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Transmission mode is associated with environment type and taxa across bacteria-eukaryote symbioses: a systematic review and meta-analysis

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ABSTRACT

Symbiotic associations between bacteria and eukaryotes exhibit a range of transmission strategies. The rates and distributions of transmission modes have not been thoroughly investigated across associations, despite their consequences on symbiont and host evolution. To address this empirically, I compiled data from the literature on bacteria-multicellular eukaryote associations for which transmission mode data was available. Of the total 528 analyzed symbioses, 21.2% were strictly horizontally transmitted, 36.0% exhibited some form of mixed mode transmission and 42.8% were strictly vertically transmitted. Controlling for phylogenetically independent symbiosis events revealed modes were approximately equally distributed among the 113 independent associations, at 32.1%+/−0.57% horizontal, 37.8%+/−1.4% mixed mode and 31.1%+/−1.3% vertical transmission. Binning symbioses by environment revealed an abundance of vertical transmission on land and a lack of it in aquatic environments. The naturally occurring uneven distribution of taxa among environments prevented controlling for host/symbiont phylogeny. However, the results were robust over a large number of independently evolved associations, suggesting that many vertically transmitted bacteria are capable of mixed mode transmission and barriers exist that reduce the rate of horizontal transmission events. Thus, both the environment type and host/symbiont taxa influence symbiont transmission mode evolution.

Keywords: symbiosis; transmission mode; evolution

INTRODUCTION

An enormous diversity of eukaryotes host bacterial symbionts for a range of functions, from nutrition to defense. In many cases, these associations have allowed both bacteria and hosts to colonize and adapt to radically new niches compared to their ancestral lifestyles. In the nutritional symbioses of sternorrhynchan and auchenorrhynchan insects (Hemiptera), bacterial symbionts allow their hosts to live on diets based solely on phloem and xylem, respectively, and have likely enabled the great radiations of diversity in these groups (Sudakaran, Kost and Kaltenpoth 2017). As an even more striking example, symbiosis with chemosynthetic sulfur-oxidizing bacteria has evolved multiple times and enabled several marine invertebrate taxa to colonize reducing environments such as the hydrothermal vents. These habitats provided no nutritional or energetic content to the host ancestors, which lacked symbiotic organisms containing the genes for sulfide oxidation and

carbon fixation (Stewart, Newton and Cavanaugh 2005), underscoring the necessity of these associations for the host's niche. Defensive symbionts often provide contextual benefit to their hosts, for example, by providing antibacterial defense during embryogenesis (Kaltenpoth *et al.* 2010; Flórez *et al.* 2017) or protection against parasitoids in environments where the threat is present (Oliver *et al.* 2010). While some symbionts perform multiple functions (*e.g. Steinernema carpocapsae* of pathogenic nematodes which are involved in host nutrition and development (Goodrich-Blair 2007)), the vast majority appear to specialize in a task. Alternatively, manipulative associations do exist in which symbionts do not necessarily provide a benefit, but tie themselves to host reproduction and so have to act within host interests (Werren, Baldo and Clark 2008; Sullivan 2017).

Regardless of the function, every symbiotic association is posed with the same challenge: How do symbionts establish contact with host offspring during host reproduction? A range of solutions have been adopted across the diversity of symbioses, but little is known about the factors influencing when each of these solutions evolve. Categorically, two modes of symbiont transmission exist: horizontal transmission of symbionts through the environment and vertical transmission of symbionts through host tissues to offspring (Bright and Bulgheresi 2010). Horizontal transmission is most certainly the ancestral mode for all symbioses because all known symbionts, even mitochondria and chloroplast organelles, have free-living ancestors (Gray, Burger and Lang 1999). Many associations have kept with this strategy (*e.g.* hydrothermal vent tubeworm symbionts (Nussbaumer, Fisher and Bright 2006)), however, a great many others have evolved elaborate mechanisms of symbiont inheritance ranging from egg-smeared secretions (*e.g.* stink bugs (Hosokawa *et al.* 2013)) to incorporation of symbionts in developing oocytes (*e.g.* vesicomyid bivalves (Ikuta *et al.* 2016)) or embryos (*e.g.* parthenogenetic aphids (Braendle *et al.* 2003)).

A third mode that incorporates both categorical modes, termed mixed mode transmission, has gained appreciation recently because many associations exhibit evidence that they use both strategies (Bright and Bulgheresi 2010; Ebert 2013). Conceptually, this is not surprising because bacterial symbionts are often in clades of bacteria with a high propensity for host-association (*e.g. Burkholderia* (Kikuchi, Hosokawa and Fukatsu 2011; Silva *et al.* 2018)) and some are closely related to free-living bacteria (*e.g.* Enterobacteriaceae stinkbug symbionts (Hosokawa *et al.* 2016)). Furthermore, symbionts often use cell-to-cell transfer strategies for colonizing host tissues (Sacchi *et al.* 1988; Allen *et al.* 2007; Koga *et al.* 2012; Dan *et al.* 2017), which may be co-opted for horizontal transmission. Mixed modes can arise via either the occurrence of horizontal events in a faithfully vertically transmitted association (*e.g.* solemyid bivalves (Russell, Corbett-Detig and Cavanaugh 2017; Russell, McCartney and Cavanaugh 2018)) and aphid facultative symbionts (Vorburger, Siegrist and Rhyner 2017)) or one in which vertical transmission fails at a high rate (*e.g.* chinch bug vertical transmission fails 70% of the time (Itoh *et al.* 2014)), placing more importance on the horizontal transmission strategy. Thus, symbiont transmission modes are best conceptualized on a spectrum between strict horizontal and strict vertical transmission (Fig. 1A).

Genomic data has enabled more sensitive detection of mixed-mode transmission, as rare horizontal events substantially impact bacterial symbiont population genetics (Russell, Corbett-Detig and Cavanaugh 2017), even if they are not informative predictors of host-symbiont cooperation (Fisher *et al.* 2017). In strictly vertically transmitted associations such as those between sap-feeding insects such as aphids and spittlebugs and their symbionts *Buchnera* and *Sulcia*, respectively, host restriction and genetic drift over millions of years has resulted in severe genome erosion (Moran and Bennett 2014). Many symbiont lineages even lack the genes needed for the association, requiring the acquisition of a secondary symbiont with complementary functions (*e.g.* spittlebugs (Koga and Moran 2014)). While a vast number of symbioses fit this model, most of which are hemipteran-associated, some associations exhibit vertical transmission without such severe genome reduction (*e.g.* (Woyke *et al.* 2006; Newton *et al.* 2007; Dmytrenko *et al.* 2014)). Given that many of these aberrant associations occur in marine habitats, and vertical transmission has been reported to be rare in this environment (Normark and Ross 2014), habitat type may shape the cost/benefit tradeoffs for transmission modes. Intuitively, this is reasonable as water is a much easier medium for a bacterium to navigate through than air.

