Review

Cite this article: Douglas AE. 2020 Housing microbial symbionts: evolutionary origins and diversification of symbiotic organs in animals. *Phil. Trans. R. Soc. B* **375**: 20190603. <http://dx.doi.org/10.1098/rstb.2019.0603>

Accepted: 17 February 2020

One contribution of 16 to a theme issue 'The role of the microbiome in host evolution'.

Subject Areas:

evolution, microbiology

Keywords:

bacteriome, light organ, symbiosis, symbiotic organ, trophosome

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Housing microbial symbionts: evolutionary origins and diversification of symbiotic organs in animals

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In many animal hosts, microbial symbionts are housed within specialized structures known as symbiotic organs, but the evolutionary origins of these structures have rarely been investigated. Here, I adopt an evolutionary developmental (evo-devo) approach, specifically to apply knowledge of the development of symbiotic organs to gain insights into their evolutionary origins and diversification. In particular, host genetic changes associated with evolution of symbiotic organs can be inferred from studies to identify the host genes that orchestrate the development of symbiotic organs, recognizing that microbial products may also play a key role in triggering the developmental programme in some associations. These studies may also reveal whether higher animal taxonomic groups (order, class, phylum, etc.) possess a common genetic regulatory network for symbiosis that is latent in taxa lacking symbiotic organs, and activated at the origination of symbiosis in different host lineages. In this way, apparent instances of convergent evolution of symbiotic organs may be homologous in terms of a common genetic blueprint for symbiosis. Advances in genetic technologies, including reverse genetic tools and genome editing, will facilitate the application of evo-devo approaches to investigate the evolution of symbiotic organs in animals.

This article is part of the theme issue 'The role of the microbiome in host evolution'.

1. Introduction

An animal host comprises many habitats that can be used by microorganisms, and the fitness of both the microorganisms and animal host can be dictated by the location of the microorganisms in the host. In one site, a microorganism may be benign or beneficial but, in a different site, it can be deleterious to the host or fail to persist. For many associations, the favoured location is a specialized organ (or part of an organ) that functions to control the conditions and resources available to the microbial cells and to ensure the host derives benefit from the microbial services. These organs are absent from related animal species that do not associate with the microorganisms and, for many associations, the organs develop only in response to colonization by compatible microorganisms. I refer to these host structures as symbiotic organs.

Research on symbiotic organs of animals has traditionally focused on functional anatomy. We have a broad understanding of the structural features of various symbiotic organs, including how these features facilitate colonization by appropriate microbial taxa and delivery of microbial services to the animal host. Nevertheless, the genetic basis of the evolutionary origins of symbiotic organs is largely unknown.

The specific purpose of this article is to explore the opportunities to investigate the evolutionary origins of symbiotic organs by applying the principles of evolutionary developmental biology (evo-devo), which uses comparative developmental biology to illuminate the evolutionary origins and diversification of

Figure 1. The squid light organ. (a) Localization of light organ in mantle cavity of the squid *Euprymna scolopes*. (b) Structure of *E. scolopes* light organ. (c) Phylogenetic distribution of bacterial light organs in cephalopod molluscs (indicated by asterisks (*)). Redrawn from figure 1 of Pankey *et al.* [3].

morphological features [1,2]. I focus on four symbiotic organs. The light organ of squid and the midgut ceca in heteropteran insects bear extracellular bacteria; and the bacteriomes of various insects and trophosome in some marine invertebrates harbour intracellular bacteria. In §2, I summarize current knowledge of these symbiotic organs from the perspectives of their developmental biology and functional morphology. Then, I review the evolutionary insights gained from the study of the underlying molecular processes, including the contributions of conserved genes to the development of symbiotic organs. Section 4 addresses the contribution of convergence and conserved functions in the evolution of symbiotic organs in different animal taxa. I conclude with a brief discussion of outstanding questions and opportunities for further research.

