Symbiont-driven niche expansion shaped the adaptive radiation of insects

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Symbiont-driven niche expansion shaped the adaptive radiation of insects

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For over 300 million years, insects have relied on symbiotic microbes for nutrition and defence[1,2]. However, it is unclear whether specific ecological conditions have repeatedly favoured the evolution of symbioses, and how this has influenced insect diversification[1,3,4]. Using data on 1844 microbe-insect symbioses across 400 insect families, we found that symbionts have allowed insects to radiate into a range of feeding niches deficient in B vitamins, including phloem, blood and wood. In some cases, such as herbivorous insects, the shift to a new niche has resulted in spectacular species proliferation. In other niches, such as strict blood feeding, diversification has been severely constrained. Symbioses therefore appear to solve universal nutrient deficiencies for insects, but the consequences for insect diversification depend on the feeding niche invaded.

Main text

Across the tree of life, microbial symbionts have enabled organisms to harness new forms of energy, access unobtainable nutrients, and outsource critical functions such as defence[1,2]. So valuable are symbiotic partnerships that they have repeatedly led to organisms becoming obligately dependent on each other for survival[5]. Such interdependence between hosts and symbionts has led to the evolution of new levels of organismal complexity that have ultimately shaped the diversity of life on earth[3,6].

The essential services provided by symbionts have enabled hosts to expand into previously uninhabitable environments[1,7]. For example, sulfur-oxidizing bacteria enable giant marine tubeworms to live in deep-sea vents, root-associated fungi helped plants colonize land, and nutrient-supplementing symbionts have allowed insects to live solely on the imbalanced diets of plant sap and vertebrate blood[2,8,9]. However, it is unclear whether there are key ecological factors that allow us to make generalisations about how and why obligate symbioses evolve.

Insects are an excellent system to study the evolution of obligate symbiosis, as microbes have been recruited by a diverse set of insect families for a range of functions, including defence and nutrition[10]. Obligate symbioses, defined here as hosts not being able to survive without symbionts, are strongly associated with insects feeding on specialized resources, such as plant-sap, blood (hematophagy) and wood (xylophagy)[2]. It is therefore widely accepted that symbiotic partnerships have opened up new ecological niches by solving a variety of nutrient limitations that have contributed to the incredible diversification of insects[7].

Previous work, however, has consistently focused on single groups of insects at a time. As a result, the extent that we can generalize about the ecological causes and consequences of obligate symbiosis across different groups of insects and microbes is unknown. For example, have obligate symbioses solved the same or different nutritional deficiencies across divergent feeding niches, and how has this influenced the diversification of different insect lineages?

We address these questions by examining macro-evolutionary patterns of symbiosis across 1844 microbe-insect combinations from 400 insect families. Data were collated across bacteria, fungi and protist symbionts with nutritional and defensive functions (Supplementary Tables 1-4). First, we estimated how often insect lineages within different feeding niches have evolved obligate symbiosis. Obligate dependence was determined by the presence of morphological structures exclusively associated with obligate symbiosis (e.g. bacteriocytes[10]). Where information on symbiont housing organs was lacking, data on the impacts of symbiont removal and patterns of host-symbiont co-speciation were used to determine obligate dependence (see Methods). Second, we examined the composition of insect diets to determine whether specific nutrient deficiencies have consistently led to the evolution of obligate symbiosis across different feeding niches. We differentiate between insect families that specialize on single plant-based resources (phloem, xylem, or wood) from families that exploit various plant parts (phytophagy, referred to here as herbivores), as there were large differences in the nutrients between these diets (Supplementary Tables 1 & 4). Third, we tested if the acquisition of obligate symbionts has increased or decreased host diversification after radiating into different feeding niches. We circumvent the problem of poorly resolved species level phylogenies by reconstructing the evolutionary history of obligate symbioses at the family level.

Evolutionary origins of obligate symbiosis

We found that obligate symbiosis has evolved in at least 13 independent insect lineages (Fig. 1. Supplementary Table 5). These origins were estimated on the time-calibrated phylogeny[11] to date back as far as 336 million years, allowing us to examine the long-term evolutionary consequences of obligate symbiosis for niche specialization and diversification.

Reconstructing the ancestral feeding niches of insect families showed that all obligate symbioses evolved from omnivorous (origins = 75%), herbivorous (origins = 8%) and predatory ancestors (origins = 17%. Fig. 1. Supplementary Table 5). Following the acquisition of obligate symbionts, 59% of lineages switched to a single food source (phloem = 40%, blood = 12%, xylem = 6% and wood = 1%. Fig.1. Supplementary Table 5). This pattern of food utilisation explains the current distribution of obligate symbiosis remarkably well, where over 90% of insect species feeding on blood, phloem, xylem and wood have obligate symbionts (Fig. 1. Supplementary Tables 1, 2 and 6). Conversely, there are no known cases of obligate symbioses in insect families that are predominantly predators or fungivores (Fig. 1. Supplementary Tables 1, 2 and 6).

In contrast to nutritional symbionts, we found that only four insect families had symbionts with defensive functions. This is likely influenced by sampling effort, as defensive symbionts have only been discovered relatively recently in insects[12]. However, out of the 11 microbial species shown to provide insects with protective services, nearly all maintain facultative relationships with their hosts. There is only one exception in our database, the Asian citrus psyllid, *Diaphorina citri*, that has evolved obligate dependence on a defensive symbiont, which is housed in bacteriocytes alongside a putative nutrient provisioning symbiont[13]. While more work is clearly needed, these data support the hypothesis that selection for protection against natural enemies is too inconsistent across generations to favour the evolution of obligate dependence[5,14].

Nutrient deficiencies and obligate symbiosis

Our results show that the evolution of obligate symbiosis in insects is associated with transitions to specialized feeding niches (Fig. 1). However, it is not clear if there are common nutrient deficiencies that explain the evolution of obligate symbiosis across these diverse niches. To test this idea, we extracted the nutrient compositions for the 19 diet types of insects in our dataset, estimating levels of carbohydrates, fats, proteins and vitamins A, B, C, E and K.

We found that only one dietary component was consistently associated with the evolution of obligate symbiosis across all feeding niches: low levels of B vitamins (Fig. 2. Phylogenetic correlation (phylo *r*), Credible Interval (CI) = -0.39 (-0.59, -0.21), pMCMC = 0.001. Supplementary Table 7). This pattern held across hosts with very different feeding niches, that had highly variable compositions of carbohydrates, proteins, fats, and vitamins in their diets (Extended Data Fig. 1. Supplementary Table 7). Other nutritional deficiencies were associated with obligate symbiosis, but these were restricted to specific feeding niches (Supplementary Table 8. Extended Data Fig. 1). For example, insects feeding on phloem and wood had significantly less protein in their diets compared to background levels across all other niches (Phloem (CI) = -0.76 (-1.03, -0.37), pMCMC = 0.001. Wood (CI) = -0.74 (-1.36, -0.28), pMCMC = 0.002. Supplementary Table 8).

Examining different types of B vitamins further showed that the evolution of obligate symbiosis was significantly associated with low levels of B5 and B9 vitamins, and weakly related to B6 vitamins (Fig. 2. Supplementary Table 9). Vitamins B1, B2 and B3 were highly correlated with B5 (Pearson's correlation coefficients $r > 0.90$) and B6 is correlated with B9 (r=0.77), indicating that sets of B-vitamins are often concurrently absent from insect diets (Extended Data Fig. 2). Data on vitamins B7 and B12 had >30% missing data and so were not analysed. No other macro- or micro-nutrients were significantly correlated with obligate symbiosis across all insect families (Fig. 2. Supplementary Table 7).

Our results are consistent with detailed studies that have demonstrated the fitness consequences of providing B vitamins to specific insect species. For example, the fitness of tsetse flies depends on B9 and B6 vitamins provided by *Wigglesworthia* bacteria[15,16], and *Buchnera* supplements aphids with B5 and B2 vitamins, with B5 having a particularly strong effect on host survival[17]. Dietary studies have also confirmed that mutualistic *Wolbachia* provide essential B-vitamins for Cimex bed bugs[18]; and metabolic homeostasis is restored in symbiont-free *Dysdercus* cotton stainers when B-vitamins are supplemented, or hosts are reinfected with their actinobacterial symbionts[19].

Evolutionary transitions to nutrient deficient diets

Our results suggest that B vitamin deficiency is of widespread importance for the evolution of obligate symbiosis in insects. There are, however, two competing explanations for why such transitions occur. One possibility is that insects feeding on diets low in vitamin B recruited symbionts to supply B vitamins. The alternative is that insects first acquired obligate symbionts that could synthesise B vitamins, possibly for some other benefit, which then enabled them to invade ecological niches where B vitamins were scarce. The question is therefore whether the evolution of obligate symbioses were triggered by low B vitamins in diets or whether they facilitated specialisation on these diets.

