Vertical transmission of sponge microbiota is weak and inconsistent

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Abstract

Classic evolutionary theory predicts that if beneficial microbial symbionts improve host fitness, they should be faithfully transmitted to offspring. More recently, the hologenome theory of evolution predicts resemblance between parent and offspring microbiomes, and high partner fidelity between host species and their vertically transmitted microbes. Here, we test these ideas for the first time in multiple host species with highly diverse microbiota, leveraging known-parent offspring pairs sampled from eight species of wild marine sponges (Porifera). Contrary to the hypothesis that vertical transmission is an adaptation that allows sponges to faithfully transmit intact microbial consortia to offspring, we found that vertical transmission is weak and incomplete. Further, we found no evidence that siblings consistently receive the same microbes from their parents, nor that vertically transmitted microbes show high degrees of host species fidelity. Finally, while we show that monophyletic groups of microbes with known symbiotic features and capabilities are more common among vertically transmitted microbes than in the consortia of horizontally acquired microbes, the signature of this vertical transmission is only detectable on the level of Porifera as a whole.

Our study demonstrates that common predictions of vertical transmission that stem from species-poor systems are not necessarily true when scaling up to diverse and complex microbiomes.

Introduction

All animals are colonized by microbes. These microbes live in communities, called microbiomes, that often exhibit astonishing diversity and complexity and can have profound effects on host health and fitness [1, 2, 3]. However, despite their importance, we still do not understand how most organisms acquire their microbiomes: are they largely inherited from parents via vertical transmission or acquired horizontally from the environment? In the last five years, the literature has provided widely divergent answers to this question [4, 5, 6]. Yet understanding the degree to which vertical versus horizontal transmission dominate microbiome assembly across the animal tree of life is necessary to learn how environments shape host phenotypes via host-microbe interactions and whether natural selection can act on hosts and their microbiomes as a unit (i.e., the hologenome theory of evolution) [7, 8, 9, 10, 11].

Classic evolutionary theory predicts that if microbial symbionts are beneficial, they should be vertically transmitted. Moreover, the more an animal host depends on its microbial partners, the higher the expected incidence of vertical transmission [12, 13, 14]. If microbes provide services that animals depend on, and if successful parents have well-functioning symbioses with microbes that lead to increase performance and fitness, then parents should be selected to ensure faithful transmission of these symbionts to offspring. In support, strict vertical transmission through the germline occurs in many well-known symbiotic systems, including *Buchnera*-aphid, *Rhizobia*-legume, and *Wolbachia*-arthropod [15]. A recent comparative study even found that the removal of vertically transmitted microbial symbionts resulted in a larger reduction of host fitness compared to the removal of horizontally transmitted symbionts [5]. However, despite this well-developed theory, evidence for horizontal transmission is increasingly common–at least in hosts with relatively simple microbiota [6, 16, 17, 18]. Two examples include the bioluminescent *Vibrio*-squid symbiosis [19], and the symbiosis between chemolithoautotrophic bacteria and the hydrothermal vent tubeworm *Riftia pachyptila* [20]. Recently, Mushegian and colleagues demonstrated that, in water fleas (*Daphnia magna*), microbes that are essential to host functioning are acquired from the environment and not maternally derived [21]. In general, caution should be taken when extrapolating patterns and processes from species-poor systems to highly diverse microbiomes: with increasing community complexity, do parents transmit a representative sample of the whole microbial community or select only a critical set of the most beneficial microbes? How does vertical transmission interact with other community assembly processes shown to be important in complex communities, including ecological drift,

52 priority effects, and environmental selection?

53 Studies that test vertical transmission in host species with diverse microbiota are needed to resolve these ques-
54 tions. The present study is, to our knowledge, the first in-depth analysis of the strength and consistency of vertical
55 transmission in multiple host species from an animal phylum with diverse and complex microbiomes. Moreover, by
56 characterizing signatures of vertical transmission in multiple, related host species, we also test, for the first time, part-
57 ner fidelity between vertically transmitted microbes and their hosts. Partner fidelity is predicted by the hologenome
58 theory of evolution because if vertically transmitted microbes occur in multiple host species, this weakens the coher-
59 ence of the unit of selection [10]. Here we test these ideas in marine sponges, an evolutionary ancient phylum with
60 a fossil record dating back over 600 million years [22]. Indeed, Porifera are the oldest metazoan group with known
61 microbial symbioses [23]. Marine sponges are filter-feeders with a simple body plan consisting of canals embedded
62 in an extracellular matrix called the mesohyl. Within the mesohyl, sponges maintain diverse microbial communities
63 that contribute to host functioning by cycling nitrogen, fixing carbon dioxide, producing secondary metabolites, and
64 acquiring and converting dissolved organic matter—tasks that, in many cases, the sponge cannot perform without
65 microbial symbionts [23, 24, 25].

66 While the prevailing transmission model in marine sponges include both horizontal and vertical transmission [26],
67 at least three lines of evidence suggest that vertical transmission plays an important role in the assembly of sponge
68 microbiota. First, sponges appear to have coevolved with a unique set of microbial symbionts that form so-called
69 *sponge-enriched* 16S rRNA gene sequence *clusters* [27, 28]. These *sponge-enriched clusters* span 14 known bacterial
70 and archaeal phyla many of which are highly specific to the phylum Porifera (e.g., phyla such as Poribacteria, Chlo-
71 roflexi and PAUC34f) [27, 28]. Unlike any other group of animal associated microbial symbionts described to date,
72 each sponge-enriched cluster is monophyletic, indicating that microbes assigning to these clusters have diverged from
73 their free-living relatives [27, 28]. Second, electron micrographs have revealed that sponge oocytes, embryos, and
74 larvae contain free-swimming or vacuole-enclosed endosymbotic bacteria that are morphologically identical to those
75 found in the mesohyl of the parent [29, 30, 31, 32]. The mechanisms for microbial selection and transference to the
76 oocytes vary between sponge species [32], as does the density and diversity of microbes that are incorporated into the
77 oocytes [33, 34, 35]. Third, multiple studies, largely based on non-high-throughput sequencing methods, have found
78 similar microbial phylotypes in adults and larvae from the same species [36, 37, 26, 38, 39]. One study also found
79 that three pre-selected bacterial taxa that were present in the embryos of the tropical sponge *Corticium sp.* persisted
80 throughout development and were consistently detected in adult samples over a period of three years [40]. These
81 lines of evidence altogether strongly suggest that vertical transmission may be a frequent phenomenon that ensures the

assembly of a functioning and beneficial microbiota in many species of marine sponges.

