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1

Introduction

THE DIVERSITY OF INSECTS AND THEIR MICROBIAL SYMBIONTS

The insects are a supremely successful group of animals, particularly in terrestrial habitats. By the criteria of number of individuals, number of species, ecological importance, and functional diversity, the insects dwarf all other terrestrial animals.

The basis for this book is that associations with beneficial microorganisms make an important contribution to the success of the insects. For example, the role of the leafcutter ants as the dominant herbivores in many Neotropical grassland and forest habitats is founded on their cultivation of plant-degrading fungi in their nests; the obligate blood-feeding lifestyle of bedbugs, sucking lice, and tsetse flies is enabled by internal bacteria that produce vitamins in short supply in vertebrate blood; and many insects, including bumble bees and honey bees, harbor gut microorganisms that protect these insects from virulent pathogens.

In general terms, insect associations with beneficial microorganisms are not exceptional. Most animals host beneficial microorganisms, from which they derive nutrients, protection from natural enemies, or other services (McFall-Ngai et al., 2013). There are, however, two ways in which microbial associations in insects are special. The first is the remarkable diversity of form and function of associations in insects, as discussed in chapters 2–4. The second way relates to the significance of insects to humans. Some insects are pests and disease vectors of agricultural, medical, and veterinary importance, and others are valuable to humans, for example, as pollinators and biological control agents. As our knowledge of insect associations with microorganisms develops, it is becoming increasingly evident that this understanding can be harnessed for

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novel strategies to control insect pests (chapter 5) and as model systems in biomedical research (chapter 6).

The purpose of this chapter is to provide an overview of the insects and their microbial partners. Section 1.1 introduces the reader to the diversity of insects and microorganisms. Section 1.2 describes the essentials of insect form and function, with an emphasis on the insect structures that provide habitats for microorganisms and insect traits that facilitate and limit microbial colonization. (An extended consideration of insect structure and function is provided by Simpson and Douglas (2013).) In section 1.3, I consider the terminology used to describe interactions involving beneficial microorganisms. Finally, section 1.4 outlines the contents of this book.

1.1 Naming the partners

1.1.1 The insects

There is overwhelming molecular and morphological evidence that the insects are a monophyletic group, comprising some 28 orders grouped within five subclasses (table 1.1 and fig. 1.1A) (Misof et al., 2014). The common ancestor of insects was likely terrestrial, and the earliest fossil insects are from the early Devonian period (ca. 410 million years ago). The class Insecta, together with the classes Collembola (springtails), Protura (coneheads), and Diplura (two-pronged bristletails), comprise the subphylum Hexapoda, which is defined by the possession of three pairs of thoracic legs; insects differ from other hexapods in having external mouthparts. The sister group of the Hexapoda is an obscure group of marine Crustacea, the class Remipedia, with fewer than 30 known species that are apparently restricted to coastal aquifers. In other words, the insects have evolved from within the Crustacea (crabs, lobsters, shrimps, etc.).

Many aspects of the biology of insects are shaped by their body plan, comprising a segmented body with paired jointed appendages (fig. 1.1B), supported by a versatile exoskeleton. This body plan permits rapid and precisely controlled movement, which has facilitated the evolution of complex behavior. It is also well suited to small size, which is associated with high reproductive rates and specialization to a multitude of ecological niches unavailable to larger animals. In addition to these ancestral traits, two evolutionary innovations account for the success of the insects. The first innovation was the origin of flight early in the diversification of the group, with the oldest fossils of winged insects at 324 million years ago in the early Carboniferous era. All extant insects

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TABLE 1.1. Classification of insects

Insect order[1]	Trivial name	Estimated number of species	Habits	Examples of significance to humans as pests and beneficials
Subclass Apterygota[2]				
Archaeognatha	Jumping bristletails	5×10^2	Nocturnal insects in leaf litter and under stones	
Zygentoma	Silverfish and firebrats	6×10^2		
Subclass Palaeoptera				
Ephemeroptera	Mayflies	3×10^3	Aquatic nymphs and short-lived adults (often less than a day)	
Odonata	Dragonflies and damselflies	6×10^3	Aquatic nymphs; adults are aerial predators	
Subclass Polyneoptera				
Blattodea	Cockroaches and termites	7.5×10^3	Cockroaches are omnivores or saprophages in leaf litter and soil; termites are “social cockroaches” that feed on wood or soil	A few cockroach species are pests of human habitations; some termites are pests of wooden buildings and other infrastructure
Dermaptera	Earwigs	2×10^3	Mostly nocturnal scavengers or omnivores	
Embioptera	Webspinners	5×10^2	Gregarious scavengers, mostly tropical	
Grylloblattaria	Rock crawlers	32	Flightless insects restricted to high altitudes in Northern Hemisphere	
Mantodea	Mantises	2.5×10^3	Diurnal predators	
Mantophasmatodea	Heelwalkers	≤20	Flightless predators, only known in south and east Africa	

(*continued*)

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TABLE 1.1. (*continued*)

Insect order[1]	Trivial name	Estimated number of species	Habits	Examples of significance to humans as pests and beneficials
Orthoptera	Grasshoppers and crickets	2×10^4	Mostly diurnal herbivores (some species are partly or entirely carnivorous)	Gregarious grasshoppers (locusts) are major pests of crops and vegetation
Phasmatodea	Stick insects	3×10^3	Slow-moving herbivores, mostly nocturnal	
Plecoptera	Stoneflies	4×10^3	Aquatic nymphs are predatory or herbivorous; adults are short-lived and herbivorous or nonfeeding	
Zoraptera	Angel insects	≤50	Gregarious inhabitants of rotting wood, leaf litter, etc.; scavengers, especially on fungal hyphae	
Subclass Paraneoptera				
Hemiptera	**True bugs**	$\mathbf{1 \times 10^5}$	Mostly plant-feeding, especially on plant sap; some predators and blood-feeders	Major crop pests (aphids, whiteflies, planthoppers, etc.) and medical pests (bedbugs, kissing bugs)
Psocodea	Bark and true lice	1×10^4	Bark lice in soil, leaf litter, bird nests etc.; true lice are ectoparasites of birds and mammals	True lice are parasites of poultry, livestock and humans; include vectors of various disease agents
Thysanoptera	Thrips	6×10^3	Feed on fungi or plants	Few species are major horticultural pests
Subclass Endopterygota				
Coleoptera	**Beetles**	$\mathbf{3.9 \times 10^5}$	Terrestrial and aquatic; scavengers, predators, and herbivores	Major crop pests; also predators used in crop pest management