While recent work by Fisher *et al.* (2017) showed that host dependence is correlated with transmission mode and symbiont function, they did not evaluate mixed transmission modes or test for an impact of environment type or transmission route. Thus, a wide-scale analysis of symbiont transmission mode distribution across the full diversity of bacteria-eukaryote symbioses was warranted. First I sought to quantify the rates of horizontal, mixed and vertical transmission modes in nature from the literature to date on symbiont transmission. Next, using these data, I tested whether factors such as the environment in which a symbiosis lives, the route symbionts take between host tissues and the function of the symbiosis have an impact on the distributions of these transmission modes. These results will be informative about processes such as gene flow between symbionts and free-living bacteria and symbiont uptake/deposition of eDNA, which should both increase as horizontally transmitted or mixed-mode associations increase in abundance in a community. Evolutionarily, these results will shed light on the variables that correlate with, and thus may be involved in, determining what modes are adopted in and among associations over time.

MATERIALS AND METHODS

Literature search

To assess the rate of mixed mode transmission among bacteria-eukaryote symbioses, I obtained references from searches through Google Scholar and PubMed, using no time limits and using keywords such as 'transmission mode', 'inheritance', 'infection', 'colonization', 'transovarial transmission', 'symbiont uptake', 'bacterial/symbiont phagocytosis', 'bacterial/symbiont endocytosis', etc., which were collected in a Zotero database. Searches for new literature to include in the analysis continued until November 7, 2018. Following PRISMA systematic review and meta-analysis guidelines (PRISMA-P Group *et al.* 2015), the number of records in this database totaled 6872, and after duplicates were removed 6757 records remained. Of these, 1509 records directly pertaining to symbiosis were selected and full-text articles were read to identify journal articles containing transmission mode data of either a direct or indirect nature. This resulted in 325 papers included in qualitative and quantitative synthesis, producing 528 symbiont-host associations for the transmission mode analysis (Fig. 1B; Table S1, Supporting Information).
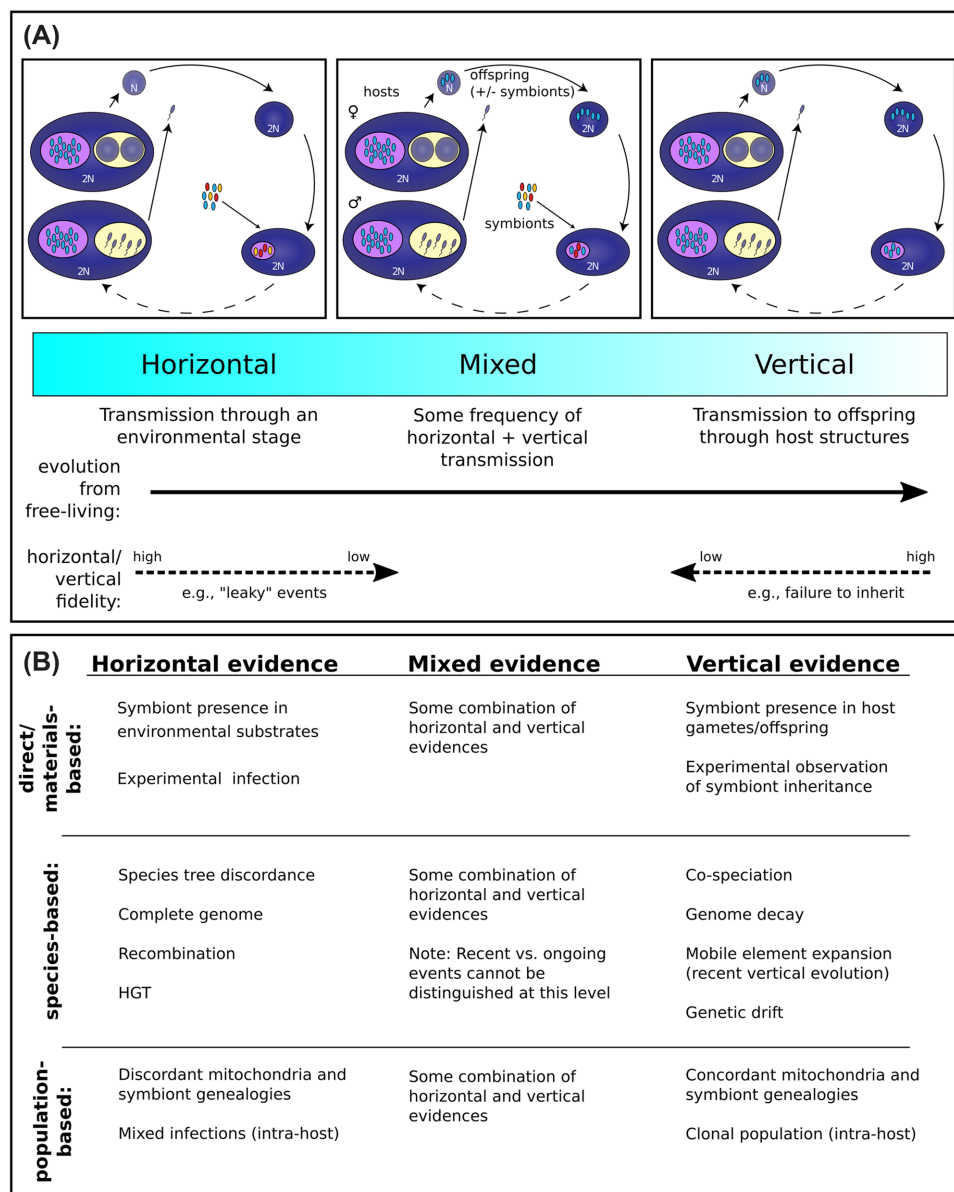
Figure 1. Transmission mode spectrum definitions and influential parameters. A) Symbionts evolve from free-living ancestors, and are thus initially horizontally transmitted through an intermediate environmental stage. Some associations evolve vertical transmission through host tissues, either obligately or in conjunction with some amount of horizontal transmission. B) Criteria for binning symbioses into each transmission mode based on data from the literature.

Data collection

Data from each publication were manually entered and tallied in Table S1 for the symbioses that met the following criteria:

- Associations between bacteria/archaea and multicellular eukaryotes only (*e.g.* no fungal symbionts or ciliate hosts).
- Mutualisms and commensalisms (no parasitisms).
- Species or strain-level symbiont identification (opposed to microbiomes comprised of complex, poorly characterized bacterial mixtures).
- Associations with data informative about the symbiont transmission mode.