Despite this compelling evidence for vertical transmission, no studies have yet used high-throughput sequencing to test for evidence of vertical transmission by comparing microbial sharing in known parent-offspring pairs from wild sponges. We fill this gap and test three broad hypotheses about the strength and consistency of vertical transmission in sponges that are generalizable to any host-microbe system. First, we test the hypothesis that vertical transmission is comprehensive, such that microbiomes in larval offspring are either a perfect replica of, or a subset of, the microbes found in their adult parents. Alternatively, vertical transmission might be incomplete or undetectable; if incomplete, larval offspring will share only a fraction of their microbes with their parent, but this proportion will be higher than the proportion of microbes they share with other adults of the same species. If vertical transmission is undetectable, then larval offspring will be just as likely to share microbes with other conspecific adults as they are with their parents. Second, we test the consistency of vertical transmission between parents and offspring. We hypothesize that if a specific set of symbionts have co-evolved with their sponge host, and if it is adaptive for parents to transmit this specific set of symbionts, then all offspring from the same parent should receive an identical or highly consistent set of beneficial symbionts. Alternatively, if consistent vertical transmission is not important to parental fitness, or if parents benefit from transmitting different symbionts to each offspring (e.g., if larvae settle in variable environments where only a subset of symbionts is beneficial), then we might expect larvae to receive a variable or even random subset of microbes from their parents that is inconsistent between siblings. Third, we test whether vertically transmitted taxa exhibit partner fidelity. If symbionts have coevolved with a particular sponge species, then conspecific sponge adults and larvae should share more vertically transmitted microbes with each other than with heterospecific individuals. Lastly, we test a hypothesis specific to marine sponges, that vertically transmitted microbes assign to *sponge-enriched clusters* more frequently than the consortia of horizontally acquired microbes. Overall, our results help to shed light on the prevalence and importance of vertical versus horizontal transmission in an animal phylum with diverse microbiota that has important ramifications for understanding co-evolution between hosts and their associated microbiota in general.

Results and Discussion

Taxonomic diversity is distributed along a *sponge-specific axis*

To establish parent-offspring relationships for wild sponges, we placed mesh traps around adult sponges living close to the Islas Medas marine reserve in the Mediterranean Sea. We sampled 24 adults from a total of eight sponge species (Table S1) and collected 63 larval offspring from 21 of these adults (1 to 5 larvae sampled per adult; Table S2). To

characterize environmental microbes, we simultaneously collected seawater samples from seven locations within the sample area near where the adult sponges were found.

After quality control, we obtained 11,375,431 16S rRNA gene amplicon reads from these 94 samples (mean=121,015 reads per sample; min=1116, max=668,100 reads), resulting in 12,894 microbial ASVs (Amplicon Sequence Variants). Of these, 9,030 ASVs were present in the 24 sponge adults, 5,786 were found in their 63 larval offspring, and 9,802 ASVs occurred in the seven seawater samples. The 12,894 ASVs were classified to over 30 bacterial phyla and candidate phyla, five of which were only detected in the surrounding seawater. One class of Proteobacteria was unique to the sponge adults, and two phyla, Deferribacteres and Fibrobacteres, were especially enriched in larval offspring albeit present in low abundances in the other two environments (Figure 1A). While several phyla (classes for Proteobacteria) were shared between all three environments (circles close to the center in Figure 1A), likely representing horizontally acquired ASVs, a large fraction of the observed taxonomic diversity was only shared between sponge adults and larvae, distributed along a *sponge-specific axis* (left-hand side of the ternary plot in Figure 1A). These included many common sponge-associated phyla, such as Poribacteria, Chloroflexi, and PAUC34f, but also more arcane phyla like Tectomicrobia and SBR1093 (Figure 1A). Many of the sponge-associated phyla include microbes with known symbiotic features and functional capabilities. For example, members of Poribacteria and Chloroflexi harbor eukaryote-like protein domains which are suspected to be involved in preventing phagocytosis by the sponge host [41, 42]. Several genomic features in Chloroflexi are related to energy and carbon converting pathways, including amino and fatty acid metabolism and respiration, that directly benefit the sponge host [42]. Microbes from PAUC34f have the capacity to produce, transport and store polyphosphate granules, likely representing a phosphate reservoir for the sponge host in periods of deprivation [43]. This type of evidence strongly suggests that microbes from these phyla indeed represent beneficial symbionts for sponge hosts.

The ASVs we found also assigned to 105 different *sponge-enriched clusters* from 13 different bacterial phyla, of which Proteobacteria, Chloroflexi and Poribacteria represented the three most common (PAUC34f came in 5th place) (Figure 1B). These *sponge-enriched clusters* accounted for 9.6% of the total ASV richness and 25.5% of the total sequence count across samples. 94 *sponge-enriched clusters* were found in seawater, however, these only accounted for about 5% of the ASV richness and 0.23% of the total number of sequences in seawater. Out of these 94 *sponge-enriched clusters*, only 4 were not detected in the sponge hosts, supporting the idea that a rare biosphere functions as a seed bank for colonization of sponge hosts [44]. While very few *sponge-enriched clusters* occurred in all three environments (circles close to the center in Figure 1B), 62 were distributed along the sponge-specific axis (with a relative abundance of <0.01% in the seawater). Sponge larvae do not filter feed prior to settlement and metamorphosis

[45]. Concurrently, very little taxonomic diversity, just one phyla and two *sponge-enriched clusters*, was shared between larvae and seawater only (bottom axis of the ternary plots in Figure 1B), showing that, at least at these higher taxonomic levels, there is a signature of microbial dispersal and posterior enrichment between adults and larvae.

