Interhost dispersal alters microbiome assembly and can overwhelm host innate immunity in an experimental zebrafish model

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Edited by Jeffrey I. Gordon, Washington University School of Medicine in St. Louis, St. Louis, MO, and approved September 7, 2017 (received for review February 15, 2017)

The diverse collections of microorganisms associated with humans and other animals, collectively referred to as their "microbiome," are critical for host health, but the mechanisms that govern their assembly are poorly understood. This has made it difficult to identify consistent host factors that explain variation in microbiomes across hosts, despite large-scale sampling efforts. While ecological theory predicts that the movement, or dispersal, of individuals can have profound and predictable consequences on community assembly, its role in the assembly of animal-associated microbiomes remains underexplored. Here, we show that dispersal of microorganisms among hosts can contribute substantially to microbiome variation, and is able to overwhelm the effects of individual host factors, in an experimental test of ecological theory. We manipulated dispersal among wild-type and immune-deficient *myd88* knockout zebrafish and observed that interhost dispersal had a large effect on the diversity and composition of intestinal microbiomes. Interhost dispersal was strong enough to overwhelm the effects of host factors, largely eliminating differences between wild-type and immune-deficient hosts, regardless of whether dispersal occurred within or between genotypes, suggesting dispersal can independently alter the ecology of microbiomes. Our observations are consistent with a predictive model that assumes metacommunity dynamics and are likely mediated by dispersal-related microbial traits. These results illustrate the importance of microbial dispersal to animal microbiomes and motivate its integration into the study of host–microbe systems.

microbiome | dispersal | metacommunity | zebrafish | innate immunity

The communities of microorganisms associated with animals, referred to as the "microbiome," are highly diverse and have the potential to strongly influence host health. Understanding how microbiomes contribute to host physiology, and how to manipulate this relationship to promote host health, requires a comprehensive understanding of the mechanistic drivers of microbiome variation across hosts. Unfortunately, it has been difficult to identify consistent host factors that can explain the large amounts of the variation in microbiome composition across individual hosts, despite large-scale sampling efforts (1). At best, only a small fraction of variation across hosts can be explained by individual host factors, leading to the perception that the rules governing microbiome assembly are idiosyncratic. However, unlike many other attributes of an animal's biology that impact its health and fitness, an animal's microbiome is subject to dispersal of microorganisms from other hosts. If the influence of microbial dispersal among hosts is substantial, then a comprehensive model of microbiome dynamics must include consideration of not just the factors associated with individual hosts but also the population of hosts with which they exchange microbiome members.

Dispersal is increasingly recognized as an important determinant of the structure and function of both experimentally assembled (2, 3) and naturally occurring bacterial communities (4, 5), and there is mounting evidence that dispersal is also important to the assembly of nonpathogenic, animal microbiomes. Biogeographic patterns have been observed for microbiomes associated with natural populations of animals (6–8), consistent with predicted effects of dispersal. Social interactions among hosts, a possible facilitator of microbial dispersal, have been shown to correlate with the composition of animal microbiomes, with hosts tending to share more members of their microbiome with the microbiomes of individuals with whom they interact frequently (9–11). Dispersal has also been hypothesized to explain differences in the microbiomes of humans in economically developed and developing regions (12). Studies of laboratory animals often report that the microbiomes of animals housed together are more similar than those in different housing units. These so called "cage effects" routinely explain significant amounts of microbiome variation, as well as variation in phenotypes known or suspected to be mediated by the microbiome (13–15). Interestingly, experiments studying the innate immune system have often shown that cohousing of healthy and immune-deficient animals can transfer phenotypes associated with immune pathway mutants, including increased inflammation and colitis (16, 17).

Significance

Manipulating the microbial communities associated with animals to improve host health requires a comprehensive understanding of the mechanisms driving microbiome variation, which a strict focus on host-specific factors has been insufficient in providing. We performed an experiment to test whether the movement, or dispersal, of microorganisms among zebrafish hosts could alter the effects of important host factors, using a dispersal-based model to guide the interpretation of results. We observed that interhost dispersal can alter the diversity and composition of microbial communities and overwhelm the effects of the host's innate immune system. These findings suggest that dispersal is an important mechanism driving microbiome variation and should be considered in future microbiome research.

