5.0×10^{9}
		1	1.1×10^{-2}	9.0×10^{7}	8.2×10^{9}
		1	1.2×10^{-2}	1.2×10^{8}	1.0×10^{10}
	midgut	1	3.5×10^{-2}	7.3×10^{5}	2.1×10^{7}
		1	5.7×10^{-2}	9.0×10^{5}	1.6×10^{7}
Lepidostoma costalis	hindgut	5	3.7×10^{-4}	1.5×10^{5}	4.8×10^{7}
	midgut	5	1.6×10^{-3}	4.6×10^{4}	2.8×10^{7}

* larger guts were counted individually, smaller guts were pooled and counted
gut volume is the average of separate measurements, numbers/gut is the average of the pooled determination

Table 2 (cont'd)

Animal	Location	No. of Animals*	Gut Volume(ml)	No. Bacteria/ Gut	No. Bacteria/ ml of Gut Contents
Pteronarcys pictetti	hindgut	1	5.7×10^{-3}	3.7×10^{5}	6.5×10^{7}
	midgut	1	4.1×10^{-2}	1.5×10^{5}	3.6×10^{6}
Gammarus pseudolimnaeus	total gut tract	3	1.0×10^{-3}	3.8×10^{4}	3.8×10^{7}
Brillia sp.	hindgut	9	5.5×10^{-5}	1.4×10^{4}	2.5×10^{8}
	midgut--bacteria clump	9	8.3×10^{-5}	7.2×10^{5}	8.7×10^{9}
	midgut--anterior	9	7.0×10^{-4}	2.4×10^{5}	3.4×10^{9}
	total midgut	9	7.8×10^{-4}	9.6×10^{3}	1.2×10^{7}
Hydropsyche bronta	hindgut	6	4.8×10^{-4}	1.5×10^{7}	3.1×10^{10}
	midgut	6	1.3×10^{-3}	2.5×10^{4}	1.9×10^{7}
Hexagenia limbata	hindgut	4	9.6×10^{-4}	1.7×10^{6}	1.8×10^{9}
	midgut	4	2.1×10^{-2}	3.5×10^{5}	1.7×10^{7}
Brachycentris occidentalis	hindgut	8	5.1×10^{-5}	1.9×10^{4}	3.6×10^{7}
	midgut	8	3.2×10^{-4}	8.3×10^{3}	2.6×10^{7}
Stictochironomos annulicrus	hindgut	10	1.8×10^{-5}	2.8×10^{4}	1.5×10^{9}
		7	6.8×10^{-5}	1.8×10^{6}	2.7×10^{10}
	midgut	10	1.9×10^{-4}	2.5×10^{5}	1.3×10^{9}
		7	7.8×10^{-4}	1.3×10^{6}	1.6×10^{9}

* larger guts were counted individually, smaller guts were pooled and counted
gut volume is the average of separate measurements, numbers/gut is the average of the pooled determination

Table 2 (cont'd)

Animal	Location	No. of Animals*	Gut Volume	No. Bacteria/ Gut	No. Bacteria/ ml of Gut Contents
Glossosoma nigrior	hindgut	7	1.4×10^{-4}	1.4×10^{4}	1.0×10^{8}
	midgut	7	2.4×10^{-4}	1.6×10^{4}	6.9×10^{7}
Nigronia serricornis	hindgut	1	1.6×10^{-2}	1.8×10^{8}	1.1×10^{10}
	midgut	1	2.1×10^{-3}	1.5×10^{5}	7.1×10^{7}
Corydalus sp.	hindgut	1	7.1×10^{-2}	3.2×10^{8}	4.5×10^{9}
		1	7.6×10^{-2}	5.0×10^{8}	6.6×10^{9}

* larger guts were counted individually, smaller guts were pooled and counted
gut volume is the average of separate measurements, numbers/gut is the average of the pooled determination

Table 3. Morphologies of bacteria observed in Petroff-Hauser counts

Animal	Location	No. of Animals*	% Rods	% Rods With Spores	% Filaments	% Spiral-Shaped Organisms
Tipula abdominalis	ileum to anus	1	79	16	2	3
	(posterior	1	90	7.4	1.3	1.3
	hindgut)	1	89	8.3	1.5	1.2
		1	86	9	1.6	3.4
		1	94	4		2
		1	88	10		2
	pylorus	1	100			
		1	99	1		
	midgut	1	100			
		1	100			
Pycnopsyche guttifer	hindgut	1	100			
		1	99.6	0.4		
		1	95	2.4	2	0.6
		1	98.6	1.4	0.7	0.3
		1	96.5	2.3	0.6	0.6
	midgut	1	100			
		1	100			
Lepidostoma costalis	hindgut	5	100			
	midgut	5	100			
Pteronarcys pictetii	hindgut	1	100			
	midgut	1	100			