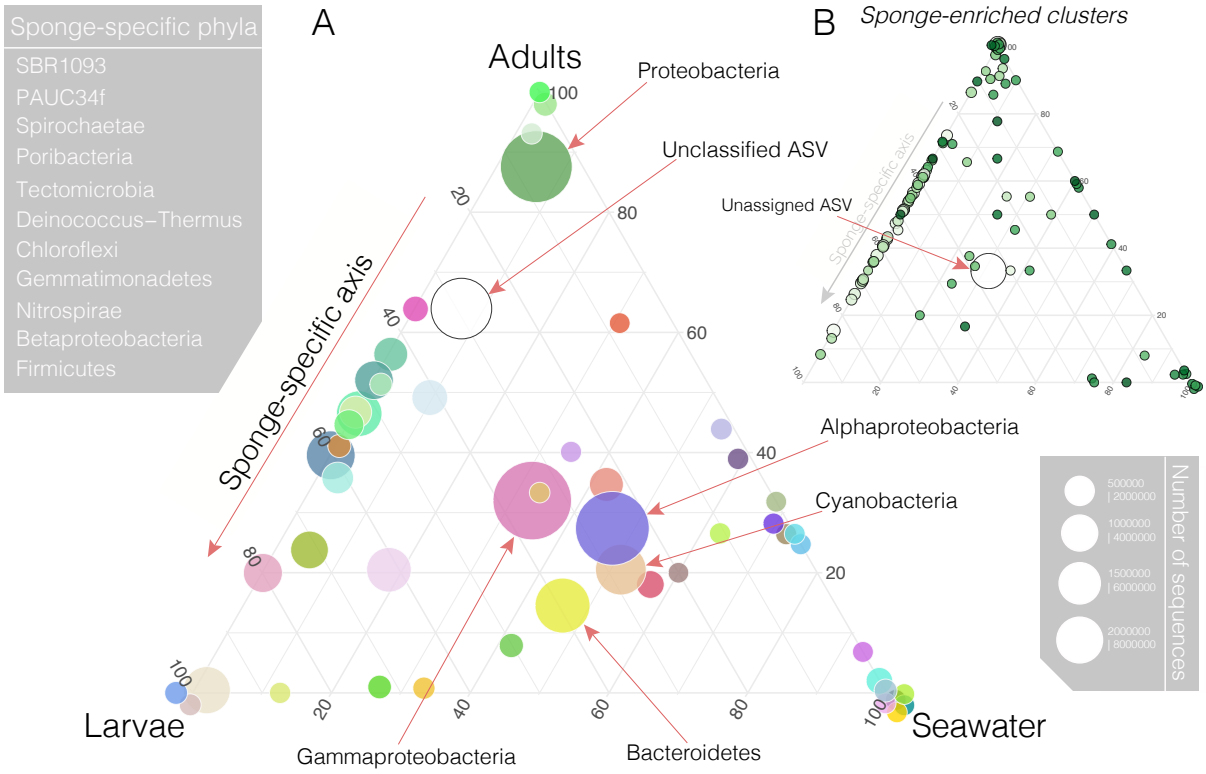

Figure 1: Ternary plots indicating the fraction of (A) all phyla, and (B) *sponge-enriched clusters* present in three environments: seawater (bottom right corner); sponge adults (top corner); and larval offspring (bottom left corner). Plot (A) shows the distribution of all microbial ASVs at the phylum level (class level for Proteobacteria). Plot (B) shows the diversity of all ASVs assigning to *sponge-enriched clusters*. ASVs that classify to phyla and *sponge-enriched clusters* that are unique to any of the three environments occur in their respective corners (100%); ASVs that classify to phyla and *sponge-enriched clusters* that are shared between any two environments occur along their focal axis. ASVs that classify to phyla and *sponge-enriched clusters* that are present in all three environments occur in the center of the ternary plots. Circle size corresponds the number of sequences that are classified to a given phylum or *sponge-enriched cluster*

Vertical transmission in sponges is detectable, but weak and incomplete

To help characterize patterns of vertical transmission, we built three bipartite networks that we hypothesized would reflect increasing host-microbe specificity (Figure 2A-C): an *overall* network containing all ASVs detected in adults, larvae, and the seawater (Figure 2A); a *sponge-specific* network containing ASVs harbored by adults and larvae, but

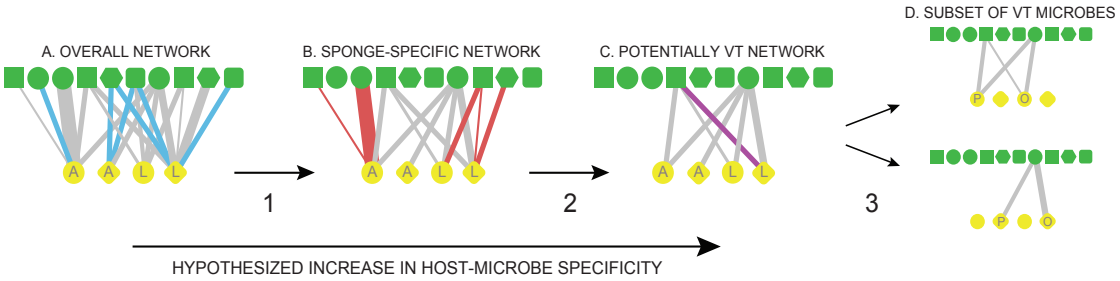

Figure 2: A conceptual diagram of the three different bipartite networks that we constructed (A through C) and an illustration of how we defined vertically transmitted microbes (networks under D). We hypothesize that these networks increase in host-microbe specificity as you move towards the right in the figure. Correspondingly, the microbial communities (i.e., the top level of each network) increased in community similarity with increasing host-microbe specificity (Figure S1). Sponges are in yellow and microbes in green; shapes represent different species; A=adult; L=larva; P=parent; O=offspring that are connected by edges that correspond to the relative abundance of microbes harbored by hosts. Network (A) corresponds to an *overall* network that contains all ASVs detected in adults, larvae, and the surrounding seawater. At arrow 1, we remove microbes present in the seawater (blue edges) to create network (B), which represents a *sponge-specific* network that contains ASVs only harbored by adults and larvae. At arrow 2, we remove microbes that are only present in adults or larvae, but not both (red edges) to create network (C), which corresponds to a *potentially vertically transmitted* network that contains ASVs found in at least one adult and one larva for a given sponge species. Note that these ASVs can still be present in multiple sponge species. At arrow 3, we subset the potentially vertically transmitted network to only include ASVs shared between a focal offspring and its parent (the purple edge is the only one that does not meet this criteria) to create subsets of vertically transmitted microbes. Note that vertically transmitted microbes can only be identified for one parent at a time. We analyzed these three networks (A-C) either as one network per sponge species, or one network containing all species.

not present (or below our detection limit) in the seawater (Figure 2B); and finally a *potentially vertically transmitted* network containing ASVs found in at least one adult and one larva for a given sponge species (Figure 2C; note that these ASVs can still be present in multiple sponge species). From the *potentially vertically transmitted* network, we further defined the subset of vertically transmitted ASVs as those shared between a focal offspring and its parent (Figure 2D).

We first tested whether vertical transmission in sponges was detectable, and if so, whether it was comprehensive or incomplete. A visual inspection of taxonomic profiles of the microbiota between parents and offspring indicated that offspring often harbor similar microbial phyla to their parents, as well as to non-parental conspecific adults (Figure 3). However, this similarity at the phylum level was superficial and largely disappeared when we re-focused our analyses to the level of individual ASVs. Specifically, while signatures of vertical transmission were detectable, they were very incomplete. For instance, across all sponge species, larvae shared, on average, only 1.43% of their *overall* ASVs with their adult parents (Figure S3). This percent of sharing was not different than the percent of ASVs larvae shared with conspecific adults living nearby (MD=0.081, 95% CI [-0.039,0.211]) Figure 4A), indicating that, at the level of all the microbes found in larvae, vertical transmission is essentially undetectable.

However, the analysis above included ASVs found in seawater, which may represent transient microbes passing through the host that are not consistent or important members of the sponge microbiota. Indeed, the detectability of vertical transmission increased as we partitioned the data into networks with increasing host-microbe specificity, but the proportion of vertically transmitted ASVs varied considerably both within and between species (Figure S3). By pooling samples across species, sacrificing resolution for statistical power, offspring shared a slightly higher proportion of vertically transmitted ASVs with their parents than with the non-parental conspecific adults in both the *sponge-specific* and *potentially vertically transmitted* network (sponge-specific: MD=0.326, 95% CI [0.014,0.626]; Figure 4B, *potentially vertically transmitted* network: MD=0.623, 95% CI [0.082,1.177]; Figure 4C). At the level of each individual host species, we only observed evidence for vertical transmission in two sponges: *O. lobularis* (*sponge-specific*: MD=0.80, 95% CI [-0.005,1.454]; *potentially vertically transmitted*: MD=1.27, 95% CI [0.12,2.33]; Figure S4a), and *C. crambe* (*sponge-specific*: MD=0.88, 95% CI [0.211,1.467]; *potentially vertically transmitted* network: MD=2.40, 95% CI [0.522,4.503]; Figure S4B).