Author contributions: A.R.B., K.G., and B.J.M.B. designed research; A.R.B. and M.A. performed research; E.M., A.S.R., K.M.-M., and S.S. contributed new reagents/analytic tools; A.R.B., E.M., and A.S.R. analyzed data; and A.R.B., K.G., and B.J.M.B. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The data reported in this paper have been deposited in the National Center for Biotechnology Information BioProject database, https://www.ncbi.nlm.nih.gov/bioproject/ (accession no. PRJNA378677).

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1702511114/-/DCSupplemental.

Similar investigations of the link between innate immunity and microbiomes have led to conflicting or inconclusive results, with some finding little to no effect of innate immune pathways on microbiome composition or diversity, especially in cases where both wild-type (WT) and immune-deficient animals were housed together or from the same litter (18–20). These examples are particularly interesting given the role the immune system plays in direct interactions between animals and their resident microorganisms, suggesting dispersal of nonpathogenic microorganisms may have important consequences to animal hosts.

Research on host-associated microbiomes has increasingly utilized frameworks from general ecological theory to guide experiments and interpret patterns such as those described above. Metacommunity theory in particular focuses on dispersal among multiple discrete "local" communities (21) and is thus potentially well suited to describing host–microbe systems, where hosts act as environments that are home to local communities of microorganisms linked by interhost dispersal (22, 23). Dispersal, as well as metacommunity theory specifically, has been invoked to explain many patterns in microbiome diversity and composition (e.g., ref. 12). While the results of these studies are often consistent with the predicted effects of dispersal, these studies are not designed to directly test the importance of interhost dispersal on the assembly of host-associated microbiomes and often struggle to disentangle the effects of dispersal from other confounding factors. Furthermore, they provide limited insight into the mechanisms by which dispersal processes result in such patterns.

Here, we describe an experiment, guided by a quantitative predictive framework, that explicitly manipulates interhost dispersal to test its role in microbiome assembly and gain insights into the underlying mechanistic processes. Specifically, we tested whether dispersal of microorganisms among hosts can influence or overwhelm the effects of individual host factors, namely the innate immune system, on the composition and diversity of zebrafish (*Danio rerio*) intestinal microbiomes. The relatively simple husbandry and large clutch sizes of zebrafish allowed us to manipulate the transmission of microorganisms among a large number of replicate individuals through cohousing and isolation at a scale not feasible in humans or other vertebrate models, while their genetic malleability made it possible to focus on the effects of host innate immunity through the generation of immune-deficient mutants. To generate specific predictions and to guide the interpretation of our results, we created a computational model assuming metacommunity dynamics across hosts. We observed that the effects of dispersal among zebrafish on microbiome composition and diversity are largely consistent with our model and can overwhelm the effects of host innate-immune activity.

Results

We generated an immune-deficient *myd88*$^{-}$ mutant zebrafish line and raised homozygous mutant with WT zebrafish under three housing conditions designed to either allow or restrict dispersal among hosts: "solitary" conditions in which each individual zebrafish was in isolation with no exposure to other individuals (i.e., no interhost dispersal), or cohoused, either with members of the same genotype only ("separated") or with members of both genotypes ("mixed"; Fig. 1). The myeloid differentiation primary response gene 88 (MyD88) encodes a universal adapter protein in the Toll-like receptor (TLR) pathway and is responsible for activating several immune responses in response to signaling from the microbiota, including the production of proinflammatory cytokines and antimicrobial peptides and the detoxification of the bacterial product lipopolysaccharide (24–26). The germline mutation in MyD88 was generated using CRISPR/Cas9, and the resulting *myd88*$^{-}$ mutant was confirmed to have the expected phenotype of low neutrophil abundance in the intestines of conventionally reared larvae, with abundances similar to those of WT larvae raised germ-free, as previously described for zebrafish injected with a MyD88 morpholino (24) (*Generation and Verification of a myd88 Mutant Zebrafish* and Fig. S1). To isolate the effects of innate vs. adaptive immunity, we raised fish to 21 d postfertilization (dpf), as adaptive immunity does not become active in zebrafish until ~28 dpf (27). At 21 dpf, we killed the fish and characterized the microbial communities associated with their intestines and those associated with their food and flask water by 16S amplicon sequencing. As one might expect given the importance of host immunity to defense against pathogens, *myd88*$^{-}$ fish had higher mortality rates and, notably, their mortality rates were higher in the cohoused treatments compared with the solitarily treatment (Fig. S2). Interestingly, mortality rates for WT fish were also higher when cohoused, especially when cohoused with *myd88*$^{-}$ fish. Because of this, by the end of the experiment, the number of fish in each flask were no longer equal. However, we did not observe a significant effect of the ultimate number of fish per flask on microbiome composition within treatments [permutational multivariate ANOVA (PerMANOVA): $P > 0.05$].

