RESEARCH ARTICLE

Ontogenetic Differences in Dietary Fat Influence Microbiota Assembly in the Zebrafish Gut

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ABSTRACT **Gut microbiota influence the development and physiology of their animal hosts, and these effects are determined in part by the composition of these microbial communities. Gut microbiota composition can be affected by introduction of microbes from the environment, changes in the gut habitat during development, and acute dietary alterations. However, little is known about the relationship between gut and environmental microbiotas or about how host development and dietary differences during development impact the assembly of gut microbiota. We sought to explore these relationships using zebrafish, an ideal model because they are constantly immersed in a defined environment and can be fed the same diet for their entire lives. We conducted a cross-sectional study in zebrafish raised on a high-fat, control, or low-fat diet and used bacterial 16S rRNA gene sequencing to survey microbial communities in the gut and external environment at different developmental ages. Gut and environmental microbiota compositions rapidly diverged following the initiation of feeding and became increasingly different as zebrafish grew under conditions of a constant diet. Different dietary fat levels were associated with distinct gut microbiota compositions at different ages. In addition to alterations in individual bacterial taxa, we identified putative assemblages of bacterial lineages that covaried in abundance as a function of age, diet, and location. These results reveal dynamic relationships between dietary fat levels and the microbial communities residing in the intestine and the surrounding environment during ontogenesis.**

IMPORTANCE **The ability of gut microbiota to influence host health is determined in part by their composition. However, little is known about the relationship between gut and environmental microbiotas or about how ontogenetic differences in dietary fat impact gut microbiota composition. We addressed these gaps in knowledge using zebrafish, an ideal model organism because their environment can be thoroughly sampled and they can be fed the same diet for their entire lives. We found that microbial communities in the gut changed as zebrafish aged under conditions of a constant diet and became increasingly different from microbial communities in their surrounding environment. Further, we observed that the amount of fat in the diet had distinct age-specific effects on gut community assembly. These results reveal the complex relationships between microbial communities residing in the intestine and those in the surrounding environment and show that these relationships are shaped by dietary fat throughout the life of animal hosts.**

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Starting at the beginning of their lives, the intestinal tracts of animals are colonized by microbes acquired from the surrounding environment (1–3) which assemble into communities as the host ages (4–6). The resulting gut microbiotas exert influences on diverse aspects of host development and physiology (7) which can vary as a function of gut microbiota composition (8–10). Alterations to microbiota composition during early life stages are associated with effects on microbiota and host phenotypes at adult stages (2, 3, 11, 12). An improved understanding of processes governing gut microbiota assembly during early life stages is therefore warranted. Gut microbiota assembly typically occurs in the context of host development and age-associated diet alterations, with ample opportunities for microbial exchange between the gut and environment. However, an understanding of how these factors combine to influence gut microbiota assembly has remained elusive.

The nutrient environment is known to be a potent force shaping microbial communities. Feeding status and diet composition have been correlated with different gut microbiota compositions (13–17). Dietary fat is a key nutrient class often associated with changes in gut microbiota (15, 16, 18) and is a rich source of energy and substrates that potentially influence both gut and environmental microbial ecologies. However, most prior studies examining the impact of different levels of dietary fat on gut microbiota have focused on relatively short-term diet alterations (16, 18, 19) and have been conducted in mammals, where nursing limits the experimental capacity for diet manipulations during critical early postnatal stages (20). To date, no studies have examined the

impact of differences in dietary fat levels during ontogenesis (i.e., from first feed to adulthood) on the process of gut microbiota assembly. Additionally, the impact of ontogenetic differences in dietary fat levels on microbiota assembly in the host's environment, and on the relationship between gut and environmental microbiotas, remains unexplored.

The zebrafish (*Danio rerio*) is a model organism that permits analysis of relationships between diet composition, the gut microbiota, and the surrounding environmental microbiota (i.e., the communities of microorganisms found in the surrounding water, on abiotic surfaces, and in food). In this system, the animal host is fully immersed in an aqueous medium that can be easily sampled and the microbial community characterized. Additionally, the high fecundity of zebrafish allows for high biological replication. We recently showed that gut microbiota in zebrafish subjected to standard husbandry and age-associated diet changes undergo compositional alterations, increased interindividual variation (6), and increased selective pressure as fish age (21–23). Although standard zebrafish husbandry incorporates age-associated dietary changes (23), zebrafish can be raised on a single diet for their entire lives, allowing rigorous control over the exogenous nutrient environment. Here we report the first analysis of microbiota assembly in the zebrafish gut and environment in the context of constant life-long diet composition. This study was also the first to use zebrafish to study the effects of dietary fat on the gut microbiota. We further compared environmental microbiota from tanks with or without fish to evaluate the degree to which gut microbiota influence environmental microbiota assembly and to explore whether changes in gut microbiota might stem from environmental changes or vice versa. In addition to studying individual bacterial taxa, we identified groups of bacteria that covary in abundance (assemblages) and that may therefore be under similar ecological pressures. These results reveal dynamic relationships between dietary fat levels and the microbiotas residing in the intestine and the surrounding environment during ontogenesis.