We tested these competing hypotheses by estimating the amount of B5 and B9 vitamins in ancestral diets prior to, and following, transitions to obligate symbiosis. We found little evidence that levels of B5 and B9 vitamins were reduced in the diets of insects before they acquired obligate symbionts (Fig. 3. Supplementary Tables 10-11). Instead, we found that hosts that recruited obligate symbionts subsequently evolved to specialise on diets with low levels of B5 and B9 vitamins (Fig. 3. Supplementary Tables 10-11). Once obligate symbioses evolved, shifts to diets deficient in B vitamins were much more frequent, particularly for B5, where transition rates to low B vitamins were 16 times higher than for lineages without obligate symbionts (Supplementary Table 11).

The key role of B vitamins in driving obligate symbioses was further supported by the loss of obligate symbionts when insects switched to diets with elevated levels of B vitamins (Figs. 3. Supplementary Tables 10-11). Insect lineages with above average levels of B5 and B9 vitamins were more likely to lose their obligate symbionts (Differences in transition rates (q): B5 = -2.05 (-3.35 , -0.47), pMCMC=0.002. B9 = -2.21 (-3.76 , -0.89), pMCMC=0. Supplementary Table 11). Our results match with observations from specific taxa, where obligate symbiont losses have been associated with dietary changes in their insect hosts. In the mealybug genus, *Hippeococcus*, symbiont losses are thought to be associated with nutrient provisioning by *Dolichoderus* ants, and *Typhlocybides* plant hoppers lost their ancestrally obligate symbionts when switching from plant-sap to more nutrient rich parenchyma.[20]

Symbiont specialisation in nutrient provisioning

Given the key role of B vitamins in both the origin and breakdown of obligate symbioses, we examined whether specific lineages of symbiotic bacteria specialise in providing B vitamins to hosts. Have hosts relied on a restricted set of symbiotic partners, or have a variety of symbionts converged to provide B vitamins? To address this question, we created a phylogeny for symbionts to quantify the amount of variation in dietary B vitamins explained by symbiont ancestry and their coevolutionary relationships with hosts.

We found that hosts evolved dependence on a broad range of microbes (Supplementary Tables 12-13). Less than 1% of variation in B5 and B9 vitamins in host diets was explained by symbiont phylogeny and the coevolutionary history between symbionts and hosts (symbiont phylogeny % variance: B5 (CI) = 0.05 (0.01, 0.09). B9 (CI) = 0.01 (0, 0.02). Coevolutionary interaction % variance: B5 (CI) = 0.04 (0.01, 0.07). B9 (CI) = 0.01 (0, 0.02). Supplementary Tables 12-13). Instead, divergent symbiotic lineages appear to have become convergently associated with insects feeding on low vitamin B diets (Extended Data Fig. 3). Following the establishment of obligate symbioses, hosts and symbionts tend to coevolve, as related insect families were significantly more likely to be partnered with phylogenetically similar symbionts (coevolutionary interaction % variance (CI) = 25.55 (0, 77). Parafit: P = 0.05. Supplementary Tables 14-15). These results match with research showing that diverse symbiotic bacteria have retained the genes for synthesising B vitamins[21], and that insects whose bacteria lose the capacity to provide B vitamins recruit new symbiont lineages to compensate for the loss[22].

Obligate symbiosis and insect diversification

Finally, we examined whether obligate symbioses have influenced insect diversification rates. The current paradigm, based on observations from specific lineages, such as sap-feeding Hemipterans, is that the acquisition of symbionts opens up new niches and increases host diversification[23,24]. Host-symbiont coevolution can also generate incompatibilities between populations that may increase speciation rates[4].

Dependence on symbionts may, however, ‘trap’ hosts in specific niches, leading to the opposite prediction that symbiosis reduces diversification[4]. For example, hosts can be restricted to feeding on specific resources because of symbiont-assisted specialization[25], or limited by the sensitivity of their obligate symbionts to environmental conditions, such as temperature[26,27]. Mutation accumulation can also degrade symbiont functioning, resulting in hosts being stranded with maladapted symbionts that may increase extinction risk[28]. These competing hypotheses have not been systematically tested, generating debate over the role of symbionts in insect diversification.

We found that obligate symbionts were associated with extreme highs and lows of diversification (Fig. 4. Supplementary Tables 16-17). At the extreme high, herbivorous insect families with obligate symbionts had 10 times as many species compared to the average across families (Fig. 4. Herbivores with obligate symbionts versus background (CI) = 2.74 (1.11, 4.13), pMCMC = 0.004). At the other extreme, extraordinarily low diversification was associated with insect families feeding on blood, which had 9 times fewer species than the average (Fig. 4. Blood feeders with obligate symbionts versus background (CI) = -1.72 (-3.63, -0.17), pMCMC = 0.014). This resulted in a 92-fold difference in the number of species in herbivorous insect families with obligate symbionts versus those in blood-feeding niches. These estimates of diversification were after accounting for differences between holo- and hemi-metabolism and insect phylogenetic history, which are known to affect the number of species in families (see Methods for analyses examining robustness to extinction rate assumptions).

Diversification within feeding niches is promoted by obligate symbiosis

Across insects, patterns of diversification appear to be dominated by feeding niche (Supplementary Table 17). However, within particular feeding niches, symbionts may still systematically promote diversification if they allow species within those niches to exploit different resources. For example, in insect families feeding on more varied resources, such as generalist herbivores and omnivores, symbionts may enable resource partitioning between species, fueling the speciation process. If true, then herbivorous and omnivorous families with obligate symbionts should have higher diversification rates than families without them.

Within feeding niches, we found that herbivorous insect families with obligate symbionts had 19 times as many species as families without symbionts (Fig. 4. Families with versus without obligate symbionts (CI) = 3.32 (1.54, 4.95), pMCMC = 0.001. Supplementary Table 17). Omnivorous and wood eating families of insects with obligate symbionts also had two to three times as many species as families that lacked symbionts, although these differences were not statistically significant (Fig. 4. Omnivorous: (CI) = 0.29 (-0.97, 1.48), pMCMC = 0.668. Wood: (CI) = 1.26 (-2.59, 3.94), pMCMC = 0.68. Supplementary Table 17). These results are similar to findings from specific taxa. For example, symbionts allowed *Chrysomelide* leaf beetles, now one of the most diverse families of insects, to feed and radiate exclusively on plants[29,30]. Similarly, the success of certain highly specious ant lineages has been facilitated by nutrient provisioning symbionts that have allowed them to thrive on primarily plant-derived diets[31].

We examined the sensitivity of our analyses to a number of alternative approaches. First, we tested how inserting families (n=23) that were not included in the published phylogeny[11] influenced our results (Supplementary Table 20). Second, we examined the robustness of our results to including non-bacterial symbionts and including families that had multiple co-occurring obligate symbionts (Supplementary Tables 21-23). Third, we repeated our analyses using a second dataset restricted to only species where dependence on symbionts had been directly studied, rather than inferred from microscopy studies examining the presence of bacteriocytes within certain insect orders and superfamilies

(Supplementary Tables 24-26). The results remained qualitatively and quantitatively similar across all analyses.

Summary

Our results suggest that we can make relatively broad inferences about the causes and consequences of obligate symbioses in insects. After acquiring microbial partners, hosts are able to exploit food resources deficient in B vitamins. In some cases, such as herbivorous insects, the shift to this new niche has facilitated adaptive radiations, analogous to textbook examples such as Darwin's finches. In other cases, such as strict blood feeding, the new niche has severely constrained diversification. The intricate relationships between hosts and their nutritional symbionts therefore appear key to shaping patterns of global biodiversity.

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Figure legends

Figure 1: The evolutionary origins of obligate symbionts and their association with different feeding niches. A) The phylogenetic distribution of obligate symbionts across insect families and their feeding niches. Turquiose tips and branches represent obligate symbiosis and different coloured dots represent different feeding niches. Ancestral feeding niches and obligate symbiosis states were estimated using Stochastic Character Mapping (SCM) (Supplementary Table 5. See Extended Data Fig. 6 for tree with tip lables). B) The number of times obligate symbiosis evolved in different ancestral feeding niches of insects. C) Current rates of obligate symbioses in relation to the feeding niches of insects. The average number of species within families is given along the x axis.