* larger guts were counted individually, smaller guts were pooled and counted

Table 3 (cont'd)

Animal	Location	No. of Animals	% Rods	% Rods With Spores	% Filaments	% Spiral-Shaped Organisms
Gammarus pseudolimnaeus	total gut tract	3	100			
Brillia sp.	hindgut	9	100			
	midgut--bacteria clump	9	39.4	60.6		
	anterior midgut	9	74	23		3
	total midgut	9	47.9	51.4		0.7
Hydropsyche bronta	hindgut	6	92	0.3	1.4	6.3
	midgut	6	100			
Hexagenia limbata	hindgut	4	100			
	midgut	4	100			
Brachycentris occidentalis	hindgut	8	100			
	midgut	8	100			
Stictochironomus annulicrus	hindgut	10	97	3		
		7	99	1		
	midgut	10	86	7		7
		7	90	3		7
Glossosoma nigrior	hindgut	7	100			
	midgut	7	100			
Nigronia serricornis	hindgut	1	95	2		3
	midgut	1	100			
Corydalus sp.	hindgut	1	95.1	0.9		4
		1	96.6		0.7	2.7

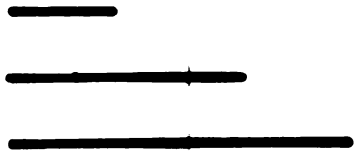
the insects which were examined with the Petroff-Hauser counting procedure, the numbers of bacteria computed, and percentages of different morphologies observed.

A summary of the feeding categories of the invertebrates examined and the gut microbiota observed is presented in Table 4. The shredder feeding category was subdivided on the basis of the observed or presumed feeding habits of the invertebrates (as explained in the "Feeding categories" section). The obligate shredders possessed an abundant microbiota in the lumen (10^9-10^{10}/ml of hindgut lumen) and associated with the hindgut wall, however lacked a significant population in the midgut (10^5-10^7/ml of midgut lumen). Facultative shredders and grazers had sparse populations of bacteria in both the midgut and hindgut (10^5-10^7/ml). More diversity in localization and types of associated microbiota was exhibited in the collector category. Some members (Hydropsyche, Stictochironomos, Hexagenia, and Stenonema) possessed a hindgut population similiar to the hindgut biota in the obligate shredders. The caddisfly Brachycentris and the blackfly Prosimulium possessed a less dense (10^8/ml) and scant (10^5-10^7/ml) hindgut population respectively. The blackfly and a midge, Stictochironomos, possessed a midgut biota that included spiral-shaped organisms and trichomycetes in addition to the rod-shaped bacteria commonly observed in the hindguts of the obligate shredders and collectors. The mayfly Leptophlebia possessed the usual rod-shaped and filamentous bacteria and was the only insect observed to harbor a hindgut population of trichomycetes. Of the two wood borers examined, the wood tunneling midge Brillia possessed a dense midgut population including spiral-shaped organisms and the localized rods (10^9-10^{10}/ml) and a less dense hindgut population (10^8/ml). The

remaining wood borer, Lara, contained a meager population in both midgut and hindgut. Of the predators, the megalopterans possessed a dense hindgut lumen population (10^{9}-10^{10}/ml) and a scarce midgut population (10^{5}-10^{7}/ml). The stonefly Paragnetina contained sparce populations in both midgut and hindgut (10^{5}-10^{7}/ml).

Table 4. Feeding categories and gut microbiota of invertebrates

Animal	Hindgut Wall	Hindgut Lumen	Midgut Lumen
OBLIGATE SHREDDERS			
Tipula abdominalis	*-F	S	
Pycnopsyche guttifer	*-F	S	
Hydatophylax hesperus	*-F		
Hydatophylax argus	*-F		
Platycentropus radiata	*-F		
Lepidostoma costalis	*		
FACULTATIVE SHREDDERS			
Pteronarcys pictetii			
Pteronarcys sp.			
Taeniopteryx parvula			
Brillia flavifrons			



* Attached biota
F Bacterial filaments
S Spiral-shaped organisms
T Trichomycetes
a lacked defined midgut and hindgut

10^{5}-10^{7} bacteria/ml of gut
10^{8} bacteria/ml of gut
10^{9}-10^{10} bacteria/ml of gut

Table 4 (cont'd)

Facu Animal	Hindgut Wall	Hindgut Lumen	Midgut Lumen
FACULTATIVE SHREDDERS (cont'd)			
Heteroplectron sp.		▬▬	▬▬
Gammarus pseudolimnaeus		▬▬ a	▬▬ a
WOOD BORERS			
Lara sp.		▬▬	▬▬
Brillia sp.	*	▬▬▬	▬▬▬ S
COLLECTORS			
Hydropsyche bronta	*-F	▬▬▬ S	▬▬
Brachycentrus occidentalis		▬▬▬	▬▬
Prosimulium sp.		▬▬	▬▬▬ S,T
Stictochironomus annulicrus	*-F	▬▬▬	▬▬▬ S,T
Hexagenia limbata	*-F	▬▬▬	▬▬
Stenonema spp.	*-F	▬▬▬	▬▬
Leptophlebia nebulosa	*-F,T	▬▬▬	▬▬