2. Developmental biology and functional morphology of symbiotic organs

(a) The squid light organ

The light organ of the sepiolid squid *Euprymna scolopes* is localized to the mantle cavity and develops in close association with the hindgut and ink sac (figure 1a). The luminescent bacteria *Vibrio fischeri* are housed within crypts, bounded by two auxiliary structures, the lens and silvered reflector, which control light emission from the animal (figure 1b). The light organ has mixed developmental origins: the crypts are lined by epithelial cells of ectodermal origin, while the lens and reflector are of mesodermal origin, derived from hindgut muscle primordia and the ventral lining of the ink sac, respectively [4–6]. The light organ develops in the squid embryo but further developmental changes occur after hatching, triggered by the symbiotic bacteria. Notably, the hatchling squid bears two pairs of ciliated epithelial appendages that function to draw bacteria in the

water column to the pores leading to the light organ crypts [7]. Once the bacterial symbionts have colonized the crypts, the ciliated appendages undergo cell death and are lost [8]. The lens also develops post-natally.

The light organ has parallels with two other squid organs. Developmentally, it is allied with the accessory nidamental gland, which arises from the same body region (figure 1a) and also bears bacteria (but not *V. fischeri*) [9]. Functionally, the light organ is similar to the eye, in that both organs control the passage of light, albeit endogenously produced in the light organ and of exogenous origin for the eye. The functional parallels between the light organ and eye extend to shared physiological and biochemical traits, including photo-transduction by opsin proteins [10], abundant production of the same specialized reflector proteins [4,11], and the same lens crystallin protein, aldehyde dehydrogenase [5].

(b) The midgut ceca of heteropteran insects

Phylogenetic reconstructions [12,13] indicate that the ancestor of heteropteran insects of infraorder Pentatomomorpha was morphologically specialized to house symbiotic bacteria in the most distal region of the midgut known as M4 (figure 2a). This symbiotic organ comprises two rows of ceca that run proximal-to-distal along opposite sides of M4 (figure 2b). The symbiotic bacteria within the ceca are required for sustained insect growth and fecundity [14].

Analysis of the functional morphology of the midgut of the bean bug *Riptortus pedestris* has revealed that the M4 ceca are linked to M3 via a narrow mucilage-filled duct, called the constricted region [15] (figure 2b). The insect nymphs feed readily on various bacteria, but only the native *Burkholderia* symbiont and related strains penetrate through the constricted region to M4 [15,16]. Successful colonization of M4 has been attributed to an unusual mode of locomotion in the native symbiont, comprising forward rotation in a corkscrew trajectory with the flagellum wound around the cell body [17]. Furthermore, the specialization of M4 as a symbiosis organ that does not mediate the function of processing ingested food is made possible by the assimilation of food-derived nutrients and water across the gut wall of M3 to the hemolymph, with waste delivered back to the hindgut and feces [15] (figure 2b). This arrangement is well-suited to the purely liquid diet of these insects, a trait of all Hemiptera. Insects feeding on solid food and generating solid waste would not be able to combine the efficient handling of ingested food with a midgut symbiotic organ whose function is incompatible with food processing.

(c) Insect bacteriomes

An insect bacteriome is an aggregation of cells (bacteriocytes) with cytoplasm densely occupied by bacteria, usually individually enclosed in a membrane of host origin. Generally, the bacterial symbionts contribute essential nutrients to the host and they are transmitted vertically, via transfer from maternal bacteriomes to the ovaries, resulting in congruent phylogenies of bacteria and insect hosts over evolutionary timescales up to 100 million years or more [18]. Nevertheless, bacteriomes have evolved independently multiple times in insects, and they vary widely in morphology and location across different insect hosts [19,20].

Recent studies have revealed unexpected complexities in the development of bacteriomes in hemipteran insects

Figure 2. Microbial symbioses in insects of the order Hemiptera. (a) Phylogenetic distribution of symbioses. Bacteriomes bearing intracellular bacteria are the likely ancestral condition of suborders Sternorrhyncha, Auchenorrhyncha and Coleorrhyncha (*) but not Heteroptera. Within the Heteroptera, a symbiotic organ comprising ceca in the distal midgut (M4 region) is ancestral to four superfamilies of the Pentatomomorpha, and harbours either β -proteobacteria of the *Burkholderia* group (Lygaeoidea, Coreoidea and Pyrrhocoroidea) or γ -proteobacteria (Pentatomioidea). This association has been replaced by a M3 gut symbiosis in some Pyrrhocoroidea and by bacteriomes in some Lygoidea. (b) Functional anatomy of the gut of *Riptortus pedestris*, showing the M4 symbiotic organ and the divergent fate of ingested food and *Burkholderia*.

