



Symbiotic solutions to nitrogen limitation and amino acid imbalance in insect diets

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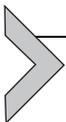
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Abstract

Although comprising ~80% of the Earth's air, nitrogen (N), in a usable form, is a limited resource for herbivorous insects. Additionally, much of the consumed dietary N is lost through-excretion, further challenging such insects, while limiting their growth and reproduction. To meet this challenge, many insect groups have evolved symbiotic relationships with microbes, enabling success within otherwise inhospitable niches. These mutualistic microbes contribute to their hosts' N-economies through nitrogen recycling, fixation and/or upgrading, using divergent metabolic strategies with varying precursors and physiological requirements. In this chapter we highlight each strategy, organizing our discussion by insect taxonomy, while enumerating the various microbes and innovations that have converged upon N-centric, nutritive functions. Our overview places a large emphasis on N-recycling, due in part to a lack of recent reviews on this topic. In reviewing N-fixation, we take a termite-centred approach, capitalizing upon an extensive bank of research performed across several decades. We also emphasize essential amino acid (EAA) and precursor biosynthesis, discussing non-fixing, non-recycling mutualisms in which symbionts make up for dietary shortcomings and missing pathways for nutrient biosynthesis in their hosts. In these explorations, we discuss the specificity and evolutionary histories between herbivorous insect hosts and their often ancient N-metabolizing symbionts. We focus, further, on correlations between dietary evolution and altered symbioses, which cast light on the causes and consequences of these nutritional relationships. We, finally, describe the evidence supporting prior arguments for N-centric mutualisms, emphasizing how a pairing of genomics and experimentation can uncover mechanism, while pinpointing just how symbiont metabolism shapes the fitness and N-budgets of diverse, herbivorous hexapods.



1. Introduction

Nutrient-limitation, especially nitrogen (N), is central to the ecology of many organisms. Comprising 78% of dry air, this element is environmentally plentiful, but not in forms usable by plants and animals. Needed in large quantities to build cells, tissues, and their building blocks, N is primarily found in the form of proteins, and their constituent amino acids (AAs) when measured inside living organisms (Behar et al., 2005; Janssen et al., 2017; Nardi et al., 2002). Other N-containing molecules consist of nucleotides, hormones, vitamins, and—for many arthropods—the chitin molecules that are used to build the cuticle (Elser et al., 1996; Finke, 2007, 2015). In the face of high demand, a scarcity of usable N can be a limiting factor for growth, reproduction and survival (McNeil and Southwood, 1978).

While potentially limiting under some conditions for predators (Denno and Fagan, 2003), N-limitation is paramount for many insect herbivores,

whose plant-based diets lack sufficient overall quantities, or sufficient quantities of essential N-metabolites that animals cannot make themselves (Mattson, 1980). In response, insects, like other animals have evolved a range of mechanisms for efficient acquisition and conservation of this element (summarized in Mattson, 1980; McNeil and Southwood, 1978; for a few examples see Fig. 1). These include prolonged development, high rates of feeding (Kheirallah, 1978; Slansky and Feeny, 1977), as well as manipulations of food plants (e.g. galls) that engender higher nutritive content (Sandström et al., 2000). Some insects use seasonal shifts in consumed plant varieties, or in the tissues of preferred food plants, to coincide with peak periods of N-availability (McNeil and Southwood, 1978). Others increase dietary N through diet mixing, including coprophagy (faecal consumption),

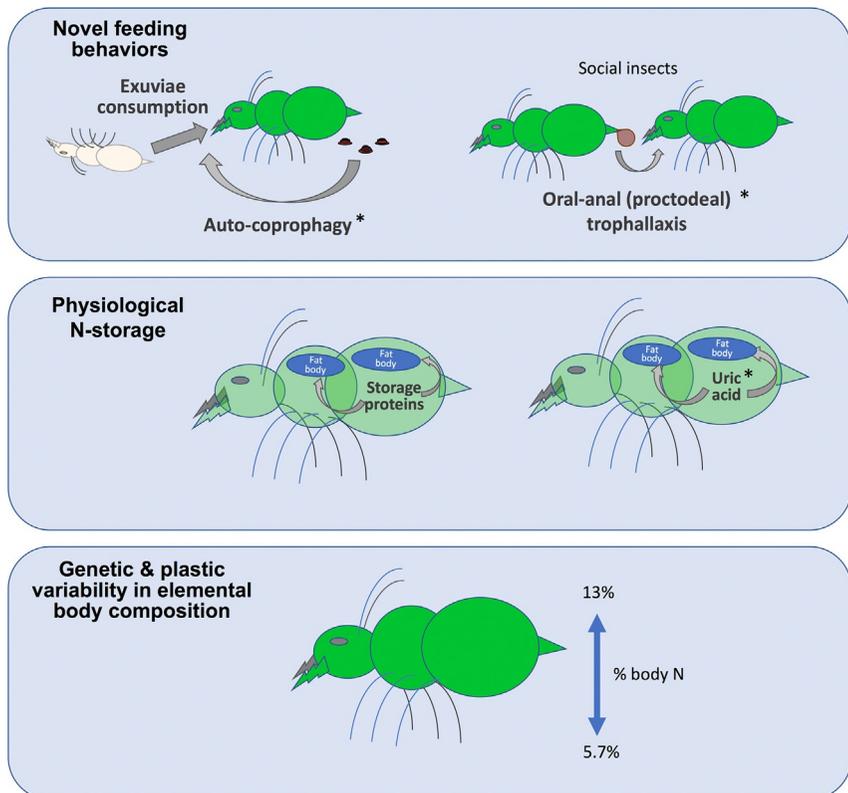
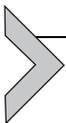


Fig. 1 Insect-evolved strategies to improve N-economies. Asterisks indicate strategies that evolved in the context of interactions with microbes, often internally housed symbionts. Several other host-evolved N-conserving and N-acquiring mechanisms are discussed in the text and are reviewed elsewhere (e.g. Mattson, 1980).

proctodeal trophallaxis (consumption of liquids from the anus), supplementary predation or cannibalism, and consumption of shed cuticles (Denno and Fagan, 2003; Machida et al., 2001; Mira, 2000; but see Clay et al., 2017). Physiological mechanisms aiding this endeavour include the storage of proteins or uric acid in the fat body (Burmester, 1999; Martinez and Wheeler, 1994; Mullins, 2015) and modestly flexible N content of the insect body plan (Fagan et al., 2002).

While endogenously controlled, the efficacy of some aforementioned mechanisms relies on exogenous parties, namely microbial symbionts. Housed either within the insect gut, hemolymph, or specialized host cells, and often involving eubacteria or fungi, such symbionts draw from biochemical pathways that are common in microbes but absent from insects. These metabolisms allow symbionts to concentrate, conserve, and improve the forms of N-containing nutrients to the direct benefit of insects (Douglas, 2009). Tightly integrated, occasionally ancient, and highly specialized nutritional mutualisms applying these mechanisms are posited as major evolutionary innovations for several herbivorous insects (Bennett and Moran, 2015).

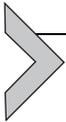
In this chapter we discuss the diversity of ways by which microbial symbionts acquire and metabolize N, and how their insect hosts obtain symbiont-synthesized N-containing compounds. We highlight the dietary niches that are correlated with the maintenance of such symbioses, along with the physiological, anatomical, and behavioural modifications that support them. We also discuss how varying methodologies have shaped our understanding of established or hypothesized N-provisioning symbioses, highlighting key knowledge gaps in need of further study.



2. Symbiotic solutions: A primer

Nutritional symbionts are common in insects with N-imbalanced or N-limited diets, whether they consume live or dead plant tissue. The mechanisms enabling symbiont contributions to host N-budgets vary, necessitating different morphological and physiological adaptations, but at least three general strategies are utilized (Fig. 2). In *nitrogen recycling*, bacteria or fungi conserve nitrogenous wastes obtained through the insect's metabolism, or the diet, converting waste products into forms accessible to insects. In another strategy, *nitrogen fixation*, bacteria derive forms of N accessible to insects through energy-intensive fixation of atmospheric dinitrogen (N_2). In the third, *amino acid (and precursor) provisioning*, symbionts synthesize essential amino acids (EAAs) or precursors for these molecules, which their hosts

cannot make, occasionally through use of a process termed N-upgrading. N-fixers and N-recyclers often fulfil this latter function, after assimilating inorganic nitrogen, but intracellular symbionts from numerous hemipteran insects make major contributions through EAA and precursor biosynthesis without apparent fixation or recycling of N. In doing so, they address amino acid imbalance but potentially not the overall shortage of N in host diets.



3. Nitrogen recycling symbioses

One major way by which microbes improve host N-economies is through conservation. Towards this end, some symbionts plug into their hosts' waste management systems to acquire, catabolize, assimilate, and upgrade compounds otherwise destined for excretion (Fig. 3). Others utilize typical insect N-wastes obtained from the diet to scavenge N from molecules that would otherwise pass through undigested. Like other animals, insects

Fig. 2 Symbiont contributions to host N-budgets and amino acid pools require varying means of physiological integration. *Top panel*—Some bacteria or fungi colonizing the gut lumen (*or hemolymph or fat body) recycle N-waste products obtained from the diet or host metabolism. Such activity may be mediated by delivery of wastes through Malpighian tubules (mt) into the hindgut or ileum (Ile). But for fat body bacteriocyte-colonizing symbionts (e.g. *Blattabacterium* of cockroaches), host derivation of urea from fat body stores of uric acid may be key. Delivery mechanisms for another N-recycler, midgut (mg) bacteriocyte-colonizing *Blochmannia*, remain unclear. *Middle panel*—The first example of AA/precursor provisioning. Bacteriocyte (Ba) colonizing microbes receive metabolites from hosts, including glucose, that are transformed (e.g. via glycolysis) into essential amino acid (EAA) precursors using metabolic pathways absent from animals. After hand-offs back to hosts, insect-encoded enzymes add N to complete the terminal steps in EAA synthesis. We use the analogy of two chefs, making lemonade together and providing varying ingredients/materials. *Third panel*—The second example of AA/precursor provisioning. Hosts provide trans-aminating N-donor molecules to symbionts, including non-EAAs (e.g. glutamate or glutamine), through bacteriocyte-expressed transporters. Symbionts use these to make EAAs in a process termed N-upgrading. This is common for symbioses with intracellular bacteria in hemipterans. We use the analogy of 1 chef (the symbiont) making lemonade out of lemons (with the aid of sugar and a juicer). *Bottom panel*—Bacteria in the gut lumen (*and possibly the hemolymph) fix atmospheric N, provisioning fixed N to hosts as AAs. Innervation of gut tissues by tracheae (tr) delivers atmospheric gas containing N, but also nitrogenase-inhibiting O₂, suggesting the importance of O₂ consumption by gut wall-associated microbes. For all extracellular gut symbioses, regardless of N-metabolic mechanisms, hosts can obtain nutrients by gut absorption after symbiont export. But some hosts obtain nutrients by digesting symbiont cells.