Figure 2: Nutrient deficiencies and the evolution of obligate symbioses. A) Macro-nutrients were not consistently associated with the proportion of species within families that had obligate symbionts. B) Insect families with diets deficient in B5 and B9 vitamins had significantly more species with obligate symbionts than families feeding on diets with high levels of B vitamins (B5 phylo *r* (CI) = -0.43 (-0.56, -0.22), pMCMC = 0.001. B9 phylo *r* (CI) = -0.64 (-0.78, -0.44), pMCMC = 0.001). Vitamin B6 was also weakly related to obligate symbiosis (B6 phylo *r* (CI) = -0.25 (-0.42, -0.02), pMCMC = 0.056). Values of macro- and micro-nutrients are standardized amounts per gram (see Methods for details). Vitamin B concentrations

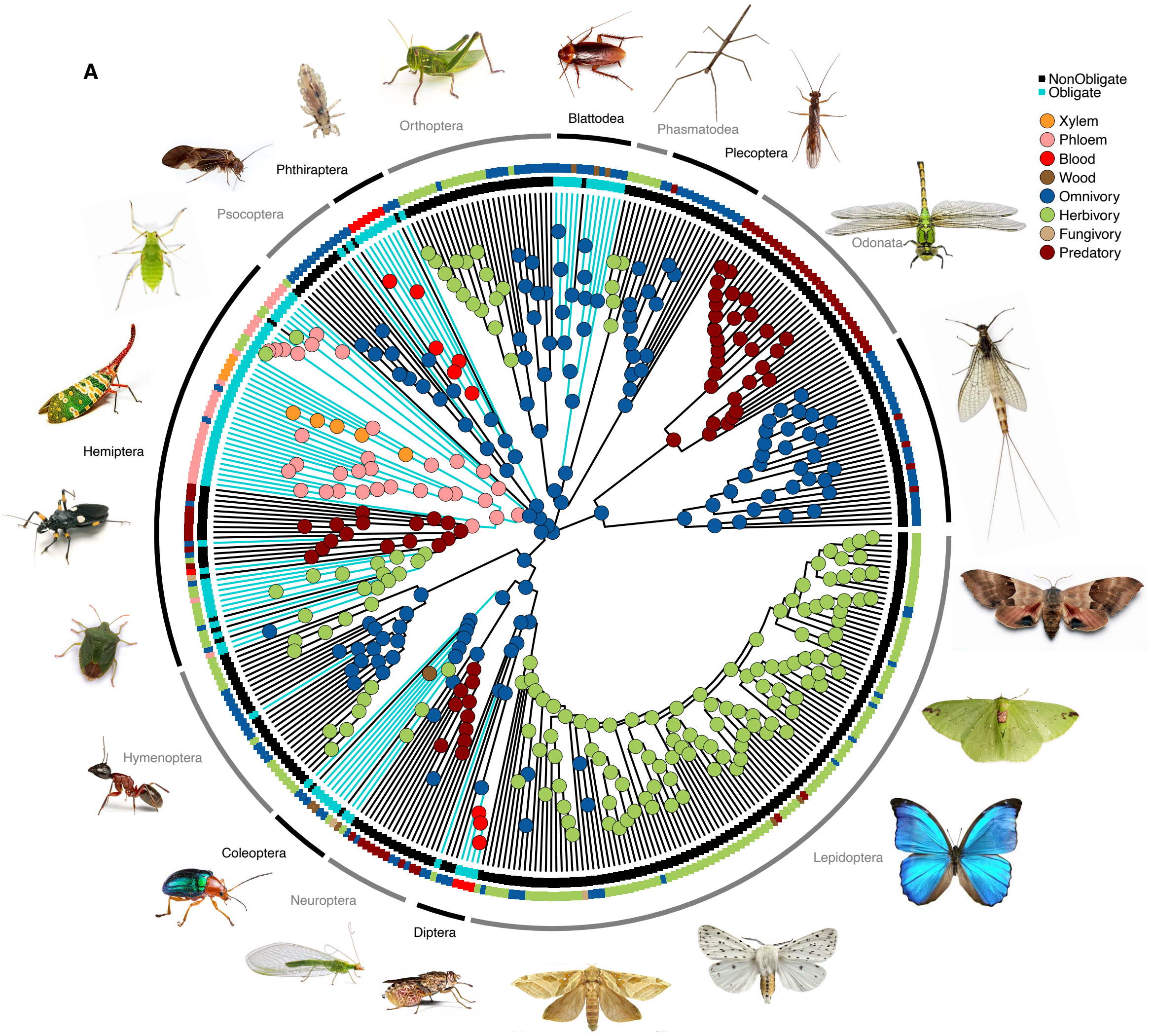
were log transformed and mean centered (see Methods for details). The size of points represents the mean number of host species (log transformed) examined for obligate symbionts per family. Lines represent logistic regressions with 95% confidence intervals plotted for illustrative purposes.

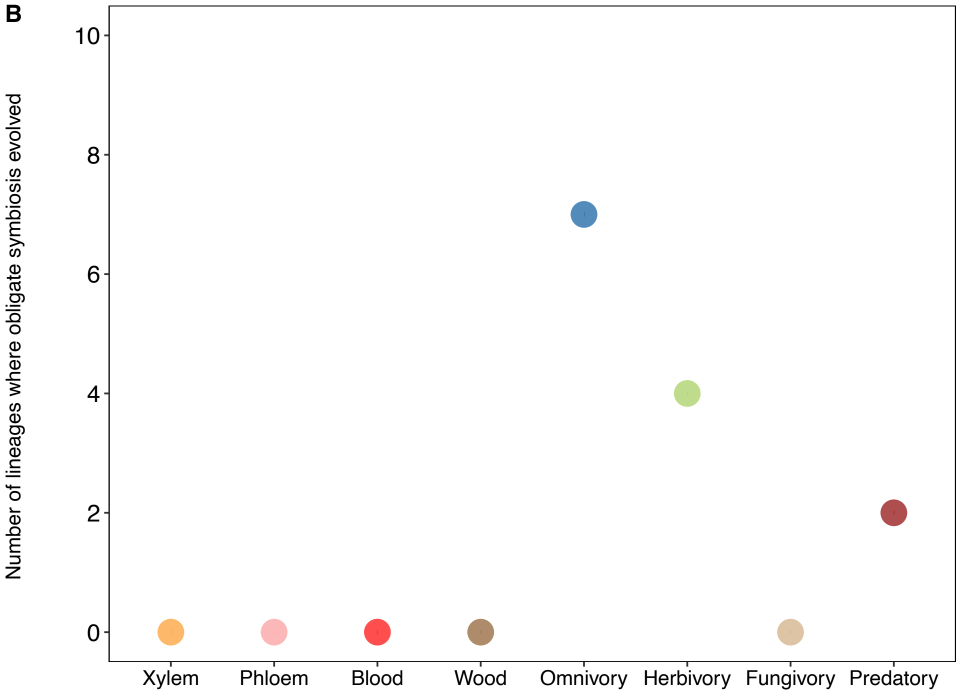
Figure 3: Obligate symbioses enable evolutionary shifts to diets deficient in B vitamins. The ancestors of lineages that evolved obligate symbioses (Non to Ob) had similar levels of B5 (A) and B9 (B) vitamins in their diets to lineages that did not evolve obligate symbioses (Non to Non). However, after acquiring obligate symbionts lineages switched to diets with significantly lower levels of B vitamins (Non to Ob versus Ob to Ob). The evolutionary loss of obligate symbiosis was also associated with increases in dietary levels of vitamin B5 (Ob to Ob versus Ob to Non). Vitamin B concentrations were log transformed and mean centered (see Methods for details). Violin density curves represent the posterior distribution of estimated ancestral levels of B5 and B9 vitamins (1000 samples) estimated using a BPMM (Supplementary Table 10). The width of the violin corresponds approximately to the most likley estimate of B vitamins. Significant differences between transitions are indicated by * pMCMC <0.05, ** pMCMC <0.01, *** pMCMC <0.0001.

Figure 4: Obligate symbioses and the evolutionary potential for diversification. Diversification was measured as the number of species within families controlling for family age. The highest and lowest number of species per family were associated with feeding niches that had obligate symbionts. Herbivorous insect families with symbionts had significantly more species than those without, whereas blood feeding insects, which exclusively had obligate symbionts, had the lowest number of species. Significant differences versus background numbers of species per family are indicated by * pMCMC <0.05, ** pMCMC <0.01, *** pMCMC <0.0001 obtained from a BPMM (Supplementary Table 17).

Figures

Figure 1




B
Number of lineages where obligate symbiosis evolved
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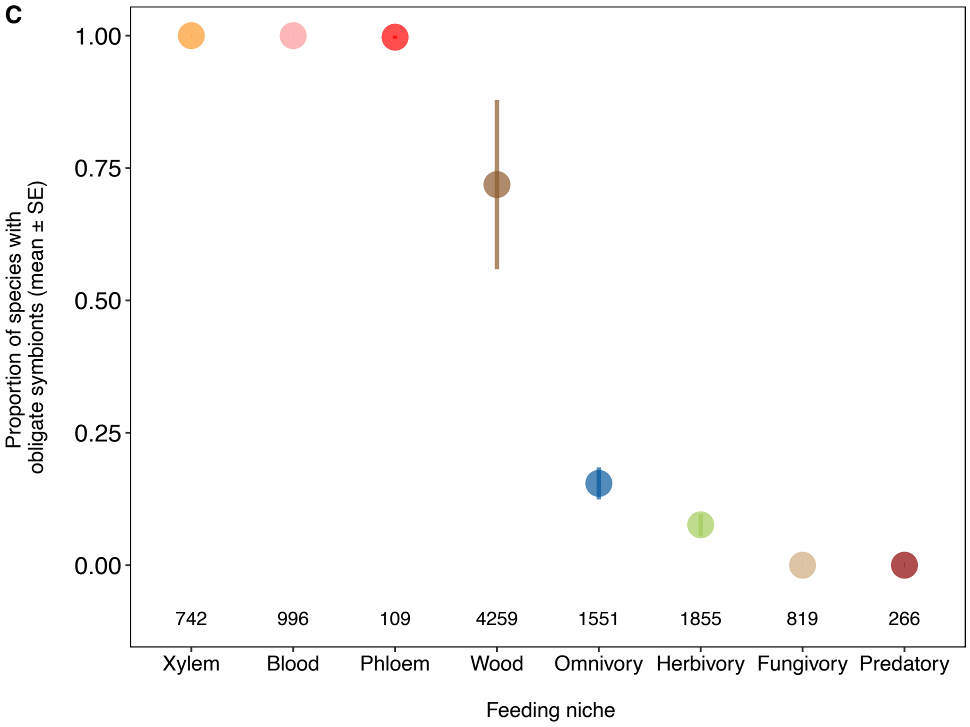
8
6
4
2
0
Xylem
Phloem
Blood
Wood
Omnivory
Herbivory
Fungivory
Predatory