RESULTS

Gut and environmental microbiota compositions quickly diverge early in animal development. To compare microbial community assemblies in the gut and environment under conditions of constant long-term exposure to different levels of dietary fat, we raised zebrafish under controlled conditions using one of three sterilized custom diets: a low-fat (LF) diet, a control (Ctrl; containing fat levels characteristic of standard fish diets) diet, or a high-fat (HF) diet (see Table S1 in the supplemental material). We performed deep sequencing of bacterial 16S rRNA genes from gut samples and from three types of samples from the tank environment: at an early prefeeding stage (5 days postfertilization [dpf]) and at three subsequent feeding stages during zebrafish development (10, 35, and 70 dpf) (see Fig. S1 and Table S2 in the supplemental material). Previous studies reported age-associated changes in zebrafish gut microbiota when animals were progressed through conventional changes in dietary regimens (4, 6). To test whether such changes still occurred when experimental diets were administered and held constant throughout life, we combined results from fish fed the three different diets and compared gut microbiotas of fish at different ages. We found that community evenness (as measured by the Shannon index) underwent little variation (Fig. 1B), whereas community membership varied markedly between ages (Fig. 1A) and community richness (estimated using the Chao1 estimator) increased with age (Pearson $r = 0.25$, $P < 0.0001$; Fig. 1C). These age-dependent changes in community membership were still observed analyzing each diet separately (see Fig. S2A to C). Age-associated changes in the prevalent bacterial taxa in the zebrafish gut in this study (see Fig. S2D) largely reflected those described in previous studies (4, 6). Together, these results reveal associations between fish development and gut microbiota composition in the absence of alterations in diet composition.

We next took advantage of the zebrafish model to investigate the relationship between gut and environmental microbiotas. We therefore tested whether the presence of zebrafish is associated with changes in their environmental microbiotas. Given the opportunity for microbial exchange between zebrafish and environmental microbiotas, we hypothesized that the composition of environmental microbiota in tanks with fish would be different from that in tanks without fish. In support, we observed significant fish-dependent differences in beta diversity, measured by pairwise Bray-Curtis dissimilarities, at earlier but not later time points (Fig. 1D). The decline in Bray-Curtis dissimilarity was accompanied by a decrease in the number of bacterial taxa that were differentially abundant based on whether or not fish were present (see Table S3 in the supplemental material). This suggests that fish altered the microbiota composition of their surrounding environment but that these effects diminished as fish aged. The causes for this diminished effect remain unclear but could include altered properties of environmental microbiotas or increased water turnover at older stages.

We next compared gut and environmental microbiotas and found that environmental microbiota evenness and richness were higher than those of gut microbiotas at all time points (Fig. 1B and C). Additionally, assessment of beta diversity (Fig. 1D) revealed compositional differences between gut and environmental microbiotas at each age. Considering all ages together, variation among individual guts was significantly higher than variation among individual environmental samples (unpaired *t* test with Welch's correction; $P < 0.0001$). These results indicate that the compositions of zebrafish gut and environmental microbiotas rapidly diverge early in host development even when diet is held constant. They further highlight the relatively large degree of variation between gut microbiota in different hosts, some of which may be attributed to the age-associated variation discussed above (Fig. 1A).

To determine whether age impacted the degree of interindividual variation between gut microbiota samples, we compared interindividual Bray-Curtis dissimilarity values for each age group. In addition to observing decreased intratank compared to intertank Bray-Curtis dissimilarities at all ages except 10 dpf (see Fig. S4 in the supplemental material), we found that interindividual variation between gut microbiota was highest at 5 dpf (analysis of variance [ANOVA], $P < 0.001$; all Bonferroni post-tests, $P < 0.0001$). This was salient when comparing the relative abundances of bacterial classes from individual guts at each age (see Fig. S3). Interestingly, gut microbiota also displayed greater similarity to environmental microbiotas at 5 dpf than at any other age (Fig. 1D). Environmental microbiotas at this early age formed two clusters as defined by principal coordinate analysis (PCoA) of Bray-Curtis distances, with water and floor samples clustering separately from wall samples (Fig. 1E). Some gut microbiotas at this early age clustered with those from water/floor or wall communities, while other gut microbiotas were separate from envi-