(figure 2a). For example, the aphid bacteriome is reported to have two developmental sources: vitellophage nuclei in the blastula embryo, and cells derived from the posterior end of the dorsal germband that migrate to the more anteriorly located vitellophage-derived bacteriocytes [21]. At present, it is uncertain whether the latter population of cells gives rise to the bacteriocytes that are colonized by symbiotic bacteria [21] or to the symbiont-free sheath cells that bound the bacteriome [22]. In whiteflies, the symbiosis is exceptional in that maternal bacteriocytes, not isolated bacterial symbionts, are transmitted to the eggs in the ovary, prior to fertilization of the egg [19,23]. In some species, e.g. *Trialeurodes vaporariorum*, the cargo of bacteria is transferred to bacteriocytes that differentiate in the late embryo and the maternal bacteriocytes are destroyed [19,24]. In *Bemisia tabaci*, however, the bacterial symbionts are retained in the maternal bacteriocyte through embryonic development to hatching and, thence, through the life cycle, thereby representing a genetically distinct lineage from the other cells of the insect body [25]. Degradation of the maternal bacteriocyte in the embryo (as in *T. vaporariorum*) is the likely ancestral condition. Bacteriocyte retention may have evolved via loss of the developmental programme mediating maternal bacteriocyte death in embryos, or by replacement of the ancestral bacteriocyte lineage by a new cell lineage with immortalized properties (i.e. not subject to senescence) [24].

(d) The trophosome

The trophosome is a large organ comprising cells, known as bacteriocytes, whose cytoplasm is packed with bacteria, usually with chemosynthetic metabolism. (The description of

the aggregation of bacteriocytes as a trophosome in chemosynthetic symbioses and bacteriome in insect symbioses (§2c) reflects the different conventions in the two fields and does not imply any fundamental difference in function.) Representatives within three animal phyla are described as bearing a trophosome: the family Siboglinidae (Annelida) [26], two genera of peltospirid gastropods (Mollusca) [27] and flatworms of the genus *Paracatenula* (Platyhelminthes) [28]. The bacterial chemosynthetic metabolism meets the carbon requirements of the host, with carbon dioxide fixation fuelled by the oxidation of reduced compounds (e.g. sulfide, methane). These associations are restricted to the interface between reducing and oxidizing environments, for example, in marine sediments, hydrothermal vents and hydrocarbon seeps.

The trophosome in the three animal phyla have different developmental origins. All described specimens of *Paracatenula* are gutless, and their trophosome occupies up to 50% of the body volume [28]. The constituent bacteriocytes are derived from bacteria-free stem cells, also known as neoblasts, and the presumptive bacteriocyte is colonized by bacterial cells transferred from pre-existing bacteriocytes [29]. The symbiosis is perpetuated when the worm reproduces by asexual fission (sexual reproduction is unknown), yielding codiversifying lineages of host and symbionts [29].

In the siboglinids and peltospirid gastropods, the association is formed *de novo* between each individual host and bacteria derived from the external environment. In both groups, the larvae feed and have a functional gut but no trophosome. Subsequently, as the animal acquires its complement of symbionts from the external environment, it stops feeding, its gut degenerates and the trophosome is formed. In

the siboglinid *Riftia pachyptila* (and presumably other siboglinids), the trophosome is of mesodermal origin [30], while in the peltospirid gastropods it is an ectodermal structure, derived from the oesophageal gland [27,31]. The siboglinid trophosome is widely accepted to have evolved once in the common ancestor of this family [26], but the peltospirid trophosome likely arose independently in the two genera, *Gigantopelta* and *Chrysomallon*, based on differences in the timing and detail of trophosome development [27].

3. Insights from genetic analysis of symbiotic organs

A key rationale of evo-devo research is that we gain understanding of the evolutionary origins of a novel structure from study of the genetic basis of the development of the structure [1,2,32]. These genetic factors may be individual genes, potentially including master regulatory genes, or gene regulatory networks (GRNs) comprising multiple interacting genes [33–35]. A triumph of the evo-devo approach is the discovery that relatively small numbers of conserved genes have been recruited in different combinations and contexts to the development of diverse structures [1]. For example, the demonstration that the same GRN is required for the development of the paired fins and the evolutionarily ancient median (unpaired) fins of fish indicates that the paired fins likely evolved by recruiting key elements of the median fin developmental programme [36]. Similarly, the finding that the same set of genes defines both the initial steps in the development of horns on the first thoracic segment (T1) of dung beetles and the dorsal developmental field of wings on T2 and T3 suggests strongly that the beetle horns have evolved by the co-option of the wing-related GRN to T1 [37]. Some developmental patterns, however, are founded on taxonomically restricted genes (TRGs). For example, the *hym301* gene family is unique to freshwater polyps of the genus *Hydra*, and species-specific patterns in expression of these genes dictate the among-species variation in timing and order of hydra tentacle development [38]. TRGs can arise from noncoding regions of the genome, by duplication and divergence of pre-existing genes, and by horizontal transfer, e.g. of retroviral sequences [39–41].