C
Proportion of species with obligate symbionts (mean ± SE)
1.00
0.75
0.50
0.25
0.00
742
996
109
4259
1551
1855
819
266
Xylem
Blood
Phloem
Wood
Omnivory
Herbivory
Fungivory
Predatory
Feeding niche

405 **Figure 2**

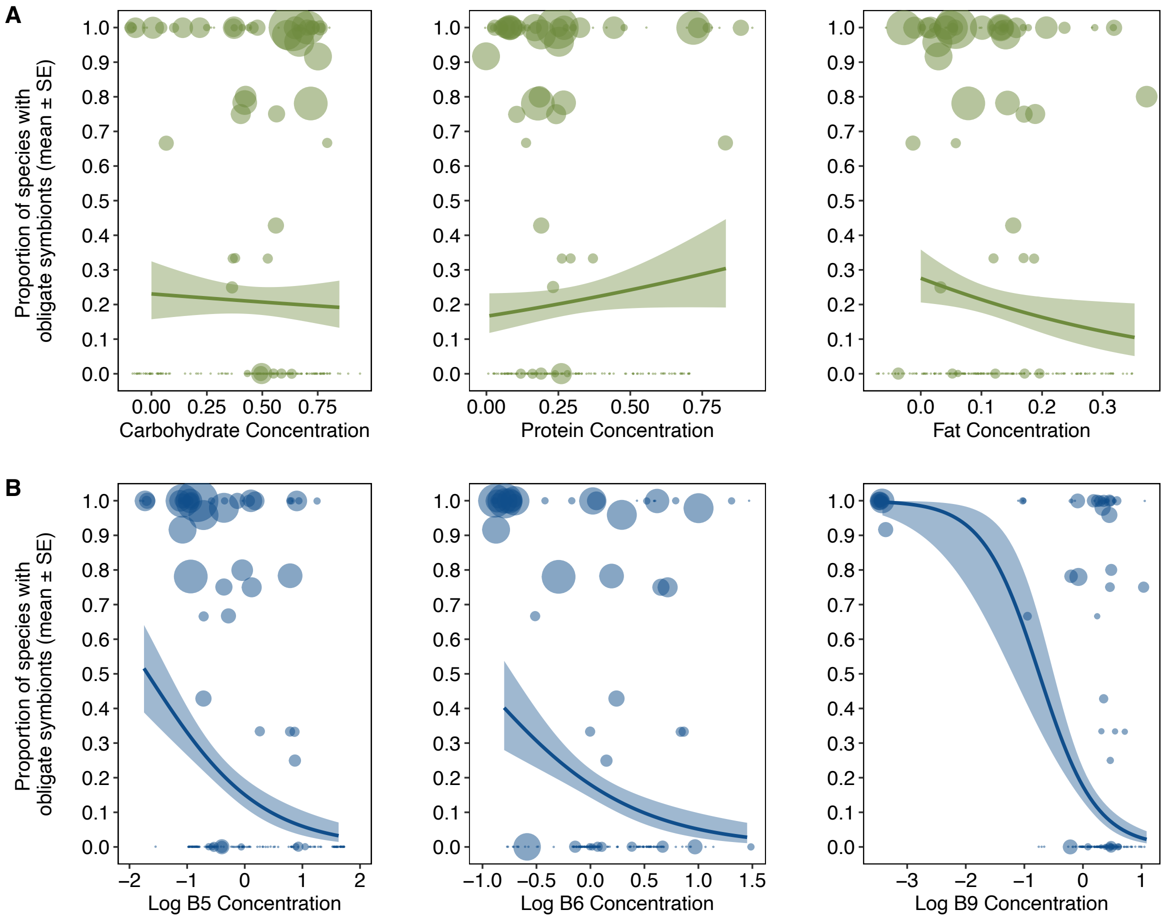


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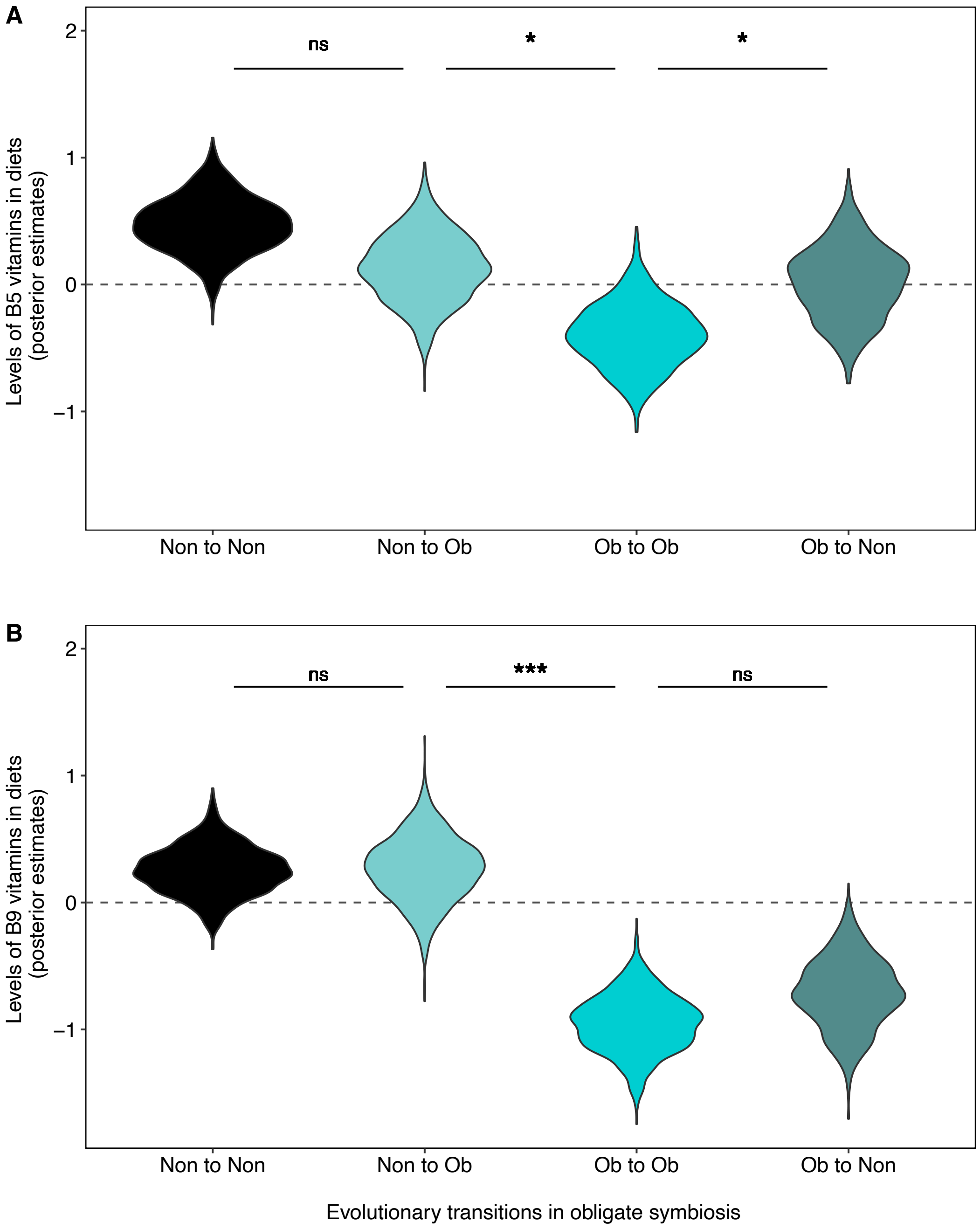
Figure 3