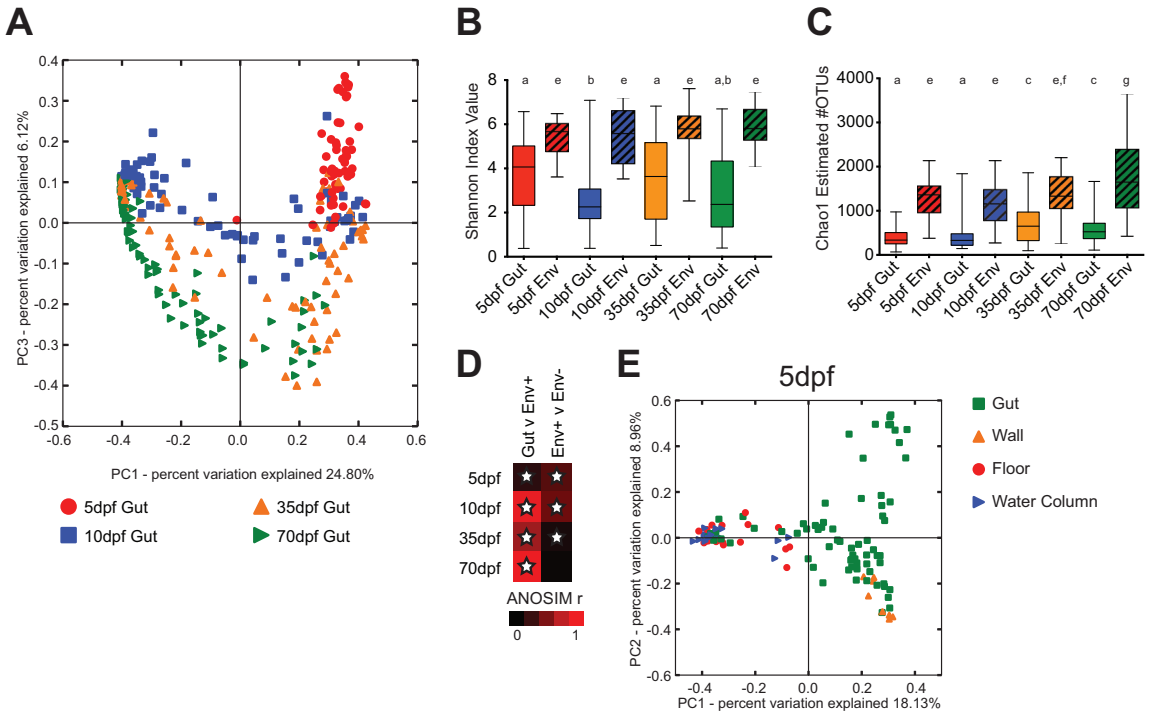

FIG 1 Alpha and beta diversity between zebrafish gut and environmental microbiotas. (A) Bray-Curtis dissimilarities between gut microbiota samples visualized by PCoA along the 1st and 3rd axes. Samples are colored according to age. (B and C) Alpha diversity in gut and fish-containing-environment (Env) microbiotas at each time point, as measured by Shannon index (B) and Chao1 estimate of richness (C) values. Whiskers indicate minimum and maximum values. Statistics comparing gut or environmental microbiotas at different ages were calculated by ANOVA with Bonferroni posttests. Groups of data with the same letters are not significantly different. Data in panels A to C represent the results of gut comparisons. Data in panels E and F represent the results of environment comparisons. Statistics comparing gut and environment were calculated by Student's t-test. Shannon diversity values were statistically significantly higher for environment samples than for gut samples at all ages except 10 dpf. Richness values were statistically significantly higher for environment samples than for gut samples at all ages except 35 dpf. (D) ANOSIM effect sizes for Bray-Curtis comparisons of gut versus environment samples with fish (left column) and environment samples with versus without fish (right column). Stars indicate $P < 0.05$. (E) PCoA plot of Bray-Curtis dissimilarities between 5 dpf gut and fish-containing-environment samples. Plot colored by sample type.

ronmental microbiotas (Fig. 1E). This raises the possibility that developing gut microbial communities are initially seeded from distinct environmental sources. These gut-environmental microbiota clusters further suggest that there may be distinct bacterial taxa that cooccur as assemblages (24) within individual zebrafish guts and associated environmental microbial communities.

To operationally identify groups of cooccurring bacteria, we used established methods (16) to cluster bacterial operational taxonomic units (OTUs) observed in this study into 145 assemblages (see Fig. S5 and Table S4 in the supplemental material). We then determined whether the phylogenetic diversity of these assemblages was greater or lesser than what would be expected if OTU members were chosen at random. Increased or decreased phylogenetic diversity relative to the null expectation could be used to infer the likely ecological mechanisms maintaining these assemblages (e.g., lower phylogenetic diversity would be consistent with a strong effect of host selection on the assemblage, among other factors). In many of these assemblages, the observed phylogenetic diversity of OTUs was lower than the expected phylogenetic diversity (Fig. 2A). In contrast, no assemblage had observed phylogenetic diversity significantly higher than the expected diversity. Analysis of these assemblages across gut and environmental microbiotas revealed striking localization and temporal patterns (Fig. 2; see also Table S5 in the supplemental material). For example, several assemblages were more abundant in the gut than in the environment, suggesting relatively increased fitness in the gut habitat (Fig. 2B). One of these, assemblage no. 4, was gut enriched at all ages and less phylogenetically diverse than expected (Fig. 2A), containing 9 of the 11 OTUs from our data set in the order *Aeromonadales* (see Table S4), whose members are commonly observed in zebrafish guts and aquatic environments (6, 25, 26). Further, assemblage no. 4 contained the only OTU that was observed in all gut microbiota samples in this study (*Aeromonadales* OTU no. 839072). Other assemblages exhibited transient enrichment in the gut. For example, assemblage no. 75 was gut enriched only at 10 dpf and 35 dpf, was less phylogenetically diverse than expected, and was rich in *Clostridia* (phylum *Firmicutes*) and *Bacteroidetes* OTUs (see Table S4). Several other assemblages, such as assemblage no. 139, were enriched in environmental microbiotas compared to gut microbiotas at all time points. Of the 9 OTUs in assemblage no. 139, 5 are in the order *Sphingobacteriales*, with 4 of these in family *Chitinophagaceae* (see Table S4). This suggests that members of this bacterial family may have relatively low fitness in the zebrafish gut relative to the environment.

Focusing on changes in the ratio of gut relative abundance to environmental relative abundance may mask changes in relative abundances in one or both niches. Therefore, we proceeded to compare changes in assemblage relative abundance between successive time points in the gut (Fig. 2C). In some cases, consistent increases in environmental relative abundance were concomitant