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Diptera	**Flies**	$\mathbf{1.6 \times 10^5}$	Larvae are terrestrial or aquatic; adults predominantly terrestrial. Very diverse habits: scavengers, herbivores, predators, parasites, and parasitoids.	Agricultural pests, e.g., tephritids, crane fly larvae; vectors of medically important disease agents, e.g., mosquitoes, sandflies; urban pests, e.g., house fly, blow fly. Beneficial pollinators and predators of agricultural pests, e.g., syrphids
Hymenoptera	**Ants, bees, and wasps**	$\mathbf{1.5 \times 10^5}$	Herbivorous, predatory, or parasitic, including many parasitoids; sociality evolved multiple times	Important pollinators; household pests
Lepidoptera	**Butterflies and moths**	$\mathbf{1.6 \times 10^5}$	Mostly herbivorous larvae (caterpillars) and nectar-feeding adults	Herbivorous larvae are major agricultural pests; many adults are important pollinators
Mecoptera	Scorpionflies	4×10^2	Generalist feeders in damp woodlands; few predators	
Megaloptera	Alderflies	4×10^2	Aquatic carnivorous larvae; nonfeeding adults	
Neuroptera	Lacewings	5.5×10^3	Mostly predators and crepuscular or nocturnal	
Raphidoptera	Snakeflies	2×10^2	Mostly predators; terrestrial in temperate woodland	
Siphanoptera	Fleas	2×10^3	Adults are wingless ectoparasites of mammals and occasionally birds (larvae feed on detritus in host nest or den)	Vectors of various disease agents
Strepsiptera	Stylops	6×10^2	Endoparasites of other insects	
Trichoptera	Caddisflies	1.5×10^4	Filter-feeding aquatic larvae; nocturnal adults feed on nectar or nonfeeding	

Source: The classification of Misof et al. (2014) is displayed here and used throughout this book.

[1] The relationships among most of the subclasses are resolved, but some phylogenetic problems remain. The five major orders (with >100,000 described species) are indicated in bold.

[2] Subclass Apterygota may be artificial, with evidence that Zygentoma is more closely related to other insects than to Archaeognatha.

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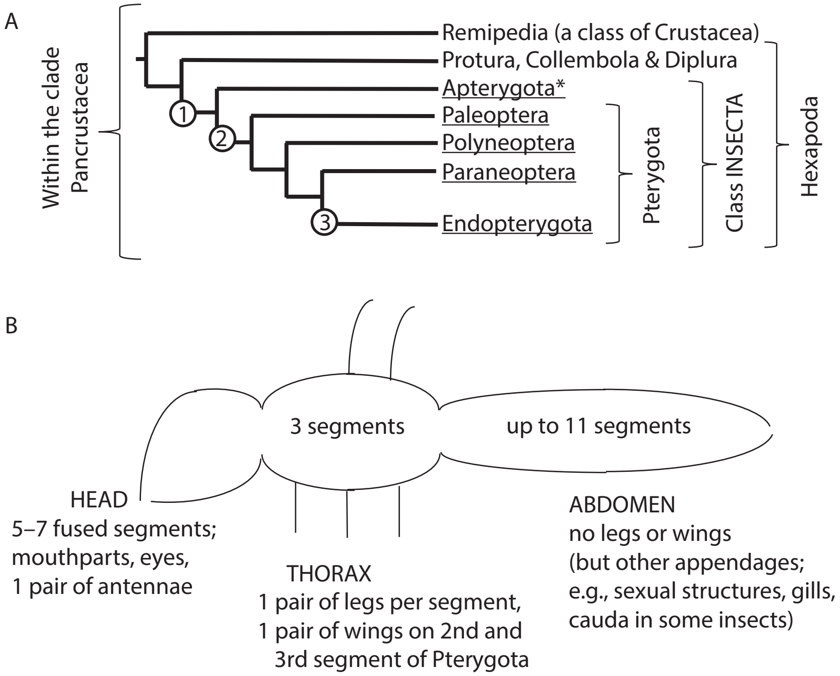

FIGURE 1.1. Insects. A. Insect relationships. The class Insecta is assigned to the subphylum Hexapoda (arthropods with six thoracic legs). The sister group of the Hexapoda (Remipedia) is a class within the Crustacea, meaning that the Crustacea is paraphyletic with respect to the Hexapoda; the clade Pancrustacea has been erected to encompass both Crustacea and Hexapoda. 1: origin of insects; 2: origin of winged insects (Pterygota, comprising four subclasses, underscored in the figure); 3: origin of complete metamorphosis (morphologically different larval and adult stages). The insect orders are listed in table 1.1. The insect body plan comprising three regions: the head, thorax, and abdomen.

other than the jumping bristletails and silverfish have wings or have evolved from winged insects; a few groups, including the parasitic lice (within the order Psocodea) and fleas (order Siphonaptera), have secondarily lost their wings. The second key innovation was complete metamorphosis, also known as holometabolism, which evolved in the common ancestor of the subclass Endopterygota. All insects of this subclass have an immature stage (larva) that is morphologically and functionally different from the adult, and a quiescent, nonfeeding stage (the pupa) within which the internal tissues are remodeled to generate the adult form. Insects other than the Endopterygota are described as hemimetabolous. These insects have no pupal stage, and in most hemimetabolous insects the juvenile stages, usually described as nymphs, are

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morphologically and ecologically similar to the adult, apart from the lack of functional reproductive organs and wings.