Table 4 (cont'd)

Animal	Hindgut Wall	Hindgut Lumen	Midgut Lumen
GRAZERS			
Glossosoma nigrior			
Goniobassis sp.		a	a
PREDATORS			
Paragnetina sp.			
Nigronia serricornis	*-F	S	
Corydalus sp.	*-F	S	

* Attached biota
F Bacterial filaments
S Spiral-shaped organisms
T Trichomycetes
a lacked defined midgut and hindgut

10^{5}-10^{7} bacteria/ml of gut

10^{8} bacteria/ml of gut

10^{9}-10^{10} bacteria/ml of gut

DISCUSSION

Gut Morphology

Ross (1965) has organized the insects into an evolutionary scheme based upon morphology of larval and adult stages of modern and fossil forms. The listing in Table 1 follows Ross' scheme, i.e. the order of increasing morphological complexity and more recent evolution is Ephemeroptera, Plecoptera, Coleoptera, Megaloptera, Diptera, Trichoptera. Ross (1956) has also pointed out that building an evolutionary scheme on the basis of either the larval or adult stages alone without consulting the other is incorrect since it is possible that one stage evolved more rapidly than the other in a particular group of insects. Either the larval or adult stage could have retained primitive characteristics while selection pressures for increasing complexity acted upon the other stage. The fact that so many of the larvae examined possessed a simple gut morphology, regardless of their position in Ross' family tree suggests that this process of differential evolution of larval and adult stages may have been or be operative.

Prior to initiation of this study it was considered that gut morphology, phylogeny, gut microbiota and feeding habit of the insects examined might correlate. For example, the cranefly possessed a morphologically complex gut. The fermentation chamber apparently served as an adaptation for maintenance of a microflora which aided in the nutrition of the harboring insect. Insects ingesting a more nutritious diet

would derive all the necessary nutrients from the food and therefore, would not have evolved a morphological adaptation containing a microbiota. The results of the study, however, do not support this notion since animals with simple guts possess microbiota as well as those with more complex gut morphologies.

Similarly, gut morphology correlates poorly with the phylogeny of the insects examined since as many examples of non-correlation can be cited as examples of correlation. That morphology of larval stages of the insects examined does not strictly follow phylogenetic lines is exemplified by the dipterans. Of the five dipterans only the cranefly and blackfly exhibited a morphologically complex gut. Among the trichopterans, the most recently evolved order of insects, the only genus to possess a somewhat complex gut was Hydropsyche; the remaining eight trichopterans had simple gut morphologies. A member of the fairly primitive plecopterans, Paragnetina, possessed a complex gut with gastric cecae and a proventriculus, while three other stoneflies examined possessed a simple gut morphology. Examples of similarities of gut morphologies among members of a taxonomic group include the mayflies, midges, and trichopterans other than Hydropsyche.

Correlation of gut morphology of insects with feeding category may, however, be a useful correlation in some cases. The predators examined possessed a more complex gut morphology than did shredders, collectors, and grazers, which generally had morphologically simple guts. Possibly the proventriculus is useful (or was useful at some time in the course of evolution) as an adaptation to a predator for grinding the chitinized portions of the prey.

No correlation exists between gut morphology and presence of gut microbiota. Microbiota was found in the midgut or hindgut of insects

with simple or more complex guts. A fermentation chamber or other morphologic adaptation is not a prerequisite for possession of gut microbiota among aquatic insect larvae.

The most striking correlation to be made is between feeding category and gut microbiota. With some exceptions, insect larvae that consume detrital material possess a microbiota while larvae living on more nutritious diets have sparse microbial populations in their guts.

Gut Microbiota

Numbers of bacteria in the lumen as determined by the Petroff-Hauser method are subject to error, particularly at 10^7 or less. A considerable amount of small debris was encountered in some of the samples and some difficulty resulted in differentiating the bacteria from debris. This could account for the seemingly high counts in areas of guts thought to contain low bacterial numbers prior to counting in the chamber. Mistaking a detrital particle for a bacterium in samples where the bacteria were sparse would result in a more inflated figure than the same error in a dense population. One organism in 25 fields (1/20,000 mm^3 = volume of Petroff-Hauser) was equivalent to 5×10^4 cells/ml, so a 10^7 figure was based on only a few bacteria/field. The 10^7 figure for sparse populations may be less in reality and the 10^9-10^{10} figures are probably more reliable estimates for dense lumen populations.