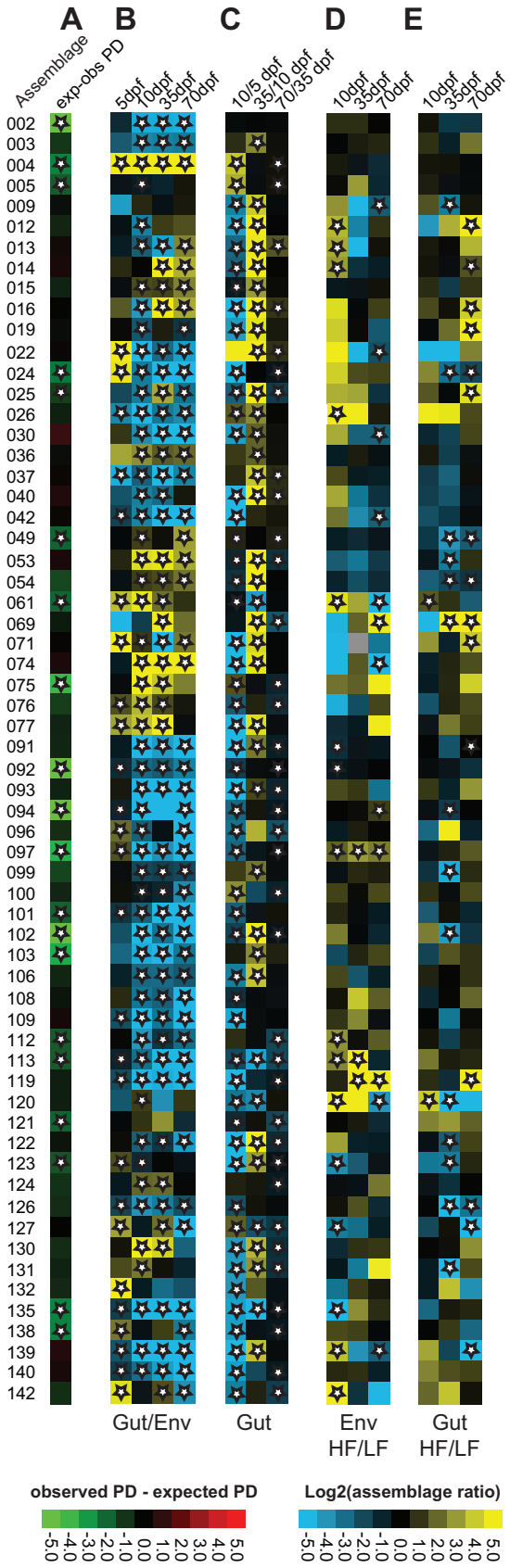

FIG 2 Differences between different experimental groups in relative abundances of assemblages. (A) Heat map showing differences between observed

(Continued)

TABLE 1 ANOSIM effect sizes comparing HF and LF microbiota based on Bray-Curtis dissimilarity matrices[a]

Sample type	Age (dpf)	Tank site	*r*	*P*
Gut	10	NA	0.0313	0.122
	35	**NA**	**0.0998**	**0.007**
	70	**NA**	**0.0977**	**0.028**
Environment with fish	10	**All tank sites**	**0.2621**	**0.001**
		Water column	**0.8093**	**0.004**
		Tank floor	**0.3306**	**0.004**
	35	All tank sites	0.0272	0.247
		Water column	0.1987	0.05
		Tank floor	0.2923	0.074
	70	**All tank sites**	**0.3277**	**0.007**
		Water column	**0.8315**	**0.002**
		Tank floor	**0.3160**	**0.037**
Environment without fish	10	**All tank sites**	**0.1535**	**0.004**
		Water column	**0.3287**	**0.009**
		Tank floor	0.0500	0.247
	35	All tank sites	0.0223	0.3
		Water column	0.1176	0.126
		Tank floor	0.1107	0.19
	70	**All tank sites**	**0.2856**	**0.008**
		Water column	**0.8176**	**0.002**
		Tank floor	−0.0063	0.503

[a] Comparisons for which *P* values were <0.05 are highlighted in boldface. NA, not applicable.

with progressive decreases in gut relative abundance and vice versa. For example, assemblage no. 135 was always environmentally enriched but progressively decreased in relative abundance in the gut and environment over the course of the experiment. This assemblage contains many OTUs from *Sphingomonadales* (*Alphaproteobacteria*) and *Sphingobacteriales* (*Bacteroidetes*) (see Table S4), suggesting that bacteria that produce sphingolipids may have increased fitness in the environment compared to the gut.

Dietary fat density impacts environmental microbiota compositions. We next used our data to test whether differences in dietary fat levels influenced environmental and gut microbiota assembly. We first examined the impact on the environmental microbiota to determine whether diet-dependent differences in the tank environment might influence diet-dependent differences in the gut. We found that the dietary fat level does not significantly impact microbial evenness or richness in the water column, on the tank floor, or on the tank wall at any age (data not shown). In contrast, Bray-Curtis distances indicated that the dietary fat level significantly impacted environmental microbiota composition at 10 dpf and 70 dpf (Table 1). Interestingly, the impact of dietary fat was generally larger in the water than at the tank floor, and the

Figure Legend Continued

and expected phylogenetic diversities (PD) of assemblages with at least 3 OTUs. Stars indicate differences greater than the variance in expected PD. (B to E) Heat maps show fold differences between 2 experimental groups in relative abundances of assemblages. Stars indicate a statistically significant change according to White's nonparametric *t* test followed by FDR correction using a cutoff of 5%. (B) Gut versus fish-containing-environment microbiotas at each time point. (C) Changes in gut microbiota between 2 consecutive time points. (D) HF versus LF fish-containing-environment microbiotas at each feeding time point. (E) HF versus LF gut microbiotas at each feeding time point.

impact in the water was generally more significant when fish were present (Table 1). These results suggest that dietary fat has different effects on microbiota in distinct locations of the tank environment and that this is influenced by the presence of fish. It is possible that different individuals may be more likely to sample microbes from distinct tank sites. Therefore, the different impacts on microbiota from different tank sites may lead to interindividual variations in gut microbiota responses to differences in dietary fat levels.