Definition of bacteria-eukaryote symbioses

The associations included in this analysis were those between an identified bacterial symbiont strain and a eukaryotic host

that are either mutually beneficial (*i.e.* mutualisms), or are beneficial for one partner and are commensal to even slightly costly for the other. Outright parasitisms, however, were not considered. Pathogen transmission modes are heavily determined by the dynamics of host-pathogen conflict, often resulting in selection against strict vertical transmission (Kover, Dolan and Clay 1997). This makes it difficult, if not impossible, to ascertain the influence of other aspects of the associations (*e.g.* environment types) on transmission mode from metadata alone. However, in an attempt to ascertain the impact of symbiont-derived host-benefit on transmission mode distribution, I included associations near the mutualism-commensalism-parasitism boundary. These associations require hosts for transmission and do not always provide a benefit, but instead have functional mechanisms to manipulate host reproduction to favor transmission (Toft and Andersson 2010; Douglas 2016; Sudakaran, Kost and

Kaltenpoth 2017). I assigned the function 'manipulative' to symbioses in this category because this is the predominant term used in the literature (*e.g.* Werren, Baldo and Clark 2008; Correa and Ballard 2016).

In addition to categorizing symbioses by their general level of symbiont-derived host-benefit, I also tallied beneficial associations according to the general function provided to the host, as this is a common approach for binning symbioses (*e.g.* Toft and Andersson 2010; Douglas 2016; Sudakaran, Kost and Kaltenpoth 2017)) and provides insight into host-symbiont interaction. Associations were binned into either 'nutrition', such as chemosynthetic or photosynthetic carbon fixation, nitrogen fixation, or amino acid synthesis, 'defense', such as viral or parasitoid protection, antibiotics, or bioluminescence, 'multiple function' for the associations that perform both a nutrition and a defense role (*e.g. Serratia symbiotica* provides both defensive and nutritional functions in aphids (Oliver *et al.* 2010; Burke and Moran 2011)), or 'unknown' for mutualisms that have not been functionally characterized.

To test whether there is an association between environment type and transmission mode, I collected data on symbiont/host habitats for the included symbioses. An environment type of either marine, terrestrial, or freshwater was noted for each association, as the medium (air vs. water) may impact the evolution of transmission modes (Normark and Ross 2014). Given that only four freshwater associations were found with transmission mode data and the medium, water versus air, is the critical factor, marine and freshwater tallies were pooled as 'aquatic' associations for most analyses.

I included as many associations in this dataset and analysis as possible, given data availability and the scope of the project. Only bacterial symbionts were considered, as the body of literature required for this group alone was immense (1509 papers) and bacteria are one, if not the, dominant group of eukaryote-associated symbionts (Sachs, Skophammer and Regus 2011). However, it should be noted that replacements with yeast symbionts have occurred in the planthoppers (Bennett and Moran 2015), cnidarian algal zooxanthellae exhibit a range of transmission modes (Quigley *et al.* 2018), and ubiquitous, horizontally-transmitted fungal mycorrhizae root symbionts help plants acquire nutrients from soils worldwide (Parniske 2008). Microbiome-type associations were not included because the bacteria involved are rarely identified at a fine enough scale (*i.e.* sub-Phylum) to uniquely identify symbionts and permit comparisons among datasets. Only associations with multicellular hosts were included because complex tissue structures enable sequestration of symbionts further from the environment than a single-celled host environment does, offering more regulation for controlling transmission processes. Lastly, it should be noted that both intracellular and extracellular symbioses were included, as it has been shown that the cellular environment does not prevent the evolution of traits thought to to be unique to an intracellular lifestyle, such as genome reduction (Nicks and Rahn-Lee 2017; Salem *et al.* 2017).

Some symbiotic associations exhibit complex localization patterns, often with nested structures consisting of one symbiont residing within another. In these situations, the association between each bacterium and the largest, most encompassing, multicellular host was recorded. For example, in the case of termite-bacteria associations involving additional protozoan symbionts, the single-celled eukaryotes were not considered, although they do tend to vertically transmit their symbionts with high fidelity (Ikeda-Ohtsubo and Brune 2009) and they cospeciate with their bacterial symbionts (Noda *et al.* 2007).

Definition of symbiont transmission modes and routes

As discussed in the Introduction, transmission modes exist on a continuum from strict horizontal transmission to strict vertical transmission, with mixed modes describing the strategies in between (Fig. 1A). While it would be ideal to treat transmission mode as a continuous character, reporting on mixed modes is still in its infancy and the lack of data on the exact amounts of horizontal/vertical transmission precludes more quantitative analyses. For example, few papers do more than show symbiont presence in host reproductive tissues or infer a multi-species phylogeny. Thus, for the purposes of this work, transmission mode is defined as a discrete trait with three values, 'horizontal', 'mixed', and 'vertical'.

The route vertically transmitted symbionts take through host tissues to gametes or offspring may influence opportunities for horizontal transmission, and thus the incidence of mixed mode transmission. Thus, I tallied whether the route through host cells/tissues is external (*e.g.* symbiont transmission via secretions applied to eggs (Hosokawa *et al.* 2013)) or internal (*e.g.* transfer to oocytes before fertilization (Sacchi *et al.* 1988; Perotti *et al.* 2007; Ikuta *et al.* 2016; Dan *et al.* 2017)).

Transmission mode frequency estimation

Few papers directly report mixed transmission modes, in part because appreciation for this intermediate mode has grown recently (Bright and Bulgheresi 2010; Ebert 2013) and the assessment of mixed modes involves multiple data/experiment types. Far more common are papers that report either a vertical or horizontal transmission mode based on a limited dataset. However, for the more thoroughly studied associations, additional papers often exist with more data either in support of the hypothesized transmission mode or in conflict with it (*e.g.* genealogical or phylogenetic con-/discordance, unexpected genome size, etc.). In this second case, strong data for both vertical and horizontal transmission in an association suggests the actual mode of transmission is some form of mixed mode. As genetic evidence of vertical transmission is quickly eroded by even exceedingly low rates of horizontal transmission (Brandvain, Goodnight and Wade 2011), combining datasets offers a powerful way to inform on the transmission processes of well-studied associations.

To arrive at an estimate for the rate of horizontal, vertical and mixed mode transmission among symbiotic associations, I collected and synthesized data relevant to transmission modes from the literature as outlined in Fig. 1B. Transmission evidence was binned into one of three categories: A) direct/material-based evidence, B) indirect species-level evidence and C) indirect population-level evidence (see Fig. 1B).

Direct, or materials-based, evidence included any observation of symbionts in the reproductive tissues (vertical transmission) or at high abundance in the environment (horizontal transmission). In a few cases, symbiont experimental infection studies had been performed on cultures in the lab, providing the most direct data supporting horizontal (*e.g.* Gonella *et al.* 2012) and vertical transmission (*e.g.* Braendle *et al.* 2003).

Indirect data were categorized at two levels: species-level evidence and population-level evidence. Species-level data was by-far the most common, as a great number of studies PCR-amplified and Sanger sequenced symbiont markers (*e.g.* the 16S ribosomal RNA gene) and host markers (*e.g.* the mitochondrial cytochrome oxidase 1 gene) to infer host-symbiont co-evolutionary relationships. However, advances in sequencing technologies over the past 15 years have permitted many symbiont genomes to be sequenced, providing data on whether they exhibit genomic evidence of strict vertical transmission,

i.e. genome size erosion, gene loss, AT-bias, accelerated substitution rate, etc. relative to free-living relatives (Andersson and Kurland 1998). These studies have also revealed evidence of rampant recombination in some vertically transmitted symbiont genomes (Baldo *et al.* 2006; Mouton *et al.* 2012; Russell and Cavanaugh 2017; Russell, Corbett-Detig and Cavanaugh 2017), which requires some amount of horizontal transmission to be observable.