A
2
1
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−1
ns
*
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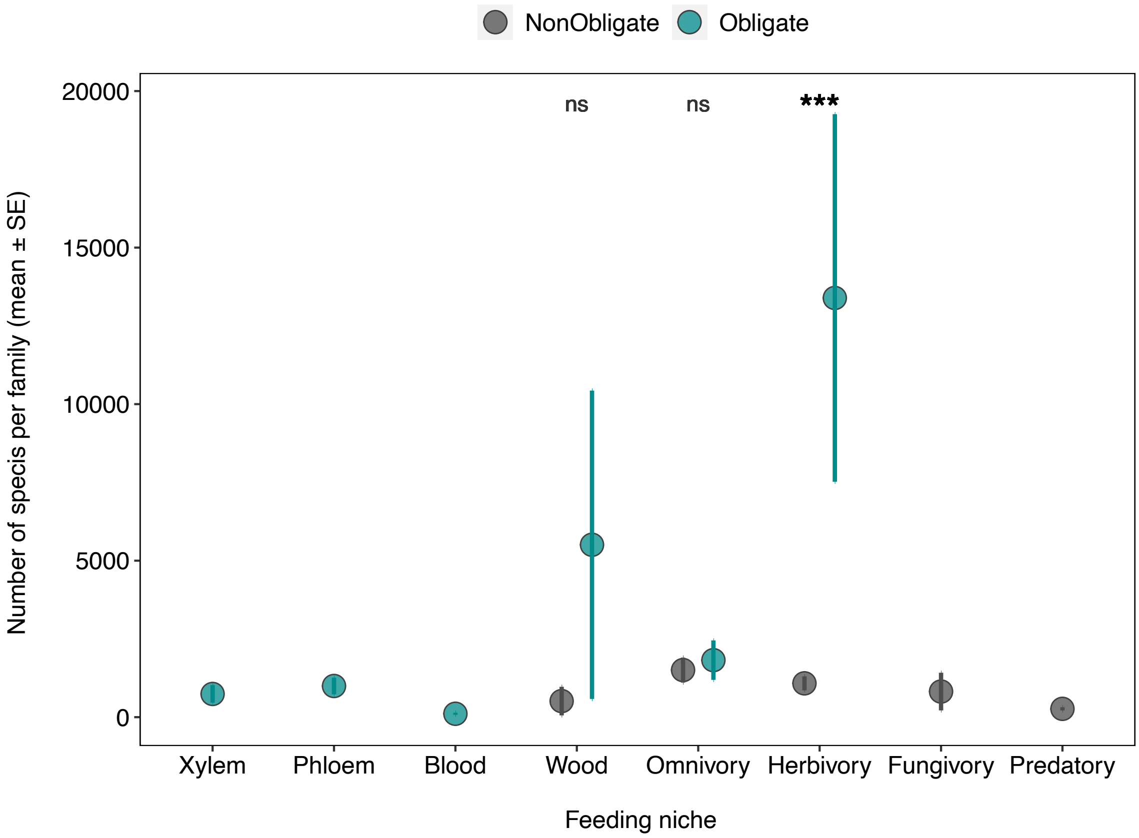
Levels of B5 vitamins in diets (posterior estimates)
Non to Non
Non to Ob
Ob to Ob
Ob to Non
B
2
1
0
−1
ns
***
ns
Levels of B9 vitamins in diets (posterior estimates)
Non to Non
Non to Ob
Ob to Ob
Ob to Non
Evolutionary transitions in obligate symbiosis

Figure 4



● NonObligate
● Obligate
Number of specis per family (mean ± SE)
20000
15000
10000
5000
0
ns
ns
***
Xylem
Phloem
Blood
Wood
Omnivory
Herbivory
Fungivory
Predatory
Feeding niche

Methods

1. Data collection

1.1 Insect and symbiont data

Literature searches

We complied a database on insect-microbe symbioses by: (1) searching published literature using the following key words [order name] OR [family name] AND "symbio"* using the search engines Web of Science and Google scholar during 2015-2017 and again in 2020, (2) searching several prominent reviews (e.g. Ries 1931[32], Schneider 1939[33], Müller 1962[34], Buchner 1965[20], Douglas 1989[35], Abe *et. al.* 2000[36], Bourtzis and Miller 2003[37], 2006[38] and 2009[39], Baumann 2005[40], Baumann *et. al.* 2013[41]), and (3) forward and backward searches from the resulting papers. A full list of the papers screened can be found in Supplementary Table 2.

The insect families included in the literature search were those listed in Bouchard *et. al.* 2011[42], Davis *et. al.* 2010[43], and Rainford *et. al.* 2014[11], and those included in published phylogenies investigating insect biodiversity: Hedges et al. 2015[44], Misof et al 2014[45], and Rainford et al 2014[11]. For symbiont detection, we only considered studies using methods capable of capturing phylogenetically diverse bacteria species (e.g. deep-coverage sequencing, or cloning, using 'universal' 16S rRNA primers), or microscopy studies investigating whole insects for the presence of symbionts.

Specific clades of insects are known to carry the same obligate symbionts due to strict vertical transmission (see Supplementary Table 2 ‘reference obligate criteria’). We therefore searched Genbank to recover all insect species that have been associated with specific vertically transmitted symbionts (identified taxonomically by symbiont genus name in most cases) in order to increase our coverage of host-symbiont associations (Supplementary Table 3). Search results were checked manually to ensure host species belonged to the insect clade known to harbour the symbiont (Supplementary Table 3). In families that have species both with and without obligate symbionts, we only considered species directly studied for obligate symbiosis. Note that vertically transmitted symbionts were only included in analyses of host evolution (section 4.1-4.3 & 4.5), not in analyses of host-symbiont coevolution (section 4.4).

Data inclusion and exclusion

The aim of our paper was to investigate the evolution of beneficial obligate symbioses. We therefore excluded studies: (1) on parasitic symbionts, such as those that manipulate host reproduction (e.g. *Spiroplasma*, *Cardinium*, *Wolbachia*) that have not evolved beneficial functions; (2) that failed to screen the entire insect (e.g. only performed insect gut analyses); and (3) on symbionts with presumed beneficial functions, but that lacked data needed for our obligate criteria (see below). Fungal and protist symbionts were included where data on host dependency was available. Analyses of host-symbiont coevolution were restricted to symbionts for which a phylogeny could be constructed (bacteria with 16S rRNA genetic data: see section 2 for details).

For each insect-microbe association we collected data on: the insect species; juvenile and adult insect diets; whether insects were holo- versus hemi-metabolous; the identity of symbionts (if known); symbiont domain; whether symbionts were intra- or extra-cellular (where known); whether symbionts were housed within specialized structures (e.g. bacteriocytes); and whether insects were obligately dependent on symbionts (see below for assessment criteria).

Criteria for assessing obligate symbiosis

Obligate dependence on endosymbionts would ideally be established from studies that have measured changes in insect fitness before and after the experimental removal of the microbes. However, such experiments are challenging and so these studies are relatively rare. We therefore defined obligate dependence of insects on symbionts using proxy measures that fulfilled one of the following criteria:

1. Insects have a bacteriome (or mycetome) with bacteriocytes (specialized symbiont-housing cells) containing symbionts, as these specialised organs have been shown to only evolve in insects with obligate symbionts[10].

2. Insect-symbiont phylogenies are concordant and symbionts are universally present in reproductive females.

3. Symbiont removal results in reductions in host fitness and symbionts are universally present in reproductive females.

Consequently, species that lack specialized symbiont organs, or where symbionts are not universally present in females, were classified as not having obligate symbionts. If symbionts were universally present, but cophylogenetic and/or host fitness data were unavailable the relationship was classified as unresolved.

Data on individual species were used to estimate the proportion of species in each family that have evolved dependency on symbionts, which is summarized in Supplementary Table 1. Data on each insect species examined, their associated symbionts and the criteria to assess dependency are in Supplementary Table 2.

1.2 Feeding niche classification

The feeding niches of species were classified using information on their diets. Omnivores were defined as species that feed on both plant and animal matter, or those that scavenged on detritus material. Due to large differences in the nutrient contents of different plant tissues, insect species that specialize on phloem-, xylem- and wood-feeding (xylophagy) were considered separately from species that exploit non-vascular/non-woody plant tissues (e.g. leaves, flowers, fruits, seeds, and/or root tips), which we refer to as generalist herbivores (or phytophagous).

Species feeding niches were subsequently used to classify each family into a feeding niche (Supplementary Table 1). Families were described as having omnivorous diets, if they contained species that were omnivores/detritivores, or if species fed in more than one of the following niches: fungivory, hermatophagy, carnivory and phytophagy or phloem-feeding. Families were assigned to the feeding categories of hematophagy, phloem-feeding, xylem-feeding and predatory where the vast majority, if not all, known species in the family fed exclusively on those resources. Families assigned as xylophagous were those where the majority of species fed on wood as their primary food source. Families containing species that fed on multiple plant tissues were classified as generalist herbivores (or phytophagous).In cases where species-specific diets were not available, we based diets on family-level feeding habits published in books and reviews listed in Supplementary Table 2.