The insect orders vary widely in their taxonomic diversity, from fewer than 100 described species in three orders to more than 100,000 species in five orders: Coleoptera (beetles), Lepidoptera (butterflies and moths), Diptera (true flies), Hymenoptera (ants, bees, and wasps), and Hemiptera (true bugs, including cicadas, aphids, planthoppers, and shield bugs). Collectively, these five orders account for 91% of all insect species, and our knowledge of most aspects of insect biology derives predominantly from research on representatives of these groups.

1.1.2 The microorganisms

Whereas *insect* is a phylogenetically meaningful category (section 1.1.1), *microorganism* is an informal term that, in common parlance, refers to organisms that cannot be seen by the human eye. Because most very small organisms are unicellular, biologists describe taxa that are unicellular for all or most of their life cycle as microorganisms.

Three instances illustrate the conventions that define the scope of the term *microorganism*. First, the smallest adult insects, including *Nanosella* (Coleoptera: Ptiliidae), a genus of beetles of length 300 μm, and various parasitic wasps (140–300 μm long), are not regarded as microorganisms, even though they are smaller than some unicellular microorganisms, such as many amoebae (Polilov, 2015). The fungi provide a second example. The fungi include both unicellular forms, e.g., yeasts, and multicellular forms comprising a network of hyphae of varying complexity. The unicellular fungi and fungi that form small or structurally simple mycelia (including taxa such as *Candida* species, which are dimorphic, i.e., with both yeast and hyphal growth forms) are generally considered as microorganisms, even though the mycelia can often be seen with the naked eye; but there is no consensus whether fungi that form complex macroscopic structures, such as the fruiting bodies of many fungi of the phylum Basidiomycota (mushrooms, toadstools, etc.), should be treated as microorganisms or not. My final example is the viruses, which fit to the definition of microorganisms by the criterion of size (they are 0.02–0.4 μm in dimension) but are, arguably, not living organisms at all because they require the cellular machinery of a host cell for reproduction. Nevertheless, viruses are important drivers of certain interactions between insects and microorganisms, as is considered in several contexts in this book.

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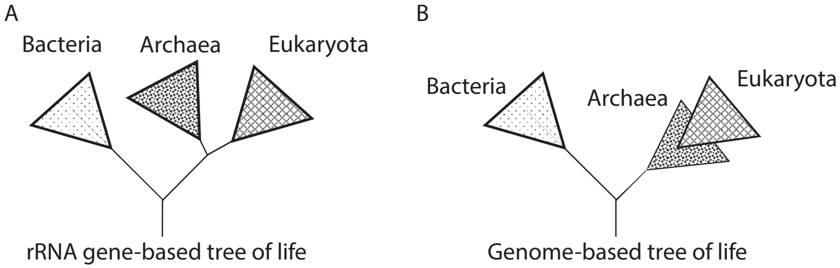

FIGURE 1.2. Relationships among living organisms. The Bacteria and Archaea are all microorganisms, and most eukaryotic phyla are also microorganisms. A. Tree based on ribosomal RNA (rRNA) sequences. B. Tree based on the sequence of multiple concatenated genes.

Most organisms are microorganisms. Phylogenetic analyses based on the small subunit ribosomal RNA gene yield three domains of organisms: Bacteria and Archaea, all of which are microorganisms, and the Eukaryota, most of which are microorganisms (fig. 1.2A). However, the validity of the three-domain scheme is in doubt. This is because analyses using the sequence of multiple concatenated genes, e.g., genes coding ribosomal proteins, yield two domains, with the Eukaryota branching from the Asgard lineage within the Archaea (fig. 1.2B) (Imachi et al., 2020). Dating these very ancient evolutionary events is extremely difficult, but there is some consensus that the first microbial life evolved at 3.6–3.9 billion years ago, and that microbial eukaryotes evolved at ca. 1.8 billion years ago. The eukaryotic microorganisms are often referred to as protists, the Protista or Protozoa, but these terms describe the grade of organization and have no phylogenetic meaning. The several groups of large, multicellular eukaryotes (i.e., nonmicrobial eukaryotes), including the animals, terrestrial plants, and red algae, evolved from different microbial lineages. The important implication for insect-microbial interactions is that insects evolved and diversified in a world that had been dominated by microorganisms for more than 3 billion years.

Which taxa in the great diversity of microorganisms associate with insects? Metagenomic analyses (i.e., sequencing all the DNA in a sample) assign most microbial sequences to Bacteria in most insect samples. The diversity and taxonomic identity of the microorganisms vary widely, but several phyla of Bacteria are well represented: Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes. Relatively few Archaea are known to be associated with animals, including insects, and the principal representatives are methanogens, especially of the

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order Methanobacteriales within the clade Euryarchaeota—found, for example, in the guts of termites and cockroaches (Blattodea) and scarab beetles (Coleoptera: Scarabaeidae). However, the incidence and diversity of Archaea in insects and other animals may be underestimated, linked to the difficulties in culturing many archaeal taxa and inadequate molecular resources to identify and quantify Archaea (Borrel et al., 2020).

Among the eukaryotic microorganisms associated with insects, the fungi are best studied. Yeasts are very commonly detected in insect samples. The term *yeast* refers to unicellular fungi, and most yeasts are members of the phylum Ascomycota, while a small number of Basidiomycota also display the yeast growth form. In addition, various insects enter into specialized associations with specific Basidiomycota that form extensive mycelia and large fruiting bodies, e.g., *Termitomyces* (Lyophyllaceae) associated with fungus-growing termites (subfamily Macrotermitinae) and *Leucocoprinus gongylophorus* (Agaricaceae) associated with leafcutter ants of the genus *Atta* (Hymenoptera: Formicidae). Other eukaryotic partners of insects include trypanosomatids (the order Trypanosomatida within the phylum Euglenozoa), which, although widely viewed as pathogens, have been reported in healthy insects, especially Drosophilidae (Diptera) and Hemiptera (Podipaev, 2001); and obligately anaerobic ciliates (phylum Ciliata) and flagellate protists (of the phyla Parabasalia and Preaxostyla) that inhabit the anoxic guts of some insects, notably various cockroaches and termites (Blattodea) (Brune, 2014).