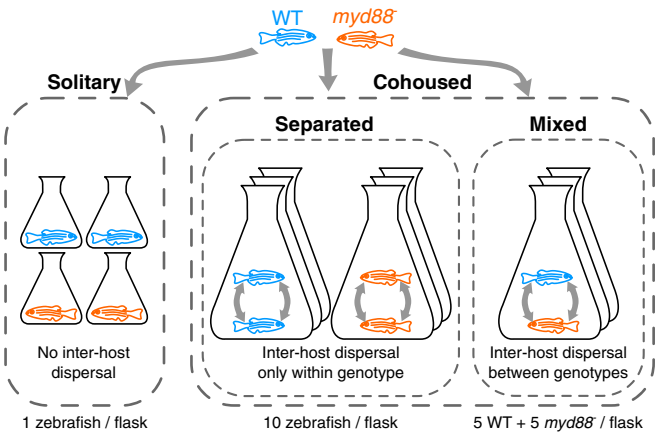

Fig. 1. Experimental design. WT and *myd88*$^{-}$ zebrafish were raised in one of three housing conditions to manipulate the degree of interhost dispersal: housed alone with no interhost dispersal (solitary), cohoused with only individuals of the same genotype (separated), or cohoused with individuals of both genotypes (mixed).

To determine whether the observed effects of housing treatments on microbiome diversity and composition were consistent with interhost dispersal being the primary driving mechanism, we compared our experimental results with predictions from a computational model assuming metacommunity dynamics (Table 1). In this model, individual hosts are home to local communities of microorganisms that are connected by dispersal to form a metacommunity comprised of all of the hosts within a population/flask (Fig. S3). Additional details regarding model construction and generation of predictions is available in *Computational Metacommunity Model*.

Interhost Dispersal Overwhelms Host Factors. Overall, there was a significant difference in the composition of microbiomes associated with WT and *myd88*$^{-}$ zebrafish, but the effect of host genotype was weaker than the effect of housing treatment across the entire dataset (Fig. 2*A* and Table 2). We predicted that the effect of host factors such as innate immunity would depend on the degree of interhost dispersal, due to the homogenizing effects of exchanging microbial taxa among host types (Table 1). In agreement with our predictions, we observed a strong interaction between housing and genotype. Specifically, there was a much greater difference in microbiome composition between genotypes when hosts were raised in solitary compared with cohoused treatments, either within or across genotypes (Table 2).

Table 1. Predictions from a metacommunity model of the effects of interhost dispersal treatments on the diversity and composition of host-associated microbiomes

Microbiome structure	Solitary	Separated	Mixed
Correlation with host environment	Strong	Strong between/weak within genotypes	Weak
α-Diversity (within host)	Low	Intermediate	High
β-Diversity (interhost)	High	Intermediate	Low
γ-Diversity (across host)	Low	Intermediate	High
Abundance of dispersal specialist	Low	High	High

To further investigate whether the effects of host factors were being weakened by dispersal at a finer, individual host level, we measured the relationship between attributes of each host and the composition of their microbiome within each housing treatment. We hypothesized that dispersal among heterogeneous hosts would dilute the effects of local host factors, and therefore the relationship would be strongest for solitary hosts and weakest for separated and mixed cohoused hosts. We first measured the standard length of each zebrafish (Fig. S4*A*), which is known to be an overall indicator of fish development and health (28), and which we had previously shown was a strong predictor of intestinal microbiomes across zebrafish development (29). We also characterized the level of innate immune activity of each host by measuring the transcriptional levels of two immune genes: one, *c3*, in the MyD88-independent complement pathway, and another, *il-1β*, in the MyD88-dependent pathway. As expected given their genotype, we found expression of *il-1β* to be lower in *myd88*$^{-}$ compared with WT hosts, while expression of *c3* was similar between the two genotypes (ANOVA on effects of genotype: F statistic = 13.9, $P < 0.001$ for *il-1β* and F statistic = 0.11, $P = 0.74$ for *c3*; Fig. S4 *B* and *C*). Despite having a strong effect on the microbiome diversity and composition, housing conditions had no clear effect on host innate immune response (ANOVA on effects of housing: $P > 0.05$ for both *il-1β* and *c3*). This reaffirmed our assumption that interhost dispersal primarily altered the degree of filtering of the microbiome by the local host environment rather than changing the host environment itself. To test this, we performed a redundancy analysis to determine the unique and shared contribution of each host factor to explaining the variance in community composition. Consistent with our hypothesis, a greater amount of variance in microbiome composition was explained by host factors in solitary than in separated or mixed hosts (Fig. 2*B*).