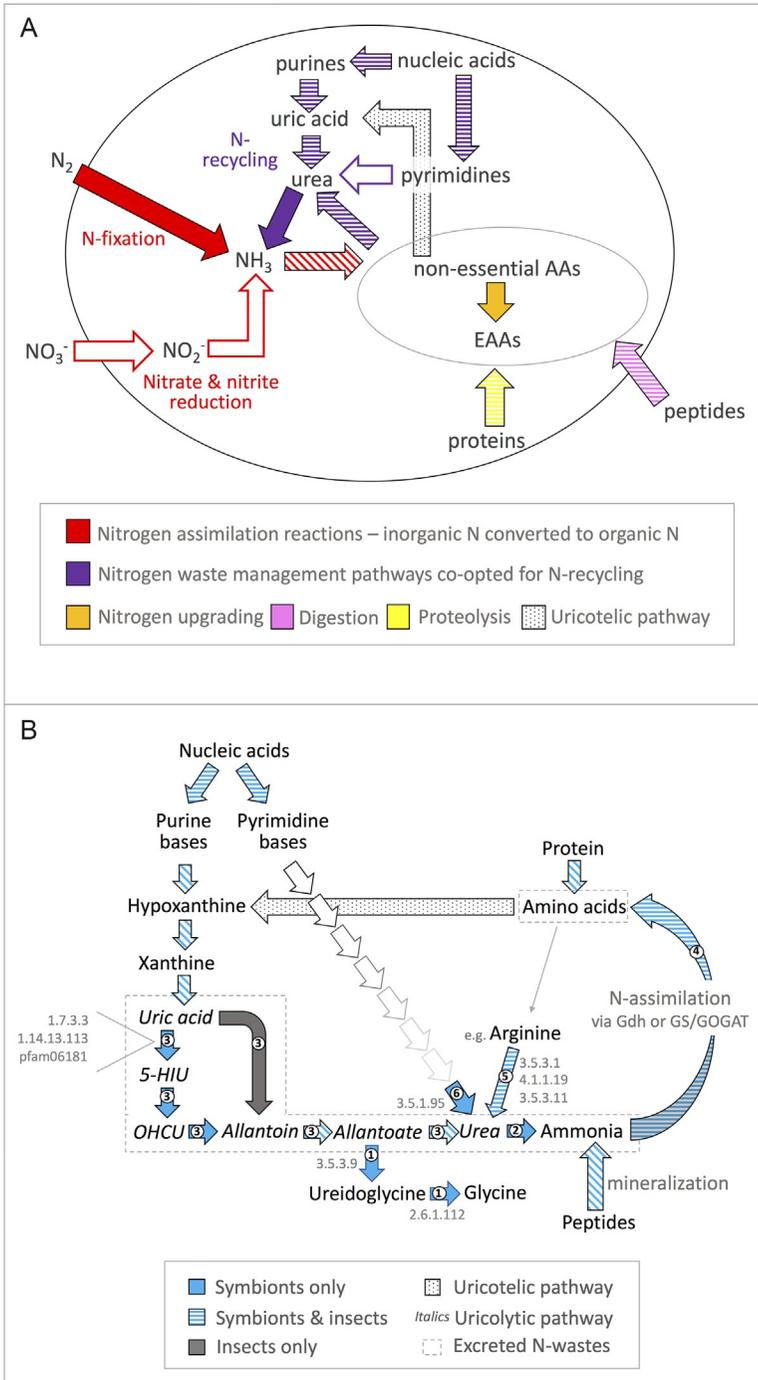


Fig. 3 See legend on next page.

produce a range of metabolites enabling removal of excess N (Bursell, 1967). Uric acid is one of the most common forms of waste, but some insects produce allantoin or allantoate, transporting these metabolites to the hindgut through Malpighian tubules, for eventual removal in the excreta (O'Donnell, 2008). A subset of insects can also derive urea through uricolytic pathways (Scaraffia et al., 2008) or through arginine catabolism, as part of the urea cycle (Fig. 3; Panfilio et al., 2019). Although uric acid may serve as an antioxidant in some species (Souza et al., 1997), the aforementioned waste products are of limited nutritional use to insects due to their inability to reacquire the constituent N. Microbial metabolism, in contrast, can clear such hurdles, due to urease capacities to derive ammonia and to other parts of the purine catabolism pathway assisting with the re-assimilation of waste-product N (Fig. 3). Collectively, these activities suggest several avenues for symbiont-mediated N-recycling. The range of possibilities expands with the knowledge that ammonia can also serve as the primary form of insect N-waste.

Fig. 3 Key aspects of insect and symbiont N-metabolism. (A) Terminology used in the manuscript, showing symbiont-contributed functions (coloured arrows) using inorganic (red arrows—assimilation), or organic (yellow, pink, orange, and purple) forms of N. For several metabolic transformations, insects lack the necessary enzymes (solid arrows), and for others, at least a subset can participate (hatched arrows—e.g. a subset of insects can generate urea from uric acid). Symbiont-mediated N-fixation, N-recycling, and N-upgrading are diagrammed and are of demonstrated importance to insects. Contributions through nitrate and nitrite reduction and peptide-enabled digestion, in contrast, are not yet known to be significant. (B) Aspects of N-waste/N-recycling pathways controlled by symbionts and/or hosts. The use of colour and hatched arrows generally follows the use in (A). Shown is the uricolytic pathway to derive urea from ammonia (*italicized font*) and the uricotelic pathway (polka-dot arrow), a mechanism by which amino acids and various C-metabolites are combined to make hypoxanthine. Rather than nucleic acid breakdown, this is posited to be the main way by which insects derive N-wastes including uric acid and its derivatives (Bursell, 1967). Dashed box highlights compounds excreted in some insects as waste products. Numbered circles highlight: (1) Glycine derivation from a uricolytic product, allantoate, as possibly fulfilled by symbionts of olive flies. (2) Urease activity, not encoded by insects, but fulfilled by some symbionts. (3) The uricolytic pathway encoded by a subset of symbionts and, partially, by some insects. (4) The assimilation of ammonia by GS-GOGAT or glutamate dehydrogenase, resulting in glutamine and/or glutamate for direct use or transamination (i.e. N-upgrading). (5) Arginase or arginine decarboxylase/agmatinase activity to derive urea. (6) A fairly under-studied enzyme, ureidomalonase, found in Actinomycetales bacteria, that makes urea from through pyrimidine catabolism. For these examples EC numbers are provided for key steps, when available (Pfam IDs provided, when not).

3.1 Nitrogen-recycling in termites

Among the best-studied and most fascinating groups of insects engaging in N-centric symbioses are those from the Blattodea, commonly known as the cockroaches and termites. The diets of most termites consist of dead wood or grass, but those in the “higher” termites (family Termitidae) have expanded to include cultivated fungi, detritus, and soil organic matter (Donovan et al., 2001). Arguably less is known about the diets of cockroaches. Presently, most are thought to be detritivores (Bell et al., 2007), despite common beliefs that most are omnivorous. With the exception of soil-feeding termites, and their peptide-rich diets, many cockroaches and termites require adaptations for a balanced N-economy. Conspicuous concentrations of microbes in both groups were recognized decades ago (Buchner, 1965; Leidy, 1881), and have long been hypothesized to fulfil nutritional services (e.g. Brooks and Richards, 1955).

One of the first demonstrations of nutritive symbiont function in the Blattodea was shown for the lower, wood-feeding termite, *Reticulitermes flavipes*. This insect was known to store large quantities of uric acid within the fat body, the primary site of uric acid synthesis; and uric acid stores showed impressive capacities to accumulate over time (Potrikus and Breznak, 1980a). Surveys of other “lower” termites, from the Kalotermitidae and Rhinotermitidae, showed that uric acid comprised ~1–14% of dry body mass and ~4–38% of total termite N (Potrikus and Breznak, 1980a). Since uric acid was extremely rare (0.04% of dry faecal mass) in termite excreta (Potrikus and Breznak, 1980a), and uricase activity was not found in microbe-free termite tissues of *R. speratus* (Potrikus and Breznak, 1981), gut microbes became prime suspects in a hypothesized N-recycling symbiosis.

In a seminal test of this hypothesis, Potrikus and Breznak (1981) detected high levels of uricase activity in hindgut luminal contents of *R. flavipes*, and suppression of this activity with bacteria-targeting antibiotics. The authors also cultivated uric acid-degrading bacteria from termite hindguts. And they, further, detected labelled N—derived from dietary uric acid—within termite tissues, via isotope ratio mass spectrometry. These findings illustrate that N-recycling is occurring in termites, with the authors positing that hosts mobilize and deliver their own N-wastes to gut symbionts, by way of hemolymph and Malpighian tubules. This delivery route, however, has gone unconfirmed, leaving some to hypothesize that cannibalism of uric acid-rich siblings is the key means of N-waste delivery (Slaytor and Chappell, 1994).

This above work cemented a symbiont-mediated role in the re-use of insect nitrogenous waste (Bursell, 1967). But nearly 40 years since this discovery,

the generality of this phenomenon across termites remains largely unknown. In one exception, uric acid-degrading bacteria were isolated from the guts of eight additional termite species (Thong-on et al., 2012). These termites spanned four of the six families, including the higher termite grouping Termitidae, expanding the range of hosts in which microbe-mediated N-recycling may be important. Lacking still, however, have been efforts to quantify recycling in vivo or the abundance of N-recycling bacteria within the termite gut. Also observed is that cultivated uricolytic bacteria from termite guts show high relatedness to free-living bacteria (Potrikus and Breznak, 1981; Thong-on et al., 2012). So, despite evidence for ancient specialization of many gut-associated termite symbionts (Hongoh et al., 2005), and evolved trophallaxis behaviours promoting partner fidelity (Nalepa et al., 2001), it is unclear whether N-recycling is carried out by coevolved, termite-confined microbes. Additional questions remain, as well. For example, based on NCBI searches, the genome of *Coptotermes scandus* (Kalotermitidae) encodes a uricase enzyme (accession #: XP_023702356.1; XP_023702357.1). No uricase genes were reported in the genomes of two other termite species, *Nasutitermes exitiosus* (Termitidae) and *Zootermopsis nevadensis* (Termopsidae), and our efforts to identify these through BLAST searches, using the aforementioned termite uricases, have accordingly come up negative. This suggests the potential for a variety of entry, or hand-off points, for N-recycling gut symbionts utilizing the uricolytic pathway, in vivo, within these eusocial insects (Fig. 3B).