Another source of error in the Petroff-Hauser estimates is in the calculated gut volumes. Guts did not have a uniform diameter and a visual "average diameter" was chosen. The smaller the resulting radius, the smaller the gut volume and the larger the bacteria/ml figure (Table 2). The discrepancies are greater in the smaller insects than the larger ones.

The numbers in Table 2 represent a conservative estimate of the populations present, since only the lumen population was counted. Organisms that remained attached were not counted, as evidenced by the fact that pieces of tissue from macerated preparations were occassionally examined and had as many bacteria adhering as were observed in the vortexed preparations. This is further indicated by the observation that characteristic attached filamentous bacteria were rarely seen in the macerated preparations (Table 3). The presence of filamentous bacteria in the macerated preparations is not thought to be due to the maceration since occassional filament fragments are observed in wet mounts of feces of the caddisfly Pycnopsyche and the cranefly Tipula. Filament fragments and spores apparently are broken from the wall and probably spend some time in the lumen prior to defecation by the insect.

Coprophilic feeding is presumably the mode of dispersing and transmitting bacteria among members of the insect populations. Coprophilic feeding is probably by chance among the obligate shredders since active feeding of feces to members of the population by other members has not been observed. Among the collectors coprophilic feeding is common due to the fact that many fine particles are derived from shredder and collector feces.

The caddisfly Lepidostoma initially was thought not to possess a wall-attached biota. Critical phase microscopy at 1600X revealed the anterior half of the rectum to be colonized with an apparent monoculture of rods. The possibility arises that some of the other small insects which are difficult to dissect and obtain muscle-free wall preparations may also possess a wall-attached biota. The wood boring midge Brillia and the caddisfly collector Brachycentris were reexamined with phase

microscopy, but no definite conclusion was reached in the case of Brachycentris. Rods were, however, found adhering to the rectum of the wood borer. Reexamination of other larvae (bark beetle and blackfly) by more critical microscopy is needed to ascertain whether an inconspicuous rectal wall population of rods is present.

Staley (1968) described a group of bacteria with appendages and proposed the term prosthecates. The term prostheca(e) he defined as:

> a semirigid appendage extending from a procaryotic cell with a diameter which is always smaller than that of the mature cell and which is bounded by a cell wall.

The cells in Figure 5 fulfill these requirements. However, they are not similar enough to any of Staley's photographs to warrant the statement that they are the same organisms. Staley's isolates were from freshwater and only one had the capacity to grow anaerobically. The anaerobic nature of the gut of the cranefly is suggested by a large percentage of obligate anaerobes (Klug, pers. comm.), thus the prosthecates observed would at least have to be facultative anaerobes.

In addition to prosthecates, electron microscopy of microbiota from other insects is likely to reveal other morphologically unique bacteria. A row of cuboidal bacteria adjacent to the hindgut wall of the caddisfly Pycnopsyche was noted in a preliminary examination.

The filamentous bacteria observed are similar to Breed's descriptions of two genera Arthromitis and Coleomitis, in the order Carophalales. The original description of these organisms was by Leidy in 1850 and they were observed in gut tracts of millepedes, termites, cockroaches and tadpoles. The Arthromitis group was deleted by Gibson and Gordon (1974) except for a brief paragraph under the heading "endospore forming bacteria of uncertain taxonomic position" in the introduction to the

Bacilliaceae. Attempts in the course of this study to isolate the filamentous bacteria using aerobic and anaerobic techniques on a variety of media have repeatedly failed. The end-on attachment of bacteria to the gut wall is not limited to the filaments and their host insects. End-on attachments have been observed in intestines of mice (Davis and Savage, 1974), rats (Wagner and Barrnett, 1974), and the hindgut of cockroaches (Fogelsong et al, 1975). In the murine systems the bacteria have been observed to adhere in indentations of the gastrointestinal wall. In the cranefly no indentation or crypt in the wall has been noted as the site of attachment of the bacteria, possibly due to the presence of the chitinous intima. The slightly electron-dense material surrounding the bacteria and apparently acting as a cement to hold the bacteria to the wall has been suggested to be a mucopolysaccharide. Efforts by Breznak and Pankratz (pers. comm.) using electron microscopy and ruthenium red, a stain specific for acid mucopolysaccharide, were inconclusive in investigations of the attached microbiota in termite paunches.

