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An emerging paradigm of colony health: microbial balance of the honey bee and hive (*Apis mellifera*)

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Abstract Across the globe, honey bee populations have been declining at an unprecedented rate. Managed honey bees are highly social, frequent a multitude of environmental niches, and continually share food, conditions that promote the transmission of parasites and pathogens. Additionally, commercial honey bees used in agriculture are stressed by crowding and frequent transport, and exposed to a plethora of agricultural chemicals and their associated byproducts. When considering this problem, the hive of the honey bee may be best characterized as an extended organism that not only houses developing young and nutrient rich food stores, but also serves as a niche for symbiotic microbial communities that aid in nutrition and defend against pathogens. The niche requirements and maintenance of beneficial honey bee symbionts are largely unknown, as are the ways in which such communities contribute to honey bee nutrition, immunity, and overall health. In this review, we argue that the honey bee should be viewed as a model system to examine the effect of microbial communities on host nutrition and pathogen defense. A systems view focused on the interaction of the honey bee with its associated microbial community is needed to understand the growing agricultural challenges faced by this economically important organism. The road to sustainable honey bee pollination may eventually require the detoxification of agricultural systems, and in the short term, the integrated management of honey bee microbial systems.

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> "There's no limit to the complexity of interaction in nature. If humans can think of a scheme, the chances are nature's already implemented it." Thomas Eisner.

Symbiotic microorganisms

Symbiosis is widespread in eukaryotes, wherein both microbial and host elements work synergistically to maintain proper nutrition, health, and immunity (Gill et al., 2006). As evidenced by the recent explosion of studies of the human gut microbiota using metagenomic approaches, many researchers are already convinced of the importance of microbial communities for the expression of phenotypes traditionally attributed solely to the host organism (Zilber-Rosenberg and Rosenberg, 2008). In some well-studied solitary insects, endosymbiotic bacteria and fungi can manipulate host reproduction, contribute to nutrition or provide defense against pathogens (Douglas, 1998; Moran and Telang, 1998; Feldhaar and Gross, 2009; Gibson and Hunter, 2010). The nature of beneficial endosymbiosis in insects varies from obligate and intracellular in modified cells to facultative and extracellular within the gut lumen (Kikuchi, 2009). As a classic example, aphids have protein deficient diets, but harbor bacteria in specialized cells (bacteriocytes) that supply the amino acids essential for host survival (Douglas, 1998). While much research has focused on obligate intracellular endosymbioses, difficulties with culturing such specialized bacteria and their host tissues have been a consistent barrier for the investigation of host–

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symbiont molecular interactions. In contrast, facultative symbionts occurring in the gut are often cultivable, lending themselves to genetic manipulation and a wider variety of experimental approaches. Facultative symbionts have a much wider niche breadth, can occur in a range of host tissues, and are often distributed sporadically throughout host populations (Ishikawa, 2003). Historically, facultative symbioses have been considered commensal or parasitic, but recent studies have demonstrated host fitness benefits in some species (Scarborough et al., 2005).

In social insects, host–symbiont interaction must be considered in the context of the colony microenvironment, a complex and dynamic arrangement of biotic and abiotic interactions evolved to protect future generations and preserve and/or process nutrients (Turner, 2000, 2004; Hughes et al., 2008). Commensal bacteria or fungi adapted to one niche (e.g. host gut) are likely to be found ephemerally or consistently in another hive niche (e.g. host food stores). As an extreme example, fungus growing termites cultivate a fungal food, a process that contributes to colony disease resistance, nutrient upgrading, gas exchange, and water balance (Turner, 2000; Rouland-Lefèvre et al., 2006). The termites ingest fresh wood, grass or leaf mulch that is then inoculated with fungal spores while in transition through the termite gut (Batra and Batra, 1979). The resulting fecal pellets consist of plant forage slowly digested by fungal hyphae, the principal food source. In a similar system, fungus-growing ants employ mutualistic bacteria to protect their fungal food from pathogens. Remarkably, the glands and exoskeleton of the ant have evolved to nurture bacterial mutualists in thoracic crypts (Currie et al., 1999; Currie et al., 2006; Little and Currie, 2008). Additionally, some fungus-growing ants selectively prune their fungus garden, removing spores of pathogenic fungi that accumulate in a pouch-like structure under their tongue. This pocket houses a community of bacteria that functions as a sterilization device, killing spores of the fungal garden parasite (Little et al., 2006). Such discoveries suggest that social insects with stored or processed food stores are likely to possess many behavioral and physiological traits that have evolved as a direct consequence of symbiosis with beneficial microbes.

Given the depth of understanding in the aforementioned systems, it is alarming to consider that virtually nothing is known about the beneficial microbial symbionts of the honey bee, a social insect vital to the worlds food supply. Many thousands of different microbial strains have been cultured from honey bee colonies (Gilliam, 1997), yet we know virtually nothing of honey bee microbial ecology. In this review, we present a description of the currently available information about potentially beneficial microorganisms recovered from the honey bee alimentary tract (gut) and hive environment. Due to the increasing incidence and limited understanding of colony collapse disorder (CCD) we feel that a new understanding of microbial ecology in honey bees is both necessary and timely. We present several points of view to showcase honey bees as a model system for investigating the functional complexity of beneficial microbial communities in the context of health, nutrition, disease and management. We discuss the potential for microbial interactions within and between the gut and hive environments, and in developing new strategies for honey bee management and sustainability. In the broadest sense, research on honey bee microbial systems will contribute to the understanding of general microbial ecology with potential applications to both human and agricultural disease management. In the most immediate sense, such research will lay the foundation for honey bee microbial health and disease management in the context of commercial beekeeping and the persisting trend of CCD (Cox-Foster et al., 2007; Ribiere et al., 2008; van Engelsdorp et al., 2009; Johnson et al., 2009; Runckel et al., 2011).