3.2 Nitrogen-recycling in cockroaches

The N-recycling symbioses of cockroaches show several deviations from those seen in termites. To begin, the focal contributors are intracellular *Blattabacterium* symbionts, hailing from the order Flavobacteriales (phylum Bacteroidetes). Confined to cockroaches and the basal termite lineage Mastotermitidae, phylogenetic analyses have shown that these specialized bacteria have largely cospeciated with their hosts (Lo et al., 2003). After the origin of termites from within the cockroach lineage, and retention by early-branching termites, *Blattabacterium* was lost from the crown group of these eusocial insects (Bandi et al., 1995). The symbiont has, contrastingly, been retained by the majority of cockroach lineages, including asocial and subsocial species (Evangelista et al., 2019).

Blattabacterium cells colonize fat body-associated bacteriocytes, and are passed transovarially (reviewed in Mullins, 2015), supporting partner fidelity across this ancient, >200 million-year-old symbiosis

(Evangelista et al., 2019). Discovered in the late 1800s by Blochmann (1887), early research hinted at N-recycling, including the observation that *Blattabacterium*'s bacteriocyte domiciles were found next to fat body cells containing urate (uric acid) crystals (Park et al., 2013). These N reserves were depleted in cockroaches fed N-poor diets (Cochran, 1985; Mullins and Cochran, 1974), suggesting their use to sustain host N-requirements. Additional observations showed uric acid accumulates in large quantities in cockroaches deprived of *Blattabacterium*, further implicating these microbes in the breakdown of nitrogenous waste (Valovage and Brooks, 1979).

So, do *Blattabacterium* recycle N-waste to make derived products that are useful for the host? Support for this role was obtained upon sequencing the *Blattabacterium* genome (Sabree et al., 2009). While lacking genes for the breakdown of uric acid, the bacterium encodes a urease enzyme that catalyses the hydrolysis of urea into ammonia and carbon dioxide (Fig. 3B). This transformation represents the only task within the uricolytic pathway that insects are universally unable to fulfil. In this same study, the authors reported that cockroaches encode enzymes to convert uric acid into urea. Since this time, research has documented the expression of all required host-encoded N-recycling genes—uricase, allantoinase, and allantoicase (Fig. 3B)—in fat body tissues, observing their upregulation in cockroaches consuming low N diets (Patiño-Navarrete et al., 2014). Enzymatic urease activity has also been reported in the symbiont-housing cockroach fat body (López-Sánchez et al., 2009), consistent with symbiont conversion of unusable urea into usable ammonia.

To summarize, the body of work to date shows that *Blattabacterium*: (1) can generate ammonia through the metabolism of host-derived urea, (2) that it can likely assimilate this ammonia into glutamate (via glutamate dehydrogenase, *gdhA*), and (3) that it can then use glutamate to synthesize up to 10 EAAs, in part through use of this glutamate-incorporated, recycled N (Fig. 3; Henry, 1962; Sabree et al., 2009). While this detailed understanding has transformed the cockroach-*Blattabacterium* interaction into an exemplar N-centric symbiosis, important work remains. For example, while isotope-labelled N in dietary uric acid is detectable in amino acids found in cockroach oothecae (Mullins et al., 1992), it is not clear if these amino acids were confined to cells of *Blattabacterium* queued up for transovarial transfer, or if they were instead confined to cockroach tissue—indicating host acquisition. Experiments more directly testing for such acquisition, the identities of acquired nutrients that are synthesized from recycled N, and the conditions favouring such provisioning, will be useful areas for future study.

3.3 Nitrogen-recycling in ants: *Blochmannia*

The above examples in termites and cockroaches illustrate two distinct mechanisms by which symbionts recycle forms of N commonly derived from host waste metabolism. The symbionts of other insects may recycle similar waste products, or based on known variability in N-waste metabolism (Bursell, 1967), utilize other host-delivered metabolites. Ants and their symbionts help to illustrate this. In their ~150 million-year evolutionary history (Moreau and Bell, 2013), these insects (Hymenoptera: Formicidae) have evolved a variety of lifestyles during their rise towards dominance of terrestrial ecosystems (e.g. Nelsen et al., 2018). While frequently viewed as predators or omnivores, behavioural observations and stable isotope analyses suggest that many arboreal ants feed on a range of N-poor or N-inaccessible diets (Blüthgen et al., 2003; Davidson et al., 2003). Such diets include sugar-rich extrafloral nectar and hemipteran honeydew, which show either a paucity or imbalance of amino acids (Auclair, 1963; Baker et al., 1978; Fischer et al., 2002). In addition, some ants are attracted to vertebrate excreta, including N-waste-rich bird droppings and urine (Powell, 2008). This led to the hypothesis that such “herbivorous” ants may rely on nutritional activities of symbiotic bacteria (Davidson et al., 2003), and the separate origins of these diets raised the prospect of functional convergence in symbiont roles (Russell et al., 2017).

The first symbiosis used to test this hypothesis involved ants in the tribe Camponotini (i.e. carpenter ants and relatives) and their *Blochmannia* symbionts (Gammaproteobacteria: Enterobacteriales). Colonizing bacteriocytes associated with the midgut cells of their hosts, these transovarially transmitted bacteria (Sauer et al., 2002; Schröder et al., 1996) have cospeciated with camponotines for an estimated 40 million years (Degnan et al., 2004; Sauer et al., 2000; Wernegreen et al., 2009). Sequencing of the first *Blochmannia* genome revealed a capacity to synthesize several EAAs and to convert urea to ammonia via urease activity (Gil et al., 2003). Full gene sets for functional urease enzymes are encoded in all five *Blochmannia* genomes that have been subsequently sequenced (Williams and Wernegreen, 2015). In an important demonstration of this symbiont’s contributions, Feldhaar et al. (2007) showed that *Blochmannia*-infected *Camponotus floridanus* metabolize ¹⁵N-labelled urea, and that the urea-derived N is used to make EAAs and non-essential amino acids (non-EAAs) found circulating in ant hemolymph.

Capacities for amino acid biosynthesis are supported by genome sequencing across five *Blochmannia* species bracketing the diversity within

the host Camponotini (Gil et al., 2003; Williams and Wernegreen, 2015). For instance, all strains can synthesize 9 of the 10 EAAs (all but arginine), with the exception of one lacking the *ilvA* gene for isoleucine synthesis. Curiously, while most can process ammonia derived from urease activity, glutamine synthetase was lost independently in two separate *Blochmannia*. The absence of glutamate dehydrogenase and glutamine oxoglutarate aminotransferase (GOGAT) suggest a potential inability to assimilate the ammonia derived from urea catabolism (Fig. 3B)—making these symbionts highly unusual in comparison to other bacteria (Williams and Wernegreen, 2010). In examining other ammonia-adding steps from the KEGG pathways that could possibly make-up for this absence, we saw only one AA biosynthesis gene that could fulfil this capacity in *Blochmannia*—cysteine conjugate beta lyase. To our knowledge, cysteine is not a major amino group donor in the synthesis of other amino acids, unlike glutamate and glutamine, suggesting a novel means of ammonia processing in some camponotine-*Blochmannia* symbioses. Host involvement may be key, based on the precedent from the pea aphid-*Buchnera* system in which symbionts receive host-derived glutamate and glutamine for use in N-upgrading and EAA synthesis (Hansen and Moran, 2011).

Additional studies in the camponotine system give insight into aspects of host N-demand making *Blochmannia* useful. To begin, *Blochmannia* show peak abundance during pupation and immediately after eclosion (Wolschin et al., 2004). Patterns of gene expression reveal the symbiont's investment in N-recycling during and around metamorphosis. Importantly, urease genes, agmatinase (involved in deriving urea from arginine; Fig. 3B), and glutamine synthetase genes show peak expression late in larval development through early adulthood (Stoll et al., 2009; Zientz et al., 2006). Since aromatic amino acid biosynthesis genes show peak expression in mid-to-late pupation, and symbiont numbers decline in mature adults, *Blochmannia*'s N-metabolic activities have been argued to contribute towards construction of the camponotine cuticle.

As a likely N-recycling symbiosis, questions arise as to how hosts deliver N-wastes and in what form. The host *Camponotus floridanus* genome (Bonasio et al., 2010) encodes uricase, allantoinase, and allantoicase enzymes (NCBI accession #s: XP_011255385.1, XP_019883740.2, XP_025268351.1) that would collectively enable ants to generate symbiont-usable urea (Fig. 3B; Scaraffia et al., 2008). While dietary urea can reach these symbionts (Feldhaar et al., 2007), *Blochmannia*'s intracellular midgut localization, and absence from the hindgut and fat body, raise the question of whether urea

produced from host waste metabolism is the main ingredient for recycling, due to unresolved routes for urea delivery to bacteriocytes. Future research on these questions is warranted, as is work on the importance of host- and symbiont-encoded enzymes that derive urea from arginine (or its derivatives), including arginase (accession #: XP_011264771.1) and agmatinase (Stoll et al., 2009).

3.4 Nitrogen-recycling in ants: Beyond *Blochmannia* and the camponotines

Two recent studies have further supported the hypothesis that transitions to low N diets in arboreal ants have been facilitated by convergent N-metabolic activities of symbiotic bacteria (Bisch et al., 2018; Hu et al., 2018). In the first, researchers focused on *Cephalotes* turtle ants (Hu et al., 2018), which have diets resembling those of camponotines (Davidson et al., 2003), including foods with limited N (e.g. plant wound secretions, extrafloral nectar) and inaccessible forms of N (urine, bird droppings—e.g. de Andrade and Baroni Urbani, 1999). Using stable isotope labelling in manipulative dietary experiments on *C. varians*, Hu and colleagues found that N from ^{15}N -labelled dietary urea was used to make at least seven EAAs and eight non-EAAs that were eventually acquired by workers. Suppression of symbiotic gut bacteria with antibiotics erased these recycling, upgrading, and acquisition capabilities. The authors found no evidence for symbiont contributions through N-fixation, and detected only subtle degrees of symbiont N-upgrading via diet-derived glutamate.

Extracellular symbionts from *Cephalotes* ants colonize the gut environment, including the hindgut (a.k.a. ileum; Roche and Wheeler, 1997), where metabolic N-wastes from the hemocoel are delivered by Malpighian tubules. Phylogenetic analyses have illustrated that these bacteria are generally host clade specific (Hu et al., 2014), with symbiont community similarity dendrograms showing partial, but significant matching with the host phylogeny (Sanders et al., 2014). This pattern is consistent with host-microbe cospeciation and is perhaps mediated through social transmission-enabled partner fidelity (Lanan et al., 2016). Shotgun metagenomic analysis of the specialized bacteria from 17 *Cephalotes* species (Hu et al., 2018) revealed a strong degree of conservation, both in the bacterial lineages colonizing these ants and the presence of key N-recycling genes encoded in specific symbiont lineages. Bacteria from the ubiquitous symbiont genus *Cephaloticoccus*, for instance, universally encoded urease genes, allowing those tested to derive ammonia, from urea, in *in vitro* assays. Metagenomics also demonstrated that at least 13 microbiomes harboured unnamed members of the

Burkholderiales (Betaproteobacteria) encoding uricase genes. Several encoded full uricolytic pathways enabling the synthesis of urea; and cultured *Burkholderia* isolates from multiple *Cephalotes* could synthesize urea from the uric acid derivative allantoin.