1.1.3 Describing the taxonomy of insect and microbial partners

The taxonomic position of species of insects and microorganisms described in this book is summarized after first mention of a species in each section. For insects, I provide the order and family, e.g., *Drosophila melanogaster* (Diptera: Drosophilidae). For most microorganisms, I provide the phylum and family, e.g., *Bacillus subtilis* (Firmicutes: Bacillaceae) and *Saccharomyces cerevisiae* (Ascomycota: Saccharomycetaceae). Exceptionally, members of the bacterial phylum Proteobacteria are described by class (which is a Greek letter-Proteobacteria), e.g., *Escherichia coli* (γ-Proteobacteria: Enterobacteriaceae).

Specific issues of nomenclature arise for the many unculturable microoorganisms associated with insects. Formal taxonomic assignments require isolation of microorganisms into culture, and unculturable microorganisms are referred to as *Candidatus* taxa. For example, whiteflies bear the unculturable bacterium *Candidatus* Portiera aleyrodidarum. For convenience, I follow the

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convention of abbreviating these *Candidatus* names to the genus name, e.g., *Portiera* in whiteflies.

A further taxonomic issue concerns microorganisms that have been associated with insects for many millions of years. The phylogenetic status of some microbial symbionts is uncertain or disputed, with particular concern that the published assignments of some ancient bacterial symbionts with very small genomes may be an artifact of long branch attraction (the erroneous interpretation that two or more distantly related taxa are closely related because they are different from other taxa in the phylogeny). For example, the intracellular bacterial symbiont of most aphids, *Buchnera aphidicola,* can be assigned to the order Enterobacterales (γ-Proteobacteria) with reasonable confidence, but a more precise placement of this taxon is not assured (although some authorities assign it to the family Enterobacteriaceae). In this book, I indicate this uncertainty by the designation of *Buchnera* as "γ-Proteobacteria: Enterobacterales *incertae sedis*" (*incertae sedis* means "uncertain placement"). Similarly, the bacterial symbiont of cicadas is *Sulcia muelleri* (Bacteroidetes: Flavobacteriales *incertae sedis*), but the bacterial symbiont of whiteflies, for which phylogenetic signal to the family level is assured, is referred to as *Portiera aleyrodidarum* (γ-Proteobacteria: Halomonadaceae). With the application of increasingly sophisticated phylogenomic methods, many of the current phylogenetic uncertainties and assignments of microbial taxa to *incertae sedis* status are likely to be resolved in the coming years.

1.2 Insect habitats for microorganisms

Most insect-associated microorganisms are localized to the cuticle, gut, hemolymph, or within cells of insects. In this section, the biology of these insect habitats is described. The characteristics of insect-microbial associations in the different locations are considered in detail in chapter 2.

1.2.1 The external coverings

The insect body is bounded by a light, waterproof cuticle (fig. 1.3) that protects the underlying soft tissues against mechanical damage, desiccation, and microbial colonization. The cuticle is a composite material of chitin fibrils in a protein matrix, and its mechanical properties (strength, flexibility, etc.) are dictated largely by the degree of protein cross-linking by quinones. The cuticle is largely inextensible, and sustained growth of an immature insect (nymph or

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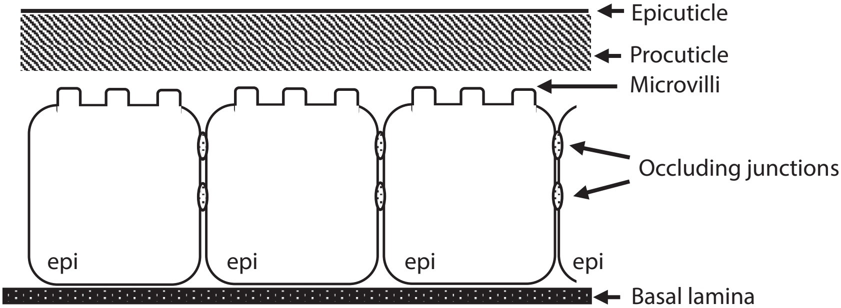

FIGURE 1.3. The insect integument. The insect body is bounded by a layer of epidermal cells (epi), which secrete most of the constituents of the acellular cuticle. The cuticle comprises the procuticle of chitin fibrils and cuticular proteins, many of which are cross-linked to form a tough, inextensible structure; and the epicuticle, which confers waterproofing and is made of lipoproteins, waxes, hydrocarbons, etc. The epidermal cells adhere closely via lateral occluding junctions (including adherens junctions and septate junctions) to form a coherent sheet of tissue that rests basally on the noncellular basal lamina.

larva) requires the insect to undergo molting. This is a complex process that requires the formation of a new cuticle under the old cuticle, then shedding of the old cuticle (a process known as ecdysis), and finally the expansion and then hardening of the new cuticle.

The insect cuticle is, generally, an inhospitable habitat for microorganisms. It has low availability of water and nutrients, and it is protected further by both microbicidal secretions from the underlying epidermal cells and specialized glands (Yek and Mueller, 2011) and nanoscale cuticular structures that impede microbial adhesion (Watson et al., 2017). Microbial cells detected on the insect cuticle are generally transient (Zhukovskaya et al., 2013), being dislodged by insect grooming and lost with cuticle shedding at ecdysis.

Despite the general inhospitality of the insect cuticle for microbial colonization, regions of the cuticle in some adult insects are specialized to facilitate colonization by specific microorganisms. For example, the cuticle of many attine ants (Hymenoptera: Formicidae of subtribe Attini) is modified to bear a dense colony of the bacterium *Pseudonocardia* (Actinobacteria: Pseudonocardiaceae) (see section 4.5.1). Other insects have cuticular structures that function specifically for microbial storage and transport. These structures are known as mycangia (singular, mycangium), following their initial description in insects harboring fungal symbionts. However, some mycangia contain both fungi and bacteria or exclusively bacteria (see sections 2.1.3 and 2.1.5). The

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structural organization of mycangia varies from simple pits in the cuticle surface to anatomically complex invaginations with a narrow opening to the exterior that is often bounded by protective setae (chitinous hairs). The diversity of mycangia has been studied most extensively in ambrosia beetles (Coleoptera: Curculionidae of subfamilies Scolytinae and Platypodinae), in which these structures have evolved multiple times (Hulcr and Stelinski, 2017).