Interhost Dispersal Increases Diversity. The strong independent effect of housing treatment on microbiome composition suggests dispersal fundamentally alters the structure of host-associated microbiomes. Our model predictions of the effects of dispersal on diversity were qualitatively in agreement with the predictions of general ecological theory (30, 31): dispersal among hosts increases α-diversity (i.e., within-host diversity) through the maintenance of taxa in hosts where they would otherwise go extinct, decreases β-diversity (i.e., variation among hosts) through the homogenizing effects of sharing individuals, and increases γ-diversity (i.e., across-host diversity) by allowing dispersal-specialized taxa to evade competitive exclusion at the metacommunity scale (Table 1).

Our experimental results were largely consistent with these theoretical predictions. Both within-host α-diversity (Fig. 3*A*; ANOVA on effects of housing: F statistic = 36.6, $P < 0.001$) was lower in the solitary treatment, relative to mixed and separated, with the mixed and separated treatments indistinguishable statistically. Variation among hosts, or β-diversity, was significantly greater in solitary hosts than mixed hosts, with separated hosts overall more similar to solitary than mixed hosts (Fig. 3*B*; ANOVA on effects of housing: F statistic = 26.5, $P < 0.001$). Across-host γ-diversity (Fig. 3*C*) was lower in the solitary treatment, relative to mixed and separated. While housing treatment had a large impact on microbiome diversity at multiple scales, there was no detectable difference between genotypes, with the exception of β-diversity within separated hosts (Fig. 3; ANOVA: $P > 0.05$). The most notable deviation from our predicted effects of dispersal on diversity was the effect of the separated housing treatment, which we anticipated would show a response intermediate to the solitary and mixed treatments. In contrast, we observed no difference in α- and γ-diversity between the separated and mixed cohousing treatments. The simplest explanation for this discrepancy is that innate immunity has a smaller effect on diversity than expected relative to the independent effects of dispersal.

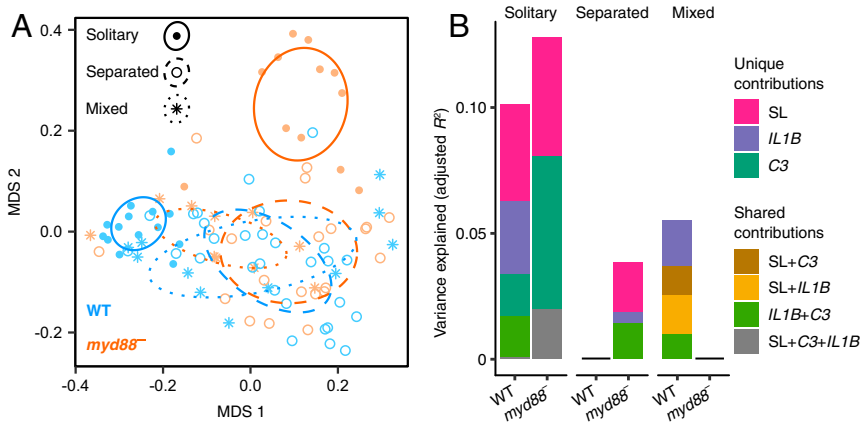

Fig. 2. Relationship between host factors and microbiome composition. (*A*) A nonmetric multidimensional scaling ordination of Canberra distances among individual microbiomes. Ellipses represent 95% CIs around the group centroid. (*B*) Variance in microbiome composition explained by individual host factors following a redundancy analysis. Shown are the adjusted R^2 values for the unique and shared contribution of multiple host factors: *il-1β* expression (*IL1B*), *c3* expression (*C3*), and standard length (SL). Negative adjusted R^2 values are treated and displayed as zeroes (considered as null).