In the second study of relevance beyond the Camponotini-*Blochmannia* symbiosis (Bisch et al., 2018), researchers focused on ants in the genus *Dolichoderus*; a group known for their N-limited and N-imbalanced diets of sap-feeding hemipteran honeydew (Cook and Davidson, 2006). Hailing from a different subfamily (Dolichoderinae) relative to cephalotines (subfamily Myrmicinae) and camponotines (subfamily Formicinae), these arboreal ants harbour highly conserved microbiomes largely dominated by a monophyletic group of genus-specific bacteria in the Rhizobiales (Alphaproteobacteria) showing relatedness to *Bartonella*. Metagenomes from four *Dolichoderus* species characterized these midgut-colonizing Rhizobiales (provisionally assigned to the genus *Tokpelaia*) as urease-encoding N-recyclers. Their maintenance and apparent, long-term cospeciation are arguably due to proctodeal trophallactic transfer. This mechanism mirrors those used for passaging hindgut bacteria of termites and cephalotine ants. But the localization of *Dolichoderus* symbionts raises questions about the mechanisms of waste N-delivery, like those raised for *Blochmannia* and camponotines, as their midgut domicile lies anterior to the site of Malpighian tubule-driven N-waste delivery (Fig. 2).

Experimentation to demonstrate host acquisition of recycled N in *Dolichoderus* clearly awaits, as does work to understand the importance of urease genes in related bacteria found in predatory ants (Neuvonen et al., 2016). Also, of interest will be studies on roles of hosts and symbionts in scavenging N from large urate stores in *Dolichoderus* fat body organs (Cook and Davidson, 2006). N-recycling is a posited function of symbionts in other ants, as well (van Borm et al., 2002), including some hosting Rhizobiales symbionts with relatedness to those in *Dolichoderus* (Russell et al., 2009; Stoll et al., 2007). Interestingly, a subset of Rhizobiales found in cephalotine ants encode ureases (Hu et al., 2018). So concentrations of related Rhizobiales in at least four ant herbivore groups (also *Tetraponera* and *Cataulacus*) suggests an intriguing potential for functional convergence due to N-recycling symbioses (Russell et al., 2017).

3.5 Nitrogen-recycling in Coleoptera and Diptera

Beetles (Coleoptera) have evolved a range of diverse symbioses with microbes, and a subset have clear or hypothesized impacts on beetle N-economies. Unlike ants and cockroaches, there is not yet evidence for

ancient, N-recycling symbioses of importance to beetle biology. But a collection of findings has begun to hint at such importance. Perhaps the best illustration of bacterial N-recycling in beetles was obtained for the Asian long-horned beetle, *Anoplophora glabripennis* (Cerambycidae), a major invasive tree pest in North America. In this study (Ayayee et al., 2014), the authors used ^{15}N -urea-labelled diets, finding the heavy N signal in degutted beetle tissues and, thus, demonstrating that beetles had acquired symbiont-processed N from their gut-confined microbes. Stable isotope ratio mass spectrometry identified that this recycled N was used to synthesize several EAAs and non-EAAs. The authors also used a combination of PCR and reverse transcriptase PCR, discovering the presence and expression of urease genes (i.e. *ureC*) in eggs and larvae. In this same study the authors provided evidence for symbiont-mediated N-fixation, suggesting a multi-faceted set of mechanisms that can improve host N-economies. Since this publication, researchers have sequenced nucleic acids from gut tissue-extracts for this same beetle species (Scully et al., 2014). Amplicon sequencing of bacterial 16S rRNA genes and fungal ITS loci identified a suite of recurring bacteria and yeasts comprising the microbiome. And shotgun-metatranscriptomic analyses supported the prior finding of bacterial-encoded urease genes, while implicating symbiotic yeasts in both uricolysis and urea degradation. It was later shown that defecation into oviposition sites can transmit symbionts vertically (Mason et al., 2019), opening the potential for coevolved N-metabolic mutualisms in this invasive insect system.

On a different branch of the coleopteran phylogeny, a root- and leaf-feeding scarab beetle, *Melolontha hippocastani* (family Scarabaeidae) harbours several varieties of gut bacteria, including members of the Betaproteobacteria in the order Burkholderiales (Alonso-Pernas et al., 2017). Density gradient centrifugation coupled with 16S rRNA sequencing revealed that these bacteria incorporate ^{15}N from host-consumed dietary urea into their own DNA. Through further in vivo experiments, this labelled N was found in symbiont-free insect tissue (e.g. muscle), using isotope ratio mass spectrometry. It is seemingly not yet understood whether these are highly specialized symbioses with evolved mechanisms for transmission, or whether hosts, instead, acquire beneficial microbes from the environment to navigate their N-poor diets.

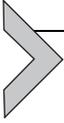
Beyond these examples, there is modest evidence for more widespread distribution of N-recycling in beetles. One potential case involves weevil species in the Scolytinae, known commonly as bark beetles. A subset of these insects harbour vertically transmitted fungi (Six and Klepzig, 2004) that

concentrate N in host tree phloem sap through unknown mechanisms. Fungal mutualist-harboured beetles also feed on fungal tissue, further alleviating dietary N-shortages (Six, 2012). Beetles with more phloem-heavy diets are expected to face greater shortfalls in dietary N and increased phloem consumption is one strategy to address this (Ayres et al., 2000). Another may involve nutritional symbiotic bacteria. In a cultivation-based study, researchers grew N-fixing and uricolytic bacteria from *Dendroctonus* bark beetles (Morales-Jiménez et al., 2013). The candidate N-recyclers were closely related to free-living microbes from three orders in the Gammaproteobacteria and made up 1.7% of the total culturable bacterial community—approximately sixfold lower than population size estimates for uricolytic bacteria from termites (i.e. *R. flavipes*; Potrikus and Breznak, 1980b). Like termite N-recycling symbionts, modest quantities of uric acid were found in the tissues and eggs of the studied bark beetles (Morales-Jiménez et al., 2013), raising the potential that these reserves could be mobilized for N-scavenging by bacteria to benefit beetle nutrition.

Diptera and Lepidoptera round out the remaining orders of holometabolous insects; and in the latter group there is no present evidence for N-recycling (or N-fixing) symbioses, to the best of our knowledge. But at least one instance of N-recycling has been reported in the Diptera, involving true fruit flies from the family Tephritidae. Larvae of the olive fruit fly, *Bactrocera oleae* feed on unripened fruit, while adults consume diets such as plant exudates, honeydew and bird excreta, which are low in EAAs or available N. Like so many of the aforementioned examples, the limiting nature of these food items suggests potential benefits through N-provisioning symbioses, and research into this possibility has focused on gut bacteria. Through a series of manipulative in vivo experiments, *B. oleae* fitness was shown to be reduced upon antibiotic suppression of its gut microbiome (Ben-Yosef et al., 2014) on diets of urea or bird droppings, but not on sucrose-only diets or diets containing all amino acids. The authors suggested that gut bacteria—including the nearly ubiquitous symbiont, *Erwinia dacicola*—play a N-recycling role, enabling hosts' use of waste N from these dietary sources. *E. dacicola* symbionts switch from intracellular stages in larvae to extracellular gut habitats in adults, and it is argued that intracellularity may allow them to survive metamorphosis (Estes et al., 2009; Hammer and Moran, 2019). They are, further, found associated with female ovipositors and, hence, arguably transmitted from mother to offspring through egg smearing. Such partner fidelity may explain why *B. oleae* harbour a monophyletic lineage of these bacteria, which is divergent from other *Erwinia* (Estes et al., 2009).

A recent study on *E. dacicola* partially supported its hypothesized N-recycling role, with two urease accessory protein-encoding genes being reported in its draft genome (Estes et al., 2018). Follow-up examination of this genome (NCBI BioProject accession #: PRJNA288714) through our own efforts identified a seemingly full set of urease genes on a single scaffold (accession #: LJAM02000124), including the core enzyme components (*ureA*, *ureB*, and *ureC*), the initially reported accessory genes (*ureF* and *ureG*), as well as two other genes playing likely accessory/activation roles (*ureD* and *ureJ*). Beyond their likely urease capacities, Estes et al. (2018) invoked additional roles in N-waste metabolism for *E. dacicola* through their discovery of two possible allantoin transporter genes. After locating the scaffold containing these genes (accession #: LJAM02000068.1), our BLAST-based study of adjacent genes suggested the existence of two others of likely importance. The first encodes an enzyme that can convert allantoin into ureidoglycine (i.e. allantoin amidohydrolase, protein accession #: RAP72056.1). The second encodes an enzyme that could upgrade ureidoglycine (i.e. ureidoglycine-glyoxylate aminotransferase; accession #: RAP72061.1), combining this molecule with glyoxylate to synthesize glycine (Fig. 3). KEGG pathway maps illustrate that glycine can be directly used to synthesize threonine or serine, also serving as a more distant precursor for amino acids like lysine, cysteine, and methionine. Its carbon skeleton can also be used in leucine, valine, and isoleucine synthesis; in these latter processes, recycled N—originally incorporated into glycine—would be freed as ammonia and made available for assimilation into other amino acids. An inspection of 51 genomes from *Erwinia* species in the IMG (Integrated Microbial Genomes) database reveals that nearly all strains encode the ability to make glycine from allantoin and to then transform it into the above-described amino acids. Most also have the capacity to make allantoin from uric acid, suggesting a potential loss of this function from *E. dacicola*. In addition to their work on this symbiont, Estes et al. (2018) reported on a symbiont from the genus *Enterobacter* that is potentially uricolytic. This symbiont is common in some populations of *B. oleae*, suggesting that a multipartite relationship may occur in at least some populations to facilitate N-recycling.

Beyond olive fruit flies, there are few clearly documented instances of N-recycling in the Diptera. For instance, a candidate yeast symbiont (genus *Meyerozyma*) from the digestive systems of blood-feeding sand flies encodes a uricolytic pathway and can use uric acid as a sole N-source (Martin et al., 2018). But low population prevalence—i.e. it is found in ~9% of surveyed *Phlebotomus perniciosus* sand fly hosts—raises questions about this symbiont's importance to the host N-economy, as does a lack of demonstrated uricolysis *in vivo*.