We next sought to identify bacterial assemblages and taxa that are indicative of low-fat (LF) versus high-fat (HF) tanks. We found that the dietary fat level was associated with differentially abundant assemblages at all 3 fed ages (Fig. 2D; see also Table S5 in the supplemental material). At 10 dpf, 11 assemblages were significantly enriched in HF environmental microbiotas from tanks containing fish, and 5 assemblages were enriched in the LF environments. HF-enriched assemblage no. 142 consisted entirely of OTUs from *Proteobacteria* (see Table S4), including 3 species in the family *Pseudomonadaceae*, which was identified by linear discriminant analysis (LDA) effect size (LEfSe) as indicative of HF environmental microbiotas (see Table S3). Moreover, LEfSe identified a specific OTU (*Pseudomonas* OTU no. 72643) within assemblage no. 142 (see Table S3) as indicative of 10 dpf HF environmental microbiotas. At 35 dpf, only three assemblages exhibited significantly different relative abundances in HF versus LF environmental microbiotas from tanks with fish, and all were enriched in the HF environment (Fig. 2D; see also Table S5 in the supplemental material). At 70 dpf, four assemblages were significantly enriched and eight assemblages were significantly depleted in environmental microbiotas from tanks with fish receiving a HF diet compared to those receiving a LF diet (Fig. 2D; see also Table S5 in the supplemental material). One of the HF-enriched assemblages, assemblage no. 97, was composed almost entirely of OTUs from the class *Betaproteobacteria*, which LEfSe identified as indicative of HF environmental microbiotas at 70 dpf from tanks containing fish (see Table S3). Together, these results reveal that the dietary fat level impacts distinct assemblages during environmental microbiota assembly.

Dietary fat density impacts gut microbiota composition. We next tested whether different dietary fat levels influence gut microbiota assembly. We found that community evenness and richness were not significantly different at any fed age based on the proportion of fat in the diet (data not shown). In contrast, comparison of Bray-Curtis distances revealed that dietary fat level had a significant effect on gut microbiota beta diversity at each fed age, with significant differences between HF and LF guts at 35 dpf and 70 dpf and the largest effect at 35 dpf (Table 1).

To identify the bacterial groups underlying these differences, we compared the relative abundances of bacterial assemblages from gut microbiotas of HF-fed versus LF-fed fish. At the first postfeeding time point of 10 dpf, only 2 assemblages, both enriched in HF guts, were significantly different (Fig. 2E; see also Table S5 in the supplemental material). Interestingly, both were also enriched in environmental microbiotas of HF diet tanks with fish (Fig. 2D). At 35 dpf, assemblage no. 69 was the only assemblage enriched in HF guts while 13 other assemblages were depleted in HF guts (Fig. 2E; see also Table S5). Assemblage no. 69 contained just three OTUs, including one *Janthinobacterium* OTU and one *Pseudomonas* OTU (see Table S4). In accordance, LEfSe analysis identified the *Pseudomonas* OTU and the entire *Janthinobacterium* genus as indicative of HF gut microbiotas at this age (see Table S3). At 70 dpf, eight assemblages were enriched and seven assemblages were depleted in HF guts compared to LF guts (Fig. 2E; see also Table S5). Interestingly, of these, assemblage no. 19 was relatively enriched in the guts of HF-fed fish and contained an OTU from *Firmicutes* class CK-1C4-19 as well as 2 *Fusobacteria* OTUs from the genus *Cetobacterium* (see Table S4). Use of LEfSe to compare 70 dpf gut microbiotas from HF-fed and LF-fed fish also identified members of *Fusobacteria*, a phylum that has been associated with adult zebrafish gut microbiotas (4, 6, 8, 25), suggesting that the HF diet may accelerate the establishment of these characteristic "adult" bacteria. Notably, this occurs despite HF-fed fish having a body size relatively smaller than that of LF-fed fish at 70 dpf (see Fig. S2B in the supplemental material). In contrast, assemblages no. 49 and no. 54, which were enriched in LF compared to HF guts, are entirely or predominantly composed of alphaproteobacteria, particularly of the order *Rhizobiales* (see Table S4). This suggests that a variety of *Rhizobiales* members may experience a competitive advantage in the guts of zebrafish fed diets containing less fat. Although the dietary fat level affected multiple OTUs and assemblages at each fed age, these differences were largely restricted to a subset of fed ages. Indeed, no assemblage demonstrated consistently different relative abundances in LF-fed versus HF-fed fed guts at all 3 fed ages, and LefSE analysis identified only 5 OTUs that fulfilled that criterion (all 5 enriched in the LF diet; see Table S3).

DISCUSSION

This report provides the first view into how life-long diets that are high or low in fat impact *de novo* gut microbiota assembly processes in a vertebrate host. This report further provides the most detailed analysis to date of the life-long relationship between animals' gut microbiotas and the microbiota of their surrounding environment. This study held diet constant, in contrast to our recent report on *de novo* zebrafish gut microbiota assembly in which animals were raised on conventional diets that were altered with ontogenetic progression (6). Here we provide definitive evidence that development-associated changes in gut microbiota occur in the absence of alterations in diet composition. We found that gut and environmental microbiotas became increasingly divergent over time but also that fish impacted the microbiota of their environment. Further, we found that different levels of dietary fat led to distinct effects on gut and environmental microbiota assembly.

To compare microbiota of different experimental groups, we employed a number of methods, including the identification and analysis of assemblages, defined here as groups of bacteria that covary in abundance (24). This observed covariance raises the possibility that the members of an assemblage form a functional network or possess similar levels of fitness within their niche. We found that assemblages tended to consist of closely related taxa (Fig. 2A), indicating that traits underlying assemblage membership are often shared among related organisms. However, the data set used to identify assemblages can impact the correlation between OTU abundances and can therefore impact assemblage builds. In this study, we operationally identified assemblages using an OTU table containing all gut and environmental microbiota samples. Furthermore, our use of hierarchical clustering to identify assemblages mandates that specific OTUs belong to only one assemblage. While this provides a useful operational approach to

predict assemblages, we recognize that a given OTU may be involved in multiple assemblages. More sophisticated modes of analysis are needed to detect how the same bacteria may participate in different assemblages under different environmental conditions or in different niches.