At the population level, evidence of vertical transmission includes clonal intra-host populations due to transmission population bottlenecks (Mira and Moran 2002; Kaltenpoth *et al.* 2010) and mitochondria-symbiont genealogical concordance due to co-inheritance (Sanchez, Arnold and Asmussen 2008). While genealogical discordance is too sensitive of a measure to distinguish degrees of horizontal transmission (Brandvain, Goodnight and Wade 2011), genetic evidence of mixed populations in host tissues is strong evidence that horizontal transmission is ongoing at a high rate (Russell and Cavanaugh 2017). Transmission evidence levels and results are presented in the 'evidence for mode' column of Table S1 (Supporting Information).

It should be noted that in the definition of mixed transmission used here, no distinction is made between horizontal and paternal transmission, as this was generally not tested for in the literature and is hard to distinguish otherwise. However, rates of paternal transmission are generally low and are often mediated via host-to-host contact (essentially a horizontal transmission process), as sperm are too small to house many bacterial symbionts (Peccoud *et al.* 2014; De Vooght *et al.* 2015). In addition, no distinction was made between ongoing horizontal transmission (*i.e.* mixed mode transmission) and the recent evolution of strict vertical transmission, both of which exhibit symbiont genomes hallmarked by extensive mobile element expansions (Plague *et al.* 2008; Newton and Bordenstein 2011). This is because complete absence of horizontal transmission is difficult to prove, and is only becomes evident in genomic data over time.

The patchiness of the available data on symbiont transmission made it impossible to use the same criteria to call transmission modes across all associations. Thus, conservative calls were made from what data could be obtained. For example, if only tissue/environmental-based data existed in support for vertical/horizontal transmission, and no genetic data was available on the fidelity of that mode, then a strict vertical/horizontal transmission mode was assigned. This conservative approach likely produced an underestimate of the frequency of mixed modes among symbioses, the extent of which was assessed via sensitivity analysis, which is described below.

Selection of phylogenetically independent symbioses

Analysis of the full, unfiltered dataset for correlative relationships is problematic because it is biased towards particular groups of bacteria and hosts, such as insects and Enterobacteriaceae, which speciated after the symbiosis evolved. Thus, I identified the independent symbiosis event to which each association in Table S1 (Supporting Information) belongs. To identify these independent events, I searched the literature for studies testing for this explicitly among either hosts and/or symbionts. This strategy was selected, opposed to one in which new trees were constructed, because the host and symbiont taxa involved in this analysis span the diversity of life, which would make gene marker selection, alignment quality, and missing data challenging problems to solve.

The evolution of a symbiotic lifestyle was considered separately for symbionts and hosts, and recorded with a unique identifier in Table S1 (Supporting Information). Independent symbiosis events were identified as unique combinations of symbionts and hosts. For example, *Sodalis* colonize several unrelated hosts, so this tallies each host group separately. Importantly, this also catches new associations between hosts with long evolutionary histories (*i.e.* symbiotic ancestors) that have recently partnered with a new symbiont (*e.g.* the 'homopteran' subset of hemipteran insects). Some associations were discovered after publication of the inclusive studies describing the groups they belong to (*e.g.* Husník, Chrudimský and Hypša 2011) or were not included in the studies. To include these associations, genetic data on the relationships of these taxa to those in the inclusive studies were used to 'root' the associations in their proper host/symbiont independent symbiosis group. Symbioses for which a separate, dedicated study has not been performed (indicated by '?' in columns 1–4 of Table S1, Supporting Information) were categorized by their host and/or symbiont family. As many of the independent symbiosis events were found to occur at or below the family level (see columns 1–4 vs columns 4 and 6 in Table S1, Supporting Information), this was deemed a conservative proxy when actual data is lacking. Furthermore, only 97/528 symbionts and 45/528 hosts had to be handled in this way.

From these data, I identified 113 independent symbiosis evolutionary events in the dataset. The symbiosis between Enterobacteriaceae group 4 and pentatomomorphan stinkbugs contained the largest number of associations at 48. To subsample the diversity of this entire group and ascertain the effect of the associations selected on the results, I resampled these 113 symbioses 48 times with replacement. Transmission mode frequency estimation and the statistical and sensitivity analyses described below were performed on these subsets of the data.

Statistical methods

I sorted and tallied data by category (transmission mode, environment, (vertical) transmission route, and function) for the total 528 symbioses and the subsampled 113 unique symbioses with a custom perl script. Plotting and statistical testing were performed in R. These nominal data were compared with Fisher's exact test to determine whether the relative transmission mode rates are independent of environment type and whether vertical transmission route, external or internal, is independent of transmission mode. Pearson's chi-squared test was used to test for an impact of symbiosis function on transmission mode. Associations between host/symbiont taxa and the tallied variables were plotted using the heatmap.2 (ggplot) heatmap plotting function in R.

Permutation tests were performed on the subsampled phylogenetically independent data to determine whether enrichments for transmission modes in the different categories were significant above noise/bias in the dataset. To do this, the data subsampled from Table S1 (Supporting Information) were read by a custom perl script and the columns were shuffled, randomizing the data for each symbiosis. The categories described above were tallied for these permuted data, and recorded. This was then repeated for a total of 100 times for each subsample to determine how many times the empirical data's value was above/below the randomized data values, and thus the probability the value could have been obtained by chance.

Sensitivity analyses

I repeated each of the four analyses, 1) transmission mode rate estimation, 2) correlations between mode and environment type, 3) correlations between mode and transmission route and 4) correlations between symbiosis functions and transmission modes, on subsets of the data as described below.

Robustness to data availability
To maximize the amount of data and the number of associations included in the analysis, I included symbioses that had at least one form of evidence among either materials-based, species-based and population-based evidence. However, this could bias the results, if a lack of data drives the dominance of one mode or another. For example, there may be a skew towards vertical transmission when only host tissues have been examined. To account for this, I repeated the analyses two more times, once on the subsets of associations that had at least two levels of evidence, and again on the subset that had all three. Subsampling by evidence level reduced the dataset to 408 associations with a minimum of two levels and 124 with all three levels.

Robustness to unequal sampling or biodiversity distribution
The accumulated dataset is enriched in terrestrial associations, which could bias results. To control for this, I randomly subsampled the terrestrial symbioses to match the sample size of aquatic symbioses (n = 71) and calculated transmission mode rates. This was repeated 100 times for each of the 48 subsampled sets of symbiotic associations from the 113 independent groups.

Resulting datafiles were plotted in R. The scripts used to perform these analyses can be downloaded from https://github.com/shelbirussell/MixedModeAnalysis_Russell2018.