4. Nitrogen-fixing symbioses

Nitrogen fixation is a bacterially driven, energy-intensive process that generates ammonia for eventual assimilation and upgrading (Figs 2 and 3). Fixation of just a single molecule of N_2 requires ≥ 16 ATP molecules, indicating that the use of a plentiful, but otherwise inaccessible form of this element might be expected for symbiont-housing animals with N-poor, energy-rich diets. Several groups of wood-feeding termites appear to meet these requirements, given the prevalence of symbiont-driven cellulolytic activity and reductive acetogenesis (Brune and Dietrich, 2015; Hongoh, 2011). And indeed, the first demonstration of nitrogenase activity in insects was obtained for termites, through use of acetylene reduction assays (Benemann, 1973; Breznak, 2000; Breznak et al., 1973). This tool has been widely applied across free-living and symbiotic systems, as active nitrogenase enzymes convert acetylene into ethylene—molecules that are easily detectable through techniques including flame ionization gas chromatography.

4.1 Experimental verification of N-fixing and its importance to termites

As pointed out by Täyasa et al. (1994), woody tissues contain 0.03–0.7% N and their C/N ratios range from 70 to 500. Wood-feeding termites, in contrast, have body tissues comprised of 10% N, with C/N ratios ranging from 4 to 12. With needs met in part by N-recycling in at least some termites, support for N-fixation has been a further revelation for this group. The first direct demonstration that atmospheric N_2 was converted to ammonia for eventual incorporation into host tissues was obtained through use of stable isotope-tracing experiments. In particular, Bentley (1984) experimented on *Nasutitermes corniger*, a higher termite (Termitidae), examining fates of newly fixed N_2 across tissues of workers and soldiers with isotope ratio mass spectrometry. Within 24 h of incubation, fixed N was detectable in head and body tissues. Greater quantities were found in body tissues of workers compared to soldiers, and soldiers received greater quantities of fixed N through trophallactic exchanges with workers. This study was important in being the first to directly show fixed N-assimilation, but also because it elucidated the social component of this N-metabolic symbiosis, suggesting that trophallactically acquired nutrients or microbial cells are necessary for termites' assimilation of fixed N.

Since this early study, other authors have estimated symbiont-mediated N-fixation contributions using similar analytical chemistry, stable isotope

profiling, and comparisons of naturally occurring isotope ratios in food vs. hosts. Fujita and Abe (2006), for instance, quantified the degree to which symbionts of a lower termite, *Reticulitermes spearatus* (Rhinotermitidae), enabled assimilation of fixed atmospheric N₂. The authors reared these insects in chambers with controlled quantities of ¹⁵N-labelled dinitrogen for up to 28 days, subsequently performing gas chromatography-coupled mass spectrometry on various tissues to detect assimilated N. Their findings confirmed Bentley's (1984) discovery that fixed N is incorporated into symbiont-free termite body tissues. Through a detection sequence ranging from first observations of fixed N in the hindgut, subsequent detection in the crop/midgut, and later ¹⁵N arrival in head plus gut-free body tissues, the authors also supported earlier arguments that fixed N is obtained by termites through trophallaxis. Separate research has added to our current understanding on the mechanisms behind this nutrient acquisition, revealing hosts obtain symbiont-derived nutrition from post-trophallactic digestion of symbiont cells in the midgut (Brune, 2014; Fujita and Abe, 2002; Fujita et al., 2001).

In other research, isotope measures in food and termite tissues were used to estimate the fraction of termite N that is derived from atmospheric N-fixation (Täyasu et al., 1994). The authors detected lower ratios of heavy (¹⁵N) vs. light (¹⁴N) stable N isotopes in *Neotermes* termite tissues (Kalotermitidae) than those detected in their food. Recognizing that insects are typically enriched for heavy N when compared to their diets (due to fractionation), and also recognizing the low ratios of ¹⁵N/¹⁴N for the atmosphere, it was concluded that substantial fractions of the N in termite tissue were derived from symbiont-enacted atmospheric N-fixation. Quantitative estimates suggested that these proportions ranged from, and perhaps exceeded, 30–50%. Since this work was done on field-collected termites, it is of heightened importance due to tendencies of lab-rearing to under-estimate N-fixation (Breznak, 2000).

Follow-up work (Täyasu et al., 1997) shows wood-feeding termites exhibit N-isotope ratios closely matched to diets, while those of soil feeders—in strong contrast—exceed those of their diets, in a more typical fractionation pattern. These results support the prediction that consumption of peptide-rich, humified foods by soil-feeding termites (e.g. Brune and Ohkuma, 2010; Mattson, 1980) should lessen the need for N-fixation. Instead, host and symbionts of soil-feeding termites appear devoted to N-mineralization activities (Ji and Brune, 2006), hinting at drastically different roles for symbioses across ecologically divergent termites (e.g. Eggleton and Täyasu, 2001).

4.2 Genetic evidence for N-fixation in the termite system

Molecular approaches have been applied to further knowledge on the termite gut microbe symbiosis. In one early example (Noda et al., 1999), researchers leveraged the predicted reliance on substantial N-fixation by the aforementioned *Neotermes* species (Täyasa et al., 1994) to develop a priori expectations for high rates of in vivo nitrogenase gene expression. Through a focus on the *nifH* (dinitrogenase reductase) family of homologous genes, they performed reverse transcriptase PCR, cloning, and sequencing, and T-RFLP analysis on nucleic acids isolated from termite guts. Estimated numbers of *nifH* transcripts across diets correlated with acetylene reduction levels for these *Neotermes* specimens; and consumption of ammonium in the diet drove expression down to undetectable levels. These mRNA-derived findings contrasted with findings of no change in *nifH* gene copy number using DNA extracted from termites under the same conditions. This suggested transcriptional plasticity by bacteria as a response to changing N levels, rather than changes in population size. It was also discovered that a majority of expressed *nifH* genes belonged to the *anfH* family of molybdenum- and vanadium-independent, “iron-only” nitrogenases. This finding was bolstered by T-RFLP analysis, which showed enrichment of *anfH* vs. other nitrogenases in the mRNA, but not the DNA fraction. Hence, while multiple N-fixers may colonize gut tissues, not all are similarly active.

Explorations for *nifH* genes have been carried out in other studies, including an early metagenomic analysis (Warnecke et al., 2007) that reported 12 nearly full-length *nifH* gene sequences in lumen-extracted DNA of wood-feeding *Nasutitermes* (Termitidae). Shortly after this report, genome sequencing of one Bacteroidetes symbiont provided evidence for a N-fixing role. After lysing a single protist cell from the gut of a wood-feeding *Coptotermes* termite (Rhinotermitidae), researchers obtained endosymbiotic bacteria, which they subjected to whole genome amplification and sequencing (Hongoh et al., 2008). Annotation of the streamlined 1.1 Mbp genome revealed genes encoding all required components for Mo-Fe type N-fixation, including the nitrogenase (NifHDK), molybdenum-iron cofactor synthesis proteins, a regulator of the *nif*-operon (NifA), and a molybdenum transporter. This microbe also encoded a urease, so its additionally encoded glutamine synthetase might assimilate NH_3 produced from both N-fixation and N-recycling. Evidence that this microbe can, further, synthesize 19 amino acids, suggests that this symbiosis may be important in N-upgrading.

Like this prior example, researchers used similar methodologies to sequence genomes of a Spirochaete bacterium (genus *Treponema*) from protists in the guts of wood-feeding *Hodotermopsis* (Termopsidae; Ohkuma et al., 2015). Isolating and sequencing individual bacterial cells, they found similar capacities for N-fixation, N-recycling, NH₃ assimilation through glutamine synthetase, and the synthesis of most amino acids. Having isolated these bacteria from protists associated with high rates of N₂-fixation, the authors argue that reductive acetogenesis, further encoded by this symbiont, couples an energy-generating process with the ATP-intensive reduction of N₂ molecules. Confinement of these symbionts to cellulose-digesting protists may further concentrate the materials sustaining these energetic requirements. It is noteworthy that this system shows strong parallels, in these regards, to the aforementioned Bacteroidetes-protist-*Coptotermes* association.

4.3 Macroevolutionary patterns inferred for termites and their nutritional gut bacteria

In a broad attempt to characterize *nifH* gene diversity across a variety of Blattodea, and to relate this to host ecology and phylogeny, Yamada et al. (2007) performed PCR with conserved primers, cloning, and Sanger sequencing. Their sampling design involved 5 termite families, including three subfamilies within the Termitidae as well as the wood-feeding cockroach *Cryptocercus*, the closest relative of termites, in which N-fixation activity was previously detected (Breznak et al., 1974). Using sequence similarity, phylogenetics, and ordination analyses, the authors found a trend whereby termites with similar diets and lifestyles harbour related N-fixing bacteria, or at least bacteria with related *nifH* genes.

The first group united by similar *nifH* gene content included termites in the Rhinotermitidae and Mastotermitidae. Enriched within four out of the five species in this paraphyletic grouping were *nifH* alleles from the III-3 phylogenetic lineage, which have thus far been predominantly isolated from termites. It was also noted that the termites in this group were all wood-feeders of the “intermediate life type,” whereby termite colonies consume the wood that they colonize, but change their joint nesting/feeding sites throughout their lifespan. Another paraphyletic grouping, united through their sharing of related *nifH* alleles, included termites from the Kalotermitidae and Termopsidae, in addition to the wood-feeding *Cryptocercus* cockroach. Five of the six studied species from these collective taxa were enriched for clade III-3 *nifH* genes, and all were enriched for *nifH* alleles in the group II clade, which included homologues from free-living

Clostridium and Spirochaetes. Termites in this group were wood-feeding, “single-piece nesters,” which colonize and consume one piece of wood for the duration of each colony’s existence. The final grouping of termites united by *nifH* similarity included seven of the nine species of sampled Termitidae. Seeming to lack, completely, *nifH* allele copies from the II and III-3 lineages, these termites were instead enriched for bacteria encoding III-2 clade *nifH* alleles, showing relatedness to those from various *Clostridium*, including one from the bovine rumen. Termitidae termites harbouring bacteria with such *nifH* were united by a lifestyle of “separate-piece nesting,” meaning that they colonize separate pieces of wood from those they eat.

It is intriguing that patterns here seem to transcend host relatedness, being better predicted by termite life type. Prior authors have hypothesized important differences for symbioses across these habits (Eggleton and Tāyasu, 2001; Higashi et al., 1992). Differences among wood-feeders were hypothesized due to diverging interests in preserving wood biomass, a strategy presumably favoured by single-piece nesters. For such insects, conservative use of C to derive energy to fuel N₂-fixation was posited as the optimal strategy for overcoming N-limitation. Separate piece foragers were, contrastingly, hypothesized to have divergent interests, investing more in metabolisms to “burn off” carbon from rampantly consumed non-nest wood, through use of fungi or prokaryotic methanogens. Through this strategy, they might uniquely solve the challenge of N-shortages through restoration of a more ideal C:N balance.