Lictwardt (1973) in a general description and key of the trichomycetes stated that they are widely distributed among marine, freshwater, and terrestrial arthropods and that the degree of colonization of a particular arthropod population varies from uninfected to completely infected. He reported that most species occur in the hindgut and a few in the midgut or foregut. That the trichomycete completes its life cycle within the gut is evidenced by the presence of asexual and sexual reproductive stages as well as vegetative hyphae in both the guts and feces of those insects possessing a trichomycete population. Furthermore, the trichomycete completes its life cycle prior to pupation or emergence to adulthood as evidenced by the observation that mayflies were heavily colonized in the winter and poorly colonized in the spring

immediately prior to emergence. Apparently, the timing of the two life cycles (trichomycete and insect) is well synchronized. However, potential microbe-arthropod interactions and possible symbiotic relationships between arthropods and trichomycetes are unknown. Species of only two genera of trichomycetes have been cultured and were found similar to typical saprobic fungi in their nutritional requirements (Lichtwardt, 1973). Differences between trichomycete-colonized and non-colonized members of an arthropod population have, apparently, not been observed.

The potential anaerobic conditions in insect guts arouse speculation as to the metabolic nature of these fungi. They produce a holdfast that attaches to the peritrophic membrane (Lichtwardt, 1973) and may derive their oxygen from the insect. Alternatively, the guts may be microaerophilic and the fungi are able to derive sufficient oxygen from their surroundings, or they may be obligate anaerobes. Incubations under anaerobic, microaerophilic, and aerobic conditions using complex media plus antibacterial antibiotics have failed to isolate these organisms.

Coprophilic feeding, again, is likely to be the mode of transmission from one individual to another in a population as evidenced by the presence of trichomycete reproductive structures in the feces and the detrital nature of the food consumed by the collectors.

Feeding Categories

Cummins (1973) has noted that generalizations of aquatic insect feeding habits are usually based on incomplete studies of a few representatives of a genus, family, or order and extrapolations are made to the remaining members of the taxonomic category. Cummins (1973) also cites examples of differences in feeding habits of geographically

separated members of the same species. The listing in Table 4 is a compilation of information on feeding behavior in relationship with the microbiota data. Categorization of the feeding behavior of the insects examined from Augusta Creek was accomplished through consultation with Dr. Cummins. Dr. Sedell was consulted concerning feeding behavior of the insects from Oregon.

The obligate leaf shredders, facultative leaf shredders and wood borers are subdivisions of the shredder category. Obligate shredders are those observed and collected in natural accumulations of leaf detritus, but seldom found elsewhere. The cranefly Tipula and the caddisfly Pycnopsyche from Augusta Creek were shown to shred leaves and grow as a result of their shredding activities (Cummins et al, 1973). Although feeding experiments have not been done with the limniphilids Hydatophylax and Platycentropus they are found in the same habitats as Pycnopsyche and the general mouthpart and gut morphologies are fairly similar, with the likeness between Pycnopsyche and Hydatophylax particularly striking. The caddisfly Lepidostoma is found only at the upstream sites of the Augusta Creek watershed and is credited with the faster processing of leaf material at these sites than at comparable downstream sites. This is particularly evident in experimental leaf packs placed in the stream in summer after Pycnopsyche has ceased to feed and Tipula is nearing pupation (Cummins, pers. comm). The obligate leaf shredders are considered to consume leaf material deriving their nutrients from this source only. Among the obligate shredders examined the correlation between the presence of gut microbiota, both lumen and wall associated, and shredder feeding behavior of the insect is good (Table 4).

Facultative shredders are possibly more omnivorous in their feeding habits than obligate shredders and wood borers. At this time considerable uncertainty exists concerning the feeding habits of the invertebrates placed in this group. The caddisfly Heteroplectron consturcts its case by hollowing out a twig. Presumably an animal capable of tearing wood would be capable of consuming and utilizing the wood. Sedell (pers. comm.) however, has suggested that possibly this insect larva derives a majority of its nutrition from algae it scrapes from the wood. The gut analysis agrees with this suggestion since quantities of algae were observed during the microscopical examination of the gut. The midge Brillia flavifrons is considered to be a collector on the basis of the collecting activities of its near relatives, but has been shown by R. King (Kellogg Biological Station, pers. comm.) to shred hickory leaves and grow on this substrate. Pteronarcys has been shown to be capable of shredding leaves (McDiffett, 1970; Cummins et al, 1973). However, this animal may also be consuming other substrates. The fact that Pteronarcys pictetti died within a week in the experiments of Cummins et al and that Pteronarcys scotti in McDiffett's experiments were shredding more leaf material than they consumed suggests that this substrate may not be optimal for this animal. Taeniopteryx in Augusta Creek is considered to live in the same manner as Pteronarcys (Cummins, pers. comm.) The crustacean Gammarus has been used in feeding preference experiments and shown to shred leaves (Bärlocher and Kendrick, 1973a, 1973b, 1975). The normal diet, however, may be considerably more diverse since sideswimmers are not only collected in leaf packs, but in quiet pools and among macrophytes where their food would be quite different. Prior to the dissections in the present study the amphipods had been

in chambers containing leaves for up to two weeks. However, the majority of the gut contents were amorphous unidentified particles with an occassional seta rather than the leaf-derived particles as observed in the obligate shredders.