We also tested whether offspring shared a higher proportion of vertically transmitted *sponge-enriched clusters* with their parents than with non-parental conspecific adults. We found that, while the proportion of vertically transmitted clusters were somewhat higher for some adults compared to others (Figure S3), offspring did not share a higher proportion of vertically transmitted *sponge-enriched clusters* with their parents than they did with non-parental conspecific

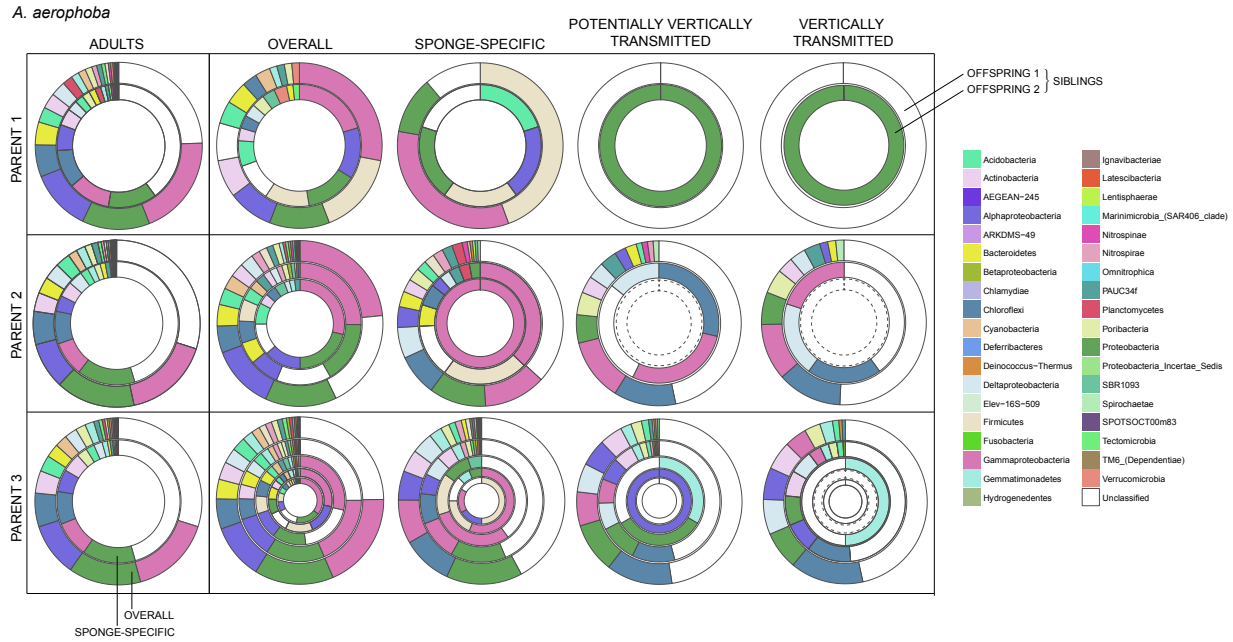

Figure 3: Donut charts showing the relative contribution of ASVs classifying to different microbial phyla (classes for Proteobacteria) in sponge parents and their offspring across the different networks in Figure 2. The left-hand column corresponds to the three adult specimens of *A. aerophoba*, while the remaining donuts depict microbial communities within their offspring across the different networks. For the adult donut charts, the inner and outer donuts represent the *overall* and *sponge-specific* networks, respectively. For the offspring donut charts, concentric donuts correspond to each offspring from the same parent (i.e. siblings). All donuts show results for the sponge species *A. aerophoba*; results for the other sponge species are in (Figure S2A-G). White donuts with a solid outline indicate a community where all the ASVs were unclassified. White donuts with a dashed outline indicate a community where the focal offspring did not contain any ASVs found in the focal network. Colors represent different microbial phyla (classes for Proteobacteria).

adults (MD=0.103, 95% CI [-0.08,0.304]; Figure 4a).

To further characterize patterns of vertical transmission, we computed modularity on weighted bipartite networks constructed for each sponge species (`DIRT_LPA_wb_plus`, [46]). In the ecological network literature, modules are groups of species that "interact" more among themselves than with groups of other species (e.g., flowers and their pollinators, and fruits and their seed dispersers). If modules are perfectly separated; that is, no species interact with species from other modules, we call them compartments. Weighted modularity has been shown to be positively correlated with network specialization (H_2'), reinforcing the idea that modules exist because some species do not interact with each other [47]. Computing modularity on weighted networks allows for weighting species by information content (here, relative abundance), which means that rare microbes are down-weighted and modules are formed around the most common host–microbe associations [47, 46]. The networks will be organized into compartments corresponding

to parents and offspring if they harbor the same set of microbes, and if those microbes are unique to those parents and offspring. We tested whether the observed modules deviated from the prior expectation of perfectly separated parent-offspring compartments using the Normalized Mutual Information (NMI) criterion [48, 49]. NMI ranges between 0 and 1, where 0 indicates complete dissimilarity between expected and observed modules, and 1 indicates that the observed modules only contain nodes corresponding to parents and offspring. While the majority of networks were highly modular (Table S3), the observed modules were not comprised of nodes corresponding to parents and offspring. The *sponge-specific* networks had, on average, the highest NMI score (Figure S5A), but these networks were still quite far from the prior expectation of perfectly separated parent-offspring compartments (Figure S5B).