Microbial frontier

While past studies have emphasized the pathogenic nature of microbes, the research focus has shifted toward an understanding of the beneficial nature of insect-microbial systems. The utility of a systems approach for this process has become clear, and will allow rapid and novel insight into the composition, function and evolution of the beneficial honey bee microbiota. A major break-through in the characterization of microbial communities was the development of high-throughput next-generation sequencing technologies (Simon and Rolf, 2009), capable of examining metagenomic material directly from environmental samples (Handelsman et al., 1998). Sequencing improvements have had a profound impact on our understanding of microbial species, for instance, two strains of the same species can differ in gene content by as much as 30% (Fraser-Liggett, 2005). Thus, the effort has turned to sequencing multiple isolates and strains that will provide new insights into metabolic diversity at the level of the prokaryotic genome and the interactive nature of entire microbial communities with their host. The rapidly expanding wealth of sequence information provides a broad context for interpreting the honey bee microbiota. Taxonomic groups related to potentially beneficial honey bee bacterial symbionts are well represented in genomic databases, including 195 strains of *Bacillus*, 183 strains of *Lactobacillus* and 50 strains of *Bifidobacterium*.

Past investigations of microbial communities employed primarily selective growth media, followed by characterization with morphology and enzymatic assays (Gilliam, 1997). More recently, 16S rDNA fragments or sequences

were used to estimate taxa abundance and diversity (i.e. terminal restriction fragment length polymorphism, degrading gradient gel electrophoresis, cloning, and 16S amplicon sequencing). However, 16S genes are too conserved to be a useful predictor of metabolic function (Berger et al., 2007). Moreover, it was suggested long ago that characterization of insect microbiota should focus on the functional aspect of host–symbiont interaction (Brooks, 1963). The introduction of new techniques has made this possible. These new tools provide insight into the functional aspect of symbioses and include niche and tissue specific metagenome sequencing, used to determine the occurrence and prevalence of microorganisms in a particular niche, and transcriptome sequencing, revealing which microbial or host genes are expressed in response to changes in individual or colony development, microbial community composition, or environment factors. A major advantage of this high throughput approach is the ability to simultaneously monitor changes in transcription and translation in both the symbiont and the host. New post genomic tools like RNA-interference and microarrays have also been developed for many insect species (see Kikuchi, 2009). Cultivable microbes can be tagged with fluorescent reporter genes to visualize distinct taxa and determine the abundance, succession and spatial arrangement of cells in the gut or hive environment. Although it is often impossible to assemble individual genomes from the metagenome of a complex microbial community, predicted enzyme function facilitates the culturing of previously uncultivable microbes via the in silico design of organism-specific media (Lemos et al., 2003). The ability to culture both symbionts and insect cell lines provides a powerful platform for the in vitro investigation of host–symbiont interactions.

Sequence-based studies have their own limitations and inherent bias including inconsistent sampling strategies and the extraction of nucleic acids from different bacterial cell types. In vitro simulations of synthetic microbial communities reveal that DNA preparation methods have biased most metagenomic experiments (Morgan et al., 2010). Both primer choice and DNA extraction method can radically alter community profiles. For example, the inclusion of lysozyme in the DNA extraction protocol reveals a honey bee bacterial community rich in gram positive bacteria (see Martinson et al., 2011), while the inclusion of 18S primers reveals the presence of a fungal community (Cox-Foster et al., 2007). Differences between culture and non-culture-based methods can be difficult to resolve, and many discrepancies can be traced to differences in methodology, a critical aspect when profiling a microbial community with DNA sequence technology. The interpretation of metagenomic datasets involves phylogenetic classification based on reference databases containing sequences of known origin and gene function. These databases are heavily biased toward readily cultivable microorganisms, and up to 90% of the sequences from a metagenomic sample may remain unidentified because they lack a reliable reference sequence (Huson et al., 2009). Fortunately, most honey bee-associated microbes can be cultivated (Gilliam, 1997), increasing taxonomic resolution and facilitating inhibition and interaction assays that examine the relationship of microbial isolates with one another and with molecules and organisms of interest.

Distinguishing a minimally functional or "core" microbial community requires differentiating transient from constitutive microbes, resulting in the description of a relatively stable climax community. While mammals have complex structures like paunches, diverticula, and caeca that can serve as niches to support a wide variety of microorganisms (Tanada and Kaya, 1993), insects such as the honey bee possess a relatively simple digestive tract, suggesting a much less complex microbiota (see Martinson et al., 2011). However, the honey bee microbiota exists at two major levels; within the relatively simple alimentary tract, and throughout the extended organism of the hive that houses the developing young and food stores. Sequence-based methods examining the adult honey bee alimentary tract suggest a relatively simple and stable bacterial flora regardless of geography (Jeyaprakash et al., 2003; Mohr and Tebbe, 2006; Babendreier et al., 2007; Cox-Foster et al., 2007; Martinson et al., 2011), but culture-based results reveal incredible microbial diversity in the extended hive environment (Gilliam, 1997). These may include transient or enduring environmental microbes acquired each generation, or those transferred from previous generations.

The adult gut as a microbial niche

Changes in structure, pH and nutrient availability throughout the adult honey bee gut will influence the establishment and persistence of the associated microbiota (Fig. 1). The pH of the social stomach is highly acidic, but also varies in accordance with the pH of ingested food products. Projecting into the social stomach is the proventriculus, a one-way valve structure that actively transports pollen grains while retaining most of the liquid (water, nectar and royal jelly), and ensures that the social stomach is not contaminated with the enzymes and microbes from the more posterior midgut (Terra and Ferreira, 1994). The cells comprising the social stomach are designed for expansion and storage, but there appear to be no molecular transport mechanisms between the honey bee hemolymph and the social stomach (Maddrell and Gardiner, 1980). The midgut (ventriculus) is the primary site of honey bee digestion, and the largest part of the alimentary tract. Digestive enzymes of the honey bee midgut can function across a range of pH but

optimally at pH = 8. Thus, the proventricular mechanism coupled with a drastic change in pH between the social stomach and the midgut demarcates two major microbial niches, one co-evolved with liquid transfer and food storage, and the other co-evolved to reside in the enzymatically active and relatively nutrient-rich midgut (Fig. 1). Following the progression of food toward the posterior midgut, pH values again decrease due to increasing uric acid content as malpighian tubules remove nitrogenous waste from the hemolymph (Terra and Ferreira, 1994). Like the social stomach, the rectum is also expandable and workers postpone defecation during the winter months, provisioning this third major gut niche with many unused nutrients.