Of the factors contributing to variation in the gut microbiota, host development has proven to be a strong correlate in diverse animal species, including mammals and fish. Despite the interindividual variations in microbiota composition observed at all ages, both mammalian and zebrafish gut microbiotas undergo broadly similar taxonomic changes with age. For example, human infant microbiotas are abundant in *Bifidobacteria* but eventually become abundant in *Bacteroidetes* and *Firmicutes* following the introduction of solid foods (5). Zebrafish larvae are abundant in *Firmicutes* and *Proteobacteria*, but adult gut microbiotas are dominated by *Fusobacteria* (6, 8, 25). Our recent cross-sectional study described the changes occurring in the zebrafish gut microbiota at multiple developmental stages in the context of standard diet transitions (6). In the present study, where diet composition was unchanged, we observed similar taxonomic alterations in gut microbiota as the zebrafish aged. Our previous studies also reported that community richness decreased with host age (6). Moreover, the earliest time point used by Stephens and coworkers—a prefeeding larval stage—yielded the largest number of distinguishing taxa (6), indicating that the greatest differences between stages were between this prefed stage and all other stages. In accord, previous studies have reported profound postfeeding changes in gut microbiota composition (13, 14). It is therefore possible that the onset of feeding may be a strong driver of change in gut microbiota. In contrast to the results of Burns and coworkers (21), we observed an increase in microbial richness as the fish aged (Fig. 1C). Importantly, unlike these recent studies, animals in the present study received the same diet throughout the course of the experiment. This underscores a confounding factor in prior studies exploring the relationship between host development and microbiota assembly: the changes in diet that typically occur at developmental milestones (e.g., weaning in mammals, feed transitions in fish husbandry). Our knowledge of the diets consumed by zebrafish in the wild at different life stages is very limited, but available studies suggest that wild larval zebrafish feed primarily on zooplankton while adults are omnivorous and feed from multiple levels of the water column (22, 27). In the laboratory, standard zebrafish husbandry incorporates age-associated dietary changes, with larvae primarily raised on zooplankton and formulated powder diets and adults on brine shrimp and formulated pellet and flake diets (23). Importantly, different diets can impact gut microbiota directly or indirectly through diet-induced alterations to host physiology (28–31), which can in turn influence host development and physiology. We therefore speculate that the observed differences between this study and that performed by Stephens et al. (6), at comparable developmental stages, may be due in part to use of a constant diet and a variable diet, respectively.

While the act of feeding on exogenous dietary nutrients may exert a strong influence on gut microbiota, altering the composition of the diet can also impact the microbiota of the surrounding environment. Lipids are a major macronutrient class and are used by both microbes and animals, including fish (32), both as an energy source and as critical structural and signaling components. Lipids may act as a source of direct microbial selection through differential microbial capabilities for lipid metabolism (33) and can also exert indirect effects through the modulation of host immunity and physiology (34, 35). For example, bile acids are released in response to fat consumption (36) and possess antimicrobial properties (37) and can be metabolized by some bacteria via bile salt hydrolases (38). Therefore, the developmental stage may exert a large impact on how different levels of dietary fat directly or indirectly impact gut microbiota assembly.

Indeed, we observed such effects of development on the correlation between dietary fat levels and gut microbiota in zebrafish. For example, *Bacteroidetes*, *Clostridia*, and *Erysipelotrichia* have been reported to be differentially abundant in the guts of mammals fed diets differing in the amount of fat (10, 16, 19, 39). In our study, these taxa also exhibited diet-associated differences in relative abundances in the gut, but we found also that the age of the fish influenced which dietary condition showed the higher relative abundance (see Table S4 in the supplemental material). Indeed, only 5 OTUs in the entire study demonstrated consistently different relative abundances in the guts of fish fed a LF or HF diet, suggesting a strong interaction between age and dietary fat that exerts a strong effect on zebrafish gut microbial ecology (see Table S3). The underlying age-dependent processes remain unclear but could include the maturation of the adaptive immune system which occurs between 10 dpf and 35 dpf, the maturation of the intestine and other digestive organs during metamorphosis (40, 41), and establishment of an anaerobic niche in the intestinal lumen. Together, these results emphasize the need to consider developmental context in studying microbiota responses to diet and other perturbations. In the future, it would be useful to define the dietary differences between wild and laboratory-reared zebrafish at different life stages and to determine how those differences and specific dietary fats contribute to microbial ecology in the zebrafish gut.

There are ample opportunities for microbial exchange between gut and environmental communities via host ingestion and excretion, but the degree to which these communities influence each other has remained unclear. In the context of dietary manipulations, one might expect to see differences in the gut reflected in the environment and vice versa. A recent study revealed that human inhabitants alter the microbiota of their homes by serving as a microbial source but reported little reciprocal impact of the home microbiota on humans (42). That report also included the observation that specific parts of homes had different microbiota compositions, indicating that location and substrate are determinants of environmental microbiota composition. Similarly, we found that environmental microbiotas differed depending on whether or not fish were present and that the impact of dietary fat changed depending on the location of the site within the tank. Whether or not fish were in the tank also impacted the diet-dependent differences in environmental microbiota (Fig. 1 and Table 1). Changes in environmental microbiota may result from proliferation of bacteria that can metabolize animal waste products such as urea or that withstand challenges such as the presence of host antimicrobial products. These are possible etiologies for the divergence we observed between environmental microbiotas from tanks with versus without fish at earlier time points (Fig. 1) and for the greater impact of dietary fat levels on environmental microbiotas from tanks with fish than on those from tanks without fish (Table 1). Despite these differences and in contrast to the results reported by Lax and colleagues (42), we found that the zebrafish

environmental microbiota was relatively stable, with intersample variation between environmental microbiotas at all time points much smaller than interindividual variation between gut microbiotas (Fig. 1). Our results suggest that most members of the environmental microbiota in a tank are relatively insensitive to the presence of fish or dietary fat variation but that the members of a small contingent of environmental microbes are highly sensitive to these factors.