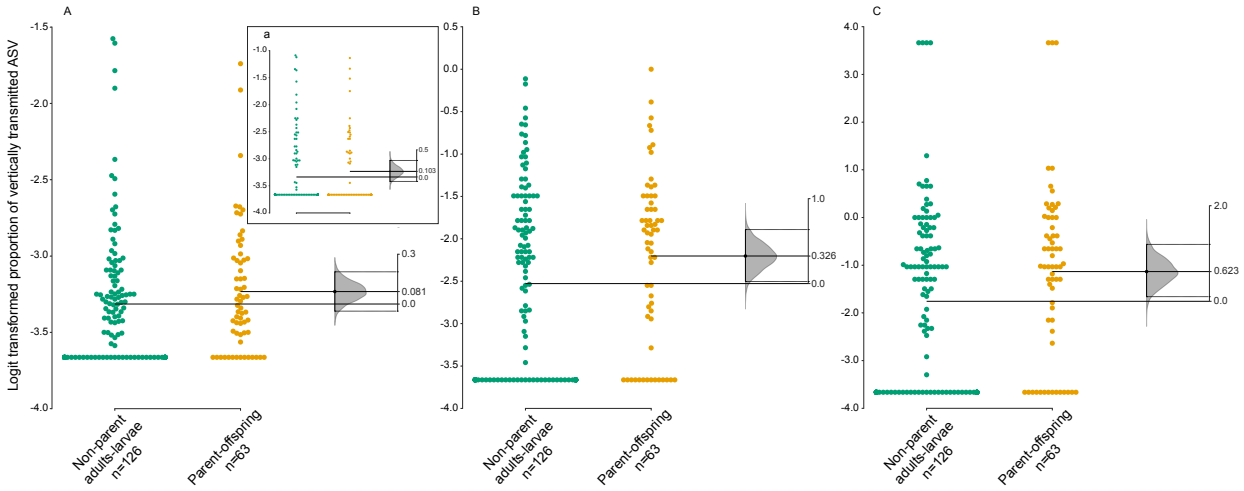

Figure 4: Gardner-Altman comparison plots of logit transformed proportions of vertically transmitted ASVs shared between sponge larvae and either (i) their known parents (orange dots), or (ii) two non-parental conspecific adults (green dots). Each plot contains comparisons for all host species (see Figure S3), and each dot represents one parent-offspring pair or one non-parent-offspring pair. Plot (A) corresponds to the *overall* network; plot (B) to the *sponge specific* network; and plot (C) to the *potentially vertically transmitted* network. Finally, subplot (a) corresponds to the *overall* network but for *sponge-enriched clusters* (the axes in this plot is the same as in A-C). Parents and offspring shared, on average, 1.5%, 10.6% and 31.3% of the ASVs present in the *overall*, *sponge-specific* and *potentially vertically transmitted* network, respectively. In comparison, non-parental conspecific adults and larvae shared, on average, 1.4%, 8.4% and 23% of the ASVs present in the same networks. Furthermore, parents and offspring, and non-parental conspecific adults and larvae, shared, on average, 0.019% and 0.015% of vertically transmitted *sponge-enriched clusters* present in the *overall* network, respectively. The axis on the right-hand side of the plots shows the mean difference distribution between the two groups, and the narrowness of the confidence interval gives a clear impression of effect size precision.

Vertical transmission is inconsistent; each offspring receives a different set of microbes from their parent

The results above indicate that vertical transmission in sponge microbiomes is incomplete. One explanation for this lack of completeness is that perhaps only a few symbiotic microbes are required to establish a functioning and beneficial microbiota; hence, parents might only transmit a few of the most important microbes to offspring. However, if these few symbiotic microbes are important, then parents should be selected to transmit the same symbionts consistently to each offspring; that is, siblings should receive the same or very similar subsets of vertically transmitted microbes. Contrary to this expectation, we found no evidence that vertically transmitted ASVs were consistent across offspring from the same parent. For instance, in Figure 3, the taxonomic profiles (at the phylum level) of vertically transmitted microbes often differ considerably between siblings. We analyzed this quantitatively by calculating Jaccard (similarity) coefficients between all larvae in our data set (Jaccard coefficients measure the overlap in ASVs shared between two hosts; a similarity of 1 indicates complete overlap, while a similarity of 0 indicates no overlap). We found that neither siblings nor non-sibling conspecific larvae shared similar assemblages of vertically transmitted ASVs (Figure 5). The average Jaccard coefficient for assemblages of vertically transmitted ASVs between siblings was 0.023±0.046, which was not different than the Jaccard coefficient between conspecific larvae that did not share the same parent (0.012±0.029; MD=0.012, 95% CI [-0.010,0.039]; Figure 5). We complemented these analyses by calculating the Jaccard coefficients between each larva for their assemblage of vertically transmitted ASVs that assigned to *sponge-enriched clusters*. Similarly, we found that neither siblings nor non-sibling conspecifics shared similar assemblages of vertically transmitted *sponge-enriched clusters* (Figure S6). The average Jaccard coefficient between vertically transmitted *sponge-enriched clusters* in siblings was 0.01±0.031, which was not different than the Jaccard coefficient between non-sibling conspecific larvae (0.001±0.005; MD=0.014, 95% CI [-0.001,0.039]; Figure S6).

The absence of a consistent set of ASVs transmitted between a given parent and its offspring could have at least two explanations. First, parents may benefit from varying the microbes transmitted to each offspring. Such variability might be important if offspring disperse long distances and settle in diverse and varying environments. In this case, larvae containing key symbionts are more likely to survive post settlement. This explanation is analogous to the idea that a genetically diverse cohort of offspring is more likely to succeed than a genetically uniform offspring (in this case, the genetic diversity is microbial, not from the host). Importantly, variation in conspecific microbiota may reflect the nature and strength of host-microbe interactions; when these microbial communities are highly similar (low variation), this indicates high specificity, where only a specific set of symbionts may be able to interact with the host. In contrast,

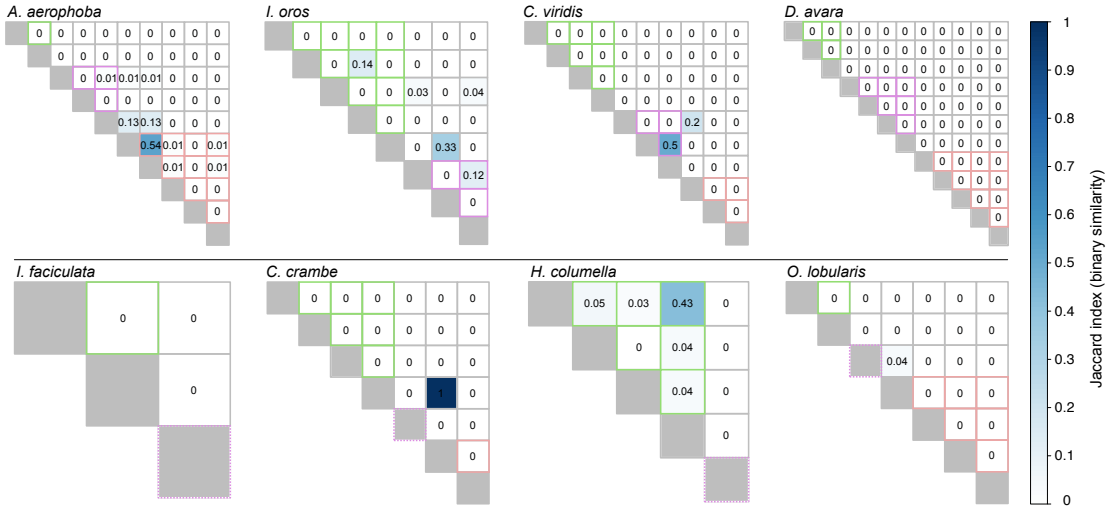

Figure 5: Siblings almost never inherit the same vertically transmitted taxa as show by pairwise Jaccard coefficients calculated for assemblages of vertically transmitted ASVs between larvae that shared the same parent. Each cell represents a larva and sets of siblings from the same parent are indicated by cells bordered by the same color (green, purple, or red). In cases where parents only had one offspring, the diagonal is bordered by a dashed line. Cells with gray boarders correspond to Jaccard coefficients calculated for assemblages of vertically transmitted ASVs between conspecific larvae that did not share the same parent. Gray cells represent the comparison with self. The Jaccard index ranges between 0 (no ASVs shared) and 1 (all ASVs shared)

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when microbiota between conspecific hosts are more variable, this may reflect a situation where specific symbionts are not required for host functioning [50].