By comparison to our understanding of the GRNs that define the morphological traits described in the previous paragraph, our knowledge of the genetic basis of symbiotic organs is fragmentary. Two studies suggest that conserved transcription factors (TFs) may have been coopted to function in the specification of insect bacteriomes. In the differentiating bacteriome in aphid embryos, three homeodomain-containing TFs, *Distal-less*, *engrailed* and *Ultrabithorax*, are strongly expressed, apparently out of context to their described developmental functions in insects (limb patterning, segment polarity and segment identity, respectively) [21]. *Ultrabithorax* is also expressed in the presumptive bacteriome of the lygaeoid bug *Nysius plebeius*. In this species, a functional role is implicated by the demonstration that bacteriome development is prevented in insects with *Ultrabithorax* expression suppressed by RNA interference [42].

Further insights come from genomic and transcriptomic analyses of the squid *Euprymna scolopes*. Members of two gene families, the reflectins and a group of heme-peroxidases, have particularly high transcript abundance in the light organ [11,43]. Both gene families comprise tandem gene clusters

generated by repeated gene duplications. Relative to the octopus *Octopus bimaculoides* which lacks a light organ (figure 1c), the gene families have expanded from two to seven peroxidase genes and from 12 to 28 reflectin genes in *E. scolopes* [11]. These genomic changes may be associated with light organ evolution, perhaps enabling the diversification of gene-specific regulatory regions for dedicated expression in the light organ, or of coding regions for optimized function in the light organ. Interestingly, the gene expression patterns in the light organ are strikingly different from the transcriptome of the accessory nidamental gland, which includes a large number of highly expressed TRGs of unknown function [11]. These data have led the authors to emphasize the likely difference in the genetic basis of the evolutionary origin of these two microbe-associated organs of the squid, despite the developmental similarities between the two organs (§2a).

A further issue is that a compatible microbial partner is required for the developmental programme of many symbiotic organs, but how the microorganisms interact with the GRN(s) underlying the developmental programme of the symbiotic organs is unknown. This applies especially to the siboglinid trophosome. Bacterial cells from the water column gain entry to *Riftia pachyptila* via the epidermis of the body wall and become incorporated into various cell types. Some animal cells, e.g. muscle and epidermal cells, respond to bacterial colonization by undergoing cell death, likely by apoptosis, but one population of mesodermal cells display a strikingly different response: these cells support the proliferation of their complement of bacteria and differentiate, forming the trophosome [30].

The identity of the microbial product(s) that trigger the genetic programme underlying trophosome differentiation in *Riftia* is unknown. In the squid light organ system, however, two critical microbial products have been identified, and their impact on the host has been described at the level of host morphology but not host gene expression. One is a monomer of bacterial peptidoglycan, known as tracheal cytotoxin (TCT), which is shed when proliferating bacterial cells remodel their cell wall. TCT triggers the regression of the ciliated appendages of the light organ, structures that facilitate colonization of the light organ but are not required for function of the established symbiosis [44]. The second is the light produced by *V. fischeri* in the light organ, which induces the swelling of the epithelial cells bounding the crypts of the light organ, resulting in narrowing of the crypt spaces and close host cell–bacterial proximity [45]. TCT and light production are traits displayed by *V. fischeri* in nonsymbiotic contexts and also by various other bacteria, and so they are described as cues; not signals, which are defined as evolving under selection for communication with the host [46,47]. (To my knowledge, no microbial signals mediating light organ development have been described in the squid–*Vibrio* association.) The outstanding issue is whether and how these microbial cues have been recruited as a switch (ON/OFF) or rheostat (modulating output) that defines the activity of key regulatory genes or GRNs underpinning defined aspects of the development of the squid light organ.