While the simple sugars present in nectar and honey may temporarily support a gut microbial community, elemental building blocks (nitrogen, phosphorous, and co-factors) from pollen protoplasm or royal jelly are necessary for microbial reproduction and persistence. The temporal and spatial availability of these nutrients will determine in part the microbial abundance and diversity throughout the hive and honey bee gut. Microbes adapted primarily to the social stomach may have limited or ephemeral access to pollen protoplasm. Evidence suggests that pollen digestion begins in the honey bee midgut, initiated in part by the transition from high to low osmotic pressure causing the pollen protoplasm to swell, a process demonstrated to rupture pollen grains in vitro (Kroon et al., 1974). However, ingested pollen is delayed while passing through the social stomach and piles up as a bulbous mass while being filtered through the proventriculus. Klungness and Peng (1984) reported that the outermost layers of dandelion pollen (*Taraxacum officinale*) were breached in the social stomach allowing access to the protoplasm. Consistent with these results, recently developed EST libraries generated from honey bee head/brain tissue indicate that cellulase is part of the honeybee exome, and expressed in the mandibular or hypopharyngeal glands of the head (Kunieda et al., 2006). Therefore, pollen digestion may begin with the application of cellulolytic saliva, and according to the ability of particular pollen species to resist enzymatic action, supply the social stomach niche with naked pollen protoplasm.

The composition and function of the microbial community inhabiting the alimentary tract may well be affected by the physiological changes and nutritional regimes associated with honey bee age and behavioral polyethism. The roles of nurse and forager are accompanied by changes in the structure and function of the salivary glands (Takayuki et al., 2009). Nurse bees possess well developed hypopharyngeal glands that synthesize major royal jelly proteins. In foragers, the hypopharyngeal glands shrink and synthesize primarily carbohydrate-metabolizing enzymes involved in food preservation such as α-glucosidase, α-amylase, and glucose oxidase all of which generate byproducts that limit microbial growth (Kubo et al., 1996; Ohashi et al., 1996, 1997). The role of the social stomach microbial community in food preservation compliments some of the functions of the honey bee salivary system. Enzymes produced by both systems split simple sugars resulting in hydrogen peroxide and a variety of organic acids, both of which possess strong antimicrobial properties. Foragers consume almost exclusively nectar and honey to meet the metabolic demands of flying (Winston, 1987). Nurse bees eat large quantities of stored pollen to meet the nutritional demands for synthesizing and secreting royal jelly to developing larva and other

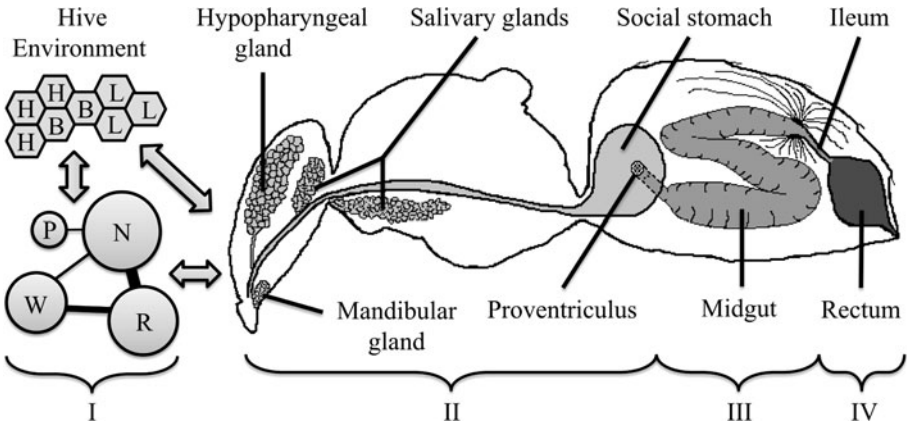

Fig. 1 The microbial niche of the hive and alimentary tract (gut). The relatively static hive environment (I) includes developing larva (L), stored honey (H) and beebread (B). The embedded network diagram (I) depicts a more dynamic niche generated through trophallactic interactions and the collection or processing by distinct worker subcastes of nectar (N), pollen (P), water (W), or royal jelly (R). Two way arrows indicate the transfer of microbes or nutrients that may support microbial growth. Niche II includes the mouth, esophagus, social stomach and associated secretory glands. The one-way valve of the proventriculus separates niche II from the digestive enzymes and more basic pH of the midgut (III). The hindgut niche (IV) is demarcated by another valve-like structure that separates the ileum and the rectum from the midgut. See text for details

adults (Crailsheim et al., 1992). This constant supply of sugars, lipids, proteins and micronutrients may supplement the microbial community of nurse bees. Alternatively, the frequency and amount of royal jelly transferred between different adult sub-castes may influence microbial persistence. While royal jelly possesses antimicrobial peptides and has inhibitory effects on the growth of some common bacteria (Romanelli et al., 2011), it is likely that bacteria coevolved to live in acidic honey bee niches can use dilute or concentrated royal jelly as a growth medium.