While the presence of fish can alter the environmental microbiota, environmental microbes seed gut microbiota. A probable result, which we recently described (6), is that gut microbiota most resemble environmental microbiota at birth. Such a phenomenon has been previously reported in humans, where gut microbiotas of infants born by C-section contain skin microbes and those of infants born naturally contain vaginal microbes (2). Intriguingly, despite high interindividual variation between gut microbiotas at 5 dpf, in this study we also observed that different subsets of gut microbiotas from 5 dpf resembled the microbiotas of different tank sites (Fig. 1). This suggests that the microbiotas of these guts may have been seeded from those respective tank sites. While differences in the initial seeding could potentially lead to differences in gut microbiota assembly and alter host physiology later in life, we observed decreased interindividual differences in the gut microbiota at the fed 10 dpf time point (Fig. 1). This suggests the differences observed at 5 dpf were superseded by changes associated with feeding. Further work is needed to test whether assemblages of cooccurring bacteria remain linked under different conditions (e.g., new diets or altered host physiology) or whether rearrangements of functional networks occur.

MATERIALS AND METHODS

Zebrafish husbandry. All zebrafish experiments were conducted in conformity with the Public Health Service Policy on Humane Care and Use of Laboratory Animals using protocols approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill. Unless stated otherwise, all fish were maintained at 28.5°C on a 14-h light cycle on a recirculating aquaculture system (Z-Mod; Marine Biotech). A total of 6 adult pairs of zebrafish (Tübingen strain), all derived from the same two parents, were allowed to mate naturally and collectively laid ~1,800 fertilized eggs. All embryos were mixed and split evenly among 9 sterile petri dishes containing fresh conditioned water obtained from the recirculating zebrafish aquaculture system (system water). Embryos were incubated in the Petri dishes in system water at 28.5°C until 1 dpf. At 1 dpf, live embryos from each dish were transferred to an autoclaved 8-liter tank containing 300 ml of system water (see Fig. S1 in the supplemental material). For the remainder of the experiment, fine mesh was secured over all points of entry into the tank to limit introduction of undesired material. Embryos were left in 300 ml static water until 5 dpf, at which the water volume was increased to 500 ml/tank. A separate set of autoclaved tanks was subjected to the same treatments except no zebrafish were ever added (see Fig. S1). All tanks were moved to the recirculating aquaculture system at 6 dpf. Drip water flow commenced at 6 dpf, and fast water flow commenced at 28 dpf. At 5 dpf, all tanks received one feeding of their assigned diet after sample collection was completed. Starting at 6 dpf, each tank received two feedings per day for the remainder of the experiment. Throughout the course of the experiment, all fish remained in the same tank in which they were placed at 1 dpf, with periodic removal of floc from the tank floor performed using sterile cell scrapers and sterile serological pipettes (see Fig. S1).

Dietary manipulations. Control (Ctrl), high-fat (HF), and low-fat (LF) diets (Zeigler Brothers, Inc.) (see Table S1) were custom formulated and ground to a pellet size of 50 to 100 μm and then sterilized by irradiation (Neutron Products, Inc.) (absorbed dose range, 106.5 to 135.2 kGy). For the duration of the experiment, starting at 5 dpf, each tank was assigned one of 3 diets: LF, Ctrl, or HF. A total of 3 fish-free tanks per diet were also maintained in parallel under identical husbandry conditions (see Fig. S1 in the supplemental material). From 5 dpf to 27 dpf, each tank received 80 mg of the assigned diet per feeding; from 28 dpf to 40 dpf, tanks received 120 mg of the assigned diet per feeding; from 40 dpf to 49 dpf, each tank received 160 mg of the assigned diet at each feeding; from 49 dpf to 70 dpf, each tank received 192 mg of the assigned diet at each feeding. For the remainder of the experiment, each tank received 160 mg of the assigned diet per feeding.

Sample collection. At each sampling time point, randomly selected fish were collected and euthanized via tricaine overdose (sterile-filtered tricaine at 0.83 mg/ml). Following euthanasia, fish were imaged for subsequent standard length measurements (see Fig. S2E in the supplemental material) (40). Intestinal tracts were dissected from each fish, placed into a tube containing sterile lysis buffer (20 mM Tris-HCl [pH 8.0], 2 mM EDTA [pH 8.0], 1% Triton X-100) and sterile 0.1-mm-diameter beads (BioSpec Products catalog no. 11079101z), and immediately frozen in a dry ice-ethanol bath. Samples were stored at −80°C until sample processing.