In the final ecological pattern from Yamada et al. (2007), the authors observed that soil- and fungus-feeding termite species (*Pericapritermes nitobei* and *Odontotermes formosanus*) hosted a low relative abundance of bacteria with functional *nifH*. Among this paraphyletic, two-species grouping, most sequenced *nifH* clones grouped into cluster IV, which is presumed to have some other function aside from N-fixation. While limited by small sample size, this discovery fits previous observations that high N-fixation activity is most pronounced in wood-feeding termites, and less so in termites consuming fungi, fungus-conditioned food, and soil (e.g. Breznak, 2000; but see Sapountzis et al., 2016). Also of importance is just how often higher termites invest in N-fixation. Altered expectations arise in part due derived tendencies towards separate-piece foraging, but also because of their loss of energy-generating cellulolytic protists.

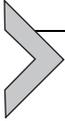
In conjunction with these questions are broader queries on the fidelity between N-fixing symbionts and their hosts. Despite similarity among

relatives (Hongoh et al., 2005), termite gut communities have undergone changes in composition across evolutionary timescales. Based on amplicon sequencing of 16S rRNA, it was posited that a subset of termite gut microbiota was harboured in the common ancestor shared with cockroaches (Dietrich et al., 2014). Using this same dataset, researchers found additional ecological and evolutionary correlates of microbiome change. Interpreting beta diversity in light of the host phylogeny, it was argued that bacterial gut communities were altered after the termite/*Cryptocercus* wood roach lineage diverged from the remaining Blattodea (i.e. cockroaches), when they acquired protists and wood-based diets. The subsequent loss of protists and shifts beyond wood-feeding in higher termites appear correlated with another major reorganization in gut bacterial composition (Dietrich et al., 2014). Within the higher termites (Termitidae), amplicon 16S rRNA sequencing has also shown a link between dietary convergence and convergence in bacterial gut microbiomes; and it has further detailed that bouts of cospeciation are ephemeral and punctuated by occasional host-switching (Mikaelyan et al., 2015). But in the Rhinotermitidae (lower termites), cospeciation has been documented among hosts, protists of the genus *Pseudotriconympha*, and their endosymbiotic bacteria from the Bacteroidetes (*Azobacteroides pseudotriconymphae*; Noda et al., 2007). The apparent N-fixing activity of the Bacteroidetes bacteria (Hongoh et al., 2008) hints that symbionts fulfilling such functions can be tightly coevolved with both termites and protist hosts, and this is likely facilitated by social, trophallactic symbiont transfer. Phylogenetic clustering of *nifH* genes from bacteria of related termites suggests similar specialization, to some degree, even though the encoding bacteria may sometimes move between distant termites with similar life types (Yamada et al., 2007).

4.4 N-fixation beyond termites

Outside the Blattodea, N-fixing symbioses have been demonstrated or hypothesized in a number of other insect systems, ranging from wood wasps, cerambycid beetles, bark beetles, cochineal scale insects, and tephritid fruit flies. Such discoveries have been recently reviewed in Ulyshen (2015) and Bar-Shmuel et al. (2019). We thus, refrain from elaboration here. Patterns emerging from these studies suggest enrichment within xylophagous insects, but there appear to be exceptions, with detection in some phloem-feeders, as well. Future studies on whether energy-rich, N-limited diets exclusively favour convergence in N-fixing symbioses will be of considerable interest

to the field. Of additional interest will be concerted efforts to test whether preliminary findings (e.g. *nifH* detection, cultivation of N-fixing bacteria) frequently translate to in vivo nitrogenase activity, with definitive impacts on host N-budgets. Of further importance will be efforts to understand whether N-fixers commonly coevolve with their hosts, or whether hosts instead acquire their diazotrophs with regularity from the environment.



5. Hemipteran symbioses: Synthesis of amino acids and their precursors as a central service

Symbioses without N₂-fixation or N-recycling can still be of benefit to host amino acid budgets, as commonly seen across sap-feeding hemipteran insects. Diets of sap-feeders include phloem and xylem. Phloem is a sugar-rich, N-poor liquid, and its limited N pools are primarily comprised of free amino acids. Non-EAAs are most predominant, with a high representation of asparagine, glutamine, or glutamate (Andersen et al., 1992; Brodbeck et al., 1990; Douglas, 2006; Sandström and Moran, 1999). Xylem sap, in comparison, has less sugar and even lower N content, with a similar amino acid imbalance (Brodbeck et al., 1990; Mattson, 1980).

These facets pose a strong challenge to sap-feeding hemipterans, and are compounded by the shortage of EAAs. In phloem and xylem, EAAs comprise an average of 20–25% of the total free amino acid concentration (Andersen et al., 1989; Sandström and Moran, 1999). But in the proteins of sap-feeding insects EAAs make up 50% of the total (Sandström and Moran, 1999). Challenges posed by animals' irreversible loss of EAA biosynthesis genes, and by these insufficient levels of EAAs must, therefore, be solved by nutritional mutualists (e.g. Bernays and Klein, 2002; Gündüz and Douglas, 2008), frequently termed primary, or obligate, symbionts. Such microbes cospeciate with hemipteran hosts (Moran et al., 2008), and are required for their growth, development, and reproduction (e.g. Fisher et al., 2017).

5.1 Tight integration of hemipteran/EAA(precursor)-synthesizing bacterial symbioses

A widespread theme for the majority of symbioses in the sap-feeding hemipteran suborders, Auchenorrhyncha, Sternorrhyncha, and the lesser-studied Coleorrhyncha (e.g. Santos-Garcia et al., 2014), is the intracellular localization of primary symbionts. These reside within host-membrane derived vesicles, inside of the cytoplasm of specialized, polyploid cells called bacteriocytes, which often aggregate to form an organ-like structure called the

bacteriome (Fig. 2; Baumann, 2005; Braendle et al., 2003; Douglas, 1997; Moran et al., 2008; Munson et al., 1991). Most bacteriomes are located adjacent to the ovarioles in the abdominal haemocoel (Braendle et al., 2003; Douglas, 1997), and these nutritional endosymbionts are transmitted to the next generation maternally and internally, mainly by transovarial infection (Salem et al., 2015). In such cases, entire bacteriocytes (whiteflies, psyllids) or exocytosed symbionts (aphids, mealybugs, planthoppers) migrate from the bacteriome to the nearby egg or ovarioles, respectively, where they are endocytosed into the egg or developing embryo (Braendle et al., 2003; Douglas, 1997; Hongoh and Ishikawa, 1997; Koga et al., 2012; Michalik et al., 2018; Salem et al., 2015; Sasaki et al., 1996; Thao and Baumann, 2004; von Dohlen et al., 2001; Wernegreen, 2002). For such symbionts, not only have hosts evolved mechanisms for faithful vertical transmission, several have also evolved regulatory mechanisms that integrate their own N-metabolism with that of their symbiont (Hansen and Moran, 2013).

Before discussing such integration, we note first that an impressive body of research has shown benefits from obligate symbionts in sap-feeding hemipterans that stem largely from EAA, or EAA-precursor provisioning (Fig. 2). Most impressive has been work performed in the aphid-*Buchnera* system though use of antibiotic treatments, artificial diets, isotope-labelling, quantification of amino acid concentrations in sap and aphid proteins, and genome sequencing (Douglas, 2006; Douglas and Prosser, 1992; Douglas et al., 2001; Febvay et al., 1995, 1999; Gündüz and Douglas, 2008; Hansen and Moran, 2013). For example, artificial diets containing labelled amino acids or sucrose have been fed to either symbiotic or symbiont-cured aphids. Inferences from these studies show incorporation of dietary N and carbon into EAAs, but only in the presence of symbiotic *Buchnera* (Douglas et al., 2001; Febvay et al., 1999). Genomes of both aphids and *Buchnera* (Gammaproteobacteria: Enterobacteriaceae) help validate that most of the leg work in the EAA biosynthesis is carried out by the symbionts, with a few key exceptions discussed below (Baumann et al., 1995; Charles and Ishikawa, 1999; IAGC, 2010; Moran and Baumann, 2000; Shigenobu et al., 2000; but see Hansen and Moran, 2013).

While the aphid-*Buchnera* system has proven adept for experimentation, many other hemipteran systems lack such tractability. Genomic data, hence, remain the central line of evidence for roles of symbionts in EAA provisioning (McCutcheon et al., 2009). Because these symbionts are irreversibly specialized, with highly eroded genomes (Moran and Bennett, 2014), retention of genes implies that they are of high functional relevance.

At minimum, they enable symbionts to sustain their own existence. But almost universally accepted is the idea that retention signifies direct benefits to hosts, given prior precedent (e.g. aphids) and known host dietary N-limitations. The importance of EAA pathways for these symbioses are further reinforced by trends of metabolic complementarity—obligate symbionts of sap-feeding insects tend to keep N-metabolic genes that hosts do not have (i.e. those for EAA synthesis), while losing genes that are redundant with those in hosts (i.e. those for non-EAA synthesis; [Wilson and Duncan, 2015](#)). When multiple obligate bacteria are harboured within the same host, metabolic complementary also exists between these “co-primary” symbionts. In these cases, a subset of one or more EAA pathways is encoded by one symbiont, while the remaining genes are encoded by its ancient co-inhabitant, in a jigsaw puzzle-like pattern ([McCutcheon et al., 2009](#)).

Another approach for analysing EAA synthesis in these endosymbiont systems involves regulatory analyses on bacteriocytes. Such methodology has been key to identifying an impressive degree of host-microbe integration in the process of EAA synthesis. In the best-studied example, the primary symbionts of many sap-feeding hemipterans are missing the terminal enzymes for several EAA pathways. It is predicted that the insect host expresses homologues of these missing enzymes to complete EAA production. This was confirmed through studies of *Buchnera* and pea aphids (*Acyrtosiphon pisum*), a model symbiotic system with published host and symbiont genomes. Through use of bacteriocyte-targeted RNAseq and proteomics, key aphid-encoded homologues for amino acid biosynthesis were shown to be up-regulated in bacteriocytes ([Hansen and Moran, 2011](#); [Poliakov et al., 2011](#)). Two key genes of particular interest were branched chain-amino acid transaminase (EC number 2.6.1.42) and aspartate transaminase (EC number 2.6.1.10), which, respectively, encode the terminal steps for fabricating the EAAs isoleucine, valine, and leucine, and phenylalanine, respectively. Transcription of these genes was 4.73- and 3.29-fold higher in bacteriocytes relative to other body tissues ([Hansen and Moran, 2011](#)). When coupled with the absence of these genes in the *Buchnera* genome, these findings helped confirm host-participation in completion of EAA biosynthesis ([Figs 2 and 4](#)), raising the likelihood that the aphids can regulate this metabolism. Similar results have been found with RNAseq in subsequent studies within mealybugs, psyllids, whiteflies, and leafhopper bacteriocytes, suggesting that the integration of insect-encoded enzymes in the production of EAAs may be widespread in Hemipteran-microbe symbioses ([Husnik et al., 2013](#); [Luan et al., 2015](#); [Mao et al., 2018](#); [Sloan et al., 2014](#)).