Honey bee microbiota

Adult gut

Using culture-based methods, Martha Gilliam (1997) was the first to note the presence of a consistent honey bee gut microbiota, occurring independent of season and geography. Nucleic acid sequencing suggests that the vast majority of these cultivated organisms represent opportunistic commensals that abound in the hive environment, but occur only sporadically within the honey bee gut. Although the molecular methods differed among the sequencing studies, results were similar, revealing a very characteristic bacterial microbiota specific to the gut (Jeyaprakash et al., 2003; Mohr and Tebbe, 2006; Babendreier et al., 2007; Cox-Foster et al., 2007; Martinson et al., 2011). The microbiota of the European subspecies *A.m. ligustica*, commonly used to pollinate US crops, was very similar to African subspecies *A. m. scutellata* and *A. m. capensis* (Jeyaprakash et al., 2003; Cox-Foster et al., 2007). The bacterial groups are unique to the honey bee, and occur independent of geography suggesting that a "core" bacterial community has coevolved with the honey bee over millions of years and now represents a relatively stable and constitutive component of healthy bees (Table 1). Additionally, the microbial abundance profile of healthy colonies was distinct from colonies afflicted with colony collapse disorder (Cox-Foster et al., 2007), suggesting that a well-balanced microbiota might be an important component of colony health.

The microbial community from the gut of the adult honey bee is composed primarily of 8 bacterial groups from five major bacterial classes that account for over 95% of the 16S rRNA sequences (Martinson et al., 2011). Three of the eight major groups are broadly classified as facultative anaerobes, tolerant of acidic environments and ferment sugars to produce lactic or acetic acid. These include two clades of the genus *Lactobacillus* (Firmicutes), which accounted for over 70% of the 16S rRNA gene sequences, and one clade of *Bifidobacterium*, estimated to be 2.8–8.4% of total bacteria in honey bees (Mrazek et al., 2008). These bacteria are considered beneficial gut inhabitants of humans and other animals and are involved in immunomodulation, interference with enteric pathogens and the maintenance of a healthy microbiota (Mitsuoka, 1992). Also abundant were two clades related to the Acetobacteraceae, one of which groups with the genus *Gluconobacter* (Martinson et al., 2011). Some of these obligately aerobic bacteria are adapted to highly acidic environments rich in sugar. They use Krebs cycle enzymes for the incomplete oxidation of sugars, ethanol, and polyalcohols, often producing large amounts of gluconic acid as end products (Ruiz-Argueso and Rodriguez-Navarro, 1975). The novel Gammaproteobacteria sequences are related to the families Enterobacteriaceae and Pasteurellaceae (Martinson et al., 2011). Members of this group are facultative anaerobes allowing them access to a wide range of host niches from aerobic and anaerobic. Some of these bacteria ferment sugars to produce lactic acid and various other end products, and many can reduce nitrate to nitrite suggesting a potential function in nitrogen metabolism within the gut. Members of the genus *Simonsiella* (Neisseriaceae: Betaproteobacteria) are strict aerobes typically found as commensals in the oral cavities of warm blooded vertebrates. These bacteria generate filaments of eight or more cells that adhere tightly to host epithelial cells. The group related to *Bartonella* (Alphaproteobacteria) has colonized many different species of herbivorous ants (Russell et al., 2009), and is considered a facultative intracellular parasite and a versatile opportunistic pathogen of animals (Jeyaprakash et al., 2003). Unlike the other bacterial groups, *Bartonella* are well adapted to the lack of carbohydrates, possessing the ability to derive carbon and energy from the catabolism of amino acids rather than glucose (Chenoweth et al., 2004).

While ignored by four of five recent sequence-based studies, fungi are often found in the adult gut. Using 18S primers, Cox-Foster (2007) identified four major groups of fungi representing a spectrum of pathogenic potential. One group consisted of well known and devastating pathogens of the genus *Nosema*, microsporidians that interfere with digestion in the mid-gut. Another is classified as Entomophthorales, a name which literally means "group of insect destroyers". Members of the other two major groups, Saccharomycotina (yeasts) and Mucoromycotina (molds), are quite common according to previous culture-based studies (Gilliam et al., 1974, 1989; Gilliam, 1979a). These groups abound in the hive environment, can often occupy the alimentary tract, and their abundance is apparently associated with seasonal change, stress and disease resistance (Gilliam et al., 1974; Gilliam, 1997). Many of these yeasts [i.e. *Candida* (= *Torulopsis*) and *Saccharomyces* spp.] frequently attain high densities in floral nectar (Gilliam et al., 1983; Herrera et al., 2009), and some possess osmotic capacities that allow them to withstand high sugar concentrations. Thus while some yeasts may be commensal, many

Table 1 Non-pathogenic microorganisms commonly sampled from the honey bee alimentary tract and hive environment

Organism	Culturable	Larval gut	Gut of adult worker	Beebread	Nectar or honey	References
Eubacteria						
Lactobacillus spp.[A]	x	x	x	x	x	a, b, c, d, e, f, h, k, l, m
Bifidobacterium spp.[A]	x	x	x	x	x	a, b, c, d, e, f, h, k
Acetobacteraceae[A]	x	x	x	x	x	a, b, c, d, e, h
Gluconobacter spp.[A]	x		x		x	a, m
Bartonella spp.[A]			x			a, b, c, d, e
Simonsiella spp.[A]		x	x			a, b, c, d, e
Gammaproteobacteria[A]	x		x			a, e, f, h
Pseudomonas spp.	x		x	x		d, h, j
Bacillus spp.	x	x	x	x	x	d, g, h, j, k
Serratia spp.	x	x	x			c, d, e, f
Streptomyces spp.	x	x			x	h
Leuconostoc spp.	x	x	x		x	d
Propionibacterium spp.	x	x				c
Enterococcus spp.	x		x	x		h, j, l
Fungi						
Penicillium spp.	x	x	x	x	x	h
Aspergillus spp.	x	x	x	x	x	h
Candida spp. *(Torulopsis)*	x		x	x	x	h, i
Saccharomyces spp.	x		x	x	x	b, h, i, k
Cryptococcus spp.	x		x	x	x	h, i
Mucor spp.	x			x		b, h

References a–g represent taxa designations based on 16S sequences. a. Martinson et al. (2011), b. Cox-Foster et al. (2007), c. Mohr and Tebbe (2006), d. Babendreier et al. (2007), e. Jeyaprakash et al. (2003), f. Olofsson and Vasquez (2008), g. Evans and Armstrong (2006), h. Gilliam (1997), i. Sandhu and Waraich (1985), j. Kachaniova et al. (2004), k. Rada et al. (1997), l. Audisio et al. (2010), m. Ruiz-Argueso and Rodriguez-Navarro (1975)

[A] Considered the "core" gut microbiota

are transported from flowers to the hive environment where they compete for sugar resources with the indigenous microbiota. Although considered opportunistic pathogens, certain yeasts may have beneficial effects, fermenting food stores when the bacterial flora has been compromised. Yeasts are also capable of synthesizing many of their own vitamins (i.e. B vitamins; Egorova, 1971), which may supplement both honey bee metabolism and the associated microbiota.