1.2.2 The insect gut

As for most animals, the gut in the great majority of insects is a tubular structure through which ingested food is passed from mouth to anus. Although insects are exceptionally diverse in feeding habits, the fundamental plan of the gut is conserved, comprising a foregut, midgut, and hindgut (fig. 1.4). The foregut is the principal site of mechanical disruption and initial processing of food, while enzymatic digestion and nutrient assimilation is predominantly in the midgut, and the hindgut mediates selective absorption of water, ions, and some metabolites. The gut lumen in the foregut and hindgut is separated from the gut epithelium by cuticle that is continuous with the cuticle protecting the external surface of the insect. As for the external cuticle, the gut-associated cuticle acts as a barrier to microbial colonization. When the insect molts, the gut cuticle is lost via the mouth and anus, and then replaced by new cuticle in the subsequent life stage of the insect.

The conditions and resources in the insect gut vary widely among species, with developmental age (particularly differing very substantially between the larva and adult of holometabolous taxa) and with location in the gut (Engel and Moran, 2013). In small insects, the gut is predominantly oxic or mildly hypoxic, due to the diffusion of oxygen from the gut epithelium, which is aerobic and well supplied with tracheae, but some regions of the gut lumen of large insects can be anoxic. For many insects, the variation in pH in the gut lumen is relatively small, in the range of 6–8 pH units, but extremely alkaline conditions (pH >11) occur, for example, in the midgut of lepidopteran larvae and the hindgut of some soil-feeding termites, and a portion of the midgut of many Diptera is highly acidic, at pH <3.

The midgut is a relatively inhospitable habitat in many insects. This region is the principal site of digestive enzyme production, often including lysozymes and other enzymes that attack the cell walls of bacteria, and it is immunologically active, with the capacity to produce antimicrobial peptides and microbicidal bursts of reactive oxygen. The midgut can also be physically unstable

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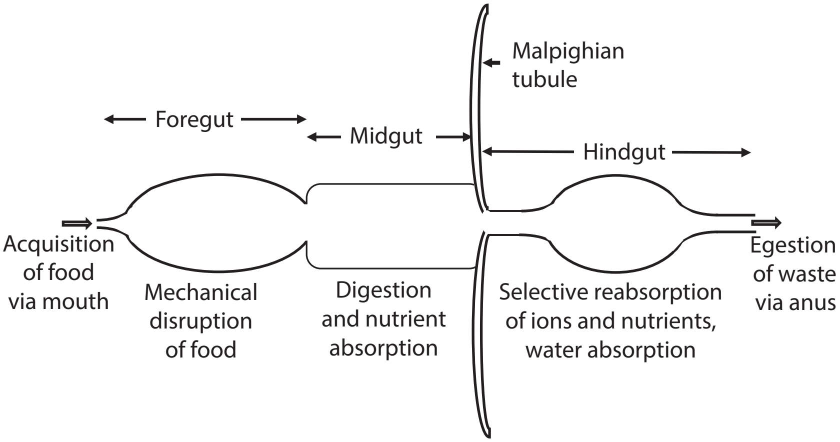

FIGURE 1.4. The insect gut. The major subdivisions of the gut are the foregut, midgut, and hindgut, with Malpighian tubules at the midgut-hindgut junction (insect species vary in the number of Malpighian tubules, from a single to hundreds of pairs). The sequential stages in processing of food, as it is transported from the mouth through the different parts of the gut to the anus, are shown. The functional morphology of the gut varies widely among insects; see Simpson and Douglas (2013) and Engel and Moran (2013) for details.

because the midgut epithelium of many insects secretes a sheet of chitin and protein, known as the peritrophic envelope, which encloses incoming food. The peritrophic envelope has a similar function to mucus in the vertebrate gut (some of the proteins of the peritrophic envelope have mucin-like domains): to protect the gut epithelial cells against mechanical and chemical damage, and to act as a physical barrier against colonization by microorganisms in the ingested food. The importance of the protective role of the midgut peritrophic envelope is illustrated by analysis of the fruit fly *Drosophila melanogaster* (Diptera: Drosophilidae) with a loss-of-function mutation in the gene *dcy* coding the protein Drosocrystallin. The peritrophic envelope in these mutant flies is permeable to many ingested macromolecules and microorganisms, and the mutant flies display heightened susceptibility to the pathogenic bacterium *Pseudomonas entomophila* (γ-Proteobacteria: Pseudomonadaceae) (Kuraishi et al., 2011).

The hindgut is the gut region that accommodates the highest-density and most persistent microbial communities in many insects. The conditions of redox and pH in the hindgut are generally favorable (although, as mentioned above, certain compartments of the hindgut are very alkaline, for example, in some termites). Additionally, the eluate from the Malpighian tubules, which

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open at the midgut-hindgut junction (fig. 1.4), provides nutrients and a suitable ionic composition for microbial growth.

An important factor limiting the persistence of microorganisms in all regions of the gut is the passage of food from mouth to anus, driven by peristalsis of the gut musculature. Protection against loss with the bulk flow of food can be provided by the boundary layer immediately abutting the gut wall, especially at loops in the intestinal tract. Some microorganisms escape from the flow of food by incorporation into gut outpocketings that are present in some insects. For example, the midgut ceca of some Coleoptera and Orthoptera and the extensible foregut diverticulum (known as the crop) in some adult Diptera have been reported to bear dense populations of microorganisms. Some insects display specific adaptations for the retention of microorganisms in the gut; e.g., the hindgut cuticle of some termites is modified to form a mesh of long cuticular spines to which many microorganisms adhere (Bignell et al., 1979).