Table 2. PerMANOVA analysis on the effects of genotype and housing on microbiome composition

Factor	MS	F	R^2	P*
Across-housing treatments				
Genotype	0.007	7.2	0.05	<0.001
Housing	0.009	9.6	0.14	<0.001
Genotype × Housing	0.008	8.6	0.13	<0.001
Within-housing treatments				
Genotype–solitary	0.067	34.1	0.58	<0.001
Genotype–separated	0.014	3.4	0.15	0.010
Genotype–mixed	0.004	2.8	0.05	0.001

*P values calculated from a distribution of 1,000 random permutations.

Interhost Dispersal Promotes Dispersal-Related Traits. We unexpectedly observed that cohousing had a similar effect on microbiome composition and diversity regardless of whether hosts were cohoused with only members of the same genotype (separated) or with members of both genotypes (mixed; Fig. 2*A*; PerMANOVA: $P > 0.05$ for both WT and *myd88*$^-$ comparisons). If dispersal acted only to homogenize microbiomes through the simple exchange of microorganisms among hosts, then we would expect that the two genotypes would maintain their distinctiveness in the separated treatment (since dispersal between genotypes is not possible). This suggested that dispersal among hosts altered the nature of selection for microbiome members, potentially by increasing the success of dispersal-adapted microorganisms. In further support of this hypothesis, both separated and mixed microbiomes were overall more diverse than solitary microbiomes (Fig. 3*A*) and were not limited to being a simple mix of taxa from solitary microbiomes. While the majority of taxa that occurred in solitary microbiomes were also detected in their cohoused counterparts (86% and 88% for solitary WT and *myd88*$^-$ hosts, respectively), a much smaller proportion of taxa that occurred in cohoused microbiomes were also detected in their solitary counterparts (59% and 64% for cohoused WT and *myd88*$^-$ hosts, respectively). Our computational model also predicted a similar increase in diversity in cohoused hosts. In particular, the model predicted that interhost dispersal would favor the persistence of species with greater dispersal rates (Fig. S5*A*), especially when a trade-off was imposed between dispersal rates and host specificity, such that species with high dispersal rates would be found in a wider range of host types, thereby homogenizing communities (Fig. S5*B*). This suggests that interhost dispersal allows for the success of taxa in the metacommunity as a whole that would not otherwise persist.

If it is indeed the case that trade-offs in the life history strategies of microbial taxa underlie the effects of dispersal in this system, then we should see this reflected in the traits of microbial taxa associated with cohoused and solitary microbiomes. To test this prediction, we asked whether taxa associated with cohoused microbiomes were enriched for traits related to dispersal and colonization ability compared with taxa associated with solitary microbiomes. The taxa most differentially abundant in cohoused compared with solitary microbiomes primarily belonged to the genera *Vibrio* ($\log_2$-fold change = 1.1, $P < 0.0001$) and *Shewanella* ($\log_2$-fold change = 0.62, $P < 0.01$). To infer the traits of individual operational taxonomic units (OTUs) in our study, we used the ancestral state reconstructions implemented in PICRUSt to estimate the gene content of our observed OTUs by matching them to a reference database (32). We then asked which gene pathways were predicted to be enriched in those taxa that were differentially abundant in cohoused microbiomes compared with those associated with solitary microbiomes. Notably, the most strongly enriched pathways in cohoused associated taxa included ones related to bacterial motility (such as flagellar assembly and bacterial chemotaxis), quorum sensing (which is often used to regulate biofilm formation and virulence), two-component regulatory systems (which facilitate responses to changes in the environment), and *Vibrio* pathogenicity (Table 3). Although these traits are merely predictions based on similarity to a known reference database rather than direct measurements, they independently support our modeling predictions that dispersal among hosts selects for taxa with life history strategies favoring dispersal and repeated colonization of multiple host types. In the case of the *Vibrio* pathogenicity-related pathways, they may also explain the observed decreased survivorship of cohoused zebrafish of both genotypes (Fig. S2).

Discussion

Dispersal among hosts had a substantial impact on the diversity and composition of zebrafish gut microbiomes in our study and was sufficient to overwhelm the effects of host-specific factors such as innate immune activity. Notably, the strong effects of dispersal in the experiment were specifically the result of "interhost" dispersal, as the overall migration of microorganisms from all other sources, such as from the microbial communities found in the flask water or food, was not directly altered. Furthermore, we only manipulated the potential for dispersal to occur by exposing hosts to one another, while the actual movement of microorganisms among hosts occurred naturally without direct