Second, an alternative explanation for weak and/or inconsistent vertical transmission is that vertical transmission is not the primary mechanism by which parents ensure that offspring acquire the symbionts they need. Indeed, adult and larval sponges lead very different lifestyles, and symbionts that are beneficial to adults are not necessarily the same as those that are beneficial to larval offspring. Hence, vertical transmission might not be an adaptation in marine sponges, and the weak signatures of vertical transmission we observed might arise via the same neutral processes that govern isolation by distance; that is, offspring are more likely to be colonized by a random subset of microbes from parents as opposed to from non-parental conspecific adults.

Vertically transmitted ASVs are not host species-specific

Because vertically transmitted ASVs were inconsistent across offspring from the same parent (Figure 5), but microbes in adults and larvae were similar at the phylum level (Figure 1), and sometimes showed signs of similarity across larvae from the same species (Figure 3), we further inquired whether a signal of vertical transmission could be detected at the host species level; that is, do conspecific adults and larvae share more vertically transmitted ASVs than they do with individuals from different host species?

Contrary to the idea that vertically transmitted microbes demonstrate high levels of host species fidelity as a result of co-evolution between microbes and host, conspecific adults and larvae did not share more vertically transmitted ASVs than they did with heterospecific individuals. For instance, pairwise Jaccard coefficients between the aggregated subsets of vertically transmitted ASVs from all species, revealed that larvae were not more likely to share vertically transmitted ASVs or *sponge-enriched clusters* with larvae from their own species as compared to larvae of other species (Figure 6). To test this beyond binary pairwise comparisons, we computed modularity on three weighted bipartite networks containing all samples. If conspecific adults and larvae harbor the same microbes and do not share those with other species, then the networks will be organized in compartments consisting of conspecific adults, larvae, and their shared ASVs. Contrary to our expectation, the *overall* network was the most modular (Q^{norm}=0.906) with the highest NMI score (MNI=0.500) followed by the *sponge-specific* (Q^{norm}=0.894; NMI=0.396) and *potentially vertically transmitted* network (Q^{norm}=0.865; MNI=0.390; Table S4), indicating that, for at least some ASVs, host species-specificity decreased with increased host-microbe specialization. In the *overall* network, apart of adults from the two species *A. aerophoba* and *I. oros* that together formed one module, all other adults, including seawater samples, formed their own species-specific modules. However, while some modules contained larvae, they rarely corresponded

to offspring or even larvae of the same species; instead, a mix of heterospecific larvae tended to form their own modules.

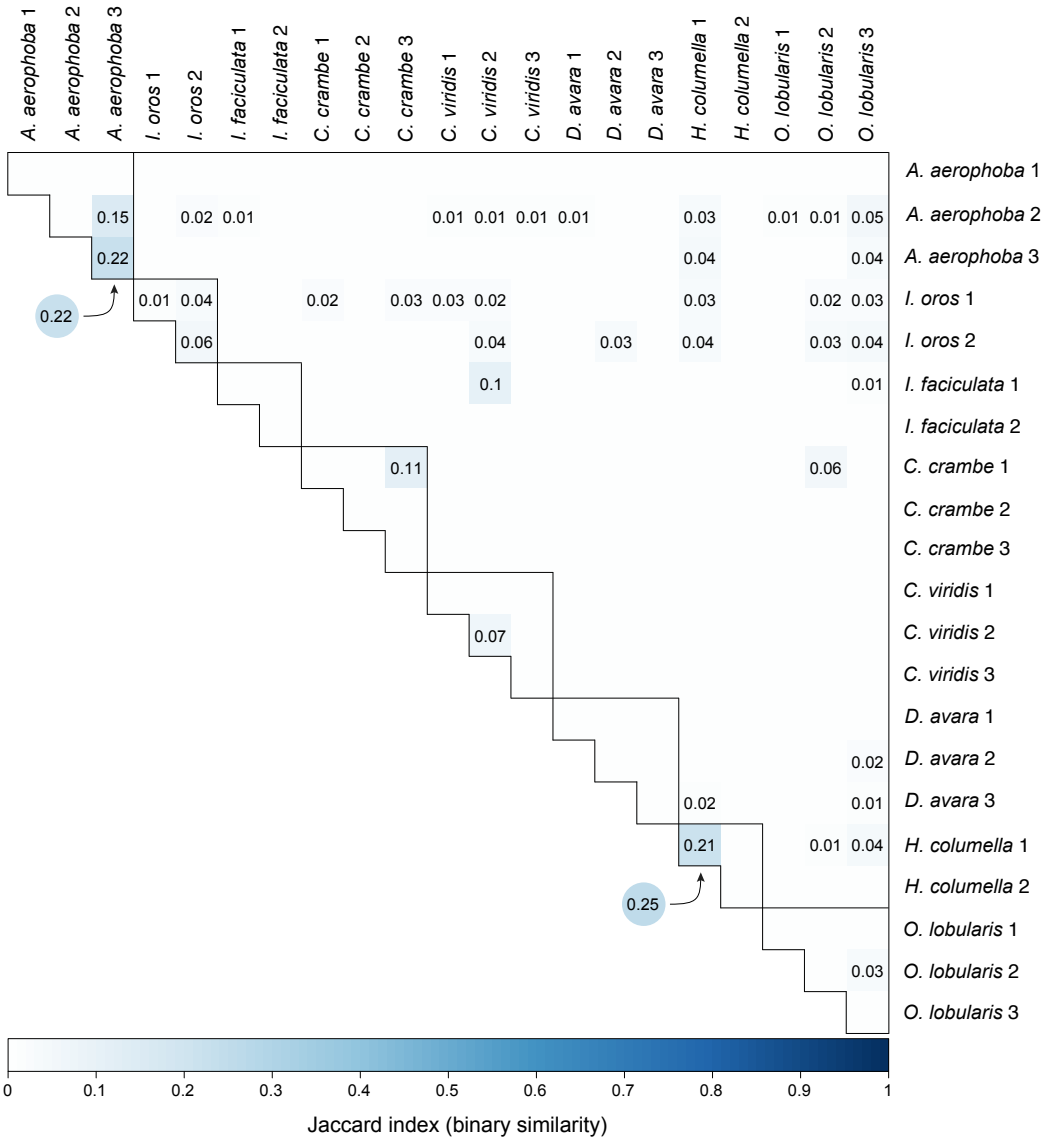

Figure 6: Jaccard coefficients between all the offspring from any two adults calculated for assemblages of vertically transmitted ASVs. The plot shows Jaccard coefficients within and between host species, as well as within single adults (i.e. siblings) on the diagonal. The Jaccard index ranges between 0 (no ASVs shared) and 1 (all ASVs shared). The two circles correspond to the only non-zero Jaccard coefficients for ASVs assigning to *sponge-enriched clusters*.