1.3 Nutrient data

We assigned insect diets into 19 categories (Supplementary Table 2) based on published literature using the search terms [species name] and [adult diet] or [juvenile diet] in Web of Science and Google Scholar. Where possible we cross-validated diet assignments using multiple published studies (Supplementary Table 2). For each of the 19 diet categories we collected data on the total carbohydrate, protein, and fats, as well as the micronutrients vitamins A, B, C, D, E, K, choline and betaine based on as many sources of the same food type as possible (range of number of of different food sources = 1 to 28) collated from nutritional databases and scientific literature (Supplementary Table 4). Nutrient contents were from a range of food types, and are therefore approximations of insect diets. Where possible, micronutrients were broken down into their sub-components, for example, individual B vitamins. There was insufficient data on vitamin D, choline and betaine for analyses (>40% of insect families missing data).

Nutrient profiles for families were estimated by taking an average for each nutrient based on the diets of species used to confirm the obligate criteria, or based on the family-level diets where species-specific diets were unavailable. To calculate nutrient values for each species, an average of adult and juvenile diets was taken, which were highly correlated (Extended Data Fig. 4). For omnivorous species, nutrients were calculated by averaging across all food sources.

Standardisation of nutrient data The data on nutrients (carbohydrates, fats, proteins and vitamins) were reported as the amount per gram, but for some dietary items this was wet weight and others it was dry weight. Wet weights were much greater than dry weights and therefore we standardized values to make nutrient values comparable across dietary items. Values were standardized by dividing each macro-nutritional component (carbohydrate, fat, protein) by the relative weight of each dietary item. The relative weight of each dietary item (*rw*) was calculated as:

$$rw = \text{total weight of dietary item} / \text{maximum total weight of any dietary item}$$

As a result, the dietary item with the greatest total weight remained unchanged ($rw = 1$) whereas foods with lower weights were increased ($rw < 1$). To calculate the relative amounts of each vitamin in each food source the same approach was used, but instead of using total weight of all components we used the total weight of vitamins (See R script "DataConstruction.R" for details).

1.4 Diversification rates

In the absence of species level phylogenies, diversification is often modelled using two different approaches: diversification rates and raw values of species richness. We examined the influence of obligate symbiosis and feeding niches on diversification using both approaches. Data on the number of extant species in insect families (species richness) were taken from Rainford et. al. 2014[11]. The ages of families were extracted from the Rainford phylogeny which was time calibrated using 86 fossils[11].

Net diversification rates were also estimated using methods outlined by Magallón and Sanderson[46] assuming different rates of extinction using the function 'bd.ms' in the R package geiger[47]. Diversification rates with different extinction fractions were strongly correlated (Pearson's correlation coefficient $r > 0.99$. Extended Data Fig. 5). Therefore, we only analysed diversification rates that were calculated assuming an intermediate extinction fraction ($e = 0.5$) (see Wiens *et. al.* 2015[48] for a similar approach).

Diversification rates are an estimate of the rate of change in species numbers over time using clade age and species richness data, typically modelled as some form of birth-death process[49]. While such analyses try to more accurately capture speciation and extinction processes, they can produce misleading results when diversity is not constant or unbounded through evolutionary time[50,51]. This is known to be the case for insect diversification where there have been rapid bursts of speciation through time[52]. Our data also support this idea, as diversification rates were weakly correlated to species richness ($r \sim 0.16$. Fig. 5).

As the aim of our paper was to examine how obligate symbiosis has influenced increases and decreases in diversification, irrespective of how fast or slow those species accumulated, we present analyses of species richness in the main text. Differences in the ages of insect families were accounted for in these analyses by including family age as an explanatory variable (see analysis section 4.5.2 below). The results of the analyses of diversification rates were quite different from those of species richness (see analysis section 4.5.3 and 4.5.4). Feeding niche and obligate symbiosis were not significantly related to diversification rate (Supplementary Tables 18-19). This highlights that in these widely divergent groups of insects feeding niches and obligate symbioses do not influence the rate of diversification across the evolutionary time scales examined here, but rather influence the number of species that have accumulated due to the long-term balance between speciation and extinction.

2. Insect and symbiont phylogenies

Insects

We used the insect phylogeny generated by Rainford et al 2014[11]. Families that lacked data on obligate symbioses were pruned from the tree. There were 23 families for which there was data on obligate symbioses but not included by Rainford. We therefore added families to the phylogeny at branches corresponding to published sister taxa (Supplementary Table 1) using the bind.tip function in the R package 'phytools'[53] (See R script 'Rainford_adding_tips.R' for details). Added families were not included for diversification analyses due to uncertainty of the age of these families.

Symbionts

We estimated the phylogenetic relationships for bacterial symbionts for which genetic data was available. A ~1,500 bp region of the bacterial 16S rRNA gene downloaded from the SILVA RNA database was aligned with MUSCLE and edited in the alignment software Geneious 8.1.8 (https://www.geneious.com). We generated a maximum likelihood phylogeny for the bacterial lineages using the on-line PhyML server[54], and the best fitting models of evolution were estimated using the Aikake Information Criterion (AIC). We bootstrapped the symbiont phylogeny 100 times and rooted to *Thermus thermophilus*, which is basal to all the bacterial lineages presented in this study.

3. General statistical methods

Data were analysed using Bayesian Phylogenetic Mixed Models with single (BPMM) and multiple response variables (MR-BPMM), Stochastic Character Mapping (SCM), and transition rate models with Markov Chain Monte Carlo estimation. In this section we provide general details of modelling approaches and in section four we outline the specific analyses conducted. All analyses were conducted in R version 4.0.2[55], apart from transition rate models that were conducted in BayesTraits V3[56]. Continuous response and explanatory variables were Z-transformed prior to analyses (mean = 0, standard deviation = 1).

3.1 Single and Multi-Response Bayesian Phylogenetic Mixed Models (BPMM & MR-BPMM)

Model construction, parameter estimates and assessing significance

To estimate phylogenetic signature, co-evolutionary relationships and ancestral trait values we used BPMMs and MR-BPMMs with Markov chain Monte Carlo (MCMC) estimation in the R package MCMCglmm[57]. The non-independence of data resulting from multiple species per family, phylogenetic relatedness between insect hosts and phylogenetic relatedness between symbiont lineages were modelled using random effects. For phylogenetic effects we fitted variance-co-variance matrix constructed from the insect and bacteria phylogenies. We estimated the amount of variation in response variables explained by random effects (RE), including phylogenetic effects, as the intraclass correlation coefficient (ICC) on the latent scale estimated as:

$$Vi / VRE + Ve$$

where Vi is the focal random effect, VRE is the sum of all random effects and Ve is the residual variance on the latent scale. For binomial error distributions Ve was calculated as the observed residual variance plus the variance associated with the link function (logit = pi^2/3. See [58,59] for discussion).

Phylogenetic and residual correlations between traits were estimated using MR-BPMMs whereby unstructured phylogenetic and residual covariance matrices were fitted as random effects. Correlations between traits were calculated as:

COV xy / square root (VAR x * VAR y)

The global intercept was removed from MR-BPMMs to allow trait specific intercepts to be estimated. Parameter estimates from models are presented as posterior modes (PM) with 95% credible intervals (CIs). P values (pMCMC) were estimated as the number of posterior samples above or below a specified value divided by the total number of posterior samples, corrected for the finite number of MCMC samples[57,60]. For correlations and fixed effects the specified value was 0, and for testing differences between fixed effects it was the number of posterior samples where one level was greater than the other.

Prior settings

For random effects we began prior selection by assessing model convergence using inverse-Wishart priors (V = 1, nu=0.002). If the mixing properties of the MCMC chain were poor, which was often the case for binomial response variables, we examined two different parameter expanded priors (Fisher prior: V = 1, nu=1, alpha.mu = 0, alpha.V = 1000) and ($\chi 2$ prior: V = 1, nu=1000, alpha.mu = 0, alpha.V = 1)[59]. For all other traits an inverse-Wishart prior was specified for residual variances (V = 1, nu=0.002). For fixed effects the default priors in MCMCglmm (independent normal priors with zero mean and large variance (10^10)) were used apart from in models with binomial response variables where a prior of mu = 0, V = $\sigma 2$units + $\pi 2/3$ was specified. This is approximately flat on the probability scale when a logit link function is defined[57], and in all cases improved the mixing of chains. The final prior settings used for each analysis are specified in the Supplementary R code (See R script "Analyses.R").

Model settings and examining model convergence

Models with Gaussian, Binomial and Poisson error distributions were run for 2000000 iterations, a burnin of 1000000 iterations and chains sampled every 1000 iterations. Binomial models were specified with logit link functions and Poisson models were specified with log link functions.

We examined the convergence of models by repeating each analysis three times and examining the correspondence between chains using the R package 'coda'[61] in the following ways: (i) visually inspecting the traces of the MCMC posterior estimates and their overlap; (ii) calculating the autocorrelation and effective sample size of the posterior distribution of each chain; and (iii) using Gelman and Rubin's convergence diagnostic test that compares within- and between- chain variance using a potential scale reduction factor (PSR). PSR values substantially higher than 1.1 indicate chains with poor convergence properties. For convergence checking see R script 'ModelCheckingCombining.R'.