RESULTS

Compiling these data and binning by variables such as transmission route, environment type, symbiont function and host/symbiont taxon revealed that transmission modes are indeed non-randomly distributed (Figs 2–4). It should be noted that there is no way to test this question appropriately with the full dataset because the distribution of taxa is itself non-random, making phylogenetically independent contrasts impossible. For example, neither bivalves nor chemosynthetic ecosystems occur on land and insects make very limited ventures into aquatic environments of any sort (exceptions include: reed beetles (Kölsch and Pedersen 2010) and the seal louse (Boyd *et al.* 2016)). However, filtering the dataset to only include independent symbiosis evolutionary events controls for a small number of associations driving correlative patterns. Overall, these results suggest that transmission mode is correlated with environment type and transmission route through host tissues, and may be influenced by these factors.

Transmission mode rates

Tallying transmission mode counts over all 528 symbiotic associations shows that 21.2% (112/528) of symbioses considered here are strictly horizontally transmitted, 36.0% (190/528) have some degree of mixed transmission and 42.8% (226/528) are strictly vertically transmitted (Figs 2A–D and 3A, black line). While these numbers suggest that there is some overall preference for associations to adopt vertical transmission relative to horizontal

transmission, it should be pointed out that the sampling distribution is highly skewed towards terrestrial associations (457 terrestrial versus 71 aquatic; Fig. 2A and B). This may either be due to sampling bias or a true excess of terrestrial species relative to marine and freshwater species in terms of biodiversity (see similar distribution in Fig. 2B vs C), as there is more extant biodiversity on land than in the ocean (Grosberg, Vermeij and Wainwright 2012; Costello and Chaudhary 2017).

Subsampling the data down to sets of the 113 independent symbiosis evolutionary events that comprise the full dataset showed that transmission modes are actually fairly evenly distributed among independently evolved associations (Fig. 3A, pink lines). In these datasets, 32.1%+/−0.57% of symbionts are horizontally transmitted, 37.8%+/−1.4% exhibit mixed mode transmission and 31.1%+/−1.3% are vertically transmitted. These subsampled datasets were also less biased towards terrestrial associations than the full dataset. An average of 28.3% of subsampled associations were aquatic, compared to 13.4% in the total dataset, leaving an average of 71.7% associations, compared to 86.6%, in terrestrial environments.

Regarding how the quantity of data used to infer transmission mode affects these results, when I required associations be supported by at least two levels of evidence (individual, species or population-level), the values remained relatively consistent with the full dataset values (Figs 3 and 4, grey dot-dashed vs black solid lines). However, when all three levels were required, the estimates for mixed modes increased in almost all cases (Figs 3A–C, E and 4B–F, grey dashed lines), suggesting that the full dataset underestimates the rate of mixed modes.

Transmission modes are non-randomly distributed among environments

Comparing the distribution of transmission modes in symbioses from different environments revealed that vertical transmission is depleted from aquatic environments and enriched in terrestrial environments. As can be seen in Fig. 2A, the raw counts are highly skewed with very few vertically transmitted associations occurring in the marine environment (Fisher's Exact Test P = 5.559e-14). These patterns remained even after controlling for independent symbiosis events (Fig. 3B, blue lines, and 3C, green lines). Aquatic environments exhibit an average of 44.3%+/−0.89% horizontal, 43.1%+/−1.7% mixed mode and 12.5%+/−2.0% vertical transmission. This low rate of vertical transmission was significantly less than expected by chance (permutation test P ≤ 0.010). In terrestrial environments, symbioses exhibit 27.3%+/−0.87% horizontal, 35.7%+/−1.8% mixed mode and 37.0%+/−1.7% vertical transmission. In contrast to the aquatic dataset, vertical transmission is elevated in terrestrial associations (permutation test P ≤ 0.010).

When these data were randomly subsampled to only include equal numbers from each environment type, these trends persisted, indicating that the interactions between variables are large-scale impacts and are not driven by a small number of associations (Fig. S1, Supporting Information).

External modes of vertical transmission may predispose associations to mixed mode transmission

Comparing transmission routes for symbiont inheritance in mixed and vertical modes of transmission revealed a deficit of internal transmission routes in associations with mixed mode transmission relative to vertical transmission. In the full dataset
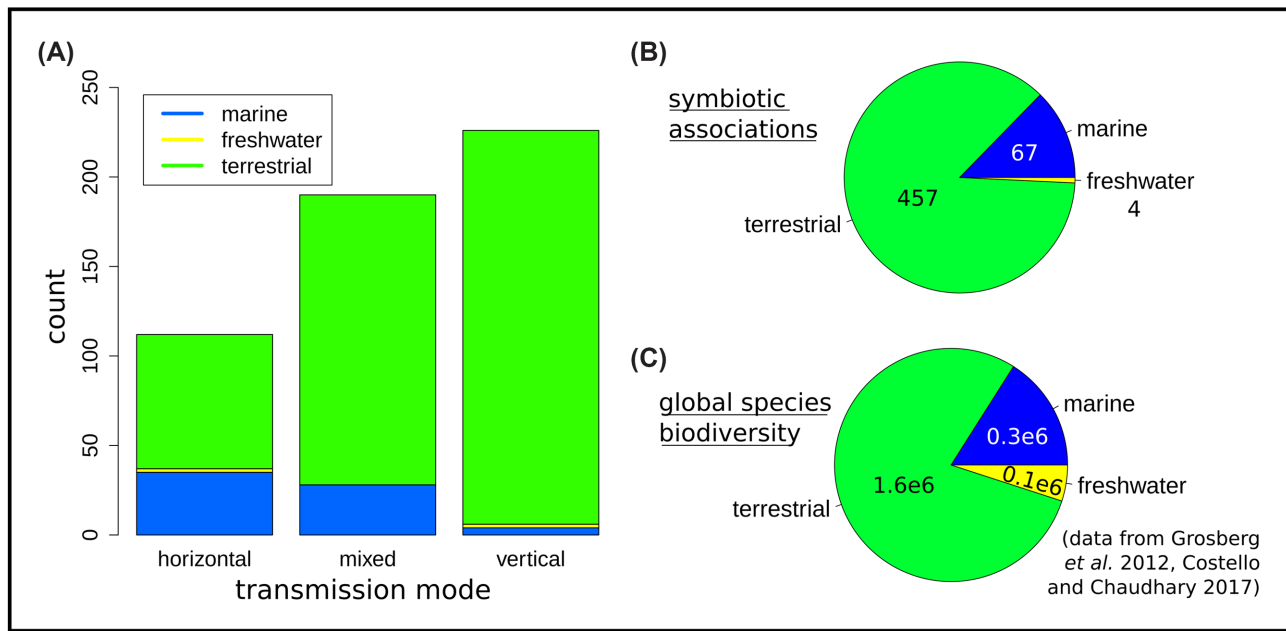
Figure 2. The distribution of symbiont transmission modes is similar to the distribution of biodiversity across marine, freshwater and terrestrial environment types. A) Barplot of associations binned by transmission mode and the environment the symbiont/host inhabit. B) Distribution of associations across environment types. C) Distribution of species across environments globally.