1.2.3 The open circulatory system and internal organs

The internal organs of insects are bathed in hemolymph, which is the principal extracellular fluid of insects. Although sometimes described as "insect blood," hemolymph differs from vertebrate blood in that it is not contained within a closed network of blood vessels and it does not transport oxygen required for aerobic respiration. Insect hemolymph is an excellent medium for microbial growth. It has a balanced ionic composition and near-neutral pH, and is rich in sugars (usually dominated by the disaccharide trehalose), amino acids, and other nutrients. Added advantages of hemolymph as a habitat for microorganisms are that the dorsal tubular heart of insects powers slow hemolymph flow around the body with minimal damaging shear forces on planktonic microbial cells, while the basement lamina bounding internal organs offers favorable sites for adhesion and biofilm formation.

Other attributes of hemolymph, however, are hostile to microbial colonization. Hemolymph is well protected by the insect immune system, which includes both hemocytes that engulf or encapsulate microorganisms and humoral effectors, such as phenoloxidases and antimicrobial peptides. Although certain pathogens can overcome these defenses and proliferate rapidly in hemolymph, causing sepsis and insect death, resident microorganisms in the hemolymph of healthy insects are widely believed to be uncommon (Blow and Douglas, 2019). Nevertheless, some beneficial microorganisms can be

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detected routinely in the hemolymph of certain insects. Examples include the bacterium *Spiroplasma* sp. (Tenericutes: Spiroplasmataceae), which protects its insect host *Drosophila neotestacea* (Diptera: Drosophilidae) against nematode parasites (Jaenike et al., 2010), and *Hamiltonella defensa* (γ-Proteobacteria: Yersiniaceae), which protects the pea aphid *Acyrthosiphon pisum* (Hemiptera: Aphididae) against parasitic wasps (Oliver et al., 2003). Other nonpathogenic microorganisms can persist in hemolymph for limited periods and, because the hemolymph is in direct contact with multiple organs, microorganisms can be transferred readily between organs—notably to the reproductive organs enabling vertical transmission to the insect offspring (see section 3.2.3).

Many of the internal organs, including the brain and nervous system and the reproductive organs, have many parallels to the equivalent organs in other animals, including vertebrates. There are, however, two insect organs, the fat body and the Malpighian tubules, which are structured to function in the context of an open circulatory system, and they are functionally different from any organs in vertebrates.

The fat body is the metabolic and immunological powerhouse of the insect (Li et al., 2019). It is a large and amorphous organ that is distributed throughout the body, often comprising loosely connected cells in the hemolymph. The fat body is the main lipid store of insects and the principal site of carbohydrate, lipid, and nitrogen metabolism. It also plays a central role in the coordination of insect growth, development, and reproduction, and serves as the key location for detoxification of xenobiotics and synthesis of antimicrobial peptides, an important component of the humoral immune system of insects. The fat body is not colonized by microorganisms in most insects, but specialized cells bearing bacteria are localized to the fat body of cockroaches (Blattodea); and yeasts in some delphacid planthoppers (Hemiptera: Delphacidae) are distributed predominantly among fat body cells (see sections 2.3.2 and 2.3.5).

The Malpighian tubules are the principal excretory organs of insects (Beyenbach et al., 2010). They are blind-ended evaginations at the midgut-hindgut junction extending into the hemolymph (fig. 1.4). The primary urine of insects is created by secretion-excretion, powered by a H^+-pumping ATPase, in contrast to the primary urine of vertebrates, which is driven by filtration-excretion, i.e., high blood pressure in the nephrons of the kidney. The principal nitrogen waste products of many insects, including uric acid and urea, as well as various xenobiotics are transferred from the hemolymph to the lumen of the Malpighian tubules and transported to the hindgut, where water, ions, and nutrients are resorbed prior to evacuation of solid or semisolid waste.

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1.2.4 Insect cells

Insect cells are similar to insect hemolymph in that they represent a nutrient-rich but strongly defended habitat that is colonized by relatively few microbial taxa. Some microorganisms, however, are specialized for the intracellular habitat in insects. These include *Wolbachia* (α-Proteobacteria: Anaplasmataceae), which has been estimated to infect 40%–60% of insect species and can colonize a diversity of insect cell types, including oocytes in the ovary of females (see section 3.2.4). In addition, insect cells with the sole known function to house and maintain required intracellular bacteria have evolved multiple times. These insect cells are known, generically, as bacteriocytes. The bacteriocyte symbioses are considered further in chapter 2 (see section 2.3.2).

The great majority of intracellular microorganisms in insects are localized to the cell cytoplasm, and they are separated from the host cell contents by a membrane of host origin, known as the symbiosome membrane (fig. 1.5). In this location, the microorganisms are protected from intracellular receptors of the host cell that would otherwise detect microbial products (e.g., cell wall constituents), triggering immunological attack. The symbiosome membrane likely plays an important role in maintaining strict host controls over the flux of metabolites, thereby regulating microbial access to its nutrient pools and likely influencing both the metabolic function and proliferation of the microorganisms.