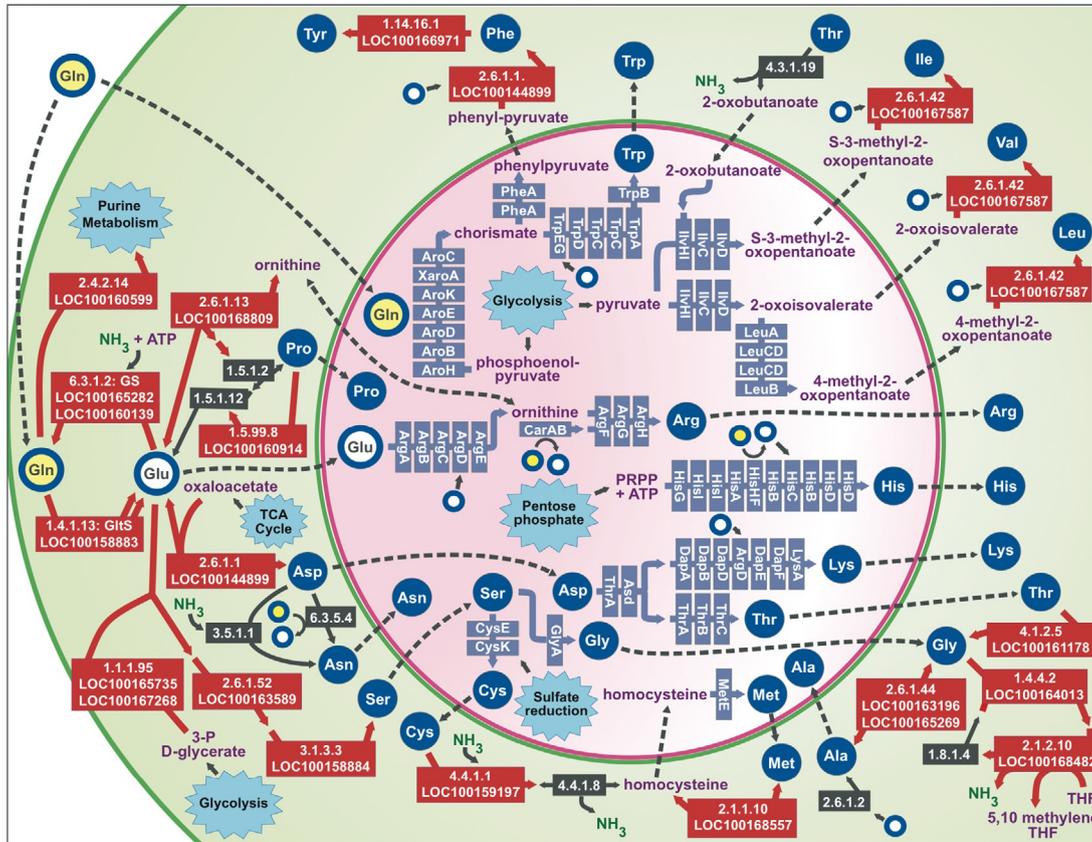


Fig. 4 A cartoon image of a specialized aphid host cell that harbours its nutritional symbiont, *Buchnera* (bacteriocyte); green = cytosol of aphid, pink = cytosol of *Buchnera*. RNAseq data reveal aphid host-encoded enzymes for amino acid pathways that are up-regulated in bacteriocytes compared to body cells (red rectangles). These are hypothesized to collaborate with *Buchnera*-encoded enzymes for essential amino acid biosynthesis (purple boxes). Aphid enzymes are labelled with Enzyme Commission and locus numbers from NCBI annotations. Brown aphid enzymes indicate that the gene is not up-regulated in bacteriocytes. Reprinted figure obtained with permission from Hansen, A.K., Moran, N.A., 2011. Aphid genome expression reveals host-symbiont cooperation in the production of amino acids. *Proc. Nat. Acad. Sci. U S A* 108, 2849–2854.

An extensive review on the presence and absence of these EAA pathways across a diversity of sap-feeding symbioses, and the complementary host-encoded enzymes, is provided in [Hansen and Moran \(2013\)](#).

5.2 Symbionts help sap-feeding hemipterans to overcome amino acid imbalance, with the aid of derived host bacteriocyte metabolism. But have hosts evolved additional features to improve their N-economies?

Measures of symbiont contributions to host amino acid budgets have been key to showing how EAA-provisioning symbionts can benefit hosts. For instance, [Gündüz and Douglas \(2008\)](#) showed that pea aphids ingested eight times their body N-requirements from diets of fava bean phloem. But ingested fluid was not sufficient to meet demands for seven of the nine examined EAAs on at least one of eight replicate plants. Using artificial diets, measures of aphid growth, and total protein content, the authors showed that aphid performance was suppressed in the absence of *Buchnera*, even more so when any of the nine studied EAAs were omitted from the diet. Variable protein growth-suppression on each of these omission diets, for aposymbiotic aphids, revealed that *Buchnera* provisions all nine studied EAAs in varying quantities, with especially large devotion to leucine. Taking a different approach, [Bernays and Klein \(2002\)](#) studied a different aphid, *Uroleucon ambrosiae*. They quantified phloem consumption, honeydew excretion, growth and reproductive rates, and the amino acid content of both body tissues and ingested food. Through a series of calculations, they then estimated the percentage of aphid requirements that must be met by *Buchnera*. Non-EAA concentrations were considerably higher than those of EAAs in phloem, with notable excesses of asparagine, alanine, glutamine, and serine. Among the EAAs, quantities of histidine, methionine, and phenylalanine in the phloem came close to meeting aphid requirements. But the remainder were in scarce supply, with estimates suggesting *Buchnera* meets 15–80% of *U. ambrosiae* body requirements for seven EAAs, most drastically tryptophan.

In overcoming dietary amino acid imbalance, by covering for the hosts' inability to produce EAAs, bacteriocyte-colonizing primary symbionts of hemipterans are clearly useful. Symbiont-provided services align with prodigious sap-consumption rates, helping hemipterans succeed despite specialization on insufficient food. Several hemipterans also manipulate plant sap diets to their benefit, receiving increased concentrations of key EAAs ([Sandström et al., 2000](#)). Beyond these strategies it is unclear whether

hemipterans, or their ancient bacteriocyte-dwelling symbionts, have evolved adaptations for elevated conservation of overall N content.

While it was thought that *Buchnera* and other obligate symbionts recycle ammonia derived from host waste metabolism (e.g. Prosser and Douglas, 1991), *Buchnera* genome sequencing revealed a limited capacity for NH_3 -assimilation (Shigenobu et al., 2000). Subsequent work using transcriptome data concluded that host-derived glutamine synthetase assimilates host-derived ammonia in bacteriocytes, and that glutamine can be further converted to glutamate through hosts' glutamine synthetase (GS) and glutamine oxoglutarate aminotransferase (GOGAT) cycle (Hansen and Moran, 2011; Fig. 4). This hypothesis was supported by modelling work and ammonia concentration measurements (MacDonald et al., 2012), suggesting that N from ammonia-derived amino acids is primarily used in host-directed transamination reactions to make phenylalanine, isoleucine, valine, and threonine.

The GS-GOGAT cycle enzymes are also widespread within other Hemipteran insects (Hansen and Moran, 2011; Husnik et al., 2013; Luan et al., 2015; Sloan et al., 2014). For example, transcriptomic data show the GS-GOGAT cycle is up-regulated in bacteriocytes compared to other body tissues in aphids, mealybugs, psyllids, whiteflies, and leafhoppers (Hansen and Moran, 2011; Husnik et al., 2013; Luan et al., 2015; Mao et al., 2018; Sloan et al., 2014), and is hypothesized to play an important role in ammonia assimilation to fuel N-upgrading in sap-feeding insects (Hansen and Moran, 2011). This collectively suggests that the insect hosts, and not the symbionts, conduct N-recycling in these symbioses (Fig. 4). The efficiency with which they shunt useful forms of N in their bodies to bacteriocytes for these purposes remains unclear, as does the exact nature of hemipteran N-waste management. These questions lie at the heart of whether aphids, and other sap-feeding hemipterans have evolved thrifty N-economies, or whether excessive feeding, host plant manipulation, and regulatory changes to bacteriocyte-localized N-metabolism comprise the bulk of host-derived solutions to N-imbalance and N-limitation.

5.3 Supplanting symbionts spur sporadic instances of N-recycling and N-fixation across the Hemiptera

In addition to findings of host involvement in N-waste management, initial discoveries that symbionts like *Buchnera* did not fix- or recycle N did not rule out the potential for symbiont-driven N-concentration across all sap-feeding hemipterans. But the accumulation of genomes across this group has revealed that N-fixing and N-recycling enzymes are generally absent

from obligate symbiont genomes. To date, the only primary symbiont encoding an enzyme with plausible N-recycling function is *Sulcia*-PSPU, which is harboured in the spittlebug, *Philaenus spumarius* (Koga and Moran, 2014). This endosymbiont encodes glutamate dehydrogenase (*gdhA*) (Koga and Moran, 2014), which may recycle ammonia and α -ketoglutarate into glutamate, an important amino donor for EAA biosynthesis. This *Sulcia*-PSPU enzyme was lost from other *Sulcia* lineages and, notably, its most closely related homologue is encoded by related *Blattabacterium* symbionts of cockroaches (Koga and Moran, 2014). Beyond this example, there is scant evidence for other N-recycling genes across hemipteran primary symbionts. The absence of urease genes is notable, but perhaps expected due to the lack of the urea cycle in many hemipterans (KEGG website: Kanehisa and Goto, 2000; Kanehisa et al., 2019; also see Panfilio et al., 2019).

In looking more broadly for N-fixation and N-recycling across insects normally dominated by EAA-provisioning symbioses, a pattern emerges whereby hemipterans—having lost their long-standing obligate symbionts—have sometimes acquired new symbionts with one or both capacities. Demonstration of N-recycling was first fulfilled for the brown planthopper *Nilaparvata lugens* (family Delphacidae), and a filamentous ascomycete fungus related to *Ophiocordyceps* (Matsuura et al., 2018; Suh et al., 2001). Previous research indicated that *N. lugens* produced substantial quantities of uric acid, which were conspicuously stored in its tissues (e.g. Hongoh and Ishikawa, 1997; Sasaki et al., 1996). The “yeast-like symbiont,” which replaced ancestral primary bacterial symbionts, expressed uricase activity, but virtually none was detected in host tissues or in aposymbiotic planthoppers (Sasaki et al., 1996). Quantities of stored uric acid increased with increasing amounts of dietary amino acids. When insects were switched from rich diets to amino acid-poor diets, uric acid stores were drastically depleted, though only in the presence of symbionts (Hongoh and Ishikawa, 1997). Colonizing fat body-localized bacteriocytes (Noda, 1977), these fungi sustain host fitness in the face of N-poor diets, perhaps due to their N-metabolic functions (Hongoh and Ishikawa, 1997).