Vertically transmitted microbes assign more frequently to *sponge-enriched clusters* than horizontally acquired ones

We hypothesized that vertically transmitted microbes would assign to *sponge-enriched clusters* more frequently than horizontally acquired microbes. Further, we hypothesized that, as host-microbe specialization increases, microbes assigning to these *sponge-enriched clusters* will increase in their contribution to both total ASV richness and total sequence count. To test these hypotheses, we defined horizontally acquired ASVs as those shared between seawater and sponge hosts (blue edges in Figure 2A). We further pooled all the subsets of vertically transmitted ASVs (Figure 2D) into one large assemblage containing vertically transmitted ASVs from all host species. Of the 7,039 different horizontally acquired and 438 different vertically transmitted ASVs, we found that 6.8% and 71% assigned to *sponge-enriched clusters*, and that these assemblages, in turn, accounted for 26.2% and 53.2% of their respective total sequence counts, indicating that *sponge-enriched clusters* indeed are more frequent and abundant among vertically transmitted microbes than among horizontally acquired ones.

The contribution to total ASV richness by microbes assigning to *sponge-enriched clusters* increased for the majority of sponge species as host-microbe specialization increased (Table S5). This pattern is expected as the denominator (number of nodes in the focal network) decreases as we move towards the right in Figure 2. The contribution to the total number of sequences by microbes assigning to these clusters increased for about half of the sponge species (Table S5). This pattern was especially noticeable for *I. fasciculata* and *A. aerophoba*, which harbored some particularly abundant *sponge-enriched clusters* among their vertically transmitted ASVs. Indeed *I. fasciculata* harbored one cluster that accounted for 89% (with n=775 reads) of the total sequence count in the *potentially vertically transmitted* network; A. aerophoba harbored a mixture of 122 rare (n=2 reads) and abundant (n=7335 reads) *sponge-enriched clusters*. Furthermore, some host species harbored a higher diversity and relative abundance of these clusters that carried over to the subset of vertically transmitted microbes, suggesting that *sponge-enriched clusters* may play a larger role in some host species than in others (Table S5). For example, *A. aerophoba* harbored over 80% of all identified *sponge-enriched clusters* across the different networks (Table S6). However, from Table S5, it is interesting to note that the number of *sponge-enriched clusters* decreased from the *overall* to the *sponge-specific* network (between 21-51% across host species), further indicating that adult hosts may acquire, at least, some of these clusters horizontally from the seawater.

Of the 105 different *sponge-enriched clusters* identified in the *overall* network, 94 were also detected in the seawater, although at very low abundances (Table S5). In light of our finding that siblings did not inherit the same *sponge-enriched clusters* from their parents, nor were these clusters consistently transmitted across conspecific larvae,

this suggests that parents may transmit a random subset of *sponge-enriched clusters* to offspring, and that the signature of this vertical transmission is only detectable when adults and larvae are pooled across species (Figure 1B). Furthermore, out of the 48 *sponge-enriched clusters* that were identified in the subset of vertically transmitted ASVs, only four were not present (or below detection limit) in seawater; three clusters belonged to the phylum Chloroflexi, and one cluster to the phylum Deltaproteobacteria. Interestingly, the latter could be further classified to Bdellovibrio–a genus of gram-negative obligate aerobic bacteria that parasitize and kill other gram-negative bacteria. This genus has previously been found in the gut microbiome of other animals, including humans, where it is associated with a healthy gut microbiome [51]. Finally, detailed -omic studies have revealed symbiotic characteristics and functional capabilities of some *sponge-enriched clusters* including, e.g., enrichment of proteins containing eukaryotic-like repeats, the capacity to degrade complex carbohydrates, and the production of secondary metabolites that are used as defenses by the sponge host [52, 41, 43]. While these results demonstrate the different but likely vital services *sponge-enriched clusters* provide to marine sponges, we can only speculate in the potential benefits of their unfaithful transmission. As previously discussed, perhaps unfaithful transmission is beneficial when offspring disperse long distances and settle in varying environments. Moreover, *sponge-enriched clusters* may be functionally versatile–the exact form of their relationship with the host may change depending on what other clusters and/or microbes are present in the microbiome, which is, at least, partly governed by priority effects–the order and timing of species arrivals [53]. Therefore, at the time of larval settlement, harboring any *sponge-enriched cluster* may strongly influence the succession trajectory and the functional development of the maturing sponge microbiome.

Conclusion

Vertical transmission is proposed to be a primary mechanism by which parents transmit assemblages of beneficial microbes to offspring in a way that maintains both these microbes' interactions with each other and the beneficial functions that emerge from their interactions [14]. However, contrary to these theoretical expectations, evidence is mounting that this classic view of vertical transmission is rare in animal microbiomes–especially when microbiomes are highly diverse (see [54] for a review). We find that marine sponges also do not fit the classic mold; while previous research based on electron micrographs has undeniably detected mechanisms by which parents pass microbes to offspring [31, 32], our findings cast doubt on the faithfulness and consistency of these transmissions. Specifically, across eight sponge species, we show that: (1) vertical transmission is detectable, but weak and incomplete such that offspring do not receive a replica of their parent's microbiome; (2) parents do not transmit the same suite of microbes to each off-

spring; (3) vertically transmitted microbes are not host species-specific and therefore unlikely to have co-evolved with particular sponge species; and (4) while vertically transmitted microbes assigned more frequently to *sponge-enriched clusters* than horizontally acquired ones, the signature of this vertical transmission is only detectable when adults and larvae are pooled across species. Furthermore, it is worth noting that measuring vertical transmission at the level of ASVs is relatively coarse and therefore conservative. A given microbial ASV may contain multiple strains; hence, while our analysis indicates that vertical transmission makes only a minor contribution to the microbiomes of larval sponges, our analysis may overestimate the relative contribution of vertical transmission to larval sponge microbiomes. Strain-level analyses will be required to truly estimate the proportion of microbes shared between sponge parents and offspring [55].

Our findings highlight the need for new theory to explain how hosts ensure the faithful transmission of beneficial microbiomes. While the classic model may sometimes work well when the microbial symbionts consist of just one or a few species [6, 18], when microbiomes are very diverse and complex, transferring thousands of microbial species such that their interaction structures and emergent functions are preserved seems highly improbable. So, how do sponge parents ensure that offspring get the microbes they need? We know that such mechanisms exist because by the time sponge juveniles reach adulthood, they have converged on highly similar and species-specific microbiomes [50]. In the absence of strong vertical transmission, at least two processes may contribute to this convergence. First, evidence from other ecological communities, including the human gut microbiome, suggests that priority effects strongly influence community assembly [56, 53, 57, 58]. Even if just a few microbes are vertically transmitted, they may pre-empt the initial host niche. Those microbes may quickly reach carrying capacity while simultaneously modifying the (host) niche in their favor, thereby altering the ability of subsequent microbial immigrants to colonize. Hence, vertical transmission of a few beneficial symbionts may, via priority effects, help build the microbiome anew generation after generation. Second, sponges likely acquire and curate beneficial microbes by filtering them from the environment. In our study, we were able to detect 90% (94 of 105) of *sponge enriched clusters* in seawater, and while these were in low abundances, sponges can filter vast quantities of water: up to 24,000 liters (24 m^3) of water per kilogram and day [59]. Once these microbes are inside the host, the innate immune defenses of some sponge species can differentiate between pathogens, food bacteria and symbionts in a manner similar to the adaptive immune system of vertebrates [60, 61, 62, 63, 64, 65]. For some microbes the host niche also provides a more favorable environment than seawater, in turn, some symbionts have molecular structures that facilitate recognition by the sponge host [41, 52]. Together, priority effects, horizontal acquisition from the rare biosphere, and active curation and cultivation of microbes by the sponge host likely combine to create adult sponge microbiota that exhibit low variation between conspecific adults,

with sometimes considerable divergence between sponge species living in the same environment [50]. However, we still do not understand how (or if) evolution has selected hosts to guide these processes, especially priority effects, to their benefit.