1.3 Insect associations with beneficial microorganisms

The basis for understanding the relationships between insects and beneficial microorganisms is that all organisms live in a fundamentally antagonistic world. Organisms encounter unfavorable conditions, including extremes of temperature, pH, water, or nutrient availability, and engage in negative biotic interactions, including competition, predation, and parasitism. One route by which many organisms enhance their fitness is to associate with other organisms that variously ameliorate those conditions or their negative effects, protect against antagonistic interactions, or improve performance in antagonistic interactions. A specific example illustrates these effects. The pea aphid *Acyrthosiphon pisum* (Hemiptera: Aphididae) bears various bacteria localized to its internal tissues. One of these bacteria *Serratia symbiotica* (γ-Proteobacteria: Yersiniaceae) enhances aphid tolerance of high temperature (Montllor et al., 2002), and a second bacterium *Regiella insecticola* (γ-Proteobacteria: Yersiniaceae) promotes

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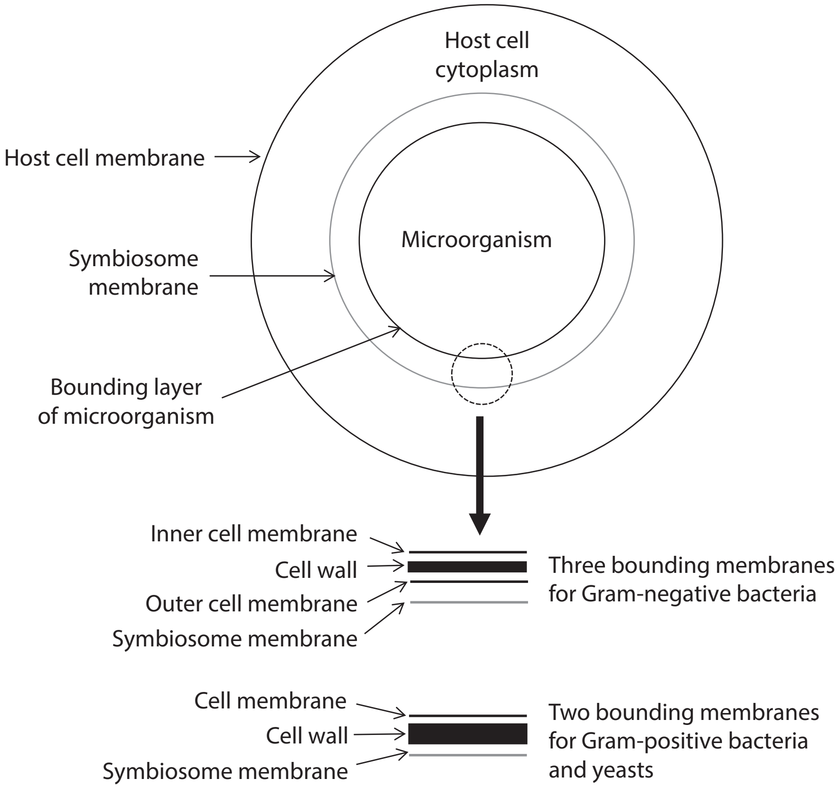

FIGURE 1.5. Localization of intracellular symbionts within a symbiosome membrane of host origin. The internal contents of the microbial cell are separated from the host cell contents by either two or three membranes (microbial membranes shown in black and the symbiosome membrane in gray). Many microorganisms additionally possess a cell wall, as illustrated.

resistance against a pathogenic fungus *Pandora aphidius* (Zoopagomycota: Entomophthoraceae) (Scarborough et al., 2005).

Insect associations with beneficial microorganisms are studied in the context of the discipline of symbiosis—the study of persistent associations between taxonomically different organisms (table 1.2). Although the vernacular meaning of symbiosis includes the criterion of mutual benefit, many biologists are hesitant to define associations in this way for two reasons. First, the fitness consequences for partners contributing to some associations can be context dependent, varying with environmental conditions, genotype, developmental age, etc. For example, the bacteria that protect pea aphids against elevated temperature and fungal pathogens (see previous paragraph) can be costly to

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TABLE 1.2. Terms used to describe associations between insects and microorganisms

Term	Definition
Symbiosis	A persistent association between taxonomically different organisms
Host and symbiont[1]	In associations involving organisms of different size, the larger partner is called the host and the smaller is called the symbiont(s); for insect-microbial associations, the term *symbiont* refers to the microorganisms
Microbiome[2]	The microbial community in a habitat, described in terms of taxonomic composition or functional composition, as deduced from gene sequencing data
Microbiota[2]	The community of microbial taxa in a habitat

[1] An alternative terminology that is not used in this book describes an association between a host and symbionts as a holobiont, the constituent partners (both host and symbionts) as bionts, and the combined genomes of all bionts as the hologenome.

[2] The terms *microbiome* and *microbiota* are used interchangeably to describe a microbial community in taxonomic terms. The term *microbiome* (but not microbiota) is also used to describe the microbial community in terms of its functional gene content.

the aphid, by the indices of reduced growth and reproductive output, at benign temperatures and under enemy-free conditions, respectively; and these costs contribute to the intermediate frequency of these bacteria in natural pea aphid populations (Oliver et al., 2010). The second reason relates specifically to associations involving microorganisms: the benefit of the association to the microbial partners is often obscure. For example, the advantage to pea aphids of high temperature tolerance and fungal pathogen resistance can readily be identified and quantified, but the advantage to *Serratia* and *Regiella* of associating with pea aphids is uncertain. This is partly because information on the incidence and fitness of free-living populations of these bacteria in the external environment is lacking, as I consider further in chapter 7 (section 7.1.2).

An important implication of these considerations is that the term *beneficial microorganisms* refers exclusively to the benefit derived by the partners of microorganisms. Research on beneficial microorganisms is focused primarily on associations involving animal or plant partners of microorganisms. In this book, I adopt the convention to describe these multicellular partners as the hosts and the microbial partners as symbionts (table 1.2).

I close this section by considering a further terminological issue: the meaning of the term *microbiome*. Research on taxonomically complex microbial communities and on microbial taxa intractable to axenic cultivation has been transformed by the development of sequencing methods that enable cultivation-independent identification and study of microorganisms. The

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"-omic" terminology that has proliferated to describe the knowledge made possible by these technologies includes "microbiome," coined to describe the catalog of microorganisms and their genes (Lederberg and McCray, 2001). Microbiome is particularly useful for describing results from methods such as shotgun metagenomics, where the genetic makeup of a microbial community is quantified without being able to assign individual gene sequences to specific taxa with confidence. The related term *microbiota* is also widely used, specifically referring to the taxa that contribute to the microbial community (table 1.2).