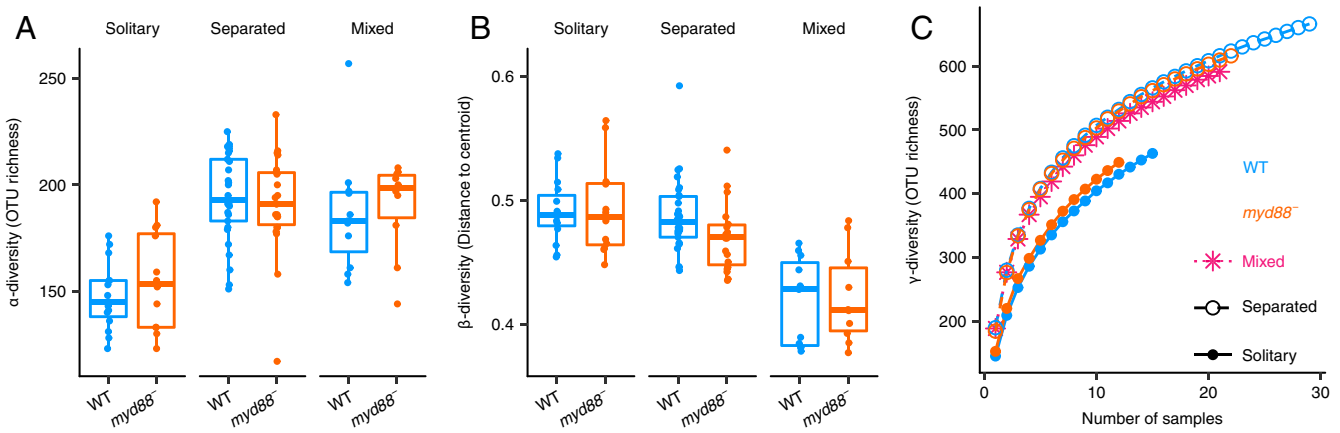

Fig. 3. Effects of host genotype and housing conditions on the composition and diversity of intestinal microbiomes. (*A*) Within-host α-diversity, measured by the Shannon diversity index. (*B*) Interhost β-diversity, measured by the Canberra distance from the group centroid (β-dispersion). (*C*) Across-host γ-diversity, measured by comparing the average gain in total OTU richness with increased sampling within each metacommunity treatment.

Table 3. The top KEGG orthology pathways predicted to be enriched in taxa that are differentially abundant in cohoused microbiomes

KEGG orthology pathway	χ^2 statistic	Adjusted *P* value
Vibrio cholerae pathogenic cycle	263	1.29×10^{-56}
Bacterial chemotaxis	131	7.27×10^{-28}
Quorum sensing	130	1.06×10^{-27}
Two-component system	130	1.14×10^{-27}
Phosphotransferase system (PTS)	100	4.93×10^{-21}
Flagellar assembly	79	2.14×10^{-16}
Vibrio cholerae infection	70	1.92×10^{-14}
Aminobenzoate degradation	70	2.42×10^{-14}
Phenylalanine metabolism	49	9.19×10^{-10}
Benzoate degradation	47	2.32×10^{-9}

manipulation (such as by gavage, injection, forced feeding, etc.). As a consequence, we do not know the actual rates of dispersal that occurred in this experiment nor how they compare with those found in natural populations of fish or other animals. It is probable that both the strength and nature of the effects of interhost dispersal differ with different rates of dispersal, and deeper investigations of this relationship will help reveal the specific circumstances under which we expect interhost dispersal to be more or less important. It is worth noting, however, that given that the rates of dispersal in this experiment were allowed to occur passively, they are unlikely to be overly unrealistic, and it is therefore reasonable to expect similar effects in many natural systems.

In addition to demonstrating the overall effects of dispersal on microbiome composition and diversity, this experiment has generated unexpected insight into the specific mechanisms by which interhost dispersal influences microbiomes. Contrary to our initial predictions, we observed a homogenization of host microbiomes regardless of whether dispersal was limited to within host genotypes (separated) or allowed to occur between host genotypes (mixed). This homogenization likely occurs because the existence of dispersal among hosts alters the viable trait space for host-associated microorganisms and selects for life history strategies favoring motility and transmission. This interpretation is supported by our genomic predictions that dispersal-related genetic pathways were enriched in the microbiomes of cohoused compared with solitary hosts, and by the ability to reproduce our results in our computational model by imposing a trade-off between dispersal ability and host type specificity. Similar phenomena are predicted in metacommunity models incorporating colonization–competition trade-offs (33) and have been investigated in communities of pathogens (34). This behavior in commensal microbiomes suggests that the consequences of interhost dispersal are likely more complex than simply the homogenization of microbiomes through the sharing of microbial taxa.