Among the facultative shredders a gut microbiota was not observed. In addition to further information concerning feeding habits of these arthropods enzymatic studies of the digestive capabilities of midguts and hindguts may yield useful information.

Wood borers are a unique and rare shredder specializing on wood as a substrate. The wood boring midge Brillia sp. burrows as much as two centimeters into waterlogged wood of a particular degree of decomposition. Little is known about the ecology of this group; appropriate logs are not readily found and considerable time and effort is required to remove them from their substrate without damaging the comparatively fragile larvae. Carpenter and Culliney (1975) have shown that marine shipworms, a group of wood boring bivalve mollusks which grow on a cellulose substrate possess a spirilla which, when cultured anaerobically, fixes nitrogen. Nitrogen fixation was not observed in an experiment with Brillia and Stictochironomos. However, nitrogen fixation was observed associated with the wood substrate from which the Brillia were collected. Presumably animals ingesting this material would derive nitrogen from microbes associated with the decaying wood. The Oregon bark beetle Lara was observed to scour the bark of sticks from Augusta Creek and in some cases to chew a few milimeters into the xylem. However, no tunneling activity was noted. The midge possessed a dense population in the midgut and a population in the hindgut, while sparse populations were observed in the elmid beetle (Tables 2, 3, 4).

Collectors are divided into two subgroups, gatherers and filterers, based on behavior and method of collecting. The gatherers inhabit areas rich in small particles. The mayfly Hexagenia and the midge Stictochironomos live in the top few centimeters of organic-rich mud deposited in quiet pools along the sides of the stream. The mayflies Leptophlebia and Stenonema gather their food from surfaces of submerged debris. Feeding experiments with Stenonema (Cummins et al, 1973) have shown that growth rates of these mayflies were faster when they were accompanied by a shredder or in crowded conditions, where more feces (small particles) were available. Growth was poorest in chambers where a few Stenonema were required to consume colonized leaf material (CPOM) or wait for the microbes to produce FPOM. The filter feeders, the blackfly Prosimulium and the caddisflies Hydropsyche and Brachycentris filter water through their mouthparts or a net they spin attached to rocks. Extensive work by Chance (1970) on mouthpart morphology and filtering capabilities of blackflies has shown that blackflies collect and ingest particles ranging from 0.5 to 300 μm by 0.5 to 120 μm. Coffman (1967) has stated that in Linesville Creek (western Pennsylvania) the net-spinning caddisfly Hydropsyche bronta is a predator. As noted in Table 1 it possesses a proventriculus which is also found in the predacious megalopterans and Paragnetina. However, in Augusta Creek this hydropsychid more commonly uses its net to filter particles from the stream rather than capture prey, though presumably it retains the capacity to consume prey as evidenced by the proventriculus.

Members of the collector category possess a microbiota, though the biota is not necessarily limited to the hindgut as in the obligate shredders. Within this group the greatest diversity in the localization

of the microflora exists with two of the seven collectors examined possessing significant midgut populations. Presumably their physiology would differ somewhat from those with a hindgut biota. The lower number of bacteria observed associated with ingested particles in the posterior midgut of the cranefly suggests that the animal is capable of lysing the ingested bacteria (Klug, pers. comm.) Conditions must be markedly different in the midgut of the two collectors and wood boring midge where the midgut contains a dense biota.

The grazers Glossosoma and Goniobassis possess mouthparts adapted for scraping algae from surfaces and in the process will also scrape other material. Amorphous detrital particles were observed in their guts as well as algae. A dense biota was not observed, which is considered to be due to the nutritious characteristics of their food.

Predacious characteristics of the megalopterans are well documented (Coffman, 1967; Peterson, 1974). Foreguts of the individuals examined were filled with dark brown fluid and midguts contained lipid. A dense biota (10^{9}-10^{10} cells/ml of gut) was observed in the hindguts of the megalopterans. In only two of ten individuals were head capsules or setae of prey observed. This lack of prey parts can be explained by Peterson's (1974) observations of Nigronia consuming its prey from the middle or posterior and not ingesting anal claws and head capsules of the prey. The suggestion has been made that some predators do not ingest the entire prey, but only suck out the body juices and gut contents of the prey (King and Cummins, pers. comm.) Some predacious insects are known to secrete proteases into the body of their prey and ingest the resulting suspension while clinging to the prey (Wigglesworth, 1974). The enzymes are sufficiently active that in a few minutes the

predator is able to discard the empty exoskeleton of the prey. In this way the predator benefits from the structural components of the prey and not just the gut contents and hemolymph of the prey. Midguts of insects hydrolyze and absorb lipids (Wigglesworth, 1974). The foreguts of the megalopterans are considerably larger than the foreguts of the other insects examined and were filled with brown watery fluid. This fact coupled with the observations of Peterson (1974) and Wigglesworth (1974) suggest that possibly the prey is digested in the foregut. Any chitinous components of the food would be macerated by the proventriculus prior to entering the midgut (though the majority of the chitin would not be ingested). Lipids would pass through the foregut, and undergo hydrolysis and absorption in the midgut. While these observations and conjectures concerning behavior and physiology explain the lack of prey parts in the megalopteran alimentary tracts they do not provide any information concerning the microbial population in the hindgut.