1.4 How this book is structured

The purpose of this book is to provide an overview of associations between insects and beneficial microorganisms, and their application both as novel strategies in insect pest control and as a biomedical model for microbiome-based therapies in human health and disease. It is structured as two complementary parts. Chapters 2–4 consider the fundamental biology of insect interactions with beneficial microorganisms, while chapters 5 and 6 address the opportunities for application. The final chapter 7 explores some of the outstanding research questions and opportunities for the application of insect-microbial associations.

This book focuses primarily on insects and their associations with Bacteria, Archaea, and eukaryotic microorganisms. Although some authorities argue that the fungi that form macroscopic structures (e.g., *Termitomyces* farmed by macrotermitine termites) are too large to be described as microorganisms, there is a long-standing convention to consider their associations with insects within the discipline of insect-microbial interactions; and I follow this convention. Associations involving beneficial viruses and arthropods other than insects are not included.

Chapter 2 provides a detailed introduction to the diversity of insect associations with beneficial microorganisms. As explained in the introduction to this chapter, insects are remarkable for the variety of ways in which they interact with microorganisms. A widely used route to categorize these associations is by the location of the microbial partners relative to the insect body. In chapter 2, I describe the basic biology of insect-microbial interactions in ectosymbioses, including the leafcutting ants, fungus-growing termites, and ambrosia beetles, then in gut symbioses, such as the wood-feeding termites, the dung beetles, and honey bees, and finally in endosymbioses, including intracellular

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bacteria that have been retained through the generations of some hemipteran insects for more than 100 million years.

Chapter 3 addresses the myriad of ways in which insects acquire and manage their complement of microorganisms. Some insects are associated with microorganisms that sustain substantial free-living populations, but many microbial taxa are transferred between insects with short or no intervening period in the external environment. Horizontal transfer between nest-mates in group-living insects frequently involves behavioral adaptations of the insects, e.g., trophallaxis (mouth-to-mouth or anus-to-mouth transfer) by recently eclosed worker honey bees and newly molted termites. Vertical transfer, usually from mother to offspring, is very widely distributed. An inoculum of microbial cells can be smeared onto the surface of the newly deposited egg, or transferred directly into the unfertilized oocytes in the ovaries of female insects. Having acquired microbial partners, the insect host has the challenge to manage the microbial populations, controlling both their location and abundance. Recent studies are revealing the contributions of neuroendocrine signaling and immune system function in shaping the acquisition and retention of beneficial microorganisms in insects.

Chapter 4 concerns microbial services, meaning the processes by which microorganisms promote the performance and fitness of their insect hosts. In very many associations, the microbial service is well defined. The microorganisms may contribute to insect nutrition by degrading lignocellulose in the diet of wood-feeding insects (such as many termites) or synthesizing vitamins and other essential nutrients in short supply, for instance, in vertebrate blood (utilized, for example, by bedbugs and anopluran lice) or in plant sap (utilized by cicadas, leafhoppers, whiteflies, aphids, etc.). They can also have defensive functions. Some beneficial microorganisms produce toxins that protect their insect host against pathogens, parasites, or predators, while others prime the insect immune system for a greater and more rapid response against pathogen challenge. Two further types of service appear, on current evidence, to be rather limited in distribution: microbial detoxification of dietary toxins, particularly plant allelochemicals ingested by herbivorous insects, and of chemical insecticides; and microbial partners as a source of pheromones mediating aggregation and mate choice in insects. Finally, I address the routes by which microbial symbionts protect insects against abiotic stress, especially elevated temperature.

How can this burgeoning understanding of the fundamental biology of interactions between insects and their beneficial microbes be applied for the public good? Chapters 5 and 6 provide an overview of two increasingly productive fields of application.

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Chapter 5 considers the opportunities to harness beneficial microorganisms in the management of insect pests and disease vectors. Much of the impetus for this approach comes from the groundbreaking discovery that certain insect-associated microorganisms, particularly *Wolbachia*, can reduce the competence of mosquitoes to vector disease agents of humans. This research has the potential to transform the control of some infectious diseases, with large-scale trials against dengue already in progress. Other opportunities are at earlier stages of development. In particular, it has been recognized for decades that highly specific microbicides against the symbionts required by insect pests could offer a most effective novel strategy to control some insect pests. Until recently, this potential has remained unfulfilled for lack of a robust strategy to identify reagents that exclusively target these microorganisms. With the advent of genomics, routes to identify taxon-specific gene products or pathways are becoming available and, simultaneously, approaches based on RNA interference are being applied both to suppress some required symbionts and to modify other symbionts for increased pathogenicity. These methods are being adopted for both agricultural pests, such as aphids and thrips, and medically important insects, including tsetse flies and triatomine bugs.

Chapter 6 addresses the second major application of insect associations with beneficial microbes: as a biomedical model for human microbiome science. Major biomedical research initiatives are in progress, founded on the evidence that the human body harbors resident microbial communities whose composition and activities profoundly affect human health. We have realistic prospects of microbial therapies to eliminate acute pathogen infections and ameliorate chronic metabolic, immunological, and neurodevelopmental/degenerative diseases. However, research on humans is necessarily correlative, and model systems are needed to determine causality, i.e., to distinguish whether changes in the microbiome are the cause or consequence of disease symptoms, and to investigate the underlying mechanisms. Building on the evidence that many aspects of microbiome-host interactions are conserved across the animal kingdom, there is increasing interest to use insect models. In comparison to rodent models, insects are cheap and plentiful, facilitating large experimental designs while reducing the animal welfare implications of biomedical research. To date, *Drosophila* is the principal insect model for microbiome research. Research on this system is revealing how individual microorganisms and perturbation of the microbial community (e.g., by antibiotics or genetic lesions in insect immunity) can have cascading effects on metabolic health, nervous system function, and the behavior of the host.

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Chapter 7, the final chapter, explores future opportunities for enhanced understanding and application of insect associations with beneficial microorganisms. Three key outstanding issues are identified: the ecology of the microbial partners, especially in associations that involve microorganisms with free-living populations; the mechanisms underlying chemical and metabolic communication between the partners; and the management of insect-associated microorganisms for improved health of beneficial insects.

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