Combining experimental studies such as this with surveys of natural systems will undoubtedly help inform both how generalizable experimental results are as well as strengthen inferences about the importance of dispersal based on observed patterns. The importance of dispersal in natural populations of hosts has often been inferred by observed increases in within-host α-diversity and decreases in interhost β-diversity (e.g., ref. 12), or by correlations between microbiome similarity and proxy measurements for dispersal, such as frequency of social interactions (e.g., ref. 10) or geographic distance (e.g., ref. 8). These inferences are frequently based on general predictions from conceptual models of community assembly or even simple intuition. By experimentally testing predictions from a mechanistic model, our study creates a stronger link between such patterns and interhost dispersal as the driving mechanism, justifying such inferences. It also provides further evidence that theories and models developed in other ecosystems may be applied to better understand the assembly and dynamics of host–microbe systems. In particular, we have demonstrated that metacommunity theory provides an appropriate framework for the study of host-associated microbial communities, as has previously been suggested (22, 23). Utilizing and combining these tools will help provide better understanding of how individual microbiomes are influenced by processes occurring at the scale of populations and communities of multiple hosts. Such a holistic understanding will improve our ability to both manipulate microbiomes and predict their responses to changes in host behavior and ecology.

Methods

Zebrafish Husbandry. All zebrafish experiments were performed using protocols approved by the University of Oregon Institutional Care and Use Committee and followed standard protocols. We generated an immunocompromised *myd88* zebrafish mutant using the CRISPR-Cas9 system (35) (*Generation and Verification of a myd88 Mutant Zebrafish*). WT AB/Tübingen fish with a fully functional immune system and isogenic mutant *myd88*$^{-}$ zebrafish were then raised in glass Erlenmeyer flasks, such that microorganisms could disperse among hosts in the same flasks, but not between hosts in different flasks. Because there was no practical or noninvasive method to reliably distinguish *myd88*$^{-}$ from WT zebrafish embryos, the embryos for each genotype were generated from two crosses of homozygous parents. To eliminate potential maternity effects, standard gnotobiotic zebrafish protocols were used to make the embryos "germ-free" (free of microorganisms) before being exposed to a shared inoculum at the beginning of the experiment.

Beginning as germ-free embryos, zebrafish were raised in flasks alone (solitary), or cohoused with 10 total zebrafish of the same genotype (separated), or 5 of each genotype (mixed), per flask (Fig. 1). Initially, 20 fish of each genotype were raised alone in solitary flasks, 40 fish of each genotypes were raised across four replicate separated flasks, and 20 fish of each genotype were raised across four replicate mixed flasks, resulting in a total of 160 zebrafish at the beginning of the study. The volume of embryo media and size of flask was scaled to the number of fish, that is, 50 mL of embryo media in a 125-mL Erlenmeyer flask for solitary conditions and 500 mL of embryo media in a 1-L Erlenmeyer flask for cohoused conditions. Doing so allowed the density of fish to be equivalent across housing conditions. Every day, ∼75–90% of the embryo media in each flask was removed and replaced with fresh, but not sterilized, embryo media. During this time, the majority of food debris and zebrafish feces, as well as any dead fish carcasses, were removed as well. Once the zebrafish fully hatched from their chorions (by 4 dpf), fish were fed live rotifers to a concentration of 20 individuals per mL, followed by the addition of live brine shrimp beginning at 10 dpf once per day.

Sampling and DNA/RNA Extractions. At 21 dpf, the juvenile zebrafish were killed and dissected to sample their intestinal microbiomes by 16S rRNA gene sequencing, as well as to characterize their innate immune response by qPCR of two genes encoding innate immune cytokines: *il-1β* and *c3*. Each individual intestine was aseptically removed and placed in a sterile 2-mL screw cap tube with 200 mL of nuclease-free water while the remainder of the zebrafish carcass was placed in a 2-mL screw cap tube with 1 mL of TRIzol (Life Technologies). Both sample types were then immediately frozen in liquid nitrogen and stored at −80 °C until DNA/RNA extractions were performed. To identify the genotype of mixed cohoused zebrafish, mixed cohoused samples were genotyped by PCR of the MyD88 gene using forward primer, 5′-GTAACGCGGAGATATACAACAAC-3′, and reverse primer, 5′-GAAGCGAACAAAGAAAAGCAA-3′.