Finally, some of our results are relevant to the predictions put forward by of the hologenome theory of evolution [7, 8, 11]. This theory proposes that there may be value in treating hosts and their microbiota as a single evolutionary unit. This comes with an important expectation: high partner fidelity–if the collection of genomes varies within and between host generations, then it is not a coherent unit of selection [9, 10]. Such tight partner fidelity is typically only found among host-microbe symbioses with obligate vertical transmission. On the contrary, we found that many vertically transmitted microbes, including many *sponge-enriched clusters*, were not faithfully transmitted by parents to offspring nor were they host species-specific. As such, their evolution is likely shaped by multiple host species across the phylum Porifera, as well as by the marine environment where the sponge hosts live. Overall, our study demonstrates that common predictions of vertical transmission that stem from species-poor systems are not necessarily true when scaling up to diverse and complex microbiomes.

Methods

We collected sponge and seawater samples between July and August 2012, close to the Islas Medas marine reserve in the northwestern Mediterranean Sea $42°3'0''N$, $3°13'0''E$ by SCUBA at depths between 5-15 m. The analyzed species are common Mediterranean sponges and were identified based on their distinct morphological features.

Larval sponge collection

We constructed larvae traps by modifying the traps used in [66] (Figure S7). In order to collect offspring from known parents, traps were mounted over individual adult sponges by SCUBA. To minimize stress to individual adults, traps were removed after one week. During this time, sample bottles were collected and replaced every day. Bottles were placed on ice in insulated coolers and transported to the laboratory (<2 hours). Larvae were identified using a stereolupe. In order to remove loosely associated microbes, larvae were carefully rinsed with filter-sterilized seawater (0.20 μm filter) before preservation in RNA later. All larval samples were stored at -80°C until DNA extraction.

Adult sponge collection

After larvae offspring were collected, three adults per sponge species were sampled. These individuals corresponded to the same adults that larvae had been collected for. However, for a few species, larvae could only be collected for two adults. In these cases, a third adult was still sampled. Specimens were sub-lethally sampled by removing a small sample of tissue. Excised tissue was placed in separate plastic tubes and brought to the surface where they were preserved in RNA later and placed on ice in insulated coolers and transported to the laboratory (<2 hours). Seawater samples were collected at 5 m depth and at 7 locations within the sampling area. All seven water samples were poured into separate, sterile 5 L jars. Aliquots of seawater (300-500 mL each, 1 aliquot per sample jar) were concentrated on 0.2 μm polycarbonate filters, and submerged in lysis buffer. All samples were stored at -80°C until DNA extraction.

DNA extraction and sequencing

DNA was extracted from ≈0.25 g of adult sponge tissue using the PowerSoil DNA extraction kit (MoBio). DNA from larvae (one larva per adult) was extracted using the XS-RNA extraction kit (Macherey-Nagel) because of its capacity to extract DNA from small samples, i.e., one larva. All DNA extractions were performed according to standard protocols. The 7 seawater samples were processed by passing 2 L (from the 5 L) of seawater through 0.2μm Sterivex filters, and DNA was extracted from these filters as described by [39]. The V4 region of the 16S rRNA gene was amplified using the primer 515FB-806RB [67] and sequenced using the Illumina HiSeq2500 platform. Sequencing was performed by the Earth Microbiome Project [68].

Identification of *sponge-specific clusters*

A representative sequence from each ASV was taxonomically assigned using a BLAST 62 search against a curated ARB-SILVA database containing 178 previously identified *sponge-specific clusters* [28]. For each BLAST search, the 10 best hits were aligned to determine sequence similarities. The most similar ASV sequence to the respective reference sequence within the database was then assigned to an *sponge-specific clusters* based on a 75% similarity threshold: (i) a sequence was only assigned to any given *sponge-specific clusters* if its similarity was higher to the members of the cluster than to sequences outside the cluster; and (ii) if its similarity to the most similar sequence within the cluster was above 75%. A majority rule was applied in cases where the assignment of the most similar sequences was inconsistent, and the ASV sequence was only assigned to the *sponge-specific clusters* if at least 60% of the reference sequences were affiliated with the cluster.

Analyses

Illumina-sequenced, paired-end fastq files were processed and cleaned using default settings in DADA2 [69] to produce an amplicon sequence variant (ASV) table. To partition data into the different bipartite networks and to find vertically transmitted microbes, we used simple set theory. Modularity was analyzed using the `DIRT_LPA_wb_plus` [46]. We computed modularity on both weighted and unweighted bipartite networks; the main difference between the two is that when calculating modularity on an unweighted network, it does not allow for any weighting by information content (here relative abundance), i.e., rare microbes are as important as abundant ones. While we found the results to be quantitatively different (as others also have demonstrated, [47, 46]), they lead to the same overall conclusion. We further used Normalized Mutual Information (NMI) criterion [48, 49] to test whether observed modules deviated from prior expectations. These algorithms were run in `R`. In the few cases where statistical analyses were performed, we used estimation statistics; a simple framework that avoids the pitfalls of significance testing that calculates a distribution of mean differences that is an approximation of the Bayesian posterior probability distribution [70]. This distribution is used to weigh plausibility over an effect likelihood size range, and is visualized in a Gardner-Altman comparison plot [71]. We used the 95% highest density interval (HDI) as a measure of statistical significance. That is, if a parameter or a pairwise parameter comparison excludes zero, then we conclude that the probability of the difference being significantly different from zero exceeds 95%. This was done in the `DABEST Python` package in `R` via the `reticulate` package. Lastly, we used the logit transformation as a variance-stabilizing transformation of proportions. The logit transformation is the log of the odds ratio; that is, the log of the proportion divided by one minus the proportion. In practice, the transformation expands the ends of the scale, such that small differences in the proportions have a larger difference on the logit scale.

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Authors' contributions

J.R.B. and J.M.M. conceived the study. J.R.B. performed the fieldwork and analyzed the data. J.R.B. and J.M.M. drafted the first versions of the manuscript, and J.R.B. and E.A. refined the ideas and wrote the final version of the paper. C.A.G. identified the *sponge-specific clusters*. All authors commented and approved of later versions of the paper.

Data and code availability

All data and code will be available on Open Science Framework with an `R Markdown` document such that all analyses and figures can be reproduced.

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