(Fig. 3D), significantly fewer mixed mode associations exhibit an internal route of vertical transmission to host gamete/offspring than strictly vertically transmitted associations (56.8% (108/190) and 80.5% (182/226), respectively; Fisher's Exact Test $P = 2.16e$-07). This trend was even stronger when the data were sub-sampled to phylogenetically independent sets of symbioses (Fig. 3E). Internal routes of transmission were exhibited in 46.9%+/−1.9% of associations with mixed mode transmission, less than expected by chance (permutation test $P \leq 0.012$). At 84.3%+/−1.9% of associations, more vertically transmitted symbioses exhibited internal routes of transmission than expected by chance (permutation test $P \leq 9.58e$-3).

Internal routes of transmission may be depleted in mixed transmission modes because they prevent horizontal transmission from occurring relative to external routes. As the aqueous environment may contribute to 'leaky' horizontal transmission events, I tested for a skew in the transmission route pattern of aquatic mixed mode and vertical associations relative to their terrestrial equivalents. Interestingly, similar numbers were obtained for both environment types: internal transmission routes occurred in 56.2%+/−1.6% of aquatic and 42.4%+/−2.6% of terrestrial mixed mode associations and in 84.0%+/−11.1% of aquatic and 84.6%+/−2.5% of terrestrial vertically transmitted associations.

## Transmission modes are non-randomly distributed among symbiosis functional types

Next, I binned symbioses by one of six functional types and evaluated the distribution of functional types among modes (Fig. 4A), revealing a highly non-random distribution (Pearson's Chi-squared test $P < 2.2e$-16). However, when symbioses were subsampled to sets of the 113 evolutionarily independent symbioses (Fig. 4B–F), this signal disappeared for all functional type-transmission mode combinations except horizontally transmitted nutritional associations, which are more abundant than

expected by chance (permutation P-value $\leq 4.38e$-3). Elevated horizontal transmission is likely driven predominantly by the chemosynthetic portion of the nutritional symbioses (47/315 total nutritional associations), as horizontal transmission is enriched in chemosynthetic associations. However, as all known chemosynthetic symbioses are marine (Cavanaugh et al. 2006), this association cannot be disentangled from that between horizontal transmission and the aquatic environments in general (Fig. 2A and E).

Although the transmission mode distributions were highly skewed in defensive, manipulative, and multifunction symbioses (Fig. 4C–E), the values were not significantly different from randomly permuted data (permuted P-values > 0.05). The insignificance of these results may be due to the small sample sizes of these datasets, as there were only 45 defensive, 23 manipulative and 30 multifunction symbioses in the full dataset of 528 associations. Furthermore, when subsampled to the 113 independent symbiotic events, these numbers reduced further. Only two lineages of manipulative bacterial symbionts, *Wolbachia* and *Cardininum*, remained among insect hosts. Compared to manipulative associations, those with multiple functions exhibited a larger diversity of symbionts, including *Serratia, Proftella, Sodalis, Xenorhabdus, Burkholderia* and some *Wolbachia* strains, as well as hosts, including homopterans, weevils and nematodes. Defensive symbioses were fairly well-distributed across bacterial taxa, spanning from gammaproteobacteria to actinobacteria. However, besides the bobtail squid and bryozoans, all other hosts of defensive symbionts were insects, which limited the number of independent comparisons.

Phylogenetic effect: transmission modes are non-randomly distributed among symbiont and host taxa

While the correlations between transmission mode and these biological and environmental variables are compelling, and it is
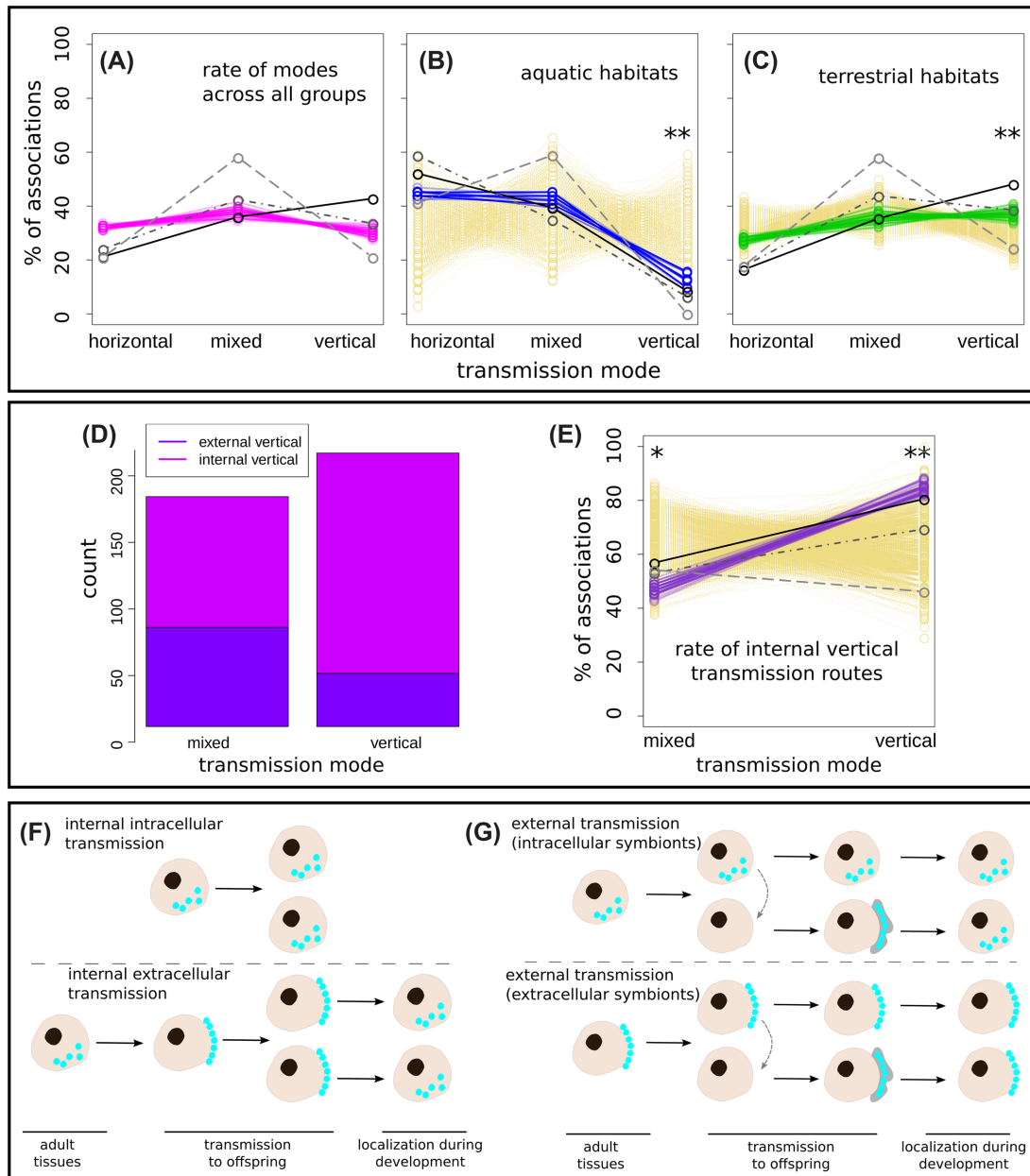
Figure 3. Environment type and transmission route are correlated with symbiont transmission mode in mutualistic/commensal symbiotic associations. A) Distribution of transmission modes across all symbiotic associations, B) aquatic associations and C) terrestrial associations. The 48 subsampled sets of the 113 phylogenetically independent symbiosis events are plotted in (A) pink, (B) blue and (C) green. The full dataset is plotted in black, with subsampled sets supported by ≥2 evidence levels plotted in grey dot-dashed lines and ≥3 evidence levels plotted in grey dashed lines. Random permutations are plotted in light yellow. The distribution of internal/external transmission routes between mixed and vertically transmitted associations are shown for D) all data and E) phylogenetically independent sets of symbioses. The lines in E are as described for A–C, except that the subsampled phylogenetically independent symbioses are plotted in purple. F,G) Illustrations of the general ways in which internal and external vertical transmission routes can occur. Permutation *P*-values: ∗*P* ≤ 0.05, ∗∗*P* ≤ 0.01.