At each sampling time point, environmental microbiota samples were gathered from the upper water column, the lower water column/floor deposits (floc), and the tank walls. For the upper water column, 50 ml of tank water was filtered using a 0.22-μm-pore-size filter (Mo Bio Laboratories, Inc.; catalog no. 14880). For the floc, tank floors were scraped with sterile cell scrapers to loosen floc, and 8 ml from the tank floor floc was filtered using a 0.22-μm-pore-size filter (Mo Bio Laboratories, Inc.; catalog no. 14880). Filters were then extracted using flame-sterilized forceps and cut in half using flame-sterilized scissors and stored at −80°C until sample processing. For wall samples, sterile cell scrapers were used to make one vertical scrape on the tank wall and swirled in 10 ml sterile phosphate-buffered saline (PBS). Scraped debris was allowed to settle, and then 200 μl of suspended debris was placed into 2-ml tubes containing sterile lysis buffer and sterile 0.1-mm-diameter beads before freezing in dry ice-ethanol and storage at −80°C.

Samples were named as follows: tank identifier (ID), followed by time point (5, 10, 35, or 70 dpf), followed by sample ID ("m" for water column, "s" for tank floor, "w" for tank wall, and the remaining letters in alphabetical order for guts) (see Table S2). For example, "2H35d" indicates that the sample was the 4th gut sample from the 2nd HF tank at 35 dpf.

Molecular biology. Genomic DNA was extracted from intestinal tract and tank wall samples using QIAamp DNA micro kits (Qiagen; modified as previously described in reference 6). For water samples, genomic DNA was extracted from one half of each filter using QIAamp DNA micro kits (Qiagen; catalog no. 56304) and from the other half using PowerWater kits (Mo Bio Laboratories, Inc.; catalog no. 14900). Amplification of the v4 region of the 16S rRNA gene was performed using a 2-step PCR amplification process as previously described (6), and sequencing was performed on an Illumina HiSeq 2000 sequencing system at the High Throughput DNA Sequencing and Genomics Facility at the University of Oregon.

Bioinformatic analysis. Preprocessing of raw sequence data was performed as previously described (6) prior to demultiplexing. We used QIIME version 1.6.0 (43) to demultiplex the reads using default parameters with the following changes: reads of less than 199 bp were discarded, and 2 primer differences were allowed in the sequences. We then used QIIME to cluster the reads, using open-reference UCLUST (44), into operational taxonomic units (OTUs) against greengenes 2012 (October update) at the 97% sequence identity level and assigned a taxonomic classification to each OTU using RDP classifier v2.2. We further required that the OTU be detected in at least 5 samples for inclusion in the analyses.

Additionally, we used QIIME to assess alpha and beta diversity in our data set. For alpha diversity analyses of all samples except tank walls (excluded here due to the use of low sequencing depths in those samples), the data were rarified such that each sample included in the analyses con-

tained 10,100 sequences. For alpha diversity analyses that included tank wall microbiota samples, we rarified the data to 100 sequences per sample to ensure adequate sample numbers. We calculated Shannon indices and Chao1 values to evaluate alpha diversity. For beta diversity analyses, we rarified the samples to 1,000 sequences per sample with the goal of retaining at least 18 gut samples/condition at each age and generated Bray-Curtis distance matrices. We used PCoA to visualize beta diversity distances and calculated effect sizes of variables using analysis of similarity (ANOSIM). To identify bacterial taxa with statistically significant differences in relative abundance between different experimental groups, we employed the LEfSe module (version 1.0) (45) available on the Huttenhower laboratory Galaxy website (http://huttenhower.sph.harvard.edu/galaxy/). For all comparisons, we used the default parameters (Kruskal-Wallis test alpha of <0.05, pairwise Wilcoxon text alpha of <0.05, and LDA score of ≥ 2.0 for significance) with the following exception: comparing gut microbiota from fish of different ages, we employed the all-against-all option in order to identify taxa for which there was a significant difference in relative abundance between at least 2 ages.

Identification of bacterial assemblages. Using the OTU table generated by open-reference UCLUST clustering at 97% sequence identity, where each OTU included was required to have been observed in at least 5 samples, we clustered OTUs into assemblages using established methods (16). Briefly, using custom Python scripts and SciPy, OTUs were ranked based on their absolute abundance and filtered such that we retained the top 95% of all. OTU counts were then normalized as described by David and colleagues (16). We then used SparCC (46), with 10 iterations, to generate pairwise correlation values for each OTU and used hierarchical clustering to cluster the OTUs and generate a distance-based tree. We arbitrarily set a depth threshold of 0.729 on the tree to delineate clusters, yielding 145 clusters. We refer to these clusters as "assemblages" according to the nomenclature of Fauth and colleagues (24). Following the identification of these assemblages, we calculated the observed and expected phylogenetic diversities of each assemblage in R using the PD and expected PD functions in the picante package, respectively. Using a phylogenetic tree trimmed to contain OTUs within the assemblage analyses, expected phylogenetic diversity and variances for each assemblage were estimated based on the number of OTUs in the assemblage. We then used STAMP (47) and/or Metastats (48) to compare the relative abundances of each assemblage containing at least 3 OTUs between the different sample groups (White's nonparametric *t* test; false-discovery-rate [FDR] threshold of 5%). Heat maps were generated using Cluster 3.0 (49) and Java-TreeView (50).

Nucleotide sequence accession number. Sequence data have been deposited under NCBI BioProject accession number PRJNA278165.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00687-15/-/DCSupplemental.

Figure S1, PDF file, 0.04 MB.
Figure S2, PDF file, 0.6 MB.
Figure S3, PDF file, 0.3 MB.
Figure S4, PDF file, 0.1 MB.
Figure S5, PDF file, 0.2 MB.
Table S1, PDF file, 0.04 MB.
Table S2, XLS file, 0.1 MB.
Table S3, XLS file, 0.6 MB.
Table S4, XLS file, 1.9 MB.
Table S5, XLS file, 0.1 MB.

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