DNA was extracted from intestinal samples using the MoBio PowerMicrobiome RNA Isolation kit (product number 26000-50) with the addition of β-mercaptoethanol (product number M3148-25ML; Sigma) using the manufacturer's suggestions. RNA was extracted from the remaining zebrafish carcasses using a standard laboratory TRIzol extraction protocol (29).

cDNA Conversion and qPCR of Host Innate Immune Genes. Extracted zebrafish RNA was converted into cDNA using the SuperScript IV reverse transcriptase kit (Invitrogen) following the manufacturer's instructions. qPCR assays were performed in 20-μL reactions with 20 ng of cDNA, and 400 nM gene-specific or control primers. Gene-specific primers were ordered from Eurofins Genomics with the following sequences: IL-1B forward primer, 5′-CATCAAACCCCAATCCACAG-3′, and reverse primer, 5′-CACCACGTTCACTTCACGCT-3′; C3 forward primer, 5′-CGGACGCTGACATCTACCAA-3′, and reverse primer, 5′-TCCAGGTCTGCTCTCCCAAG-3′. Primers for the housekeeping genes SDHA and EIF-1B (used to normalize the results) were ordered from PrimerDesign. All reactions were performed in triplicate using a Bio-Rad CFX96 Real-Time PCR (qPCR) Thermocycler. Starting concentrations of transcripts

were estimated from the resulting amplification curves using the LinRegPCR software (36). Technical replicates were then averaged and divided by the geometric mean of housekeeping genes SDHA and EIF-1B to normalize results.

16S rRNA Gene Sequencing and Processing. We characterized the microbiomes of individual samples via sequencing of the V4 region of the 16S rRNA gene using 515F and 806R primer sequences (37). We used a single-step PCR to add dual indices and adapter sequences to the V4 region of the bacterial 16S rRNA gene and generate paired-end 150-nt reads on the Illumina HiSeq 2500 platform. The resulting 16S rRNA gene sequences were assembled using FLASH (38) and quality filtered using the FASTX Toolkit (39). Zebrafish host sequences were filtered from the dataset by aligning reads to the zebrafish genome using Bowtie2 (40). OTUs were defined de novo using 97% sequence similarity in the USEARCH pipeline (41). The taxonomy of these OTUs was then assigned using the RDP classifier (42). To infer the genomic content of OTUs, we first matched representative sequences from each de novo OTU to 97% similarity OTUs in the Greengenes 13_8 database (43). We then used the preestimated genomic predictions for the Greengenes OTUs from PICRUSt to infer the genomic content of the OTUs in our study (32). Illumina sequence reads have been deposited under the National Center for Biotechnology Information BioProject accession number PRJNA378677.

Community Analysis and Statistics. Before analysis, OTU abundance tables were rarefied to 13,700 reads per sample. We measured differences in microbiome composition using the Canberra distance. To assess whether different treatments or host factors had a significant effect on microbiome composition, we performed PerMANOVA with 1,000 random permutations using these distances. To measure the overall variation in microbiome composition within groups, we performed a multivariate homogeneity of group dispersions test. Variance partitioning on microbiome composition by host factors (standard length and *il-1β* and *c3* gene expression) was done by canonical redundancy analysis to measure both the unique and shared contributions of each host factor (44). Identification of differentially abundant taxa was done using a negative binomial distribution model implemented in the DESeq2 package (45), while identification of predicted gene pathways enriched in these taxa was done by Poisson regression followed by a χ^2 test of significance. Calculation of the Canberra distance, PerMANOVA, Shannon diversity, multivariate dispersions test, and redundancy analysis were all performed in R (46) using the vegan package (47).

ACKNOWLEDGMENTS. We thank Ellie Melancon, Sophie Sitchel, Marcie McFaddon, and Tim Mason for advice on zebrafish husbandry and Rose Sockol for help in generating zebrafish populations. We also thank Keaton Stagaman and Lucas Nebert for assistance in designing and optimizing qPCR protocols, and Maggie Weitzman and Doug Turnbull of the University of Oregon Genomics Core for handling of DNA samples for 16S Illumina sequencing. This research was supported by the National Institute of General Medical Sciences of the National Institutes of Health under Awards 1R01GM095385 and 1P50GM098911. Grant P01HD22486 provided support for the Oregon Zebrafish Facility. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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