The Paragnetina observed contained partially decomposed prey in the foregut. The midgut, particularly the gastric cecae contained lipid. The ventriculus of the midgut contained insect parts, unidentified amorphous particles and diatom frustules. The pylorus contained a fairly solid bolus consisting of diatom frustules and unidentified particles. The rectum was either empty or contained more of the diatom frustules and other particles. The detritus was presumably derived from the guts or body surfaces of the prey.

The two megalopterans were the most notable exceptions to the hypothesis of feeding behavior being correlated with presence or absence of gut microbiota. Since the diets of these larvae are known to be nutritious and fairly low in fiber a dense gut microbiota was not

expected. The observance of a sparse microbiota was expected for the stonefly Paragnetina since it was known to prey on other insects.

Possible Microbe-Insect Interactions

Photomicrographs of microbiota from representative insects, the cranefly Tipula and the blackfly Prosimulium, have been presented together with assessments of the numbers of bacteria present in the lumens of various invertebrate guts. Results of the study indicate that a gut microbiota is fairly common among aquatic insect larvae and that possession of a microbiota is correlated, to some extent, with the feeding behavior of the insect. The role of the microbiota in the nutrition of the insect was, however, not examined. Several symbiotic relationships between bacterial and insect cells are possible: 1) the insect host depends upon the biota for the production of enzymes necessary to digest complex components of the food to simpler entities the insect enzymes can attack; 2) the insect absorbs products produced by the biota such as volatile fatty acids, vitamins, amino acids, or "growth factors"; 3) the biota plays a role in the internal recycling of nutrients in the insect that would otherwise be lost with the feces or urine of the host; 4) the bacterial population does not play a role in the nutrition of the host.

Prior to discussion of these possibilities information concerning the physiology of digestion in insects is presented. The diversity among insects makes generalizations regarding physiology of insectan digestion unreliable and exceptions can be cited for practically all of the ensuing statements. However, since the paucity of information concerning the physiology of the particular insect larvae studied is probably greater than that concerning their ecology, generalizations from other insects will be used in this discussion. Midguts of insects are thought to be the primary sites of both secretion of enzymes necessary for digestion

and absorption of the products of digestion, although some enzymes are secreted by the foregut or salivary glands. The hindgut functions in the absorption of water and ions (Wigglesworth, 1974).

Several physiological studies (Stobbart, 1968; Wall et al, 1970; Irvine and Phillips, 1971) agree that the hindgut of insects is involved in absorption and regulation of water and ions such as sodium, potassium, and magnesium. That the hindgut of the blowfly serves in an absorptive capacity was demonstrated by ultrastructural studies of blowfly rectal papillae (Gupta and Berridge, 1966) which reveal a tissue similiar to human kidney tissue (Sandborn, 1972) and mosquito anal papillae (Copeland, 1964), both of which are known for their absorptive and osmoregulatory functions. Thin sections of cranefly gut wall (Figure 4) bear a resemblance to these tissues. All four are characterized by parallel folds of the cell membrane extending into the interior of the cell. The insect tissues consist of a layer of cuticle, the parallel infoldings of the membrane, electron-transparent areas referred to as "canaliculi" (Copeland, 1964), numerous mitochondria, and a basement membrane. The physiological and ultrastructural evidence for the absorptive nature of these tissues is convincing, but whether the hindgut tissues of insects have the ability to absorb solutes in addition to ions has not been well documented.

For insects with a mirobiota present in the hindgut the capacity of the hindgut wall to absorb solutes in addition to ions is crucial to the first three possible symbioses stated above. Symbiosis 1) assumes an insect incapable of producing enzymes to digest its food and dependent upon a microbiota in the hindgut to carry out this function. In this case it is mandatory that the hindgut wall absorb the metabolites produced by the biota since transfer of materials from the hindgut to

midgut is not thought to occur. An insect living through symbiosis 1) would most likely house its symbionts in a mycetome connected to the anterior portion of the midgut so that the microbial enzymes could be deposited where they would be most useful and the biota would be protected from the host's enzymes.