Larval gut

The larval stage of the honey bee life cycle is the target of many major pathogens including European and American foulbrood, stonebrood and chalkbrood (Seeley, 1995). Culture-based work indicates that the minority of larva contain microorganisms, suggesting that the presence of microbes in honey bee larvae is due to unwanted contamination (Gilliam, 1971; Gilliam and Prest, 1987). However, these results may be biased due to the limitations of culture media. Under harsh conditions, some acidic acid bacteria can enter into a viable but non-culturable state (Millet and Lonvaud-Funel, 2000). Larvae are initially fed with hypopharyngeal gland secretions (royal jelly), but this secretion is subsequently mixed with pollen, glandular material and nectar (worker jelly). Royal jelly is considered highly antimicrobial possessing a pH between 3.6 and 4.2, and many peptides active against gram-positive and gram-negative bacteria, fungi and yeasts (Bilikova et al., 2001; Fontana et al., 2004). Microbes from larval feces grew best in acidified media (Gilliam and Prest, 1987), indicating that the larval niche selects for acid-tolerant microbes. Microbial communities in the larval gut differ dramatically from those of adults (Gilliam, 1997; Evans and Armstrong, 2006), but some differences may be due to the lack of direct-sequencing information from larva. Larval bees have no social stomach and fewer niches for microbial establishment. *Bacillus* spp. were the primary microorganisms detected in larvae, most of which belonged to the *Bacillus cereus* group (Gilliam and Prest, 1987; Evans and Armstrong, 2006). *Bacillus* spp. may represent an ancient co-evolved relationship for the honey bee, as DNA sequences

recovered from the abdominal tissue of an extinct stingless bee (25–40 MYA) are closely related to sequences of bacterial species associated with the modern honey bee (Cano et al., 1994). *Bacillus* spp. can survive harsh conditions by sporulation, and have antibiotic properties against bee pathogens (Alippi and Reynaldi, 2006; Evans and Armstrong, 2006), producing bacteriocins or bacteriocin-like compounds directed toward species that tend to share the same ecological niche (Katz and Demain, 1977).

The hive as a microbial niche

Although the honey bee hive has evolved to thwart the growth of pathogenic bacteria and fungi, thousands of microbial strains have been described from the hive environment (Table 1). The wax comb is a highly permeable medium that does not support microbial growth, but likely affects microbial balance. Wax accumulates particulate matter, larval feces, shed exuviae, lipophilic chemicals and environmental microbes. Thus, the wax serves as a bioindicator, because foragers experience environmental conditions over a range of many miles, (or in the case of managed bees many hundreds of miles) and import all manner of agricultural chemicals and potential pathogens back to the hive where these substances endure in the wax and food stores (Mullin et al., 2010). The honey bee lines the nest with a thin layer of plant resins mixed with saliva and wax, a substance called propolis (Meyer, 1956), with antibacterial, antifungal, and antiviral properties (Kujumgiev et al., 1999). The presence of propolis confers a type of "generalized social immunity" to individuals within the hive (Simone et al., 2009; Evans and Spivak, 2010) essentially sterilizing the nest structure that houses developing young and food stores.

Because food sources (blooming flowers) are typically ephemeral, the honey bee stockpiles massive nutrients for brood rearing and overwintering, and has evolved behaviors and physiology to protect these nutrients from microbial invasion. The honey bee derives all of its nourishment from collected pollen and nectar, and these stored food products develop according to a very specific regime. Collected pollen is mixed with saliva containing enzymes, microorganisms from the honey bee social stomach, and nectar, a mixture that becomes "beebread", a nutrient storage medium resistant to invasion by pathogenic microbes (Human and Nicolson, 2006). The growth and development of the colony relies heavily on this process because the stored beebread is later consumed by nurse bees and converted to royal jelly, a pre-digested and nutrient rich food distributed throughout the colony to the queen, workers and developing larva (Winston, 1987). Nectar becomes a stored carbohydrate source, honey, used to fuel colony metabolism. Pure honey (<18% water content) eventually kills or inactivates the growth of both beneficial and pathogenic microbes due to mechanically driven water loss from nectar resulting in unfavorable osmotic conditions (Winston, 1987) and acidic pH, a byproduct of honey bee salivary enzymes and microbial fermentation. However, honey is hygroscopic, and the microclimate created by the nest architecture and its densely packed and respiring residents counteracts evaporative water loss, providing a moist thermal refuge for many microbes. Ripening honey with as little as 30% water tends to ferment suggesting that the behaviors and physiology that contribute to evaporation are critical to the microbial balance of the hive.

Water homeostasis at both the individual and colony level can have a strong influence on microbial balance. Water is not stored in the nest, but it is collected continually for evaporative cooling and producing brood food high in water content (Winston, 1987). This delicate and dynamic water balance generates a fluctuating niche for microbial growth where moisture and nutrients are available, and osmotic conditions are more favorable than that of pure honey. There is also a genetic propensity for individual honey bee foragers to collect water or nectar (Hunt et al., 1995) helping to insure that water collection does not dilute the more concentrated nectar being collected and processed by the colony (Kuhnholz and Seeley, 1997). This indicates that relatively distinct trophallactic networks are involved in the management of water and nectar, suggesting that microbial niche and community composition may vary by behavioral sub-caste.