4. Convergence and homology in the evolution of symbiotic organs

Morphologically similar symbiotic organs have evolved more than once. In some cases, the common ancestor of symbiotic

host lineages did not bear the symbiosis. For example, light organs harbouring luminescent *Vibrio* have evolved within two families of squid, the Sepiolidae (including *E. scolopes*) and the Loligidae [3] (figure 1c), and the oesophageal trophosome has evolved twice in peltospirid gastropod molluscs [27]. In other animal groups, an ancestral symbiosis is retained in one lineage but lost in a sister lineage, only to be regained by some taxa in the latter lineage. Among hemipteran insects, symbiosis with intracellular bacteria within bacteriocytes is likely ancestral, but is absent from basal groups of suborder Heteroptera [12,19,48] (figure 2a). Nevertheless, bacteriomes have evolved in the Heteroptera, including the Cimicidae (bedbugs) and several families of Lygoidea [12,13] (figure 2a).

The molecular correlates of similar symbiotic organs in related animals have been studied in squid (figure 1c). The light organs of the sepiolid *E. scolopes* and loligid *Uroteuthis edulis* differ in the biochemical identity of the lens crystallin (dominated by aldehyde dehydrogenase in *E. scolopes* and by glutathione S-transferases in *U. edulis*) and the expression of light-detecting opsin gene only in the *E. scolopes* light organ [3]. This has led the authors to conclude that the light organs in the two species are convergent, although analysis of other structures and genes contributing to the light organs would enable a more thorough assessment of the evolutionary relationship between the light organs in the two groups.

The multiple instances of evolutionary gain and loss of bacteriocyte symbioses in insects have been investigated predominantly at the morphological level. Viewed from this perspective, the great diversity in patterns of bacteriocyte development, localization and morphology of bacteriocytes points to rampant independent origins [13,19]. An alternative viewpoint comes from the discovery (§3) that conserved TF genes are expressed in the bacteriomes of members of two hemipteran suborders, the Sternorrhyncha (aphids) and Heteroptera (lygaeoids) [21,42]. This condition could arise from the independent recruitment of these genes to orchestrate bacteriome differentiation or, alternatively, it could indicate a conserved ‘symbiosis GRN’ that, on activation, enables the animal cell to accommodate intracellular bacteria. This scenario would be compatible with the observed morphological diversity of insect bacteriomes if the putative symbiosis GRN were recruited to different cell types in different insect taxa. In §5, I consider some strategies to test for conservation of GRN structure in different insect taxa with bacteriocytes of different developmental origins.

How might a symbiosis GRN that determines the development of a symbiotic organ be activated or inactivated over evolutionary time? Consider a situation where a product of the microbial partner contributes to the regulatory circuit, but the ecology of the host then shifts, for example, to habitats that do not support free-living populations of horizontally acquired symbionts or to conditions (e.g. temperature) that are unsuitable for vertically transmitted symbionts [49,50]. This would result in loss of the symbiotic organ, but a shift back to permissive conditions would re-activate the symbiont-dependent GRN. Host genetic changes may also contribute to evolutionary switching between the presence and absence of symbiotic organs. The evo-devo literature reminds us that the genetic changes may be relatively small, involving single regulatory sequences. For example, the freshwater three-spined stickleback fish *Gasterosteus aculeatus* lacks the pelvic fin of its marine ancestor. This evolutionary change can be attributed to the loss of an enhancer sequence

upstream of a single gene, *Pitx1*; when the enhancer sequence is restored, the pelvic fin develops [51]. Insight also comes from plant–microbial symbioses. The development (and likely evolutionary origin) of root nodules that house nitrogen-fixing rhizobia bacteria in leguminous plants depends on the sequence of an intron in a gene contributing to the development of lateral roots. In plant hosts capable of forming the symbiosis, this intronic sequence includes a binding site for a transcription factor (NIN), resulting in a plant hormone-dependent diversion of the developmental programme from lateral root to nodule formation [52]. At present, it is unknown whether and how specific regulatory sequences define the presence or absence of symbiotic organs in animal systems.