clear that many phylogenetically independent symbioses contribute to these trends, it is possible that groups of host or symbiont taxa that covary with the environment type control transmission mode. Unfortunately this cannot be tested directly, as the naturally constructed phylogenetically-independent contrasts do not exist (*e.g.* chemosynthetic symbioses on land). However, the relative correlations between each of the components and the taxa can be examined and compared to qualitatively address this question.

Analysis of clustering among symbiosis traits and taxa revealed strong patterns of covariation, confirming that these

factors are not randomly distributed. As shown in Figs S2 and S3 (Supporting Information), both high and low taxonomic divisions of symbionts and hosts exhibited mutual-exclusivity and specificity for trait combinations (dendrograms on y-axes in Figs S2 and S3, Supporting Information). Importantly, while a few small groups of related symbionts cluster in groups according to shared traits (*e.g.* termite families), overall, related symbionts and hosts are fairly well dispersed across clusters. For example, defense, which ranges from the production of bioluminescent light in squid (Nyholm and McFall-Ngai 2004), to antibiotic production in beewolves (Kaltenpoth *et al.* 2010) and beetles
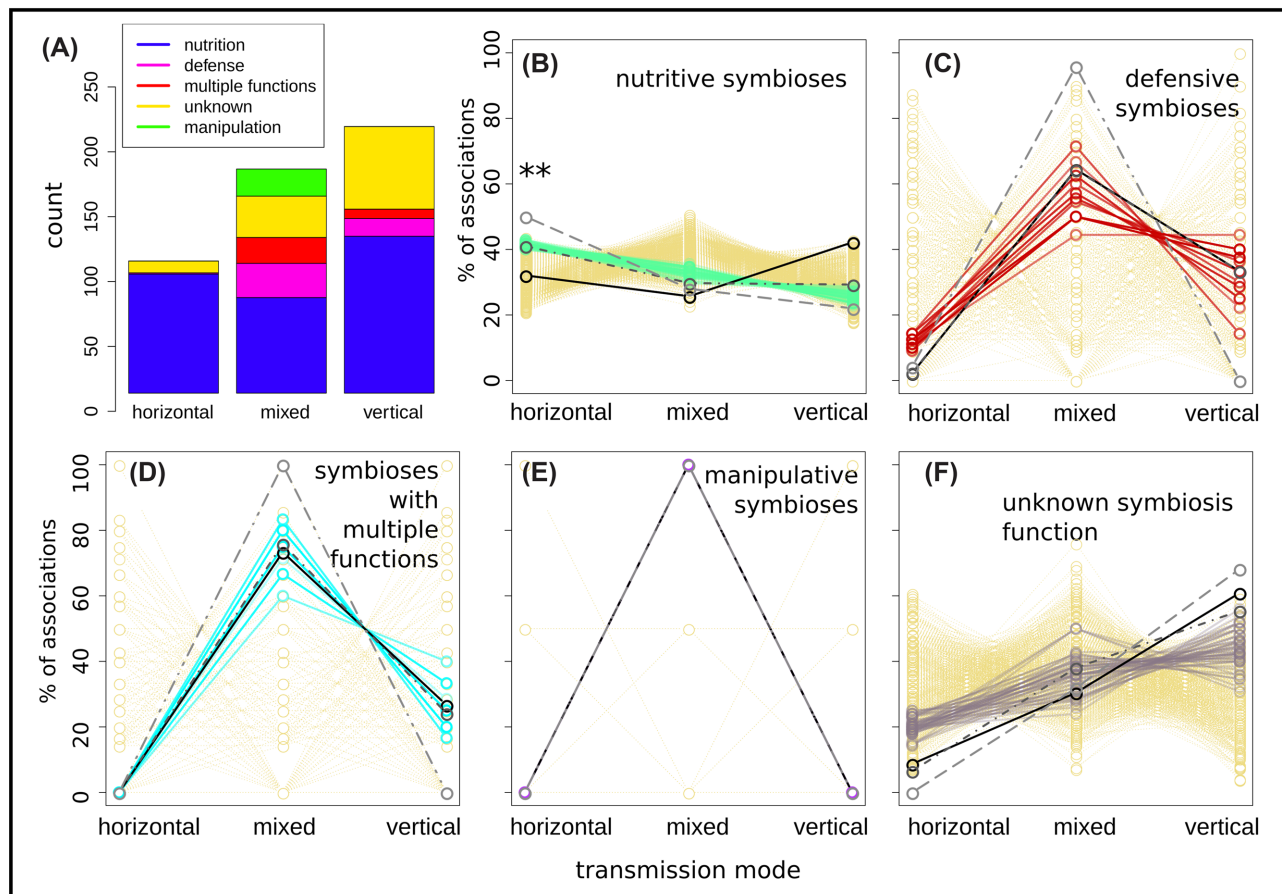
Figure 4. Symbiont transmission modes are non-randomly associated among symbiosis functional types. A) Barplot of all associations binned by transmission mode and functional type. B–F) Distribution of symbiont transmission modes in each functional type as indicated on each plot. The 48 subsampled sets of the 113 phylogenetically independent symbiosis events are plotted in B) green, C) red, D) light blue, E) dark purple and F) brown. The full dataset is plotted in black, with subsampled sets supported by ≥2 evidence levels plotted in grey dot-dashed lines and ≥3 evidence levels plotted in grey dashed lines. Random permutations are plotted in light yellow. Permutation P-value: ∗∗$P \leq 0.01$.