3.3 Stochastic character mapping (SCM)

SCM was used to estimate ancestral states of obligate symbiosis and feeding niches across the insect phylogeny in the R package 'phytools'[53]. In brief, this approach calculates the conditional likelihood that each ancestral node is in a given state that depends on the estimated transition rate matrix (Q) between states and the length of the branch associated with that node. Based on these conditional likelihoods, ancestral states at each node are stochastically simulated and used in combination with observations at the tips to reconstruct a character history along each branch. Each character history is simulated using a continuous-time Markov chain where changes between states and the time spent in each state is modelled as a Poisson process (see Bollback 2006[62] for more details).

3.4 Transition rate models

The DISCRETE module in BayesTraits V3 was used to estimate transition rates (q) between two binary traits with MCMC estimation. We used hyper priors where values are drawn from a uniform distribution with a range 0 to 10 to seed the mean and variance of an exponential prior to reduce uncertainty over prior selection[56]. We ran each model three times for a total of 11000000 iterations, a burnin of 1000000 iterations and sampled every 1000 iterations. We examined the convergence of models in the same way as section 3.2.

Bayes factors (2(log marginal likelihood of complex model – log marginal likelihood of simple model)) were used to test if models that allowed coevolution provided a better fit to the data than models that assumed independent evolution. To calculate the log marginal likelihood, we used the stepping stones procedure as described in the BayesTraits V3 manual where 100 stones were run for 1000 iterations each. Bayes factors over 2 are considered to offer positive evidence, over 5 strong evidence and over 10 very strong evidence[56].

To test whether transitions rates were significantly different from each other, we calculated the posterior mode, 95% CIs and pMCMC value of the posterior distribution of differences between transition rates (see R markdown script ‘ExtendedData.Rmd’).

3.5 Missing nutrient data

There were missing values for some nutrients in the diets of some species in our dataset (Supplementary Table 4). In BPMMs missing data is permitted in response variables and is predicted with an accuracy relative to the phylogenetic signature in traits and the magnitude of correlations between traits in the case of MR-BPMMs. This can enable missing values to be predicted with high accuracy[60,63]. All traits analysed had high phylogenetic signature (phylo $H^2 = 0.65$-0.95. Supplementary Table 7) and therefore missing nutrient values were included in MR-BPMMs models. For all other analyses and for explanatory variables in BPMMs missing data were removed.

4. Specific analyses

4.1 Evolutionary history of obligate symbioses and feeding niche colonization

4.1.1 Estimating the number of origins of obligate symbioses using BPMM

The probability of each of node in the insect phylogeny having an obligate symbiont was estimated using a BPMM with the number of species with and without obligate symbionts within each family as a binomial response variable. This accounts for variation in the number of species examined for obligate symbionts across insect families. The insect phylogeny was included as a random effect and the state of each node was estimated using the ‘predict’ function in MCMCglmm. Nodes were classified as ‘obligate’ where the posterior probability was greater than 0.5. We found support for 13 origins and 9 losses of obligate symbiosis

4.1.2 Estimating the number of origins of obligate symbioses using SCM

Insect families (n = 400) were classified as having evolved an obligate symbiosis (>0% species within families have obligate symbionts) or not (0% species within families have obligate symbionts). Data on obligate symbioses were used to build 1000 stochastic character maps across the insect phylogeny using an all-rates different Q matrix with empirical Bayes estimation. The proportion of the 1000 stochastic character maps that nodes were predicted to have obligate symbionts was used to classify the ancestral state of each node (>50% of stochastic character maps = ‘obligate’, <50% = ‘non-obligate’). Differences in state between ancestral and descendant nodes were used to identify the evolutionary origins and losses of obligate symbionts. We found support for 12 origins and 9 losses of obligate symbiosis and the relative amounts of time spent in each state were 27% obligate, 73% non-obligate (Supplementary Table 5). The estimates of ancestral states obtained using SCM were extremely similar to those from BPMM indicating our results were robust to the type of statistical techniques used (Supplementary Table 5).

4.1.3 Estimating ancestral feeding niches using SCM

Ancestral feeding niches were estimated using SCM analysis of the feeding niches of each insect family (n = 400). The settings for the model were the same as those in section 4.1.2. Each node was assigned to a feeding niche according to the niche with the highest proportion of the 1000 stochastic character maps. Transitions between feeding niches were identified where ancestral and descendant nodes were in different states (Supplementary Table 5).

4.1.4 Estimating rates of obligate symbiosis across different feeding niches using a BPMM

The probability that insects occupying different feeding niches have obligate symbionts was modelled using a BPMM with the number of species with and without obligate symbionts within each family as a binomial response variable. The feeding niche of each family was fitted as an eight-level fixed effect and the insect phylogeny was fitted as a random effect (Supplementary Table 6). To determine if rates of obligate symbiosis were significantly different across niches, we calculated the pairwise differences between niches and examined if the 95% CIs spanned 0 (Supplementary Table 6).

4.2 Nutritional deficiencies and the evolution of obligate symbiosis

4.2.1 Estimating the phylogenetic correlations between obligate symbioses and macro- and micro-nutrients using a MR-BPMM

The correlations between obligate symbiosis and nutrients within diets was estimated using a MR-BPMM with the number of species with and without obligate symbionts as a binomial response variable and Z-transformed concentrations of carbohydrate, protein, fat, vitamin A, vitamin B (sum of individual B vitamins), vitamin C, vitamin E and vitamin K as gaussian response variables. Unstructured phylogenetic and residual variance-covariance matrices were fitted as random effects (Supplementary Table 7).

4.2.2 Estimating the nutrient contents of each feeding niche with and without obligate symbionts using a MR-BPMM

Differences in the nutritional composition of different feeding niches were estimated using a MR-BPMM with carbohydrate, protein, fat, vitamin A, vitamin B, vitamin C, vitamin E and vitamin K as gaussian response variables and feeding niche (8-level factor) fitted as a fixed effect. Unstructured phylogenetic and residual variance-covariance matrices were fitted as random effects. To test whether nutrient levels in each niche differed from background rates, we re-ran models including a two-level factor of focal feeding niche versus all other niches instead of the eight-level fixed effect of feeding niche (Supplementary Table 8).

4.2.3 Estimating the phylogenetic correlations between obligate symbioses and individual B vitamins using a MR-BPMM

The phylogenetic correlation between obligate symbiosis and vitamin B was highly significant. We therefore analysed individual B vitamins (B5, B6 and B9) to examine if they varied in their association with obligate symbiosis using a MR-BPMM. The number of species with and without obligate symbionts was fitted as a binomial response variable and Z-transformed concentrations of vitamins B5, B6 and B9 were fitted as gaussian response variables. Unstructured phylogenetic and residual variance-covariance matrices were fitted as random effects (Supplementary Table 9). Data on vitamins B7 and B12 were not analysed as there were large amounts of missing values (>30% of insect families). Data on B1, B2, and B3 were highly correlated to vitamin B5 levels ($r > 0.9$), but there was more data on vitamin B5. As a result, only vitamin B5 was analysed, but it is worth noting that the associations between B5 and obligate symbioses could also be due to the effects of B1, B2 and B3.

4.3 Nutrient deficiencies and the evolutionary gains and losses of obligate symbiosis

4.3.1 Estimating ancestral vitamin B5 and B9 in diets of families that gained and lost obligate symbionts using a MR-BPMM

We examined how the levels of B5 and B9 vitamins differed between ancestors of families with and without obligate symbionts using a two-step approach: first, we used the output of the model in section 4.1.1 to classify nodes as: (i) non-obligate node with non-obligate descendants (NonOb to NonOb); (ii) non-obligate node with at least one obligate descendant (NonOb to Ob); (iii) obligate node with obligate descendants (Ob to Ob); and (iv) obligate node with at least one non-obligate descendent (Ob to NonOb); . Second, nodal classifications were entered as a four-level fixed factor in a MR- BPMM with Z transformed B5 and B9 vitamin concentrations modelled as Gaussian response variables (Supplementary Table 10). Unstructured phylogenetic and residual variance-covariance matrices were fitted as random effects with the phylogenetic covariance matrix being linked to node labels. We fitted interactions between the response trait and node classification to estimate B5 and B9 vitamin levels preceding the origin (comparison of classifications i versus ii), maintenance (comparison of classifications i versus iii) and loss of obligate symbioses (comparison of classifications iii versus iv). To account for uncertainty in our node classifications, we repeated the analysis 100 times, each time reclassifying nodes by resampling from the posterior distribution of the probability of nodes having an obligate symbiont. Posterior samples from across the 100 models were then combined. Each model was run for 1100000 iterations with a burn-in of 1000000 iterations and thinning interval of 10000 samples, which across the re-samplings resulted in 1000 posterior samples (100 re-samplings x 10 samples per resampling).