The social stomach of the honey bee has evolved to retain fluids, facilitating the sharing of colony resources among nestmates including nectar, honey, water, and protein rich jelly (Crailsheim, 1998). As a microbial niche, the collective social stomach of the hive is in constant nutritional flux due to an age based division of labor and the genetic propensity of individuals to forage for, or process, nectar, pollen or water. This niche is also the first line of defence against environmental microbes vectored from the foraging environment. The social stomach is home to a complex lactic-acid bacterial flora which is also found in larvae, unripe honey, pollen from workers' corbiculae and stored pollen (Olofsson and Vasquez, 2008). It is hypothesized that these microorganisms contribute to a number of processes throughout the hive environment including disease resistance and food storage (Olofsson and Vasquez, 2008; Audisio et al., 2010; Forsgren et al., 2010). The frequency of trophallactic exchange and the ability to sense a well balanced microbial community may actively direct some nest mate encounters. There are far more trophallactic interactions than those necessary to efficiently distribute food (Free, 1957; Korst and Velthuis, 1982), suggesting that the high rate of exchange serves a purpose beyond food distribution. It is possible that there is a honey bee sub-caste

wherein the symbiotic bacteria that aid in disease resistance and food preservation is best nurtured, and this sub-caste acts as a bioreactor for microbes that can then be exchanged with other behavioral sub-castes, regulating the microbial balance at the colony level. Additionally, individuals of a given behavioral or genetic caste may have differential capacities to upregulate salivary genes with antimicrobial and food preservation functions in response to a compromised bacteria flora. It is clear that a microbial perspective on colony health involves many levels of organization, from the proximate effects of beneficial symbiotic microbes inhabiting the gut, to the physiological and behavioral activities that build, nurture, stockpile, and maintain the hive environment.

Stored pollen or "beebread"

During times of pollen dearth, beebread stored in the hive becomes the colony's sole source of proteins, lipids, minerals and vitamins. Within the hive environment, it is primarily beebread that is microbially active. Gathered pollen undergoes a dynamic process of microbial succession to become beebread. It is largely unknown whether beebread is simply a means of preserving nutrients, or serves an important function in nutrient processing. As pollen is packed into baskets (corbiculae) for transport, it is inoculated with social stomach microbes, some honey or nectar, and honey bee derived salivary enzymes. The same species of bacteria and yeasts found in corbicular pollen also occur in beebread and the guts of workers (Gilliam, 1997). Pollen collected from a flower changes both microbiologically and biochemically immediately following collection, a process thought to occur primarily via fermentation of added sugars by *Lactobacillus*, *Bifidobacterium*, Acetobacteriaceae and yeasts (Foote, 1957; Haydak, 1958; Ruiz-Argueso and Rodriguez-Navarro, 1975; Gilliam, 1997; Olofsson and Vasquez, 2008). In the hive, corbicular pellets are quickly deposited into cells where food processing bees pack them tightly, often layering regurgitated social stomach contents between packing events. The collective result is increased sugar content, lower pH, and decreased oxygen availability. As a specific example, individual *Aloe* pollen grains swell in beebread reflecting increased water weight and carbohydrate content, but a decrease in crude protein and lipid content (Human and Nicolson, 2006). While the outermost pollen layer is lipid-rich and contains some proteins (Pacinia and Hesse, 2005), protein decreases recorded in the *Aloe* assay only seem possible if the pollen protoplasm has been somehow accessed and altered biochemically. Consistent with this assumption, bacteria and fungi isolated from beebread produce various enzymes, vitamins, antimicrobial substances, organic acids, and lipids that may contribute to the conversion or stabilization of pollen to beebread (Gilliam, 1997). Potentially beneficial fungi in the hive environment (e.g. *Penicillium* and *Aspergillus* sp.) have been shown to inhibit the growth of highly pathogenic hive fungi (Gilliam et al., 1988), and may also produce antibiotics that contribute to the beebread storage/conversion process. Yeasts subsist in beebread longer than other organisms (Gilliam, 1979a) as they are facultative anaerobes tolerant of acidic pH. Following fermentation, fungi and spore forming *Bacillus* spp. are the predominant microbes recovered from beebread (Gilliam, 1979a, b). Products produced by *Bacillus* spp. may aid in beebread preservation, while fungi may continue to slowly digest the pollen potentially altering the nutritional quality (Gilliam, 1997). The ecological succession of beebread may be analogous to the aging of certain cheeses (McSweeney, 2004), whereby secondary microorganisms (beneficial molds) colonize the surface of cheese following fermentation, and act as a microbial barrier, preserving the internal contents.

Microbial symbioses and disease resistance

The most important immune-related function of a gut microbiota may be the ability to obstruct colonization by pathogens, thereby preventing enteric infections (Berg, 1996). The relationship between symbionts, pathogens and honey bee disease resistance is complex. Of the two primary microsporidia pathogens that infect *Apis mellifera*, only one activates the immune system (*Nosema apis*), while the other (*Nosema ceranae*) actually suppresses immune response (Antunez et al., 2009). However, a number of honey bee endosymbionts inhibit the growth of *N. ceranea* (Evans and Armstrong, 2006), suggesting that endosymbionts are co-opted to battle this particular pathogen. Actinobacteria and *Lactobacillus* found in association with bees that store large quantities of honey and pollen (*Apis* and *Trigona* spp.) also inhibit the growth of major honey bee pathogens (Promnuan et al., 2009; Audisio et al., 2010; Forsgren et al., 2010). Certain immunity factors in are positively correlated with larval development, while others are not (Chan and Foster, 2008), and foragers respond poorly to bacterial challenge, showing a pronounced reduction in immune response (hemocyte number) relative to younger nest bees (Bedick et al., 2001; Amdam et al., 2005). Such differences may be associated with changes in nutrition and metabolism, as well as the microbial succession of symbionts that accompany developmental and behavioral caste transitions.