PCR and Sanger sequencing supported this symbiont’s N-recycling role through detection of symbiont-encoded uricase genes (Hongoh and Ishikawa, 2000), findings verified by subsequent genome sequencing (Xue et al., 2014). In addition to uricase, the yeast-like symbiont was also reported to encode urease genes and several intervening genes in the uricolytic pathway. Inferences from the host genome (Xue et al., 2014) reveal that planthoppers encode uricase, and at least one step in the uricolytic

pathway, but that they lack allantoicase enzymes for the synthesis of urea. Further genomic assessment revealed a glutamate dehydrogenase gene, and genes for the GS-GOGAT cycle, indicating that the symbiont can directly assimilate ammonia derived from uricolysis. The fungal symbiont also retains genes to synthesize the 10 EAAs, arguing that it can upgrade the recycled and assimilated N, perhaps through use of transaminating N-donors including glutamate (Xue et al., 2014).

A related N-recycling fungus has similarly supplanted the ancient aphid EAA-provisioning symbiont *Buchnera*. In gall-forming aphids of the tribe Cerataphidini (Sternorrhyncha: Aphididae), *Buchnera*-replacing yeast-like symbionts colonize hemolymph and, occasionally, fat body tissues (Buchner, 1965). Earlier work had detected uricase genes within these fungi (Hongoh and Ishikawa, 2000) and genome sequencing for one species revealed an intact uricase gene (Vogel and Moran, 2013). Our own efforts to detect urease enzymes, through a tBLASTn search, revealed a match with 87.5% amino acid identity to the urease enzyme of a related fungus (*Ophiocordyceps sinensis* accession #: EQK99008.1—tBLASTn vs. assembly accession #: GCA_000372705.1). This match spanned just 24% of the query, however, meaning that while the finding is consistent with symbiont-encoded urease function, this capacity—and the broader role of the symbiont in N-recycling—requires further investigation. Interestingly, the aphids' fungal symbiont does encode a full set of genes for the synthesis of all amino acids, including all 10 EAAs. It can, further, reduce nitrate and nitrite to ammonia, and it encodes a complete GS-GOGAT cycle. Given the symbionts' coevolved history with their aphid hosts, these discoveries reveal potentially impactful mechanisms for the assimilation of symbiont-derived ammonia, and subsequent upgrading for the hypothesized benefit of hosts.

Across the Sternorrhyncha in the scale insect subfamily Coccoidea, sap-feeding cochineals from the genus *Dactylopius* harbour symbionts with suspected roles in both N-recycling and N-fixation (Vera-Ponce de Leon et al., 2017). Chief among these is a betaproteobacterium, *Candidatus Dactylopiibacterium carminicum*. Colonizing hemolymph and ovaries, this symbiont was found to encode genes for the molybdenum-iron nitrogenase (*nifD-nifK*) and dinitrogenase reductase (*nifH*) enzymes. Also detected in its genome were genes encoding various N-regulatory proteins and transporters for nitrogenase cofactors, including *nifQpp-nifA* and 4Fe-4S ferredoxin. Expression of *nifH* was found in hemolymph, ovaries, and eggs; and acetylene reduction assays detected likely N-fixation in the former two tissues. The authors also reported genes in the uricolytic pathway,

including urease, revealing a second means by which the bacterial symbiont could concentrate N for its cactus-phloem-feeding host. The *D. carminicum* symbiont also encodes biosynthesis of all amino acids except arginine, further supporting an important N-provisioning role.

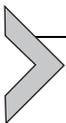
In a related study on this system, researchers focused on fungal symbionts, including those colonizing the cochineal gut and Malpighian tubules (Vera-Ponce de Leon et al., 2016). Evidence from experiments involving gene expression, metagenomics, uric acid quantification, symbiont suppression, and microscopy, suggests a role for this fungus in the recycling of host N-waste. And evidence for the symbiont's vertical transmission combines to suggest that a second mutualist with N-concentrating capacities colonizes cochineals alongside *D. carminicum*, bacterial symbionts.

The common ancestor of scale insects was likely symbiotic with EAA-provisioning bacteria (Sudakaran et al., 2017), suggesting the above example in cochineals represents an additional instance of symbiont replacement with N-recycling fungi. Bacterial symbionts have been similarly replaced in the Coccidae, or “soft scales.” In this case, amplicon sequencing of 16S rRNA identified no consistent bacterial associates across soft scale species. Instead, a mixture of sequence-, phylogenetic-, and microscopy-based assessments argued for consistent presence of vertically transferred *Ophiocordyceps*-like fungi (Gomez-Polo et al., 2017). More work remains to ascertain this symbiont's N-metabolic capacities.

Other examples of fungi replacing long-standing bacterial N-provisioning mutualists have been reported recently in cicadas (Cicadoidea), distant relatives of the delphacid *N. lugens* planthoppers within the suborder Auchenorrhyncha (Matsuura et al., 2018). In particular, several Japanese cicada lineages no longer harbour *Hodgkinia*, one of their two ancient bacterial mutualists. These cicadas, instead, contribute to the emerging theme, whereby ancient bacterial mutualists have been replaced by specialized *Ophiocordyceps*-like symbionts. These fungi are somewhat related to the yeast-like symbionts of aphids and planthoppers; and in cicadas, they colonize fat body tissue surrounding bacteriocytes, along with sheath cells enveloping the bacteriome. Microscopic observations suggest transovarial vertical transmission. And genome sequencing of one cultivated *Ophiocordyceps* symbiont revealed complete pathways for the biosynthesis of all amino acids. Importantly, the genomes were argued to encode “all synthesis pathway genes for...nitrogen recycling.” Using the uricase protein from the symbiont of *N. lugens* planthoppers (accession #: BAA89004.1), we performed a tBLASTn search against the genome assembly in NCBI (assembly accession #: GCA_002939055.1). A putative protein with

73.2% identity (191/261 identical amino acids) to the uricase query was identified. In similar tBLASTn searches, a urease gene from a *Metarhizium* fungus (accession #: XP_007823647.2) and another *Ophiocordyceps* (accession #: EQK909008.1) yielded hits with 69.3% (251/319 amino acids) and 82.4% (74/91, 26/29, and 97/119 amino acids) identity to a putative protein from the cicada symbiont, across most of their lengths. The presence of likely N-recycling genes, and the localization of symbionts to the fat body where uric acid is often synthesized, reveal the potential for a N-recycling mutualism.

Outside of sap-feeding hemipterans, the shield bug *Parastrachia japonensis* (suborder Heteroptera; Parastrachiidae) feeds on drupes (i.e. fleshy fruits), which are available for a very short time each year. Between bouts of food availability, the shield bug enters reproductive diapause, ingesting scarce amounts of food for many months. During this time, viability appears to depend on a bacterial N-recycling symbiont (Kashima et al., 2006), identified as *Benitsuchiphilus tojoi* (Hosokawa et al., 2010). This gammaproteobacterium is transmitted vertically through egg smearing and colonizes midgut crypts of its shield bug hosts. Symbiont removal with antibiotics increases mortality during this diapause, while also suppressing quantities of free amino acids in the hemolymph. But when symbionts are undisturbed, total N levels appear remarkably unchanged in these shield bugs when supplied with only water for 8 month rearing periods. Midgut extract from diapausing females revealed uricase, allantoinase, and allantoinase activity, and a re-test of uricase activity showed suppression upon antibiotic treatment (Kashima et al., 2006). These data, combined, suggest beneficial actions of yet another N-recycling symbiont. Contributions to hemipterans feeding on foods beyond phloem sap are an exception to the current rule. This raises the interesting possibility that some nutritional symbionts evolved their current host relationships due to their capacities to help hosts weather periods of dietary stress.



6. Conclusions and future directions

Nutritional symbionts contribute a range of diet-limited compounds to their hosts, and N is often at the center of such interactions. Thanks to improving technologies and, often, research into pest insects, a growing body of literature has illuminated our understanding of how symbioses improve the N-budgets of insects consuming N-limited and N-inaccessible diets. But numerous questions remain. For instance, do insects' symbionts commonly incorporate N through nitrate or nitrite reduction (Ngugi et al., 2011;

Vogel and Moran, 2013), as seen in some aquatic systems (Girguis et al., 2000)? Do symbionts make major improvements to hosts' N-budgets through assisted digestion, perhaps through protease secretion (Chu et al., 2013; Sethi et al., 2011)? How commonly do hosts invest in the strategy of "burning off" excess C to improve C:N ratios (Higashi et al., 1992)? Did fungus-cultivating mutualisms evolve for this purpose (Mueller et al., 2005), and can we hence view symbionts with particular forms of carbon metabolism as contributors to insect N-economies? Also of interest is the question of just how much our methods have limited our knowledge, whether through impacts of the lab environment on activities like termite N-fixation (Breznak, 2000), spurious impacts of artificial diets on sap-feeding insects and their symbionts (Pers and Hansen, 2019), or the over-reliance on genomics and existing gene annotations to predict function without experimental validation. Future research in these areas, and further applications of cutting-edge technologies (e.g. Volland et al., 2018), will be part of an exciting era for symbiosis research across insects and beyond.

While much exciting work awaits, we should not minimize how far we have come in recent decades. As far back as the mid-1900s, many of the patterns seen today for insect-symbiont associations had been at least roughly outlined, with one set of authors noting, "Most of the literature on endosymbiosis is of a descriptive rather than an experimental nature, and the theories on the role of the symbiotes are usually based on circumstantial evidence. Practically all symbiote-bearing insects feed on diets which are incomplete or inadequate in certain substances known to be required by non-symbiotic insects or by vertebrates," (Brooks and Richards, 1955). Clearly, the inseparable nature of many host-symbiont relationships was a serious obstacle at that time. But technological advances involving genomics and analytical chemistry have allowed researchers to circumnavigate roadblocks deriving from mutual dependence. Through the resulting hard-won discoveries on symbiont roles we have uncovered a diversity of ways that microbes have aided in the exploitation of diets rich in C, but depauperate in N. Although insects have evolved many stand-alone mechanisms for the use of such plant-based diets (Berner et al., 2005; Karban and Agrawal, 2002; Lavoie and Oberhauser, 2004), many have opted for a collaborative approach. Their microbial menageries bring diverse C- and N-metabolic repertoires, helping to exploit incredibly abundant, nutritionally challenging plant resources. In doing so, symbionts have helped to revolutionize terrestrial ecosystems, supporting lifestyles of a broad swath of the numerically dominant and hyper diverse herbivorous insects (Grimaldi and Engel, 2005).

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