4.3.2 Estimating transition rates between obligate symbioses and B5 and B9 vitamins using transition rate models

We tested if models that allowed for the coevolution between obligate symbiosis and B5 and B9 vitamins better explained our data than models that assumed independent evolution of each trait using transition rate models. Coevolution was modelled using an all rates different (ARD) Q matrix and separate sets of models were run for B5 and B9 vitamins. For these analyses only binary classifications can be modelled. We therefore transformed data into obligate (>0% species within families have obligate symbionts) and non-obligate (0% species within families have obligate symbionts) insect families, and high and low B5 and B9 vitamins. For B vitamin classifications we choose two different cut-offs to establish the sensitivity of our results to different thresholds: above and below the 25% and 50% quantile for high and low B vitamins respectively (Supplementary Table 11). It was not necessary to examine the sensitivity of our results to the classification of obligate symbiosis as 96% of 400 insect families had 100% of species with or without obligate symbionts.

4.4 Nutrient deficiencies and the evolution of host-symbiont co-specialisation

4.4.1 Estimating the effect of host symbiont interactions on the evolution of obligate symbioses using BPMMS and parafit

To examine how obligate symbioses have been influenced by the coevolutionary history between insects and bacteria, we constructed a dataset of pairwise combinations between all insect families and all symbionts. Insect families that shared a symbiont due to vertically transmission from a common ancestor were removed for these analyses. For each combination, the number of insect species within a family with a particular obligate symbiont versus the number of species without that symbiont was calculated. This enabled differences in the sampling effort across different insect-bacteria associations to be accounted for. We analyzed the number of species in insect families with and without each symbiont using a BPMM with a binomial error distribution and logit link function. Differences in the probability of forming obligate partnerships between intra- and extra-cellular symbionts was modelled by including a two-level fixed effect. We fitted three different variance-co-variance matrix as random effects to quantify the amount of variation in obligate symbiosis explained by: (i) insect hosts independent of their phylogenetic history ('h') e.g. certain hosts are more likely to form obligate relationships than others; (ii) insect hosts phylogenetic history ('[h]') e.g. certain host lineages are more likely to form obligate relationships than others; and (iii) phylogenetic interactions between hosts and symbionts ('[hs]') e.g. particular host phylogenetic lineages are more likely to form obligate symbioses with particular bacterial phylogenetic lineages (Supplementary Table 14). See Hadfield et. al. 2014 for methods on model fitting[64].

Each bacterial symbiont lineage was only found in a single insect family. The lack of replication of symbiont lineages across hosts meant that the following sources of variation in obligate symbioses were not identifiable: (iv) interspecific variation amongst symbionts independent of their phylogenetic history (s) e.g. certain bacteria are more likely to form obligate relationships than others; (v) the phylogenetic history of bacteria (‘[s]’) e.g. certain bacterial lineages are more likely to form obligate relationships than others; (vi) interspecific interactions between hosts and symbionts independent of their phylogenetic history (hs) e.g. certain insect bacteria combinations are more likely to form obligate relationships than others; (vii) particular insect hosts, independent of their phylogenetic history, being more likely to form obligate symbioses with specific phylogenetic lineages of bacteria (‘h[s]’); and (viii) particular bacterial lineages, independent of their phylogenetic history, are more likely to form obligate symbioses with specific insect families ([s]h).

To further examine whether phylogenetically related lineages of bacteria are more likely to form obligate symbioses with phylogenetically related lineages of insects we used parafit in the R package ‘ape’ (Supplementary Table 15). This tests the correlation between host and symbiont shared branch lengths against a randomised distribution generated from 1000 permutations of the data[65].

4.4.2 Estimating the effect of host symbiont interactions on dietary levels of B5 & B9 vitamins using BPMMS

To test if specific lineages of symbiotic bacteria specialise in providing B5 and B9 vitamins to hosts we used the same BPMM approach described in section 4.4.1. We estimated variation in levels of B vitamins (Gaussian responses) explained by h, [h], [s] and [hs]. Separate models were run for B5 and B9 vitamins and data were restricted to combinations of hosts and bacteria that formed obligate symbioses (>0% species within insect families with obligate symbionts) (Supplementary Tables 12-13).

4.5 Obligate symbioses and diversification

4.5.1 Estimating the relationship between species richness and obligate symbiosis using a BPMM

The relationship between obligate symbioses and diversification was estimated using a MR-BPMM with the number of species with and without obligate symbionts as a binomial response variable and species richness as a Poisson response variable. To control for older families potentially accumulating more species than younger families we included a fixed effect of family age. Family ages were estimated from the phylogenetic tree by (Rainford et al. 2014[11]), which is time calibrated. A fixed effect of whether insect families were holo- or hemi-metabolous (two-level factor) was also included as a fixed effect, as it has previously been shown to influence diversification rates[11]. Unstructured phylogenetic and residual variance-covariance matrices were fitted as random effects (Supplementary Table 16).

There were 23 insect families that were added to the Rainford phylogeny for the analyses of obligate symbioses (see below and Supplementary Table 1). It was not possible to estimate the age of these families so they were excluded from all diversification analyses.

4.5.2 Estimating the relationship between species richness and obligate symbiosis for different feeding niche using a BPMM

The diversification rates of insects occupying different feeding niches were estimated using a BPMM with species richness as a Poisson response variable. The feeding niche (eight-level factor) of each family, family age and holo-hemi metabolism were fitted as fixed effects (Supplementary Table 17). To test whether the species richness of each niche differed from background levels, we re-ran models including a two-level factor of focal feeding niche versus all other niches instead of the eight-level fixed effect of feeding niche (Supplementary Table 17).

4.5.3 Estimation of the relationship between diversification rate and obligate symbiosis using a MR-BPMM

To examine the correspondence between the rate at which species diversify versus the total number of species that accumulate in clades we repeated the analysis outlined 4.5.1. Diversification rates, calculated using the methods outlined by Magallon and Sanderson[46], were modelled as a gaussian response variable and family age was removed from the model (Supplementary Table 18).

4.5.4 Estimation of the relationship between diversification rate and feeding niche using a BPMM

To test if the rate at which species diversify differs between groups of insects with different feeding niches we repeated the analysis outlined 4.5.2 including diversification rate as a gaussian response variable and removing family age from models (Supplementary Table 19).

4.6 Verification analyses

We tested the robustness of our conclusions to several underlying data assumptions. These sensitivity analyses provided quantitatively similar results to our main analysis (Supplementary Tables 20-26).

4.6.1 Re-analysis of 4.2.1 after removing families that were added to the Rainford tree

There were 23 families within our obligate symbiont dataset that were not represented in the Rainford insect phylogeny that were added to the phylogeny (see section 2). To examine the robustness of our results when including these families we re-ran the analyses detailed in section 4.2.1 (Supplementary Table 20) with the 23 additional families excluded.

4.6.2 Re-analysis of 4.2.1 including only bacterial symbionts

Bacteria made up the vast majority of obligate symbionts (79 out of 84 insect families had bacterial symbionts = 94%). To verify that our results were not explained by a few outlying eukaryotic symbionts, we re-ran the analyses detailed in section 4.2.1 including only insect families with bacterial symbionts (n_{families}=395. Supplementary Table 21).

4.6.3 Re-analysis of 4.4.2 removing co-occurring obligate symbionts

There were 112 unique host-bacterial symbiont combinations. Of these 49% (n=55) had multiple co-occuring symbionts. It is possible that any signature of bacteria specializing in B5 and B9 vitamin production is obscured by the presence of co-residing obligate symbionts that may change nutrient provisioning roles. We therefore repeated the analyses in section 4.4.2 after removing hosts that had multiple co-occurring symbionts (Supplementary Tables 22-23).

4.6.4 Re-analysis of 4.2.1, 4.2.3 and 4.5.2 excluding obligate symbiont data inferred from microscope studies

Out of the 400 insect families included in our analyses, 260 were inferred to not have obligate symbionts based on a lack of specialized symbiont organs within certain insect clades shown from microscopy studies by Buchner and colleagues. They indicated that insects in the orders Ephemeroptera, Plecoptera, Odontata, Neuroptera, Orthoptera, Lepidoptera, superfamily Tenthredinoidea, and subclade Aculeata (excluding Formicidae) all lacked bacteriocytes and in general do not depend on endosymbionts for survival[20]. To test the sensitivity of our results to inferring the absence of obligate symbionts within these insect groups, we re-ran the analyses outlined in sections 4.2.1, 4.2.3 and 4.5.2 after removing these 260 families to focus on families and species where obligate symbioses had been studied directly (see section 1 ‘Criteria for assessing obligate symbiosis’ for more details).

The results of all verification analyses were quantitatively similar to our main analyses (Supplementary Tables 20-26).

Data and code availability

R code, BayesTraits code, data and analysis results are available at the open science framework: DOI 10.17605/OSF.IO/TYK7C. Full citations of references in supplementary tables are given in the method references[65-367].

Method references

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Ethics declarations

None

Competing interests

The authors have no competing interests.

Supplementary Information

Supplementary Tables 1 to 26 are provided in xlsx and html format in the files “SupplementaryTables.html” and “SupplementaryTables.xlsx”. Full citations of references in supplementary tables are given in the method references[48-367].

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