The complexity of an insect immune system appears to be related to many factors including life history traits, hygienic behavior and the size and composition of its symbiotic microbial community. It may be that the functional capacity of a social insect immune system is

correlated with the effectiveness of both hygienic behaviors and microbial symbionts in dealing with disease. Mounting an immune response has been shown to be costly at the level of the individual bee, as well as the colony (Schmid-Hempel, 2005; Evans and Pettis, 2005). Why would a host organism evolve an extensive immune system if symbiotic microbes have co-evolved to protect it from pathogens? For example, the pea-aphid (*Acyrthosiphon pisum*) maintains a complex community of endosymbionts that protect it from variety of pathogens (Moran et al., 2005). The genome of the pea-aphid reveals the lowest number of immune-related genes in any insect sequenced thus far. Because the aphid lacks hygienic behavior, this finding led the authors to speculate that microbial symbionts must play a large role in aphid immunity (Gerardo et al., 2010). The honey bee genome has more immune-related genes than the pea-aphid, but when compared to *Drosophila*, *Anopheles* and *Nasonia*, contains only about one-third of the genes involved in innate immunity and detoxification, roles that may be assumed by honey bee hygienic behavior and/or the beneficial microbial community (Evans et al., 2006). Additionally, a general immune function of many animals involves the production of lysozyme, which indiscriminately cleaves the peptidoglycan building blocks of bacterial cells walls (Jolles and Jolles, 1984). Genes encoding lysozyme are much reduced in the honey bee relative to organisms that have adapted to digest bacteria like *Musca* and *Drosophila* (Kunieda et al., 2006), and the expression of lysozyme in the honey bee is limited largely to the hemolymph, reducing contact with potentially beneficial gut microbes.

Anti-viral immunity is poorly understood in insects (Strand, 2008), but in humans it seems that viruses can actually benefit their host. Healthy humans harbor an abundance of latent (inactive) viruses, some of which represent symbiotic relationships that confer host resistance to major bacterial pathogens (Barton et al., 2007). There are over 25 honey bee viruses known (each with many variants), differing in their abilities to cause disease, and most occur asymptomatically in honey bee individuals and colonies (Chen and Siede, 2007; Ribiere et al., 2008; Genersch and Aubert, 2010; Runckel et al., 2011). Of queens sampled from typical apiary conditions, 93% were infected with multiple viruses without showing signs of disease (Chen et al., 2005). Conversely, viruses evolve quickly, and one widespread survey identified an emergent virus that was significantly correlated with CCD (Cox-Foster et al., 2007). Another large-scale study on migratory bee keeping identified four novel honey bee viruses and demonstrated highly episodic viral incidence (Runckel et al., 2011). Such findings suggest that viral agents are a pervading yet largely unknown and highly unpredictable component of honey bee microbial ecology. Similarly, viruses that target beneficial bacteria (bacteriaphages) represent an unstudied and potentially rich research area. Metagenomic sampling reveals that phages are likely the most abundant and diverse "organismal" classes present in any microbial community and also possess the capability to regulate microbial abundance (Shapiro et al., 2010). The propagation and rapid evolution of phages is a persistent problem for the food industry, because the activation of a typically dormant phage can result in the complete loss of the *Lactobacillus* driven fermentation process (see Shimizu-Kadota et al., 1983). Similarly, the phage-induced mortality of lactic-acid bacteria associated with the honey bee could cause major problems for the storage and nutritive value of honey and beebread, as well as the ability of the hive to fend off disease.

Managing the microbiota

Pollination by managed honey bees is critical to agriculture, but their populations have been declining for decades. While an acceptable (typical) overwintering loss in the US is around 14%, winter colony losses for the last 5 years (2007–2011) average 32% according to research by Penn State and the USDA-ARS. This 4 year trend indicates an unsustainable economic situation for commercial beekeepers. Particularly in the US (FAO, 2009) managed honey bees are under constant attack by bacterial, fungal, viral and protozoan diseases, parasitic mites (Genersch et al., 2010), environmental contaminants and stress generated by constant migration, all of which may contribute to the recent bee disappearance (CCD). Although the cause of CCD is still a mystery, research has lent credence to the hypothesis that CCD is a syndrome caused by many different factors working in combination or synergistically (Watanabe, 2008). Although the symptoms of CCD (also called disappearing disease or autumn collapse) have been documented since 1915 (van Engelsdorp and Meixner, 2009), the recent colony losses are staggering, and many anticipate even greater losses in the future. The time has come to take a fresh look at the agricultural practices of managed ecosystems, and in the case of the honey bee, the combined effects of chemical stress, nutritional state and disease progression on microbial symbioses. Many now believe that such combined stresses predispose the honey bee to viral attack, accelerating the process of colony collapse (Oldroyd, 2007).

Honey bees used for commercial pollination purposes must cope with many different biocides added directly to the hive, or applied to crops (Mullin et al., 2010) that could affect their health directly or indirectly by modifying the microbial community. Broad spectrum antibiotics and fungicides applied directly to the hive to control disease infections also reduce populations of non-target fungi and bacteria. These agents likely generate an imbalance in the beneficial microbiota of the hive (Charbonneau et al., 1992),

potentially lengthening recovery time following treatment. A recent study has documented extraordinary levels of miticides and agricultural pesticides in the bee, wax, pollen, and beebread from honey bee colonies across the United States (Mullin et al., 2010). Seven different pesticides on average were found in beebread, an alarming result considering the importance of this food storage medium for colony nutrition. Even more alarming, the synergistic effects of different biocides on the microbiota of the colony are entirely unknown (Mullin et al., 2010). The opportunistic hive microbe *Nosema* in concert with a common insecticide has been demonstrated to weaken colonies, and lead to greater mortality than either agent in isolation (Alaux et al., 2010). To lessen the potential for such toxic cocktails, commercial beekeepers can provide their bees with refugia composed of diverse and untainted pollen and nectar sources, in essence rotating bees between toxic, semi-toxic, and non-toxic environments. As a final step, a substantial decrease in the generalized nature of biocides applied to the hive and pollination environment may be needed for system sustainability.