Symbiosis 2) also requires that the gut wall be capable of absorbing more than ions and water. Examples of insects deriving vitamins or amino acids occur in insects with a midgut or mycetome biota. Rhodnius prolixus, a blood sucking hemipteran maintains a culture of Nocardia rhodnii in crypts in the midgut and the midgut presumably absorbs the necessary B vitamins the actinomycete supplies (Baines, 1956; Lake and Friend, 1968). Absoprtion of the microbially synthesized amino acids by the gut tissue in the aphid case was unnecessary since aphids possess a mycetome and the amino acids produced would presumably be secreted into the hemolymph. Thus a symbiosis in which the host derived essential amino acids from the mycetome biota was demonstrated by Mittler (1971) without the necessity for showing absorption of amino acids by gut tissue.

Absorption by hindgut tissue of vitamins, amino acids, or volatile fatty acids produced by the hindgut microbiota is an attractive hypothesis. The copius amounts of volatile fatty acids produced by anaerobic bacteria represent potential sources of energy and carbon to the insect. Alternatively, amino acids produced by the biota could be absorbed by the rectum. Ramsey (1958) suggested that amino acids and sugars were deposited in the hindgut via the Malpighian tubules and subsequently reabsorbed by the rectum. Berridge (1970) has proposed a passive transfer of these solutes based on the ultrastructural characteristics of certain rectal tissues. Apparently little has been done to test these proposals, however.

In vitro experiments by Balshin and Phillips (1971) have shown uptake of ^{14}C-glycine by desert locust rectum and they concluded that the mechanism is via active transport. Glycine is the smallest amino acid and probably more readily absorbed by the rectal tissue than the larger amino acids. In addition, it is non-essential and the insect is capable of producing it. A symbiosis based upon the production of essential amino acids by a microbiota in the hindgut would require that these essential amino acids be absorbed by the hindgut wall of the insect.

Symbiosis 3) also requires that the hindgut absorb more than ions and water in cases where compounds containing carbon or nitrogen would be derived from undigested food and waste products of the Malpighian tubules. These materials represent carbon and nitrogen which are unavailable to the insect due to the nature of their chemical bonds. Alteration of the chemical bonds and conversion to useful compounds could be accomplished by two methods: a) the microbes degrade uric acid or urea to ammonia which is absorbed by the hindgut epithelium and subsequently converted to nitrogenous compounds by the insect; or b) the microbes may be responsible for converting the ammonia to carbon-containing nitrogenous compounds which are absorbed by the hindgut wall. The majority of the urine produced by insects is in the form of uric acid (Wigglesworth, 1974). A bacterial population with the necessary enzymes to degrade uric acid through three intermediates (Wigglesworth, 1974) to ammonia could be important to an insect eating a nitrogen-poor diet. This assumes that the insect has the capacity to absorb ammonia and that ammonia is either non-toxic to the absorbing epithelium, or is quickly converted to non-toxic compounds. Alternatively, an aquatic insect may secrete its urine as ammonia, which animals living in water have been

observed to do (Lehninger, 1970). In this case the ammonia would be delivered to the hindgut via the Malpighian tubules and what was needed would be reabsorbed and converted to a less toxic form by the gut wall. However, if the insect gut wall lacked the capability to absorb ammonia and convert it to less toxic forms the microbiota would be important in converting ammonia to other nitrogenous compounds which could be safely absorbed by the gut wall. By either of these methods carbon, and probably more crucially, nitrogen would be salvaged from materials destined for execretion. Testing these conjectures would require information on the chemical composition of the urine of the aquatic insect larvae examined and the enzymatic capabilities of the gut walls.

The first three interactions suggested above represent mutualistic (beneficial and obligatory to both symbionts) or protocooperative (beneficial, but not obligatory) situations. Symbiosis 4) states that the biota does not play a role in the nutrition of the insect host. Symbiosis 4) represents a neutralistic (no benefit to either host or biota), commensalistic (flora benefitted and host not affected), or possibly parasitic interaction. If symbiosis 4) is operative the situation is presumably commensalistic since the bacteria are provided with a favorable habitat: substrates and anaerobic conditions. The parasitic situation seems unlikely since apparently all the members of the insect populations examined are infected and evidently they are not harmed either as individuals or as populations. If a neutralistic interaction is occuring the possibility of the microbiota playing a role in the conditioning of feces for collector consumption must be considered, as discussed in the "Introduction."

The correlations observed in this study between feeding behavior of aquatic insect larvae and presence or absence of gut microbiota have lead to the above suggested symbioses. Insects that depend upon detrital material for energy and carbon (obligate shredders and collectors) possess a gut microbiota. Insects living on less refractile substrates (grazers and predators) do not possess a microbiota with the exception of the megalopterans. The facultative shredders and the wood boring elmid beetle also represent exceptions to the correlation. However, with more information concerning their feeding behavior these apparent exceptions may be resolved. This observed correlation between feeding behavior and gut microbiota provides the basis and justification for further investigations concerning the symbiosis between the procaryotic and eucaryotic cells that comprise this system and their role in processing the allochthonous input in a stream ecosystem.

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