(Flórez *et al*. 2017), to cuticle hardness in beetles (Anbutsu *et al*. 2017), was broadly distributed across host and symbiont taxa. In total, these results suggest that correlated traits such as horizontal/mixed mode transmission and aquatic environments are likely not driven solely by the specific traits of particular host or symbiont clades.

DISCUSSION

Here, I show that the three symbiont transmission mode categories, horizontal, mixed and vertical are unequally distributed across environment types, transmission routes and some functional types, indicating that these factors either pose some constraint or pressure on transmission mode evolution. These results are robust, as the trends persist when the symbioses are controlled for phylogeny (Figs 3 and 4) and the excess terrestrial data, relative to aquatic data, are subsampled (Fig. S1, Supporting Information).

The most striking of these trends is the depletion of vertical transmission in aquatic environments (Fig. 3B) and the elevation of vertical transmission in terrestrial environments (Fig. 3C). This pattern may simply be an artifact of the medium in which these organisms live: water versus air (Normark and Ross 2014). With desiccation and osmolarity not a problem, symbionts can

travel through aqueous environments to new hosts if the opportunity arises and they are capable of surviving outside of the host cell. Not much fluid may be needed, as all nitrogen-fixing rhizobia root bacteria are horizontally transmitted through soil pore water (Postma and van Veen 1990). The marine environment to which most of the investigated aquatic symbioses belonged (67/71) likely further enables host-to-host transfer events via its salt and nutrient content (Rozen and Belkin 2001). Thus, vertical transmission in aquatic environments may almost always manifest as some degree of mixed mode transmission because horizontal transmission events are difficult to prevent.

Consistent with the idea that environmental opportunities for horizontal transmission drive the adoption of mixed mode transmission, I found that internal routes of vertical transmission between host tissues are depleted in mixed mode associations and elevated in vertically transmitted associations (Fig. 3D and E). As shown in Fig. 3F and G, external routes of symbiont transfer likely enable more contact with the external environment than internal routes, which could lead to opportunities for horizontal transmission between host individuals. In nature, external route strategies range from secretions applied to eggs (*e.g.* stink bugs (Hosokawa *et al*. 2013), beewolves (Kaltenpoth 2006), reed beetles (Kölsch and Pedersen 2010), earthworms (Paz, Schramm and Lund 2017) and chemosynthetic oligochaete worms (Giere 2006)), to elaborate symbiont-containing capsules

laid with eggs (*e.g.* plataspid stink bug species (Hosokawa *et al.* 2005)). Although symbionts are typically protected from the elements by some host-derived substance in these examples, this is likely not a deterrent to symbionts colonizing from other host individuals. Even obligate intracellular symbionts such as *Wolbachia* are able to survive for extended periods of time in cell-free media (Rasgon, Gamston and Ren 2006) and re-enter host cells (White *et al.* 2017), suggesting that contamination events are possible.

The influential role of environmental medium on transmission mode is highlighted by the terrestrial symbionts that have been shown to utilize plant fluids and parasitoids as strategies for transfer. Rickettsial symbionts of whiteflies can be transferred through the plant fluids the host feeds on (Li *et al.* 2017). Furthermore, plants may serve as reservoirs for symbionts, as some defensive symbionts have been found to also be plant pathogens (Flórez *et al.* 2017), including *Erwinia* and *Pantoea* symbionts of stink bugs (Frago, Dicke and Godfray 2012). Transfer between hosts can also be facilitated via infecting parasitoids, as has been reported for *Wolbachia* (Vavre *et al.* 1999). This capability is likely due to *Wolbachia's* aptitude for surviving in a diversity of arthropod hosts. While some symbionts can be transferred through these strategies, it may be a very rare process and is highly dependent on the symbiont taxon. For example, it has been shown that different mealybug species with different nested beta/gammaproteobacterial symbionts feeding on the same plant were found to maintain their native symbiont types (Koga *et al.* 2013), despite the gammaproteobacterial symbiont showing evolutionary evidence of host-switching (Gatehouse *et al.* 2012). Thus, it is likely that both the environmental medium and the host/symbiont taxon synergize to determine the specific transmission mode for the symbiosis.

The marine environment appears to represent the most-limiting situation for vertical transmission, as no associations with robust evidence (i.e. two or three evidence levels) have been found to be strictly vertically transmitted (Stewart, Young and Cavanaugh 2008, 2009; Decker *et al.* 2013; Altamia *et al.* 2014; Sipkema *et al.* 2015; Miller *et al.* 2016; Russell and Cavanaugh 2017; Russell, Corbett-Detig and Cavanaugh 2017). This pattern is not explained by the symbiont taxa present, as a diversity of symbionts found in marine environments, from Gammaproteobacteria to Betaproteobacteria, have been shown to be strictly vertically transmitted on land (see Table S1, Supporting Information). Furthermore, an internal symbiont transmission route is not a complete barrier to mixed transmission in marine environments, as vertical transmission in vesicomyid and solemyid bivalves and sponges occurs through the female germline (Ikuta *et al.* 2016; Russell, McCartney and Cavanaugh 2018) or brooded embryos (Schmitt *et al.* 2007), respectively. It should be noted, that the chemosynthetic flatworm *Paracatenula* may be strictly vertically transmitted, as it exhibits vertical transmission during asexual reproduction and shows patterns of host-symbiont co-speciation (Dirks *et al.* 2012), however, population-level data is needed to confirm this.

The non-random distribution of symbiont transmission modes among environment types, transmission routes and symbiosis functions has important implications for host and symbiont evolution. In regards to symbiont-specific impacts, the depletion of vertical transmission in aquatic environments and abundance of it in terrestrial environments likely produces an excess of reduced symbiont genomes on land relative to the ocean. While this is certainly anecdotally true (Moran and Bennett 2014; Russell, Corbett-Detig and Cavanaugh 2017), it was not tested here because genomes were not available for all

of the included symbioses, but should be investigated in the future. How the rate of horizontal transmission impacts symbiont genome evolution remains one of the most important unanswered questions in symbiosis biology.

CONCLUSIONS

The data compiled in this study on bacterial symbiont transmission modes shows a strikingly non-random distribution between terrestrial and aquatic environments, transmission routes and functions. From the analysis presented above, it is reasonable to conclude that unless restricted by a host/symbiont trait or an inhospitable external environment, bacterial symbionts tend to mix between hosts, which results in relatively high rates of mixed mode transmission across taxa (38%). However, rates are likely even higher, as a fair amount of data is needed to detect mixed modes (Fig. 3A), so it often goes undetected. Regardless of the cause, there is clearly a bias against strict vertical transmission in aquatic environments and for this mode on land. Given that host restriction significantly reduces symbiont population size and drives genome degradation in strictly vertically transmitted associations, bouts of horizontal transmission may serve to increase the effective population size and mitigate some of the deleterious consequences of such obligate host-association, altering the evolutionary trajectories of symbioses in these different environments.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSLE* online.

Conflicts of interest

None declared.

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