Commercial beekeepers may have no other choice than to constantly monitor and manage the microbial communities of their bees. Although their numbers fluctuate throughout the year, many natural honey bee pathogens appear to have a ubiquitous presence in healthy hives (Runckel et al., 2011) suggesting that the honey bee and its beneficial microbiota are subject to constant pathogen challenge. The pathogenic potential of commensal hive fungi is largely unknown, but may be mediated in part by lactic-acid bacteria. For example, the increased prevalence of yeasts may indicate a compromised microbial flora (Gilliam et al. 1974; Rada et al., 1997). *Sacchromyces* spp. and similar yeasts (i.e. *Candida*) are direct competitors for the niche occupied by *Lactobacillus kunkeei*, the bacterial strain most commonly cultured from the honey bee social stomach. This microbial battle is well-known to the wine industry wherein *S. cereviseae* fermentation is halted by *L. kunkeii* contamination. The introduction of transgenic gut bacteria is one possible strategy for controlling yeasts and other disease, and gram positive species like *Lactobacillus* are amenable to genetic transformation. However, the introduction of new genes to a poorly understood microbial system could have drastic consequences. A detailed knowledge of bacterial colonization, persistence, transmission and overall community function is needed if such a strategy is adopted. In the short term, it may become necessary to supplement or replace a compromised microbial community with beneficial microbes (probiotics) or treatments that support beneficial microbial growth (prebiotics). Researchers and probiotic hobbyists are experimenting with many substances to promote honey bee microbial health, including sprayed applications of bacteria–fungal cocktails that produce organic acids and a low pH that inhibits microbial growth. This "grass-roots" approach has merit in the sense that probiotics should be considered from the point of view of community ecology. It is typically the balanced nature of a microbial community that provides maximum benefits to the host. As such, a primarily symbiosis between a fungus and bacterial strain may have similarities to the microbial succession that processes and/or preserves nutrient-rich beebread. Given that hives treated with lactic and acetic acids prevent pathogen infections, lactic and acetic acid bacteria native to the honey bee are obvious starting places for probiotic development as they are highly effective in colonizing the sugar rich digestive system and hive environment (Hamdi et al., 2011).

Research perspectives

Present research programs are using a variety of approaches to find the cause or combat the symptoms of CCD. The recent honey bee decline calls for increased long term monitoring of both infectious (see Runckel et al., 2011) and non-infectious microbial processes within honey bee colonies. We believe that a strategy focused on how the natural occurring microbiota contributes to colony health and nutrition is paramount for the development of a sustainable system. Many questions concerning the natural state of a healthy microbial community will be difficult to address due to the putatively facultative nature of most honey bee symbioses, and the complexity and plasticity of the social system. Linking the microbiota to its functional role is critical because functional stability may be achieved despite a large variation in the microbial population size or composition. A key question becomes: which symbionts possess the capability to occupy more than one niche within the host or hive? The same bacteria may thrive and provide benefits in one niche, but barely cling to life and prove detrimental in another. Functional genomic analysis can be used to determine the genes involved in switching between different niches (i.e. food stores and the social stomach). Some points to consider are: how do microbial communities develop in concert with the dynamic nature of the abiotic and biotic environment; how does the microbial community change as a honey bee ages and the colony grows; and how does honey bee genotype, geography or diet affect microbial composition?

One of the challenges before researchers is devising novel ways to examine microbial function both in vivo and in vitro. These include new methods to culture and isolate hard to culture organisms for follow-up functional genomics studies (Tyson and Banfield, 2005). Culture media used for the isolation of gut bacteria are typically the same or modified slightly from those used in environmental or

medical studies. Media should be designed according to environmental factors typically encountered in the honey bee gut or hive environment. Many gut symbionts have very specific growth requirements, and careful manipulation of nutrients, pH and atmospheric gases are often necessary to grow and isolate such microbes. Microbes evolved to co-exist with the honey bee should multiply in a particular hive or gut niche at a rate that equals or exceeds their rate of elimination. Many microbes will only colonize or express "community living" genes when much of the community is present in a biofilm environment (Dillon and Charnley, 2002; Sauer et al., 2002), a barrier that may be overcome through the generation of synthetic biofilms. Similar to the biofilm environment, population density also alters the production of metabolic products, and has strong implications for the way in which microbial communities will be characterized (Dillon and Dillon, 2004). Given that honey bees emerge as relatively germ free adults (Gilliam, 1997), and many of their symbionts can be cultured, the in vivo introduction of simplified bacterial communities will facilitate the modeling of beneficial and antagonistic interactions. Gene expression analyses will provide information on the microbial succession of the alimentary tract, and the response of the host to colonization. These experiments can be supplemented with in vitro studies.

There is a need to develop additional diagnostic tools that not only monitor and detect the presence of pathogens, but also provide rapid information on potential disease precursors like compromised microbial communities and colony-wide nutritional status (see Feigenbaum and Naug, 2010). Within the growing field of transcriptomics, custom, PCR-based, or whole genome microarrays have dramatically accelerated many research approaches. Performed on a broad scale, this systems based approach can provide more focused hypotheses for carefully controlled experiments. Evans (2006) designed a targeted PCR-based array consisting of 48 probes to measure the expression of genes associated with honey bee individual immunity and the presence of major pathogens. Johnson et al. (2009) used a whole genome microarray across a wide geographical swath, demonstrating that expression levels clustered according to colony strength, CCD phenotype, and geography. More recently, Runckel et al. (2011) designed a custom microarray containing known bee pathogens and a wide variety of other arthropod-associated pathogens. Collectively these results suggests that to be effective, a diagnostic survey must sample numerous bee populations across space and time due to the large variation in gene expression across geography, and seasonal fluctuations in typical pathogen loads. The development of a custom microarray to assess both colony nutritional state and beneficial microbial function is in process, and will provide a timely and necessary perspective on honey bee health.

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