In the preceding paragraphs, I have compared the symbiotic organs among related animals (e.g. among squid or hemipteran insects). As the phylogenetic relationship between animals possessing similar symbiotic organs becomes more distant, so the expectation of convergent origins of the organs increases. The trophosome bearing chemosynthetic bacteria in the flatworms *Paracatenula* and siboglinid annelids appear as robust examples of independent origins. However, many years of evo-devo research have revealed that conserved regulatory circuits underpin structures that are similar but not morphologically homologous. For instance, the transcription factor Distal-less is required for the proper development of the terminal structures on the limbs of both arthropods and tetrapod vertebrates [53]. This example illustrates how structures that, at the morphological level, are designated as convergent have a conserved molecular basis to the structures; they are instances of ‘deep homology’ [54]. For now, however, whether there is a deep homology of symbiotic organs, and how that deep homology may relate to the functional traits of the microbial partners, are open questions.

5. Outlook

A priority in the initial characterization of any symbiosis is to describe the morphology of the symbiotic organ, including how it may change in form and function over the host life cycle. Despite this, surprisingly little is known about the evolutionary origins of symbiotic organs in animals. A premise of this article is that the approaches and insights of the mature discipline of evolutionary developmental biology (evo-devo) offer the symbiosis researcher valuable signposts to investigate this topic. There are two important questions.

First: what is the evolutionary relationship between a symbiotic organ and other structures in the animal host? Symbiotic organs are often described as evolutionary novelties. Although this perspective draws attention to how animals have responded to selection to sustain and optimize interactions with microbial symbionts, it also distracts from the obvious fact that symbiotic organs have evolved by modification of developmental plan(s) defining pre-existing structure(s) in the ancestral host. Developmental affinities, and likely evolutionary origins, are evident for some symbiotic organs. For example, the midgut ceca housing *Burkholderia* in various heteropteran insects (figure 2b) are a remarkable elaboration of the distal midgut, and future research on evolutionary origins of the ceca will be facilitated by knowledge of the molecular and cellular basis of midgut development and homeostasis in other insects, particularly *Drosophila* [55]. Similarly, the strong parallels in developmental origin and structure of the light

organ and accessory nidamental gland of squid point to likely overlap in the regulatory genes underlying these two organs, despite differences in their global patterns of gene expression [11]. The developmental and evolutionary origins of the siboglinid trophosome and bacteriome of many insects, both comprising specialized animal cells containing intracellular microorganisms, pose greater challenges because parallels to other organs in the host are obscure. A potentially valuable approach to address these complexities is cell lineage analysis, i.e. tracing the genealogy of cells through development. This approach offers a route to identify the progenitor of cells contributing to symbiotic organs (e.g. bacteriocytes of the trophosome and insect bacteriome, progenitor cells of the squid light organ) and the relationship between these progenitor cells and other cell types in the animal.

The second major question concerns the evolutionary relationship among symbiotic organs in different hosts. The default interpretation is that symbiotic organs are homologous across taxa with a common ancestor that bears the symbiosis, but have arisen independently in each lineage that has acquired a symbiosis. This simple dichotomy is undermined by two observations. First, lineages that have lost the symbiosis may retain key genes or GRN that determine the symbiosis and they are, therefore, predisposed for symbiosis under favourable conditions. For example, each of the two origins of oesophageal trophosomes in peltispirid gastropods and the multiple origins of bacteriomes in heteropteran insects may be founded on ancient molecular circuitry that was latent in their immediate symbiosis-free ancestor. The reverse

issue relates to the developmental constancy of symbiotic organs. This question arises for the whitefly bacteriome, where an immortalized cell may have usurped the ancestral bacteriocyte [24]. In addition, the many instances of symbiont-switching in various insect groups with bacteriomes [12,18] may have been accompanied (and possibly facilitated) by changes in the host cell type, with the replacement cell better adapted to support and control the new symbiont. We can resolve these issues most effectively by systematic application of evo-devo methodologies.

Nevertheless, there are many technical challenges to empirical testing of evo-devo hypotheses of the evolution of symbiotic organs. Suites of genetic technologies are required, including reverse genetic tools for gene knock-out, ectopic expression and gene editing, as well as cell lineage labelling and high throughput screens of the host life-stage supporting key events in the development of symbiotic organs. Comparative analysis requires knowledge of the phylogeny and symbiotic traits of hosts and easy access to representative taxa across the phylogeny for experimental study. Symbioses vary in their suitability for different questions, demanding flexibility of researchers in their choice of system for study and, in some cases, a commitment of researchers and funding agencies to develop key genetic technologies.

Data accessibility. This article does not contain any additional data.

Competing Interests. I declare I have no competing interests.

Funding. The motivation for this article arose from research funded by NSF grant no. IOS-1354743.

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